THESE

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Titre : Identification et mesures de biomarqueurs infra-individuels chez le palmier dattier (*Phoenix dactylifera*) lors d'une contamination métallique: prédiction des voies métaboliques et description des mécanismes de détoxication des métaux impliqués

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Soutenue le 21 Novembre 2017 devant le jury composé de

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À mes parents

À mon frère et mes soeurs

À Mohamed et mes beaux parents

À mes beaux frères et mes belles soeurs

À mes amis

Table de Matière

Deuxième Partie : *RESULTATS ET DISCUSSION*

Liste des abréviations

ABCC	ATP binding cassette de type C
abcc	Gène codant pour ATP binding cassette de type C
AGPI	Acides gras polyinsaturés
AHK	Histidine kinase
Al	Aluminium
APX	Ascorbates peroxydases
Ca	Calcium
CAM	Calmoduline
CaMK	Calmodulin-dependent protein kinases
CAT	Catalase
CAX	Cation Exchanger
CBL	Calcineurin B-like proteins
CBP	Calmodulin-Binding Protein
CCH	Copper Chaperone
cch	Gène codant Copper Chaperone
Cd	Cadmium
$CdCO_3$	Carbonate de cadmium
CDF	Cation Diffusion Facilitor
CDPK	Ca^{2+} dependent protein kinases
CEC	Capacité d'Echange de Cation
CIPK	CBL interacting protein kinases
CNGC	Cyclic Nucleotide-Gated non selective Cation Channels
Co	Cobalt
copt	Gène codant pour le Ctr-related copper transporter
COPT1	Ctr-related copper transporter
CP	Composés Phénoliques
Cr	Chrome
Cr(VI)	Chromate
CRK	CDPK-related kinases
CSD	Cu/ZnSOD
Csd	Gène codant pour Cu/ZnSOD
Cu	Cuivre
Cu_2S	Chalcocite
$CuFeS_2$	Chalcopyrite
DCE	Directive-Cadre sur l'Eau
EC	Cadre sur l'Eau
EC	Enzyme Commission
Eh	Potentiel redox
ERO	Espèces Réactives de l'Oxygène
ETM	Eléments traces métalliques
Fe	Fer
$FeCr_2O_4$	Chromite
Fl-OH	Polyphénols de type flavonoïdes
FSD	FeSOD
fsd	Gène codant pour FeSOD
GO	Gene Ontology

GSH	Glutathion
H_2O_2	Peroxyde d'hydrogène
Hg	Mercure
HMA	Heavy Metal transporting ATPases
hma	Gène codant pour Heavy Metal transporting ATPases
HMW	High Molecular Weight
ICP-AES	Spectrométrie d'émission atomique par plasma à couplage inductif
ICP-MS	Spectrométrie de masse à plasma couplé
IRT	Iron Regulated Transporter
ISO	Organisation Internationale de Standardisation
K	Potassium
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOG	Eukaryotic Orthologous Groups
LMW	Low Molecular Weight
MATE	Multi-antimicrobial extrusion protein MATE
mate	Gène codant pour Multi-antimicrobial extrusion protein MATE
ML	Métal Lourd
Mn	Manganèse
MnO_2	Oxydes de manganèse
MS	Masse Sèche
MS	Murashige and Skoog
MT	Métallothionéines
mt	Gène codant pour métallothionéine
NaCl	Chlorure de sodium
Ni	Nickel
Nr	Non-redondantes
Nramp	Protéine de macrophages associée à la résistance naturelle
Nramp	Gène codant pour des protéines de macrophages associées à la résistance naturelle
$O_2^{\cdot -}$	Anion superoxyde
OH^-	Radical hydroxyle
Pb	Plomb
$PbCO_3$	Cérusite
$PbCrO_4$	Crocitite
PbS	Galène
$PbSO_4$	Anglésite
PC	Phytochélatine
pc	Codant pour des phytochélatines
PCS	Phytochélatine Synthase
pcs	Gène codant pour phytochélatine synthase
PDR	Pleitropic Drug Resistance
pdr	Gene codant pour Pleitropic Drug Resistance
POX	Peroxydases
PRX	Peroxyredoxines
qPCR	Réaction en chaîne par polymérase quantitative en temps réel
RFE	Relative Fold Expression
SBP	SQUAMOSA promoter binding protein
-SH	Groupement Thiol
SIAPE	Société Industrielle d'Acide Phosphorique et d'Engrais de Sfax
SOD	Superoxyde dismutase

SOx	oxyde de soufre
SPL	SQUAMOSA promoter binding protein-like
spl	Gène codant pour SQUAMOSA promoter binding protein-like
TF	Transcription factors
TSIP	programme d'identification des sites toxiques
TSP	Triple Super Phosphate
UBC	Ubiquitin C
ubc	Gène codant pour Ubiquitin C
ZIP	Zinc Regulated Transporter
Zn	Zinc

INTRODUCTION GENERALE

I. Contexte : Méthodes de biosurveillance des écosystèmes naturels

I.1. Problématique

Les effets des polluants toxiques tels que les éléments traces métalliques (ETMs), les pesticides et les radionucléides sur la santé sont plus importants qu'on ne le pensait auparavant. En 2010, plus de 100 millions de personnes seraient exposés à des niveaux de pollution métallique supérieurs aux normes sanitaires internationales (McCartor, 2010). Il s'agit d'un problème de santé publique aussi important que la tuberculose, le paludisme et le VIH, et qui doit recevoir une attention et des ressources considérables (Bernhardt, 2015).

L'organisation environnementale Green Cross Suisse et Pure Earth (anciennement Blacksmith Institute), engagés dans la recherche de solutions aux problèmes environnementaux ont publié conjointement en 2015 un rapport alarmant sur les polluants en 2015. Ils ont décrit une situation comme jamais encore vécue dans l'histoire de l'humanité à savoir que les polluants environnementaux sont présents en quantités plus importantes que jamais dans les zones peuplées (Bernhardt, 2015). En l'absence de mesures appropriées, le nombre de personnes exposées à un niveau critique de pollution ne cesse d'augmenter. Depuis 2012, le programme d'identification des sites toxiques (TSIP) avait évalué 2400 sites contaminés par des métaux dans le monde, essentiellement par le plomb (Pb), le cadmium (Cd), le chrome (Cr), le mercure (Hg) et les pesticides riche en cuivre (Cu). Ces métaux sont non seulement présents en de nombreux endroits et en de plus grandes quantités que les autres, mais leurs effets potentiels sur la santé sont documentés (Bernhardt, 2015).

Le transport à longue distance de la pollution atmosphérique, l'important pouvoir de dispersion des ETMs facilitent la contamination des écosystèmes et leur transfert le long de la chaine alimentaire. Certains métaux peuvent atteindre des teneurs élevés dans certaines espèces consommées par l'homme, tels que des poissons et des espèces végétales accumulatrices. Ces polluants métalliques doivent faire l'objet d'une surveillance particulière du fait de leur persistance (non biodégradabilité). Même si les dangers toxicologiques et sanitaires liés aux ETMs sont relativement bien documentés, les efforts fournis pour réduire leurs émissions et sauvegarder les milieux naturels à l'échelle internationale restent fragmentaires. Il existe donc, un besoin évident de les détecter sur site dès leurs présences en état de traces, afin de prévenir leurs effets néfastes sur la santé humaine et l'environnement.

I.2. Biosurveillance au moyen de bioindicateurs et de biomarqueurs

La biosurveillance se définit comme " l'utilisation d'informations biologiques fournies à différentes niveau d'organisation (morphologique, tissulaire, physiologique, cellulaire, biochimique, moléculaire) d'un individu ou celles observées au cours des changements dans la structure d'un groupe d'individus pour prévoir et/ou révéler une altération de l'environnement ainsi que pour suivre l'évolution d'un écosystème " (Kerambrun, 2013). Il existe diverses méthodes analytiques pour détecter les fluctuations des métaux dans les milieux (Zhou et al., 2008). Cependant, les analyses physico-chimiques donnent des indications sur la présence ou l'absence d'un polluant et parfois sur la spéciation des métaux. Néanmoins, ces méthodes ne renseignent pas sur l'impact réel du polluant sur les organismes vivants. Au fil des années, la biosurveillance s'est donc imposée comme un complément intéressant des méthodes traditionnelles de mesure permettant pour apprécier la qualité des biotopes (analyses de l'air, de l'eau et des sols). La biosurveillance est devenue une approche largement utilisée, souvent basée sur la sensibilité d'un (ou quelques) organisme(s), lors d'une exposition aigue ou chronique à des contaminants (Zhou et al., 2008 ; Nachev et al., 2010). La mesure de cette sensibilité défini le concept de bioindicateur qui se traduit par des changements au niveau individuel (traits d'histoire de vie, reproduction) et supra-individuel (populations, communautés). Toutefois, ces bioindicateurs ne sont pas perceptibles rapidement, ils ne permettent donc pas une mise en évidence précoce de la pollution. Ainsi, la bioévaluation a recours à la mesure de biomarqueurs qui correspondent à des changements au niveau infra-individuel se mettant en place rapidement.

Un biomarqueur peut alors se définir comme " toute réponse biologique à un produit environnemental constatée à un niveau inférieur à celui de l'individu et qui induit un stress capable de montrer une exposition à une substance toxique (Weeks, 1995b) ". Il représente une signature biologique de l'impact ou de l'exposition à un xénobiotique. En effet, il n'existe pas de biomarqueur idéal, ni informatif sur toutes les molécules chimiques, ni prédictif quant aux effets à tous les niveaux d'intégration biologique (Flammarion et al., 2000). La mesure d'une batterie de variables biologiques « approches multi-biomarqueurs » semble être la plus efficace dans l'estimation de l'action des ETMs et l'évaluation de l'état de santé de l'écosystème (Jha, 2008 ; Ait Alla et al., 2006). Le couplage de différents types de biomarqueurs entre eux lors d'une étude permet d'avoir une meilleure vue d'ensemble de l'impact d'une pollution (Jha, 2008).

Toutefois, un effort important doit être fait afin de refléter l'intensité des expositions, intégrer les différents paramètres de l'environnement, et pouvoir déterminer les effets d'une exposition de faible intensité mais pendant une longue période. Il est apparu nécessaire de recourir en écotoxicologie à des espèces biomonitrices ou sentinelles judicieusement sélectionnées pour la surveillance des ETMs sur une période de temps étendue. Les organismes sentinelles prélevés dans un milieu doivent être représentatifs de ce milieu et remplir plusieurs conditions, telles que 1) la sédentarité sur la zone d'étude, 2) la facilité d'identification et de récolte, 3) l'abondance à plusieurs endroits géographiques dans une zone territoriale bien définie, 4) la longévité de plusieurs années et la résistance et l'accumulation de concentrations élevées de polluants sans mortalité et 5) la capacité d'établir une relation dose/effet et cause/effet (Zhou et al., 2008).

II. Objectifs

Cette thèse s'intègre dans la recherche de stratégies biologiques pour la surveillance de milieux terrestres contaminés par des métaux. Plus spécifiquement, elle a pour objectifs 1) d'évaluer le potentiel du modèle végétal tunisien ; Palmier dattier, *Phoenix dactylifera* L., comme espèce sentinelle appliquée dans le suivi de l'évolution d'un milieu face aux contaminants 2) adopter un contexte multi pollution qui cherche à déterminer les conditions et les limites d'utilisation d'une batterie de bioindicateurs et de biomarqueurs.

Pour atteindre les objectifs cités, nous avons entrepris de :

-1- faire un diagnostic, réalisé sur le terrain indiquant l'état des lieux du site d'étude à l'aide du dosage des concentrations métalliques dans les sols,

-2- recueillir des informations sur les relations exposition-réponse des végétaux du site contaminé,

-3- conceptualiser les observations faites sur site par mobilisation de la contamination métallique à l'échelle laboratoire pour mieux contrôler la réponse du palmier face aux polluants,

-4- mettre en place une grille de qualité, utilisant une batterie d'indicateurs biologiques qui reposent sur la nécessité de prendre en compte des effets de la contamination sur l'environnement à court, moyen et long terme, et définir les liens existant entre effets

toxicologiques et écologiques,

-5- apprécier la biodisponibilté réelle d'un contaminant métallique à travers l'optimisation des dosages des biomarqueurs morphologiques et physiologiques de stress chez des palmiers exposés à des concentrations croissantes en Cd, Cu ou Cr,

-6- développer une approche fondamentale qui consiste à mesurer différents biomarqueurs infra-individuels notamment le suivi du niveau d'expression des gènes de biomarqueurs spécifiques de stress métallique. L'ensemble des mesures constitue une approche « multi-biomarqueurs » permettant d'envisager une biosurveillance au cours d'une intoxication au Cd, Cu ou Cr,

-7- faciliter les extrapolations des résultats trouvés en laboratoire à plus grande échelle afin de poursuivre l'adaptation des méthodes de révélation de bioindicateurs physiologiques et biochimiques chez la plante *in vivo* et de montrer la pertinence des biomarqueurs pour la mise en évidence de plusieurs gradients de contamination au cours d'études de biosurveillance.

III. Démarche suivie

Dans la continuité des travaux visant la recherche de méthodes biologiques pour l'évaluation de la contamination métallique réelle du site d'étude, nos travaux sont orientés selon les différents axes de recherches présents dans la figure I.

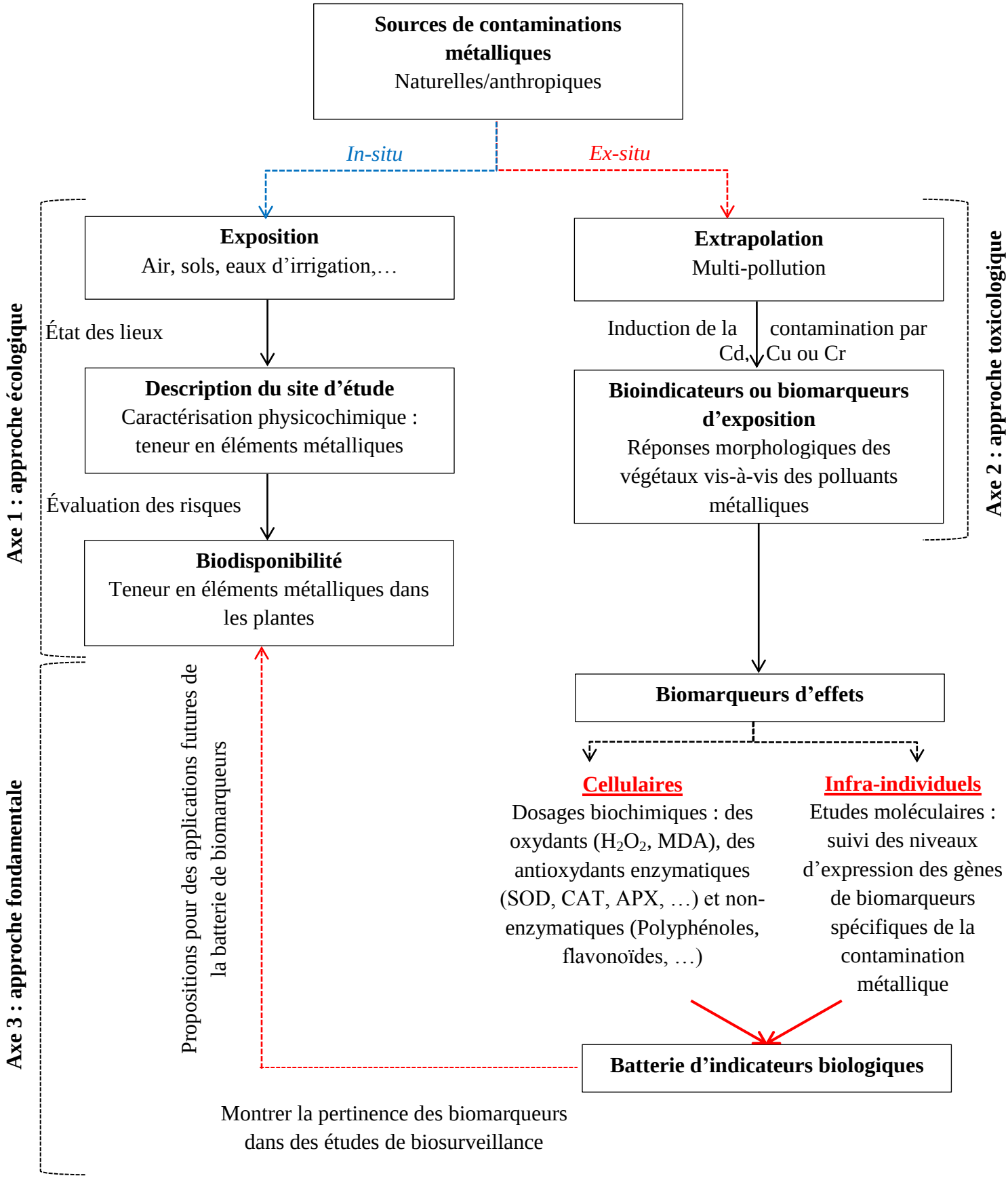

Figure I. Schéma général des axes de recherche entamés au cours des travaux de la thèse

IV. Zone d'étude

En Tunisie, il existe plusieurs sources de pollution par les ETMs provenant entre autres de la circulation routière et des activités minières au nord et au centre du pays (industrie des engrais phosphatés). Elles présentent une source majeure de dommages environnementaux dus à la migration des métaux du carburant et des digues de phosphogypse vers l'environnement (air, sol et eau) (Zairi and Rouis, 1999). Une méthode d'extraction séquentielle suivie d'analyses des propriétés chimiques des sols de certains sites du pays a révélé des concentrations élevées de métaux traces dans les échantillons de sols caractérisés par des niveaux importants de Cr, Zinc (Zn), Pb, Cd et Cu (Wali et al., 2013 ; 2015).

Les aérosols contenant des ETMs constituent l'un des polluants environnementaux les plus importants en termes d'effets néfastes sur les écosystèmes et la santé humaine (EEA, 2013). Provenant essentiellement des rejets industriels, les particules d'aérosol sont capables d'affecter le rayonnement atmosphérique, la formation des nuages ainsi que les réactions de photochimie atmosphérique. Par ces activités industrielles intenses, la région de Sfax est considérée comme la ville la plus industrialisée et de la Tunisie et est classée deuxième en terme de pollution (Bahloul et al., 2015). Les principales activités industrielles comprenaient le traitement au phosphate (usine de la Société Industrielle d'Acide Phosphorique et d'Engrais de Sfax (SIAPE)), la fabrication de savon (SIOS-ZITEX) et l'industrie secondaire de fusion de plomb (FP Sfax sud). La plupart de ces activités sont situées au bord sud de la ville et ont menacé l'environnement en raison de leur forte émission de particules notamment des oxydes de soufre (SOx) qui ont largement dépassé les normes tunisiennes (Azri et al., 2000).

L'usine de SIAPE a rejeté environ 4,5 t/jour de particules riches en ETMs, sulfate, phosphore et SOx, dépassant largement les normes d'émission autorisées (8 et 20 fois pour les particules et SOx, respectivement). Selon Azri et al. (2002), le SIAPE est la principale source d'ETMs (Cd, Zn, Cr, Cu, et Nickel (Ni)), confirmé par le facteur d'enrichissement élevé des particules en ce qui concerne ces éléments.

Face à cette intense pollution métallique atmosphérique, nous nous sommes intéressés à la biodisponibilité métallique de la région de Sfax et son effet sur les écosystèmes et les plantes.

V. Organisation du manuscrit

Ce manuscrit est composé de quatre parties :

- **La première partie** est consacrée à la *synthèse bibliographique*.

- **La seconde partie** ; *Résultats et Discussion*, dans laquelle, nous avons abordé successivement trois chapitres :

Le premier chapitre intitulé « La bioinformatiques en écotoxicologie » développe les moyens de recherche *in silico* des voies biochimiques intervenant dans la tolérance du palmier dattier cv Deglet Nour au Cd et Cu et des gènes spécifiques de la pollution métallique. Les résultats prédits en bioinformatique ont été confirmés par des suivis de l'expression de gènes candidats dans des tissues issues de la culture *in vitro* de vitroplant de palmier dattier cv Deglet Nour dans des milieux contaminés.

Le deuxième chapitre « Effets de la variabilité génétique sur la réponse métallique » est consacré à une étude en laboratoire de la tolérance des graines de palmier dattier vis-à-vis du Cd, Cu ou Cr.

Le troisième chapitre intitulé « Biodisponibilité, bioindicateurs et bio-marqueurs » est réservé aux résultats des mesures des bioindicateurs et biomarqueurs aspécifiques sur des tissus de feuilles et racines de palmier dattier collectés *in-situ* et *ex-situ*. Il présente également, la réponse moléculaire des biomarqueurs spécifiques en tant qu'outil d'évaluation réel de l'intoxication métallique.

- **La troisième partie** (*Discussion générale*), les résultats trouvés dans les chapitres I, II et III sont discutés au fur et à mesure de leur présentation dans le manuscrit et une discussion générale sur les différents axes de recherche sera reprise afin de pouvoir discuté à la lueur des apports de l'ensemble des expérimentations dans une approche écologique/écotoxicologique ;

- **La quatrième partie** (*conclusions et perspectives*) résume les apports de nos différentes approches étudiées, propose des voies d'exploitations de nos résultats dans des applications futures et dresse un bilan pour l'évaluation de la qualité des sites étudiés puis, à plus large échelle, pour la surveillance et la mise en œuvre de politiques de contrôle et de gestion plus efficaces de la contamination métallique des milieux.

Première Partie

ANALYSE BIBLIOGRAPHIQUE

I. Introduction

Le sol, ressource essentielle pour les sociétés humaines et les écosystèmes, subit de nombreuses pressions (étalement urbain et industriel, production agricole et forestière, développement des réseaux de transport…) susceptibles d'engendrer des dégradations importantes (érosion, tassement, contaminations ponctuelles ou diffuses d'origine atmosphérique, perte de biodiversité et de teneur en matière organique). Ceci est préoccupant dans la mesure où le sol n'est pas une ressource renouvelable à l'échelle de temps humaine. Les principaux contaminants d'origine anthropique sont des hydrocarbures, des polychlorobiphényles, des pesticides, des métaux mais aussi des rejets pharmaceutiques. Ces contaminants se retrouvent au sein des différents compartiments environnementaux en interaction : lithosphère, hydrosphère et atmosphère. Leur accumulation dans le sol peut aboutir à un impact non négligeable sur les biens et les services écosystémiques (Daily, 1997). Bien que les écosystèmes aient une capacité de résilience non négligeable, il est important de garder à l'esprit que certains composés comme les métaux ne sont pas biodégradables contrairement à certains composés organiques. Il est désormais nécessaire de s'assurer de la préservation de ce milieu / ressource.

II. Généralités sur les métaux lourds

II.1. Définition

« Métaux lourds (MLs) » est l'appellation couramment utilisée pour désigner les micropolluants minéraux de forte masse volumique supérieure à 5 g/cm^3 qui peuvent former des sulfures (Facchinelli et al., 2001). Ce terme sous-entend aussi une notion de toxicité. Cependant, le terme « éléments traces métalliques (ETMs) » est aussi utilisé pour décrire ces mêmes éléments, car ils se retrouvent souvent en très faible quantité dans l'environnement (Baker and Walker, 1989). Dans le manuscrit, le terme ETMs est préférentiellement utilisé. On en distingue deux types en fonction de leurs effets physiologiques et toxiques : métaux essentiels et métaux non essentiels.

- Les métaux essentiels sont des éléments indispensables à l'état de trace pour de nombreux processus cellulaires et qui se trouvent en proportion très faible dans les tissus biologiques (André, 1993). Certains peuvent devenir toxiques lorsque la concentration dépasse un certain seuil. C'est le cas du cuivre (Cu), du zinc (Zn), du nickel (Ni) et du fer (Fe).

- Les métaux non essentiels ont souvent un caractère toxique avec des effets indésirables pour les organismes vivants même à faible concentration. Ils n'ont aucun effet bénéfique connu, ne sont nécessaire à aucun processus biologiques, et sont considérés comme des poisons cellulaires pour lesquels les doses tolérables sont très faibles (Kabata-Pendias and Pendias, 2001). C'est le cas du Pb, Cd, Cr et Hg.

II.1. Origine

La présence de métaux lourds dans les sols peut être d'origine naturelle ou anthropique. Le sol est un milieu poreux, issu d'un processus d'altération des matériaux géologiques sous l'action combinée des êtres vivants et des processus physico-chimiques (solutions de percolation). Au cours de la formation du fond pédo-géochimique, des ETMs présents naturellement dans les roches mères, sont libérés dans les sols à des concentrations naturelles qui varient selon la nature de la roche, sa localisation et son âge. A cela, s'ajoute les apports de poussières et aérosols libérés dans l'atmosphère par les activités volcaniques, les embruns marins, etc… S'ajoute à ce fond pédo-géochimique les métaux d'origine anthropique, c'est-à-dire en grande partie ceux liée à l'exploitation et au traitement des minéraux, à la fabrication et l'utilisation d'engrais et de pesticides, à l'industrie chimique, aux décharges et à l'incinération de déchets ménagers et industriels, etc…

Deux types majeurs de contaminations liées aux activités humaines peuvent être distingués (Figure I-1) :

Contaminations locales : consistent en des apports massifs localisés d'origine proche qui touchent une aire relativement faible et sont dues à une ou plusieurs source(s) bien identifiée(s) située(s) à quelques mètres ou à quelques kilomètres. Ils résultent souvent d'apport anthropiques accidentels liés aux activités industrielles sans protection efficace contre la dispersion dans l'environnement et sont souvent associés aux exploitations minières, aux installations industrielles et à d'autres installations, tant en cours d'exploitation qu'après leur fermeture (Sonnet et al., 2007).

Contaminations diffuses : il s'agit d'apports diffus aériens d'origine lointaine qui affectent tous les sols, plus ou moins faiblement mais de manière généralisée. Dans ces apports diffus sont classés les poussières et aérosols provenant des chauffages, des moteurs d'automobiles, de dépôts atmosphériques et des pratiques agricoles et horticoles (épandage d'engrais, d'amendements, de boues de station d'épuration, de traitements phytosanitaires, etc…). Ce

type de contamination ne peut être totalement évité puisqu'il est la conséquence d'apports involontaires ou d'impuretés indésirables dans les apports volontaires (Sonnet et al., 2007).

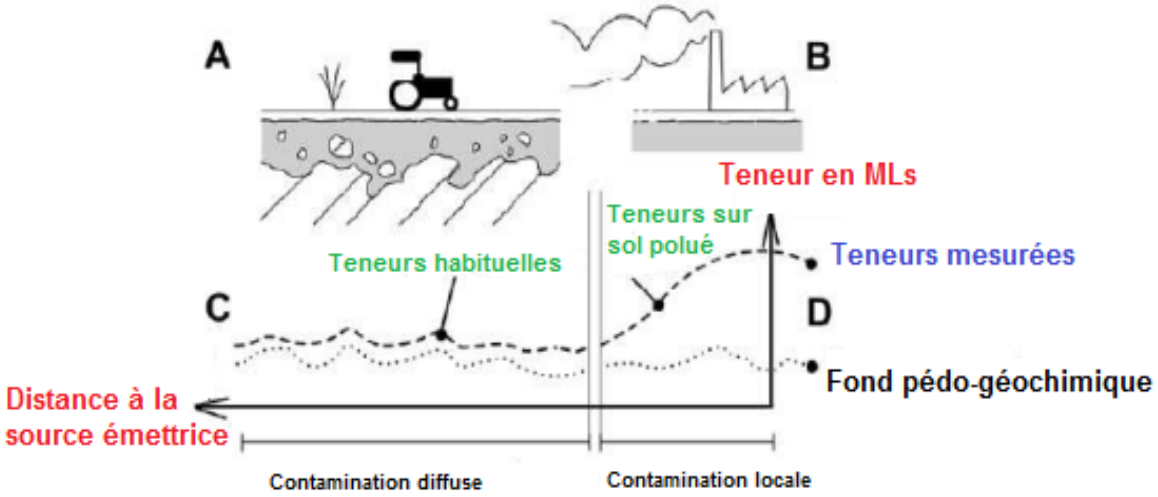

Figure I-1. Contamination diffuse et locale (d'après UZU, 2009)

(**A**) : Un sol sur un matériau parental (en grisé), développé sur des roches du substrat géologique. (**B**) : sur le même sol, présence d'une source d'émission massive de polluants. (**C**) : en pointillé : fond pédo-géochimique naturel sans contamination diffuse. En tirets, les teneurs mesurées dans le sol, qui sont qualifiées d'« habituelle » car on ne peut pas déceler l'influence d'une contamination locale. (**D**) : en tirets, les teneurs mesurées sont qualifiées de teneurs sur sol pollué parce qu'elles ont un niveau élevé résultant d'une contamination locale du sol.

II.2. Mobilité, biodisponibilité et toxicité

La mobilité d'un élément métallique constitue son aptitude à passer dans des compartiments du sol où il est de moins en moins énergétiquement retenu, souvent dans la phase liquide mais également dans l'atmosphère du sol (Juste, 1988). L'ensemble des constituants du sol forme le « complexe absorbant » (Blanchard and Wolfers, 2000). Ce complexe réalise des échanges entre la phase liquide et la matrice solide du sol, dépendant de mécanismes de sorption. Deux principaux types d'interactions entre les phases solide-liquide sont impliqués dans la rétention des ETMs. Les métaux peuvent exister sous forme d'ion libre ou sous forme liée dans six compartiments différents du sol (Huynh, 2009). Ainsi, ils peuvent être sous forme échangeable (cations ou anions), associés aux surfaces des minéraux argileux et aux matières organiques, complexés ou inclus dans les molécules organiques ou dans les réseaux cristallins des minéraux primaires et des constituants secondaires, adsorbés sur oxydes/hydroxydes et/ou

séquestrés dans les résidus végétaux et animaux. Toutefois, ils ont tendance à passer d'une forme à l'autre et leur répartition dans les différents compartiments du sol évolue dans le temps.

Dès lors, les scientifiques se trouvent confrontés à de grandes difficultés lorsqu'il s'agit de déterminer les seuils de concentration totale d'éléments métalliques considérés comme toxiques (Kabata-Pendias and Pendias, 2001) qui sont fonctions de la mobilité et de la biodisponibilité des métaux et pas de leur teneur totale. L'évaluation des conséquences de la contamination des sols par les métaux vis-à-vis des organismes vivants du sol et notamment des plantes nécessite le recours aux concepts de disponibilité et de biodisponibilité des éléments métalliques (Zhang et al., 2001; Geebelen et al., 2003). Le groupe de travail de l'ISO (Organisation Internationale de Standardisation) « ISO/TC 190 » a mené une réflexion permettant d'éviter la confusion possible entre les deux concepts (ISO, 2006). Selon ISO (2006), la biodisponibilité peut être subdivisée en trois composantes : la « disponibilité environnementale », la « biodisponibilité environnementale » et la « biodisponibilité toxicologique » (Figure I-1).

Disponibilité environnementale : regroupe les différentes formes chimiques des ETMs sous lesquelles les éléments sont présents et interagissent avec les divers constituants du sol. Cette approche tient compte de la spéciation des métaux dans la solution du sol et non seulement de leur spéciation en phase solide (Zhang and Young, 2006). Dans le cadre des interactions sol-plante, la disponibilité des métaux pourrait donc être redéfinie comme le niveau d'exposition des racines aux métaux. Celle-ci dépendrait principalement de la spéciation des métaux dans le sol mais également de la variation des conditions physico-chimiques du milieu pédologique (pH, le potentiel redox (Eh), de la capacité d'échange de cation (CEC), des concentrations ioniques, de la teneur en phosphate disponible, de la teneur en matière organique. Les activités biologiques qui gouvernent les multiples réactions d'adsorption/désorption, de précipitation/dissolution et d'oxydation/réduction influenceraient aussi beaucoup la disponibilité des métaux. Tous ces éléments concourent à modifier les concentrations métalliques ioniques et complexés dans la solution du sol et par conséquent influencent la mobilité des éléments (Geebelen et al., 2003) assimilables par les organismes vivants (Zhang et al., 2001). D'autres facteurs peuvent affecter la mobilité des ETMs dans les sols ; par exemple : les caractéristiques de l'environnement comme la température, l'humidité, la

richesse du milieu en microflore et microfaune.

Enfin, l'absorption et le transfert d'un contaminant est aussi fonction de l'espèce considérée. Chez la plante, l'espèce, la variété, la morphologie racinaire, les types d'exsudats peuvent changer la disponibilité. Les racines peuvent interagir avec le sol environnant et en modifier les propriétés physico-chimiques (concentration en solution des métaux, pH, concentration en molécules organiques…). On peut considérer que le sol au contact de la racine, i.e. la rhizosphère est une zone de sol « bio-influencée » (Harmsen et al., 2005 ; ISO, 2006). La spéciation et donc la disponibilité des métaux au sein de la rhizosphère peut donc être fortement modifiée en fonction du temps d'exposition, de l'espèce végétale et même du génotype au sein d'une même espèce (Chaignon, 2001; Michaud, 2007; Figure I-2).

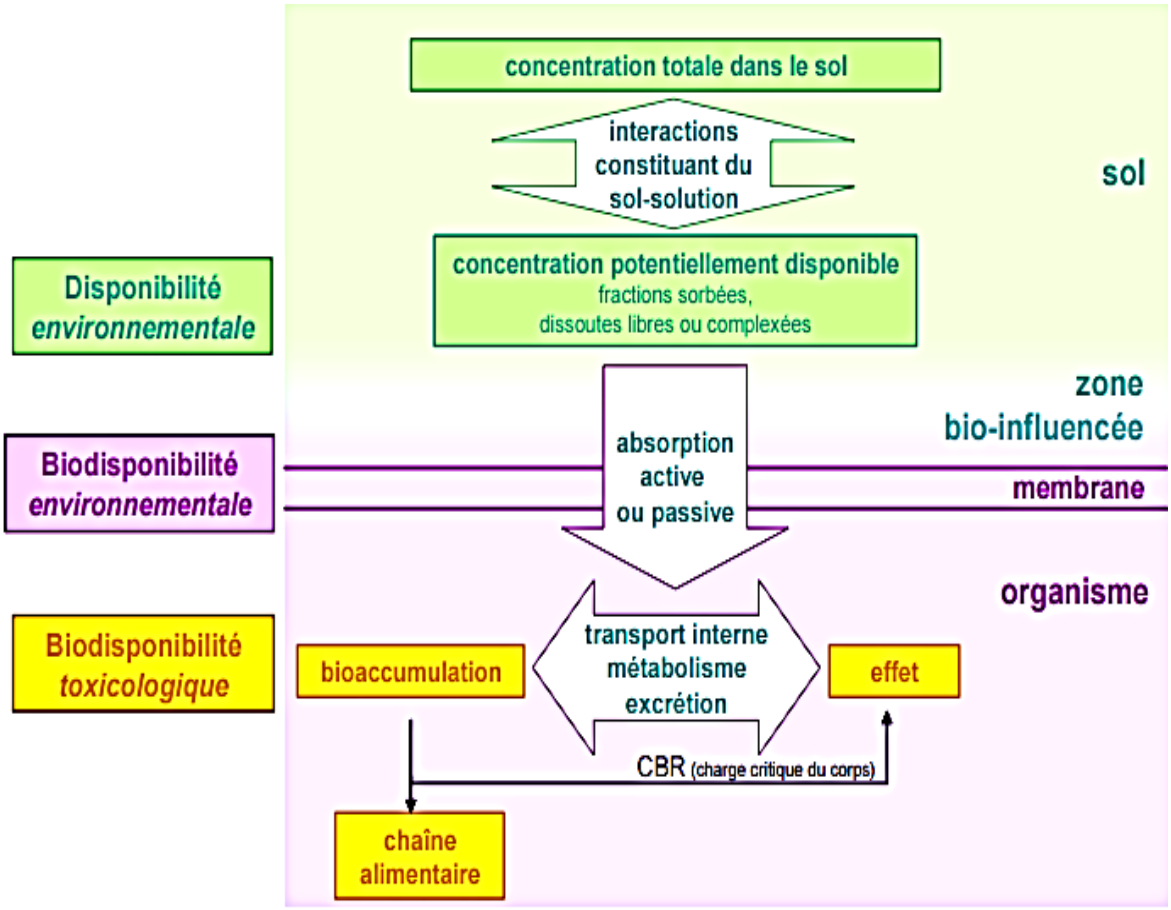

Figure I-2. Les trois composantes de la biodisponibilité des métaux pour les plantes (d'après Lanno et al., 2004)

Biodisponibilité environnementale ou biodisponibilité externe (Rensing and Maier, 2003) est la fraction disponible d'un métal qui est prélevée par la plante par des processus physiologiques d'absorption (ISO, 2006). Il s'agit donc, du flux de métal vers la surface du

système racinaire d'une plante cible pendant une durée d'exposition définie (Harmsen et al., 2005). Néanmoins, un métal n'est toxique pour la faune et la flore que s'il est sous forme libre; il est alors « biodisponible ». De l'équilibre entre les formes libres et fixées de l'ion va dépendre sa biodisponibilité, directement liée à sa toxicité.

Biodisponibilité toxicologique ou biodisponibilité interne qui concerne l'accumulation dans la plante des métaux au-delà d'un certain seuil d'accumulation dans l'organisme i.e. la charge critique du corps en résidu, et l'induction d'effets toxiques éventuels, i.e. phytotoxicité (Rensing and Maier, 2003 ; ISO, 2006) (Figure I-2).

Chez la plante, la phytotoxicité peut s'exprimer au niveau racinaire (rhizotoxicité) et/ou au niveau des parties aériennes et se caractérise par des réponses moléculaire, métabolique et/ou à différents niveaux d'organisation de la plante entière (ISO, 2006). Les principaux symptômes de toxicité observés chez les plantes suite à une exposition métallique sont une inhibition de la croissance, une chlorose foliaire, des lésions nécrotiques conduisant à un jaunissement progressif puis à un repliement ou un dessèchement du feuillage (Sanità di Toppi and Gabbrielli, 1999; Figure I-3).

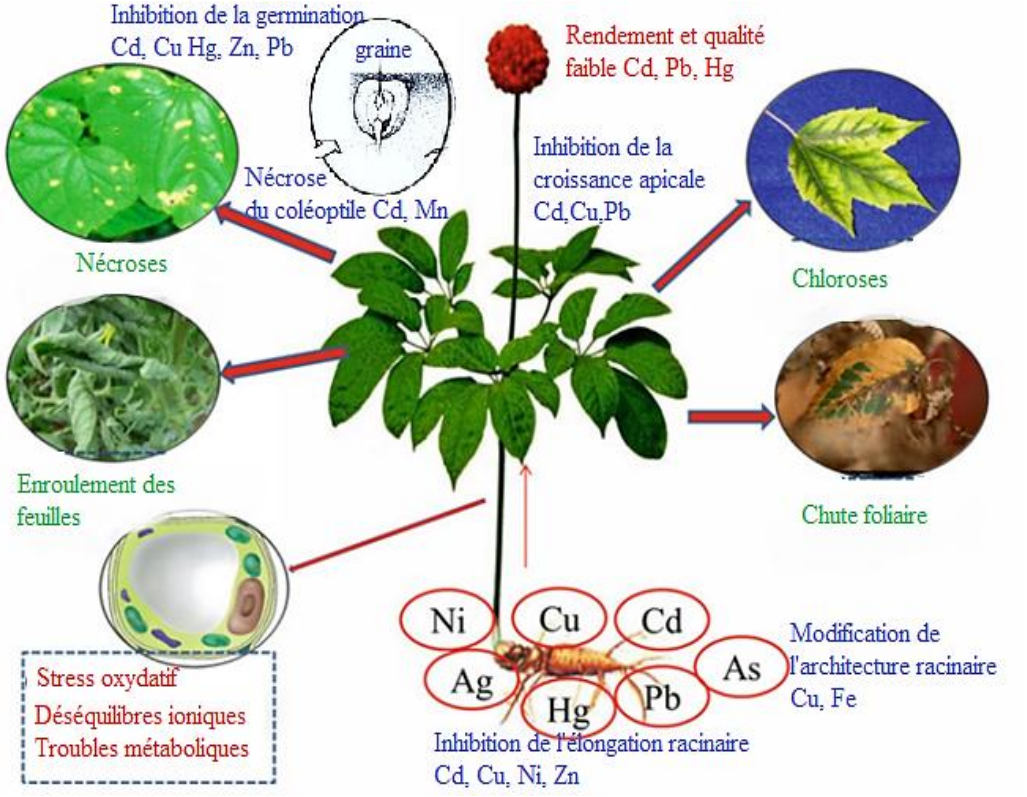

Figure I-3. Interactions métalliques lors du développement de la plante (d'après Huynh, 2009)

III. Mécanismes d'action des métaux lourds chez les végétaux supérieurs

III.1. Cas du Cadmium

III.1.1. Le Cd dans le sol

III.1.1.1. Formes du Cd

L'industrialisation croissante de ces dernières décennies est à l'origine de la libération dans la nature de quantités toujours plus importantes de Cd. Il est classé parmi les 17 substances « dangereuses prioritaires » définies par la DCE (Directive-Cadre sur l'Eau) selon la décision 2455/2001/EC (Cadre sur l'Eau) qui constitue le cadre réglementaire de la politique de l'eau au niveau communautaire. Cet élément trace assez rare dans la croûte terrestre se présente à des concentrations très variables dans le sol qui s'inscrivent dans un intervalle compris entre 0,04 et 35 µM (Sanità di Toppi and Gabbrielli, 1999). La plupart des sols présentent une concentration en Cd inférieure à 0,32 µM qui correspond à l'abondance naturelle de cet élément. Une terre est considérée comme modérément polluée si elle contient une teneur en Cd entre 0,32 µM et 1 µM. Au-delà de cette dernière concentration, le sol est décrit comme fortement pollué, rencontré souvent au voisinage des mines et des foyers industriels. Les concentrations totales de Cd dans les sols tunisiens à proximité des usines de transformation de phosphate varient de 1,2 à 6,7 mg kg^{-1} et sont donc souvent supérieures aux limites permises pour des usages agricoles qui sont de 4 mg kg^{-1} (OMS, 2012) . Cependant, Depuis décembre 2011, l'utilisation du Cd a été interdite strictement dans la totalité des matières plastiques mais aussi dans les bijoux ou encore les baguettes de brasage et ceci grâce à la découverte de nouveaux substituts (Regulation (EC), 2006). Malgré toutes ces restrictions et interdictions, le Cd persiste dans le sol car il était et est encore un sous-produit lié à certaines activités industrielles. En Europe en 2009, encore 96 tonnes de Cd étaient émises dans l'atmosphère (Haines-Young and Potschin, 2012). Les teneurs rencontrées dans des sols de sites industriels anciens peuvent être très élevées. A titre d'exemple, dans l'ancien bassin minier du Nord de la France, une teneur de 240 mg kg^{-1} de Cd a été mesurée à moins de 2 km d'une usine de production de Pb et de Zn (Douai and Franck-Dominique, 2009). Dans les horizons de surface des sols du Nord-Pas de Calais, y compris agricoles et forestiers, l'enrichissement en Cd représente le plus souvent 3 à 30 fois le fond pédogéochimique naturel (Bourennane et al., 2010; Figure I-4).

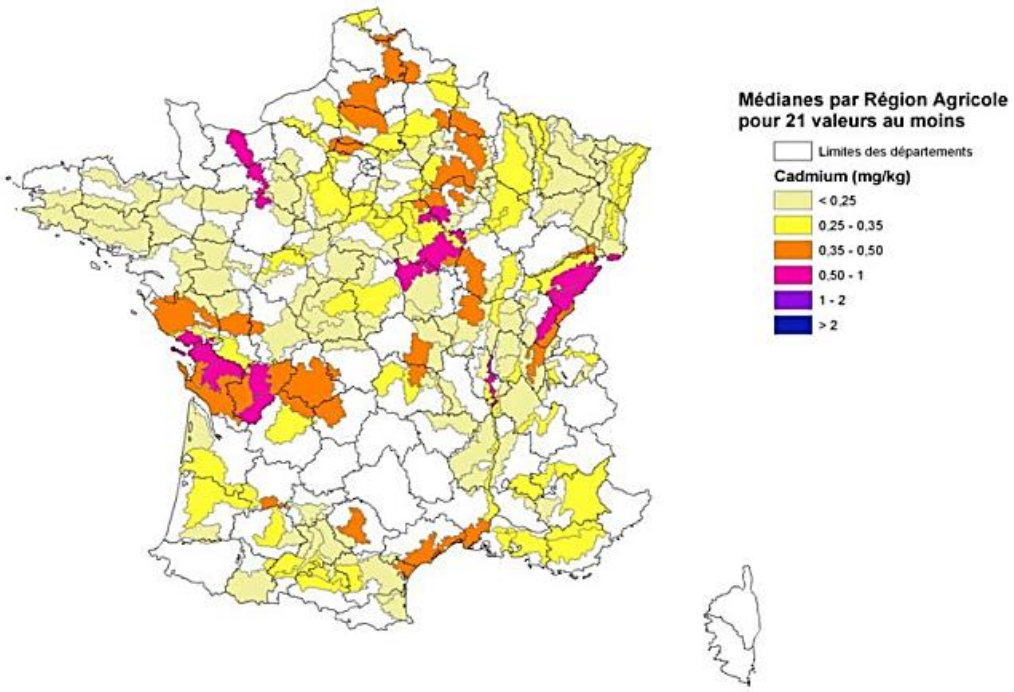

Figure I-4. Teneurs médianes en cadmium total des horizons de surface (0-30 cm) de sols agricoles cultivés par région agricole française (d'après Infosol and Orl, 2014)

III.1.1.2. Facteurs influençant la biodisponibilité environnementale du Cd

La seule fraction du Cd disponible est celle dissoute dans la solution du sol où le métal se présente sous forme hydratée et complexée par des ligands organiques ou inorganiques (Clemens, 2006). Toutefois, en l'absence de ligands organiques les principales formes du Cd présentes dans la solution du sol sont Cd^{2+}, $CdSO_4^0$, $CdCO_3$, $CdCl^+$ et $CdHCO_3^+$ (Sposito, 1989).

Dans les sols calcaires, la faible solubilité du carbonate de cadmium ($CdCO_3$) limite fortement la concentration de l'ion libre (Cd^{2+}) en solution du sol (Figure I-5). De plus, le pH du sol influence fortement la biodisponibilité du métal où les faibles valeurs de pH favorisent l'accumulation de Cd dans les tissus végétaux (Tsadilas et al., 2005 ; Yanai et al., 2006 ; Kirkham, 2003; Figure I-5). Dans ces conditions physicochimiques, les protons H^+ se trouvent en concentration élevée dans la solution du sol et sont retenus par les colloïdes à la place des ions Cd^{2+} ce qui enrichi le sol en Cd libre accessible pour l'absorption racinaire. Le pH du sol ainsi que sa teneur en eau sont liés indirectement par la température qui stimule la mobilité des métaux en déplaçant les équilibres des réactions de dissolution-précipitation et co-précipitation. Ainsi, l'augmentation de la température favorise le prélèvement du Cd par les

plantes (Deneux-Mustin et al., 2003).

La biodisponibilité du Cd dépend aussi de la richesse du sol en matière organique. Des études antérieures ont montré que la rétention du Cd par le sol était 30 fois plus élevée dans un sol riche en matière organique que dans un sol minéral (Sauvé et al., 2003). Les ions Cd^{2+} sont plus facilement retenus par les hydroxydes de fer et par la matière organique que par les argiles. Un sol riche en colloïdes organiques comme les acides humiques diminue la biodisponibilité du Cd tel est le cas de certaines cultures de maïs (Tyler and McBride, 1982), du tomate (Wolterbeek et al., 1988) et de la betterave à sucre (Greger et al., 1991).

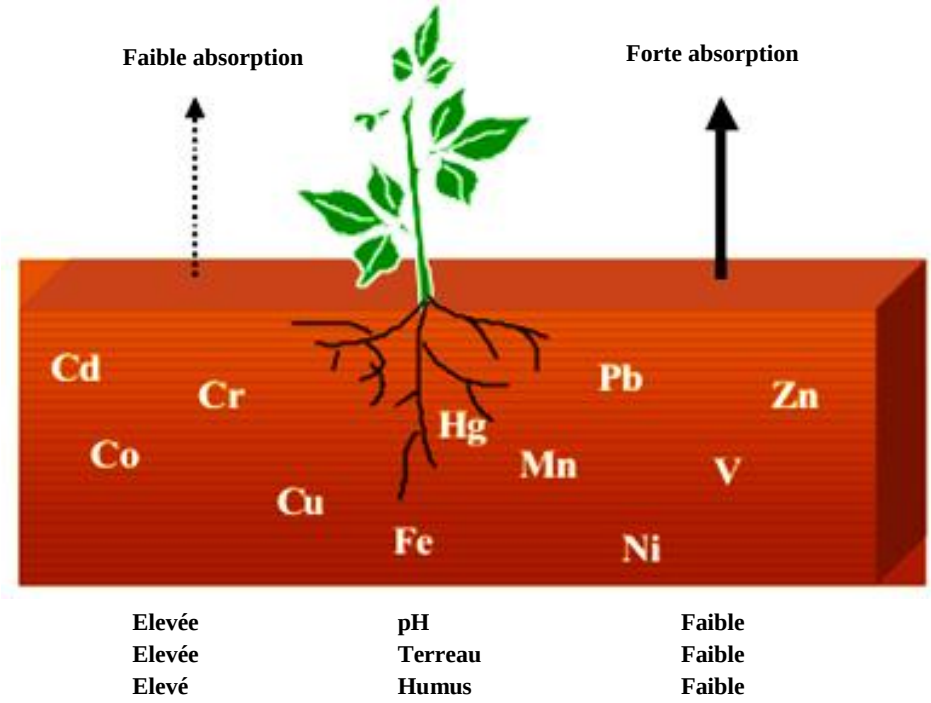

Figure I-5. Effet des propriétés du sol dans la biodisponibilité externe des métaux

De plus, les continuelles compétitions ou synergies entre les ions Cd^{2+} et les éléments chimiques du sol pour les mêmes transporteurs transmembranaires conditionnent la mobilité et la biodisponibilité du métal. Une augmentation en ions potassium (K^{+}; Clemens, 2006), calcium (Ca^{2+}), Zn^{2+} (Sarwar et al., 2010), manganèse (Mn^{2+}; Zornoza et al., 2010), Cu^{2+} (Benavides et al., 2005) diminue l'influx de Cd^{2+} suggérant une compétition au niveau de la surface racinaire. Il est également connu que l'enrichissement du sol en chlorure de sodium (NaCl) augmente la biodisponibilité des métaux lourds (Ghnaya et al., 2007) et stimule le prélèvement de Cd par les plantes (Xu et al., 2010).

III.1.1.3. Mécanismes d'absorption du Cd par la plante

Un ensemble de processus successifs contribue au transfert du Cd de la phase solide à la solution du sol puis aux tissus de la plante traduisant la « phytodisponibilité » de l'élément métallique. Le Cd dissous dans la solution du sol, qualifiée d'« offre » du sol, coexiste sous forme d'ions chimiques Cd^{2+} ou de complexes inorganiques $CdCl^{+}$ directement assimilable par les racines bien que ces complexes ne passent généralement pas la membrane cytoplasmique des cellules racinaires. Cette membrane plasmique sépare l'intérieur de la cellule (cytoplasme, vacuoles et noyau) du milieu extérieur. Elle présente un potentiel électronégatif, attribué à une ATP-ase excrétant les protons. Elle permet la diffusion des cations en solution vers la surface racinaire à la même vitesse que celle de l'eau prélevée par la plante du fait de sa transpiration et éventuellement de l'absorption active de l'eau. Les ions Cd^{2+}, facilement entrés au travers de l'apoplasme et du cortex de la membrane racinaire, sont bloqués dans la première barrière cellulaire pour la diffusion apoplastique: l'endoderme. Ils doivent alors passer dans le symplasme racinaire avant qu'ils ne puissent entrer dans le xylème.

III.1.2. Devenir du Cd dans la plante

III.1.2.1. Biodisponibilité interne du Cd

Une fois dans l'apoplaste, une partie des ions Cd^{2+} est véhiculée à l'intérieur des cellules et une autre partie se fixe aux composés des parois cellulaires et ne pénètre pas à l'intérieur des cellules. Ils circulent dans la racine par voie symplasmique et par voie apoplasmique (Figure I-6). Le Cd peut traverser la paroi par une simple absorption passive avec ou sans dépense d'énergie le long d'un gradient électrochimique des ions ou à travers les pompes à protons (exemple : pompe H^{+}). Cependant, dans le cas général, le transport des ions Cd^{2+} se fait par une absorption active impliquant des transporteurs d'autres ions divalents ayant une faible spécificité. Les transporteurs capables de véhiculer les ions indésirables seraient en particulier les transporteurs du fer ferreux IRT1 (Iron Regulated Transporter), et du Zn tel que les transporteurs de la famille ubiquitaire ZIP (Zinc Regulated Transporter/IRT Related proteins; Clemens, 2006).

Une autre famille de transporteurs de métaux permettant potentiellement l'entrée du cadmium est constituée par la famille Nramp (protéines de macrophages associées à la résistance

naturelle ; Thomine et al., 2000).

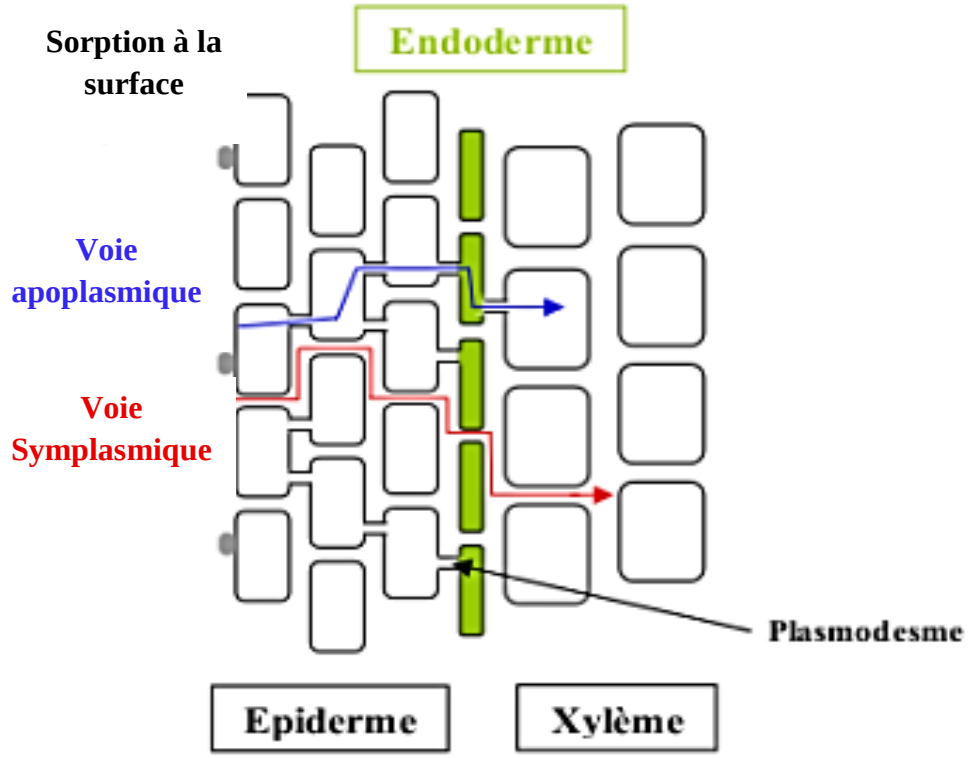

Figure I-6. Principe de l'absorption d'éléments par la plante (d'après Affholder, 2010)

III.1.2.2. Phytotoxicité du Cd

Des symptômes visibles peuvent refléter l'intoxication de la plante par le Cd. Le métal induit une inhibition de la croissance de différentes parties des plantes (Chaoui et al., 2004) accompagnée de changements anatomiques, structuraux et ultra-structuraux importants au niveau des feuilles (Sandalio et al., 2001) mais également des racines (Cosio et al., 2006). Ces changements peuvent être causés par une inhibition de la division cellulaire et de la synthèse des polysaccharides pariétaux (Punz and Sieghardt, 1993). Il a été montré que le métal se concentre plus au niveau racinaire là où les dommages génotoxiques les plus sévères ont été détectés (Gichner et al., 2004). Un retard dans le développement des jeunes pousses et une diminution de la teneur en chlorophylle causée par le Cd, peuvent se produire au niveau des feuilles. La présence de Cd dans le milieu de culture conduit à la diminution de l'absorption du fer et à la modification du rapport fer/zinc dans la feuille ce qui conduit à l'apparition du phénomène de chlorose (Cosio et al., 2006). Notons que les fruits peuvent être quasiment épargnés par la contamination (Wu and Zhang, 2002 ; Wu et al., 2004).

III.2. Cas du Cuivre

III.2.1. Le Cu dans le sol

III.2.1.1. Formes du Cu

Le cuivre, métal de transition, est un micronutriment essentiel pour la majorité des espèces des trois domaines de la vie. Avant environ 2,7 milliards d'années et lorsque les niveaux d'oxygène dans l'atmosphère étaient encore extrêmement faibles, le Cu a principalement existé sous forme de sulfures insolubles (la chalcopyrite $CuFeS_2$ et la chalcocite Cu_2S ; Kimball et al. 2009). La possibilité de son utilisation comme nutriment était alors limitée. En revanche, avec l'apparition de la photosynthèse et après l'oxygénation de l'atmosphère , le métal devenait de plus en plus biodisponible (cuivrique, Cu^{2+}; Brown et al. 1998). Les concentrations de Cu dans les sols se situent habituellement entre 3 et 100 mg/kg, mais seulement environ 1 à 20 % de Cu sont biodisponibles (Salam and Helmke, 1998). La majorité est sous forme de sels tels que $CuCl_2$, $CuSO_4$ et $Cu_3(PO_4)_2$ (Sauvé et al., 1996).

En plus des rejets industriels et urbains, d'importantes quantités d'engrais et d'amendements minéraux ou organiques appliqués chaque année sur les sols afin d'accroître leur productivité, peuvent potentiellement modifier les charges et la distribution de ce métal dans les différentes fractions des sols. Le Cu accumulé peut affecter à long terme les caractéristiques physico-chimiques des sols récepteurs et perturber les équilibres sol-solution. Les teneurs en Cu enregistrées dans les sols tunisiens au Nord du pays varient de 14,05 à 28,1 ppm. Ces sols ne présentent pas une concentration anormale en cet élément à l'inverse de zones autours des terrils de phosphogypse au centre et au sud du pays où les teneurs enregistrées dépassent la teneur maximale tolérée dans le sol fixé à 100 ppm. A titre de comparaison, les zones de vignes et d'arboriculture (actuelles ou anciennes) en France sont presque toujours contaminées par du cuivre, à des degrés divers, parfois très fortement. En effet, des traitements répétés à la "bouillie bordelaise" ont été réalisés pour lutter contre le mildiou, depuis parfois un siècle, à des doses d'apports variant entre 3 et 30 kg de cuivre par ha et par an, selon le lieu et l'époque. Les sols de vigne ainsi contaminés par le cuivre présentent aujourd'hui des teneurs totales comprises entre 50 et 600 mg/kg. De telles contaminations n'affectent que l'horizon de surface mais les plus fortes d'entre elles peuvent rendre impossible la replantation de jeunes vignes ou une culture céréalière après arrachage.

III.2.1.2. Facteurs influençant la biodisponibilité environnementale du Cu

Selon Chen et al. (2006), la capacité de fixation du Cu varie d'un sol à l'autre. Elle est affectée par le niveau de matière organique des sols, par la fraction des oxydes libres d'aluminium (Al), Fe, et Mn et par la teneur en argile des sols. Le Cu s'accumule principalement dans la fraction organique, la fraction des oxydes libres et dans la fraction labile sous forme divalente hydratée, $Cu(H_2O)_n^{2+}$. La prise en charge de la mobilité des formes cuivriques par le sol implique un faible niveau de saturation des sites de fixation. Lorsque les sites de fixation deviennent saturés par le Cu, on observe une baisse du niveau d'énergie de rétention du métal provoquant un accroissement de la fraction mobile et une mise en disponibilité pouvant conduire à la phytotoxicité. A des pH<5, l'activité de l'ion libre $Cu(H_2O)_n^{2+}$ s'accroît et le cuivre devient plus mobile et plus soluble (Figure I-6). Le métal entre en compétition pour son absorption par les racines avec des éléments chimiques comme le Ca^{+}, NH^{4+}, K^{+} et surtout Zn^{2+}. Toutefois, l'absorption du Cu augmente quand les teneurs en Fe dans des sols sont pauvres, cette augmentation étant attribuée à l'augmentation des phytosidérophores dans les exsudats (Komarek et al., 2010).

III.2.1.3. Mécanismes d'absorption du Cu par la plante

La fraction mobile ou aisément mobilisable du Cu est souvent très faible, bien inférieure à celle du Cd (Khachatryan et al., 2010). Etant un oligo-élément, le Cu avec un niveau de base de tolérance, franchit la paroi des racines par un transport actif exercé par la plante et favorisé par les exsudats racinaires (Quartacci et al., 2009).

III.2.2. Devenir du Cu dans la plante

III.2.2.1. Biodisponibilité interne du Cu

Une fois la barrière de la paroi racinaire passée, le Cu entre dans la cellule via un transporteur nommé Ctr-related copper transporter (COPT1). Au niveau intracellulaire, le Cu, en tant qu'oligo-élément, est rattrapé spécialement par des chaperonnes qui le distribuent aux protéines comme cofacteur selon les besoins des Cuproprotéines. Il est également complexé à des acides aminés, dont un des rôles est de limiter la présence d'ions libres hydratés particulièrement toxiques dans le cytosol. Le Cu peut aussi être complexé à des molécules organiques, dont la nicotianamine, afin d'être exporté via le xylème dans les tissus foliaires. La nicotianamine, pourvu de six groupements fonctionnels, permet la chélation du cuivre sous

forme octaédrique stable même dans des environnements acides dont le pH est de l'ordre de 3 (Mijovilovich et al., 2009; Trampczynska et al., 2010).

III.2.2.2. Phytotoxicité du Cu

La quantité de Cu dans les tissus des plantes vertes se situent habituellement autour de 2 à 50 mg/kg de poids sec (ppm) et ces teneurs sont largement dépendantes de l'espèce (Burkhead et al., 2009). Un excès de Cu dans le milieu environnant peut induire une rhizotoxicité visible au travers d'un épaississement et d'une coloration brune des racines, d'une diminution de la ramification et du nombre de poils racinaires et de l'élongation racinaire globale (Kopittke and Menzies, 2006). Des lésions de l'épiderme sont également observées ainsi qu'un épaississement des apex et des dommages occasionnés sur les méristèmes. D'autre part, la rhizotoxicité du Cu pourrait altérer la nutrition globale de la plante en réduisant le prélèvement d'éléments nutritifs à travers une diminution de la croissance racinaire. De plus, sa phytotoxicité est également connue pour induire dans certains cas une déficience en Fe et des symptômes de chlorose ferrique (Mengel and Kirkby, 2001). Il a été montré que les teneurs en Fe des parties aériennes diminuaient avec l'augmentation de la concentration en Cu en solution (Mengel and Kirkby, 2001) et dans le sol (McBride, 2003) chez des céréales comme le maïs et le blé, simultanément à l'apparition de symptômes de chlorose ferrique. L'excès de Cu peut induire la formation de radicaux libres et provoquer un stress oxydatif (Yruela, 2005). Ceci peut agir sur la peroxydation des lipides et entraîner l'altération des membranes plasmiques. Ce stress oxydatif peut aussi détériorer la structure et la composition des thylakoïdes et des chloroplastes, et affecter la photosynthèse (Quartacci et al., 2001; Yruela, 2005). Le contenu en chlorophylle semble aussi diminué par l'augmentation des teneurs en Cu (Ciscato et al., 1997).

III.3. Cas du Chrome

III.3.1. Le Cr dans le sol

III.3.1.1. Formes du Cr

Découvert à la fin du XVII^{ème} siècle, le chrome (Cr) pourvu de ces couleurs éclatantes, de la dureté et de la résistance à la corrosion, a trouvé un grand nombre d'applications industrielles. Dans le monde, la France a été l'un des principaux producteurs et consommateurs. Le Chrome a été extrait notamment des mines de Tiebaghi et Alice-Couse (Nouvelle Calédonie) qui

produisirent jusqu'à 60.10^3 tonnes de Cr. Ce métal est utilisé dans les industries chimiques, en tannerie, dans la peinture et la teinture, en céramique, en verrerie et en photographie, dans le traitement du bois ainsi que dans les industries papetières et surtout métallurgiques pour la production d'alliages de fer-chrome comme l'acier inoxydable, l'acier rapide, les alliages de fonte et les alliages non ferreux (Gode and Pehlivan, 2005). Compte tenu de ses utilités dans les différents procédés industriels, il n'est pas surprenant que de grandes quantités de déchets chromés aient été produits et rejetés dans l'environnement. On estime à plus de 900 tonnes par an la quantité de Cr rejeté directement dans le sol. Les eaux de surface récupèreraient environ 140 tonnes/an et 30 tonnes rejoignent l'atmosphère chaque année. La France a une place importante dans les émissions de chrome à l'échelle de l'Union Européenne. Les émissions vers l'eau et l'air représentent respectivement 66 % et 12 % du total des émissions déclarées dans l'UE (27) pour l'année 2012 (INERIS, 2015).

Dans la nature, le Cr élémentaire ne se trouve pas à l'état libre (Shupack, 1991). Il est plutôt complexé avec l'oxygène, le Fe ou le Pb, formant des oxydes comme la chromite ($FeCr_2O_4$) et la crocitite ($PbCrO_4$). Les principales mines de chromite se trouvent en Afrique du Sud et produisent jusqu'à 85 % des ressources mondiales estimées à 7600 millions de tonnes en 2010 (Delachaux, 2010). Toutefois, bien qu'il peut prendre neuf état d'oxydation différents, seuls deux valents sont connus pour le Cr ; le chrome trivalent Cr(III) et le chrome héxavalent Cr(VI). Presque tout le Cr trouvé dans les roches formant la croûte terrestre est trivalent, la forme la plus stable, peu mobile et peu disponible. La quasi-totalité (97-99 %) du Cr(III) introduit au sein de la suspension sol/solution est adsorbée après 2h de mélange avec de nombreux constituants des sols: smectites, goethite, Si-colloïdes… . Le chromate ou Cr(VI) est connus pour être plus mobile dans le sol dans certaines conditions.

III.3.1.2. Facteurs influençant la biodisponibilité environnementale du Cr

L'adsorption du Cr au sein des sols est fonction du type de phases porteuses qui contrôlenten partie sa mobilité. Il est faiblement adsorbé à la surface des phases secondaires formant des complexes très labiles. L'adsorption de Cr(III) et de Cr(VI) sur les oxyhydroxydes de Fe est maximale à pH=4 et à pH=5 respectivement. Contrairement au Cr(III) qui devient plus stable par son association aux oxydes et donc non disponible dans les environnements de surface, le Cr(VI) adsorbé est facilement déplacé par des anions comme les phosphates et les sulfates et par conséquent est considéré comme disponible au sein des sols (Becquer et al., 2003; Oze et

al., 2004). Les réactions d'oxydoréduction ont une grande importance dans la dynamique du Cr. Les oxydes de manganèse (MnO_2) sont les seules espèces assez puissantes pour oxyder le Cr(III) en Cr(VI) (Oze et al., 2007). La vitesse d'oxydation est accélérée en présence de bactéries productrices de Mn(II) et en absence de substances organiques qui se complexent rapidement avec le Cr(III) et le rendent moins disponible. Le Cr(VI) peut être réduit en présence de nombreux constituants du sol: matière organique notamment les acides fulviques, les sulfures et les minéraux contenant du Fe(II) (Mullet et al., 2007), mais aussi par l'activité bactérienne (Cummings et al., 2007) ce qui conduit à une baisse de la disponibilité du Cr toxique. Le pH est le facteur le plus important, la réduction étant plus efficace à pH acide, puisque la disponibilité en Fe(II) est plus importante (Zayed and Terry, 2003). De plus, la texture des sols qui contrôle la diffusion d'oxygène, joue aussi un rôle important pour la réduction du Cr(VI). Selon Tokunaga et al., (2001), la faible diffusion de l'O_2 combinée à la respiration microbienne peut engendrer une élévation de la concentration en Fe(II) aqueux et ainsi la réduction du Cr(VI).

III.3.1.3. Mécanismes d'absorption du Cr par la plante

La forme oxydé du Cr, Cr(VI) est toxique puisqu'elle est capable de pénétrer dans les cellules de l'organisme. L'absorption du métal ou le franchissement de la membrane plasmique des cellules racinaires des plantes est limité par le pouvoir tampon du sol pour le Cr ainsi que par le processus de transfert du métal jusqu'à la surface des racines (Brun et al., 2001). Selon Cervantes et al., (2001), l'absorption du Cr(VI) par les racines se fait par transport actif. Il implique le co-transport d'anions comme des sulfates, alors que l'absorption du Cr(III) est probablement un transport passif de Cr(III) associé à des composés organiques (Srivastava et al., 1999; Figure I-7).

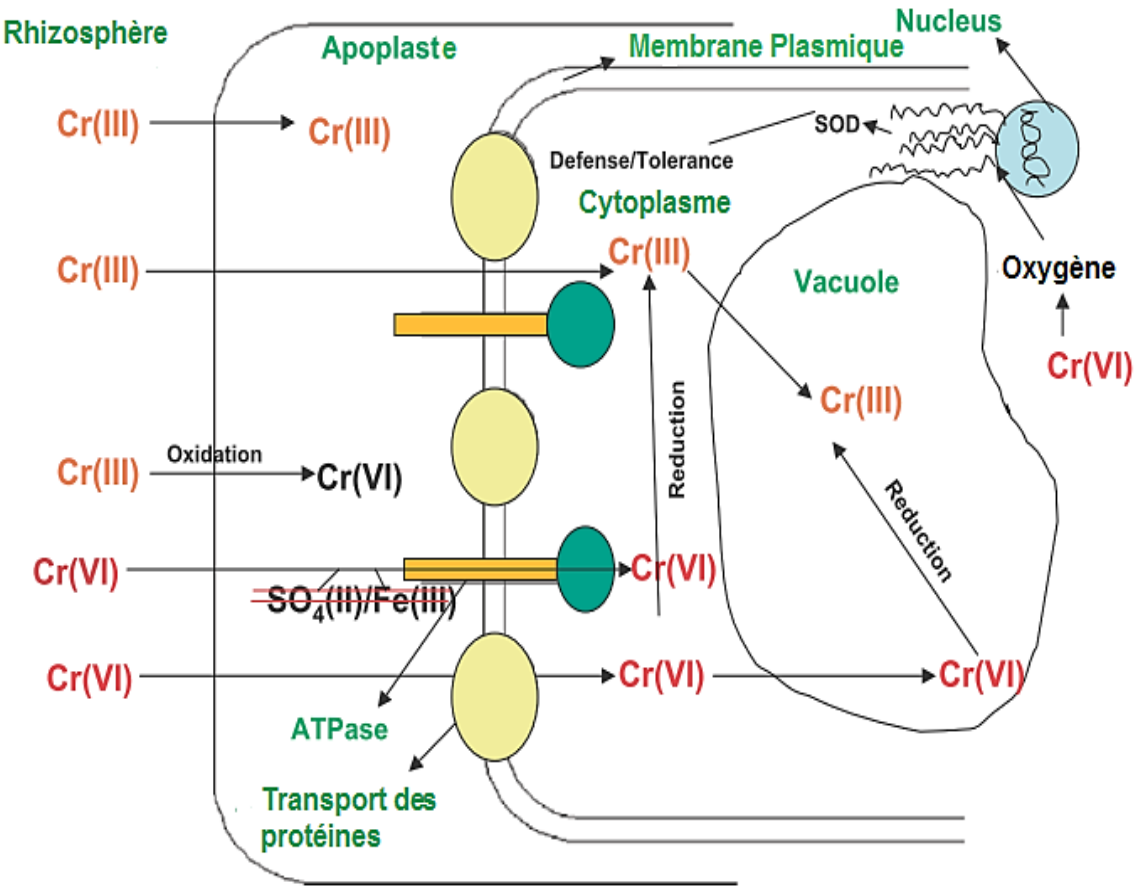

Figure I-7. Modèle hypothétique du transport et de la toxicité du Cr dans les racines des plantes (d'après Ganier, 2009)

III.3.2. Devenir du Cr dans la plante

III.3.2.1. Biodisponibilité interne du Cr

La canne à sucre et divers autres produits agricoles ont été étudiés pour leurs capacités d'absorber le chrome (Camargo et al., 2004). La majeure partie du Cr absorbé (> 99 %) reste dans les racines au niveau des vacuoles où il est rapidement réduit (Zayed et Terry, 2003), moins de 1 % est transporté vers la partie aérienne. L'accumulation du Cr au sein des vacuoles des cellules racinaires pourrait expliquer son immobilisation dans les racines et permettrait de diminuer sa toxicité (Shanker et al., 2005). Etant donnée cette forte immobilisation dans les racines, une réduction rapide du Cr(VI) au sein des cellules est associée à la production de radicaux libres et le stockage du Cr(III) au sein des vacuoles (Shanker et al., 2005 ; Figure I-7). Toutefois, d'après Cary et al. (1977), il y aurait une meilleure translocation du Cr vers la tige à partir d'une solution du Cr (VI) qu'à partir d'une solution de Cr (III). Ainsi, la notion de tolérance de certaines espèces semble plus en

adéquation.

III.3.2.2. Phytotoxicité du Cr

Le Cr et plus particulièrement le Cr(VI) est un élément très toxique aussi bien pour la faune que pour la flore. Contrairement à la forme trivalente du Cr, à ce jour, on ne connait aucun rôle biologique de la forme hexavalente chez les plantes (Bhadra and Mahananda, 2013). Il est toxique pour la majorité des plantes supérieures à partir de 100 µM/kg de masse sèche (MS) (Davies et al., 2002). Il modifie la capacité germinative des graines et engendre une diminution de croissance et de développement des végétaux (Peralta et al., 2001). Le développement racinaire s'avère plus profond dans le cas des sols dont la teneur en Cr est plus faible, suggérant une forte phytotoxicité du Cr (Banks et al., 2006).

Toutefois, Banks et al. (2006) suggèrent que les plantes prélèvent uniquement 1 % du Cr disponible au sein des sols. Pour résister à des teneurs en Cr plus élevées, certaines plantes ont développé au cours de l'évolution, des formes de résistance spécifiques leurs permettant de se développer sans problème sur des sites fortement pollués en Cr (Brady et al., 2005; Reeves et al., 2007). Les recherches traitant des interactions entre le Cr et les plantes sont relativement limitées et très peu d'espèces hyperaccumulatrices de Cr sont connues dans la littérature tels que *Dicoma nicolifera* et *Sutera fodina*.

IV. Evaluation écotoxicologique à l'aide de marqueurs biologiques : vers la caractérisation de l'état du milieu

La prise de conscience de l'opinion scientifique puis de l'opinion publique des perturbations de l'environnement a entrainé le développement des disciplines visant à déterminer l'état de la qualité de l'environnement. L'écotoxicologie est une discipline concernée par l'étude des effets toxiques des polluants sur les constituants des écosystèmes. Sa première préoccupation concerne les moyens *in* et *ex-situ* nécessaires pour l'évaluation de la toxicité potentielle de produit(s) chimique(s). Elle recherche également des indicateurs de nature biologique et/ou chimique capables de mesurer les troubles écologiques pour les utiliser dans la caractérisation de la qualité de l'environnement afin de déterminer une éventuelle perturbation et si tel est le cas d'évaluer le niveau de dégradation du milieu qui définissent le concept d'évaluation du risque. Aussi, l'écotoxicologie intervient 1- l'évaluation du succès de la remédiation et du réaménagement des milieux naturels ; 2-les transferts des contaminants vers les communautés, les populations et les organismes peuplant les sols ; 3-les effets sur les

organismes rendant des services écosystémiques et par extension sur la biodiversité des sols, impose de disposer d'outils de mesure de l'état biologique du sol ou de l'état des organismes peuplant ce sol (Blanchart, 2012).

Différentes méthodologies ont été développées afin d'évaluer la qualité de l'environnement. Toutefois, aucune méthode appliquée séparément ne peut apporter des informations suffisantes pour un bon diagnostic de l'état du milieu. L'intégration des approches chimiques pour détecter la présence de polluants et biologiques pour évaluer ses effets dans l'environnement permet de comprendre les mécanismes d'altération et d'évaluer les impacts biologiques en milieux contaminés. Seul l'outil le plus intégrateur des conditions environnementales permet d'aboutir à des résultats pertinents sur l'état de contamination chimique du milieu environnant. Malgré la complexité du monde vivant qui met en œuvre des concepts complexes de l'approche biologique, un certain nombre d'outils ou d'indicateurs ou encore, de bioindicateurs développés depuis des années sont maintenant disponibles.

IV.1. Bioindicateurs : principes et concepts

La notion de bioindicateur désigne tout organisme ou ensemble d'organismes apte à caractériser, de façon intégrée dans le temps, l'ambiance écologique et d'une manière pratique, l'état d'un écosystème et de mettre en évidence aussi précocement que possible leurs modifications, naturelles ou provoquées en se basant sur des variables biochimiques, cytologiques, physiologiques, éthologiques ou écologiques (Li and Schmidt, 2010). Généralement, tous les organismes révèlent des informations sur leur environnement, mais certains sont plus utiles pour la surveillance de la qualité des milieux que d'autres. Le bioindicateur approprié doit idéalement présenter des qualités de mesure (précision, fiabilité, robustesse), être cosmopolite et abondant dans le milieu et possède une application géographique et temporelle large des techniques de bio-indication corrélées à des fonctions de l'écosystème. Les producteurs primaires au plus proche des transferts de nutriments et d'énergie, faciles à les identifier et ayant un cycle de vie simple et court, caractérisent un bioindicateur (Figure I-8).

Toutefois, lorsque les effets observés concernent une réaction au niveau individuel, le bioindicateur sera définit comme « un simple relais ne faisant référence qu'à des effets observables au niveau de l'individu se traduisant par des altérations morphologiques, tissulaires ou physiologiques (croissance et reproduction) » (Garrec and Van Haluwyn, 2002).

Dans ce cas-là, on parlera de bioindicateurs qui intègrent des propriétés ou des processus physiques, chimiques et biologiques du sol et capable de rendre compte notamment des méthodes de gestion des sols et/ou des différents types de pollution pédologique.

Ces indicateurs sont adaptés pour déceler les effets précoces d'une contamination à travers des perturbations telles que les modifications cellulaires ou métaboliques sans pour autant présumer de changements à long terme des écosystèmes.

IV.2. Types des Bioindicateurs

Parmi les bioindicateurs, deux catégories sont distinguées (Figure I-8).

IV.2.1. Bioindicateur d'accumulation

Se définit comme tout organisme (ou partie d'un organisme ou communauté d'organismes) qui informe des concentrations en polluants des sols (Figure I-7). Grâce à leur capacité de bioaccumulation, certains bioindicateurs permettent une détection des polluants ou des perturbations environnementales (Kaiser, 2001). On parle d'espèce sentinelle si les organismes bioindicateurs sont déjà présents dans l'écosystème (surveillance passive) et d'espèce test si l'exposition se fait en laboratoire sur du sol collecté ou lors d'expériences de « caging ».

De nombreux travaux reflètent l'imprégnation d'organismes bioaccumulateurs dans l'environnement. A titre d'exemple, Rzepka and Cuny (2008) ont réalisé une revue des principales méthodologies de biosurveillance (végétales et fongiques) utilisées dans le but de surveiller les variations de concentrations des ETMs dans l'environnement. La quantification et le suivi des concentrations en polluants métalliques imposent une méthodologie adaptée (Coyne and Allen Orr, 2004). Les concentrations métalliques sont analysées par spectrophotométrie d'absorption atomique. Les teneurs métalliques dans les fractions dissoutes qui inclut des fractions ultra-fines (<0,2µm) et particulaires sont mesurées par spectrométrie d'émission atomique par plasma à couplage inductif (ICP-AES) ou par spectrométrie de masse à plasma couplé (ICP-MS). Des études ultérieures de Cuny et al. (2009) ont étudié l'accumulation de poussières à proximité de différentes sources dans l'agglomération dunkerquoise. L'observation des dépôts au microscope électronique à balayage couplé à la diffraction X a permis d'analyser la nature des poussières mais aussi de mettre en évidence leur grande diversité granulométrique.

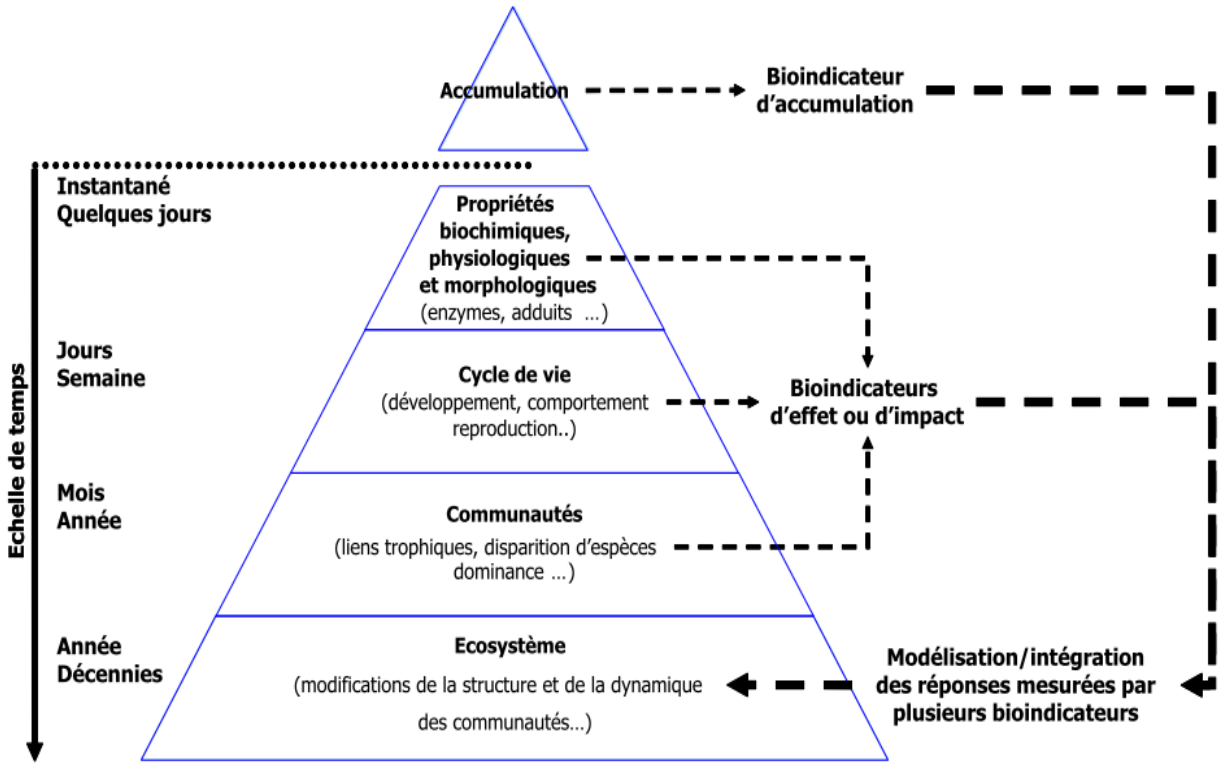

Figure I-8. Les différents niveaux de réponse des bioindicateurs (d'après Blanchart, 2012)

IV.2.2. Bioindicateurs d'exposition

Se définit comme tout organisme (ou partie d'un organisme ou communauté d'organismes) qui permet de révéler des effets spécifiques ou non lors de l'exposition à une ou plusieurs substances issues de son environnement ou à d'autres stress anthropiques ou naturels (ex: métaux, tassement, changement d'usage, statut de la matière organique). Ces effets, proportionnels ou non à l'exposition, incluent des changements environnementaux, et fournit également des informations au sujet de l'intensité d'exposition à certaines substances par des réponses "mesurables", physiologiques, biochimiques ou morphologiques (Figure I-8). Les bioindicateurs d'effets sont des effets visibles des polluants sur des espèces identifiées comme bioindicatrices. Ces effets ou encore « indices » indispensables pour caractériser certaines fonctions du sol sont très divers, par exemple : l'indice Oméga 3 chez les végétaux, des perturbations de la floraison et de la croissance, des déformations foliaires, des nécroses, des chloroses…. Par exemple, les symptômes habituellement rencontrés lors d'une carence en Cu sont une croissance réduite, une déformation ou enroulement des jeunes feuilles, une chlorose, une nécrose apicale, la fanaison des jeunes pousses et une chute des jeunes feuilles rencontrées chez les citronniers et les orangers (Hopkins, 2003). A l'échelle macroscopique, le Pb appliqué sur des plantes induit une réduction de la croissance, de la biomasse produite,

des rendements et, lorsque le stress est trop sévère, conduit à l'apparition de nécroses racinaires et foliaires, de chloroses, voire à la mort de la plante (Seregin and Ivanov, 1998; Sharma and Dubey, 2005).

IV.3. Limites des Bioindicateurs

Les analyses physico-chimiques donnent des indications sur la présence ou l'absence d'un polluant et appréhendent le devenir d'une telle molécule mais ne renseignent pas sur l'impact réel du polluant sur sa cible finale, en l'occurence le vivant. Le recours à des observations macro et microscopiques et à des niveaux distincts de la bioindication présente plusieurs avantages. Pour avoir une représentativité élevée, les conditions expérimentales doivent se rapprocher des conditions physico-chimiques et biotiques propres aux écosystèmes naturels. Cependant, elles restent insuffisantes pour évaluer la santé d'un milieu complexe, avec des pollutions toujours multiples sujettes à des phénomènes de synergie et d'antagonisme. Les bioindicateurs renseignent davantage sur la biodisponibilité des polluants plutôt que sur leur concentration totale dans le milieu (Tusseau-Vuillemin et al., 2007). Par ailleurs, par l'intervention des facteurs relatifs aux modalités et aux mécanismes de la contamination, certaines familles de toxiques échappent à la bioindication, soit parce qu'il s'agit de molécules totalement nouvelles (émergentes notamment), soit parce que le devenir de tels polluants dans le milieu est mal connu (dans ce cas, le problème est d'identifier le ou les produits de dégradation à analyser). Le choix des stratégies d'échantillonnage, les difficultés analytiques et la sensibilité des méthodes sont les principales limites à l'utilisation des bioindicateurs d'autant que ceux-ci ne sont mesurables que si l'intoxication des organismes ou plantes-test atteint une valeur ou une durée suffisante. Dans cette perspective, des « biomarqueurs » pourraient être utilisés en tant que signal d'alarme vis-à-vis d'un stress général ou particulier.

V. Méthodologies de biosurveillance

Le Conseil National de la Recherche américain a défini en 1983 l'évaluation des risques comme étant une estimation quantitative du niveau de risque, pour l'environnement et la santé humaine, associés à une exposition à des substances dangereuses. Du fait de l'incertitude scientifique liée aux difficultés d'observation de tels phénomènes et de la difficulté d'interpréter les données recueillies lors des études de la bioinduction et de la proposition des hypothèses explicatives, l'évaluation des risques s'est notamment orientée vers l'évaluation

des risques écotoxicologiques. Elle a développé la notion de biosurveillance (biomonitoring) qui peut être définie comme étant « l'utilisation à tous les niveaux d'organisation biologique d'un organisme ou d'un ensemble d'organismes pour prévoir et/ou révéler une altération de l'environnement et pour en suivre l'évolution » (Garrec and Van Haluwyn, 2002). Elle apporte des informations sur le devenir et la toxicité de ces substances dans des systèmes biologiques. Pour diagnostiquer une situation, la biosurveillance opère selon deux approches :

- Approche passive qui consiste en l'analyse d'organismes en place sur un site par rapport aux relevés de pollution de ce site et des paramètres stationnels et environnants, tout en prenant considération les variabilités génétiques du végétal étudié.

- Approche active où des végétaux génétiquement sélectionnés et cultivés dans des conditions standardisées sont mis en place sur un site pendant une période donnée. Les études expérimentales effectuées en laboratoire en conditions contrôlées ont une réplicabilité élevée. Ainsi, les paramètres expérimentaux faciles à normaliser et le suivi de l'évolution des différents niveaux de l'état d'organisation du vivant permettent également de palier en partie certaines difficultés rencontrées dans les études *in situ* et donc de traduire la pollution du site étudié (Garrec and Van Haluwyn, 2002).

Parmi les divers organismes vivants pouvant être utilisés en biosurveillance, les végétaux sont souvent privilégiés puisqu'ils répondent bien aux stress causés par la pollution. La mesure d'un stress définit le « biomarqueur ».

V.1. Biomarqueurs : principes et concepts

L'utilisation des biomarqueurs représente une méthodologie qui s'est initialement développée à des fins de biomonitoring. Ils mobilisèrent d'intenses efforts de la part d'une large communauté scientifique pendant les dernières décennies. Les nombreux travaux scientifiques autour de la thématique des biomarqueurs et la première définition d'un biomarqueur apparait dans les années quatre-vingts : un biomarqueur est la réponse d'une structure ou d'une fonction biochimique ou physiologique « normale » à la présence d'un xénobiotique. Amiard et al. (1998) définit un biomarqueur comme un changement observable et/ou mesurable au niveau moléculaire, biochimique, cellulaire, physiologique ou comportemental qui révèle l'exposition présente ou passée d'un individu à au moins une substance à caractère polluant. Les biomarqueurs utilisés en biosurveillance doivent avant tout être replacés dans un contexte physiologique et fonctionnel dans l'organisme. Avant d'être des biomarqueurs, ce sont en

effet des mécanismes physiologiques ou leur réponse. Ils représentent des variables biologiques permettant à la fois de caractériser l'état physiologique des individus dans un environnement donné et de détecter, de façon précoce, tout dérèglement pouvant avoir des répercussions à plus long terme sur les écosystèmes (populations et communautés). Ils sont mesurés sur des espèces capables d'accumuler les polluants ; bioindicateurs d'accumulation, et permettent de réaliser des études d'écotoxicité des substances chimiques (chemical testing) au laboratoire et de biomonitoring *in situ*. Le plan de gestion 2009-2013 de la réserve naturelle de l'Etang des Landes, dans son chapitre sur les objectifs liés aux acquisitions de connaissances, propose une étude portant sur la pertinence de mesures d'indicateurs biologiques ; biomarqueurs ou bioindicateurs comme des outils prometteurs pour des applications en écotoxicologie afin de développer les connaissances sur les méthodologies d'analyse des perturbations anthropiques des écosystèmes (Figure I-9).

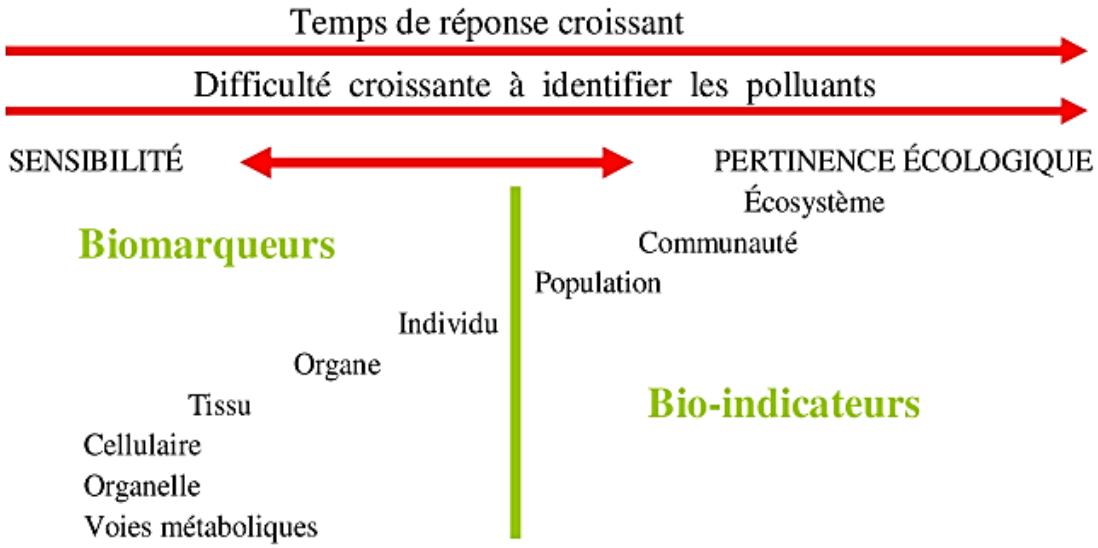

Figure I-9. Relation entre la sensibilité et la pertinence écologique des biomarqueurs choisis à différents niveaux de l'organisation biologique (d'après Guerlet, 2010)

En général un biomarqueur est dit pertinent s'il est capable de nous renseigner sur la biodisponibilité d'un polluant et s'il est capable de détecter des réponses biologiques précoces. Il représente un des moyens envisagés pour établir d'une manière fiable un diagnostic de risque environnemental. Par ailleurs, pour évaluer la robustesse et la performance d'un biomarqueur, Hamza-Chaffai, (2014) a établi six critères majeurs :

- la mesure d'un biomarqueur doit se faire sous assurance qualité au moyen de méthodes

sensibles, précises et simples de mise en œuvre ;

- la réponse d'un biomarqueur doit être rapide et sensible à l'exposition au contaminant et/ou à ses effets pour servir comme paramètre précoce d'alerte ;

- les valeurs de base du biomarqueur ; les niveaux constitutifs et les variations du biomarqueur, dues aux effets de facteurs physiologiques ou environnementaux, doivent être définis afin de distinguer la variabilité naturelle (bruit) et le stress induit par le contaminant (signal) ;

- les effets des facteurs confondants sur la réponse de ces biomarqueurs doivent être bien établis ;

- les mécanismes soulignant les relations entre réponse du biomarqueur et exposition aux contaminants (dose et temps) doivent être connus. De ce fait, la notion de biomarqueur est amenée à évoluer avec les connaissances scientifiques ;

- la pertinence écologique de la réponse du biomarqueur et la signification toxicologique du biomarqueur, c'est-à-dire les relations entre sa réponse et l'impact (à long terme) sur l'organisme, doit être établie.

L'intérêt de la mesure des biomarqueurs a toutefois été montré dans le cas d'études amont-aval ou de profil de biote. Ils présentent l'avantage d'une évaluation intégrée dans le temps, de l'exposition ou de l'effet des polluants environnementaux biodisponibles. Ils sont de plus susceptibles de refléter l'exposition des organismes, à un instant donné, des variations qualitatives et quantitatives de polluants rapidement métabolisables et peu cumulables. Ils présentent donc un caractère intégrateur important vis à vis des facteurs environnementaux, toxicologiques et écologiques. Cette indéniable avantage des biomarqueurs dans l'évaluation géographique et temporelle des effets associés à la contamination présente l'inconvénient de générer, à cause de la multitude des facteurs, un « bruit de fond » susceptible de masquer le signal d'une perturbation causée par des xénobiotiques. Ainsi, pour une application plus large des biomarqueurs dans un contexte de surveillance des écosystèmes, il est recommandé de toujours comparer les réponses entre sites de niveaux de contamination différents (emploi de sites de « relative référence »). Il est aussi nécessaire de déterminer des plages naturelles de variations des bioindicateurs qui seraient valables pour un ou plusieurs sols. Cependant, les efforts soutenus de standardisation, tant la définition de la gamme de réponses pouvant être

considérée comme « normale » pour un organisme s'avère difficile, voire impossible à établir (Brown et al., 2004a). L'utilisation d'organismes maintenus dans des conditions contrôlées peut également limiter certains des facteurs de confusion (Amiard et al., 1998).

V.2. Biomarqueurs et directive cadre pour la protection des sols

En 2006, la communauté Européenne (2004/35/EC, 2006) a adopté la directive-cadre de la protection des sols (Directive COM(2006)232). Cette proposition témoigne de la nécessité à la préservation de la qualité des sols au même titre que la protection des milieux aquatiques et aériens. Afin d'atteindre un bon état écologique des sols, il semble indispensable de positionner les biomarqueurs par rapport aux exigences de cette directive afin que ces outils puissent trouver une place légitime dans la procédure d'évaluation du pédosphère. Toutefois, les biomarqueurs doivent être considérés comme des outils complémentaires pouvant trouver une place légitime dans les différents types de programmes de contrôle de la qualité des sols permettant l'identification d'effets biologiques précoces ou de sources de contamination tout en précisant le lien entre exposition des organismes et effets sur les organismes. Une application aussi exhaustive des biomarqueurs aurait alors des implications économiques fortes en augmentant considérablement le coût des programmes de surveillance.

V.3. Catégories de biomarqueurs (Core biomarkers)

Selon Oost et al. (2003) les biomarqueurs peuvent être subdivisés en deux catégories différentes :

V.3.1. Biomarqueurs de défense

Ce sont des indicateurs liés à la capacité inhérente ou acquise d'un organisme à répondre au stress induit par l'exposition à un xénobiotique. Ils comportent les activités de détoxication de la pollution. Ces mécanismes ont potentiellement un impact positif sur les organismes, dans la mesure où ils vont leur permettre de survivre dans des zones contaminées.

V.3.1. Biomarqueurs de dommage

Appelés aussi « biomarqueurs d'effets », ce sont des révélations d'une atteinte plus au moins importante aux niveaux biochimiques, physiologiques, comportementaux ou autres au sein d'un organisme, qui en fonction de son intensité, peut l'affecter dans sa capacité de croitre, de se développer, de se reproduire, voire de survivre. L'utilisation des biomarqueurs d'effet permet de montrer que le xénobiotique est entré dans l'organisme et, qu'après avoir été

distribué entre différents tissus, a exercé un effet toxique sur une cible critique.

Une multitude de biomarqueurs des deux catégories entrent dans le concept des «core biomarkers», faisant l'objet à la fois d'une validation en laboratoire et sur le terrain (Amiard et al., 2008) et ils sont intéressants dans le cadre de la mise en place d'un biomonitoring du milieu naturel.

V.4. Cibles biologiques et biomarqueurs

Selon l'importance de la dose interne et/ou de l'exposition aux contaminants environnementaux ou aux facteurs de stress, le rôle fonctionnel des biomarqueurs intervient à différents niveaux d'organisation. Ils maintiennent l'homéostasie des ETMs essentiels (Co, Cu, Mn, Mo, Ni et Zn) dans les limites physiologiques internes des organismes, neutralisent les effets toxiques des polluants en cas de stress léger (compensation), témoignent des effets néfastes réversibles pour l'organisme à la suite d'une augmentation de la dose et/ou de la durée d'exposition (biomarqueurs de non-compensation) et enfin en cas d'insuffisance de l'action des mécanismes de compensation, les effets, irréversibles, pourront conduire à une issue fatale.

Les réponses adaptatives des mécanismes génétiques ou d'acclimatation (plasticité physiologique) des organismes aux stress environnementaux peuvent avoir lieu au niveau moléculaire et cellulaire de l'organisation biologique de l'organisme végétale.

V.4.1. Biomarqueurs moléculaires

Lors du contact entre un contaminant et un organisme, les premiers évènements mesurables ont souvent lieu au niveau moléculaire et impliquent des changements dans les profils d'expression génique. Dans ces effets précoces, on trouve les réponses compensatoires qui reflètent la réponse d'un organisme aux dommages moléculaires, présentent une capacité de prévention qui n'est pas retournée avec les autres méthodes de biosurveillance, qu'elles soient chimiques ou biologiques. Les outils de biologie moléculaire ont permis de mieux comprendre les changements qui ont lieu au niveau transcriptionel et donc d'acquérir un grand nombre de données génomiques et transcriptomiques très utiles. Comparée à l'approche enzymatique, l'étude des variations des niveaux d'expression génique permet de distinguer l'implication de chaque enzyme lors du stress oxydatif au niveau transcriptionnel. Plusieurs études transcriptomiques sur les variations d'expressions géniques ont permis d'identifier un certain nombre de gènes impliqués dans les mécanismes de tolérance et d'accumulation aux

ETMs pouvant être utilisées comme «biomarqueurs d'exposition» (Becher et al., 2004; Brulle et al., 2006, 2010; Verbruggen et al., 2009). En revanche, les gènes impliqués dans la tolérance et l'accumulation des ETMs ne sont pas des gènes spécifiques mais des gènes qui sont exprimés de façon ubiquitaire en conditions « normales » et qui sont régulés en présence d'un excès d'ETMs (Verbruggen et al., 2009). Il est peu probable que des gènes ayant une spécificité pour un métal non essentiel aient été sélectionnés au cours de l'évolution bien que selon les conditions expérimentales, une certaine spécificité puisse être observée (Brulle et al., 2010).

L'évolution des techniques de biologie moléculaire vers des méthodes plus sensibles, telles que la Puce à ADN, la PCR en Temps-Réel, le RNA-seq…, permet l'identification des changements des profils d'expression en ARNm en réponse à diverses conditions expérimentales et l'identification des gènes qui sont sur- ou sous-exprimés dans une cellule, un organe et/ou le tissu d'un organisme.

D'autre part, une analyse fine des gènes impliqués dans diverses fonctions physiologiques permet d'identifier des gènes candidats susceptibles d'être impliqués dans la réponse d'un organisme à un contaminant.

V.4.1.1. Identification et caractérisation de gènes de transporteurs de métaux lourds

a/ Absorption racinaire

Plusieurs transporteurs membranaires, impliqués dans l'entrée des métaux lourds dans les cellules de l'épiderme de la racine, ont été mis en évidence. Ils ne sont pas spécifiques des métaux lourds mais interviennent dans l'absorption des cations essentiels à la croissance et au développement de la plante. Une compétition s'installe entre les éléments minéraux et les ions métalliques présents dans le sol. Certains transporteurs voient leur expression modifiée, jouant par conséquent un rôle clé dans leur transport (Clemens, 2006; Hall, 2002).

Les transporteurs de type ZIP et IRT

Une des familles de gènes régulants l'entrée des métaux dans la cellule est la famille de transporteurs régulée par le Zn (ZIP : Zinc-Regulated Transporter) et la famille de transporteurs régulée par le Fe (IRT : Iron-Regulated Transporter ; (Krämer et al., 2007). L'expression des gènes correspondants dans les racines et dans les feuilles, produit des protéines intrinsèques des membranes (Assunção et al., 2001) suggérant leur implication à la

fois dans l'absorption racinaire et dans le transport des métaux dans la plante (López-Millán et al., 2004; Figure I-10).

Chez *A. thaliana*, il y a douze gènes codants pour des ZIP et trois pour des IRT (Hamza-Chaffai, 2014). En plus de leur implication dans le transport des cations Fe^{2+} et Zn^{2+}, ces gènes sont fortement exprimés en conditions de stress par le Cd et le Pb.

Les gènes orthologues de *IRT3*, *ZIP3*, *ZIP6*, *ZIP9* et *ZIP12* ont montré leur implication dans l'absorption du Zn/Cd chez *A. halleri* (Becher et al., 2004). De plus, Lombi et al. (2002) ont montré une surexpression d'*IRT1* chez *N. caerulescens* conduisant à une augmentation de teneur endogène en Cd. Des études ont évoqué le transport potentiel du Pb via des canaux calciques (Huang and Cunningham, 1996). Dans ce contexte, l'inhibition de l'absorption racinaire du Pb par le calcium est un phénomène connu depuis longtemps (Garland and Wilkins, 1981).

La famille des CNGC

La famille des « cyclic nucleotide-gated non selective cation channels » (CNGC) des végétaux représente des canaux à cations peu sélectifs impliqués dans le transport du calcium et du potassium (Spatz et al., 1999; Sunkar et al., 2000). Toutefois, les ADNc complets codants pour les transporteurs membranaires CNGC1 et NtCBP4 (calmodulin-binding protein) ont été isolés chez *Arabidopsis thaliana* et *Nicotiana tabacum* respectivement. La surexpression de *NtCBP4* dans des plantes transgéniques de tabac induit une augmentation de la quantité de Pb absorbée par les plantes, corrélée à une activation du transport transmembranaire du Pb (Sunkar et al., 2000).

Les transporteurs de type CDF

Impliqués dans le transport des éléments traces, les membres de la famille CDF (Cation Diffusion Facilitor) permettent l'efflux d'ions métalliques, comme le Zn^{2+}, le Co^{2+}, le Cd^{2+} et autres, du cytoplasme vers l'extérieur de la cellule ou vers les vacuoles (Gaither and Eide, 2001; Palmiter and Huang, 2004). Chez *A. thaliana*, le gène ZAT, la première protéine CDF végétale identifiée pour le transport de Zn, est induite par l'augmentation de Zn dans le milieu de culture (van der Zaal et al., 1999). Ce gène a également été identifié chez *Thlaspi caerulescens* et il est principalement surexprimé dans les racines et les feuilles des plantes poussant sur un sol contaminé par le Zn, le Cd et le Pb (Assunção et al., 2001).

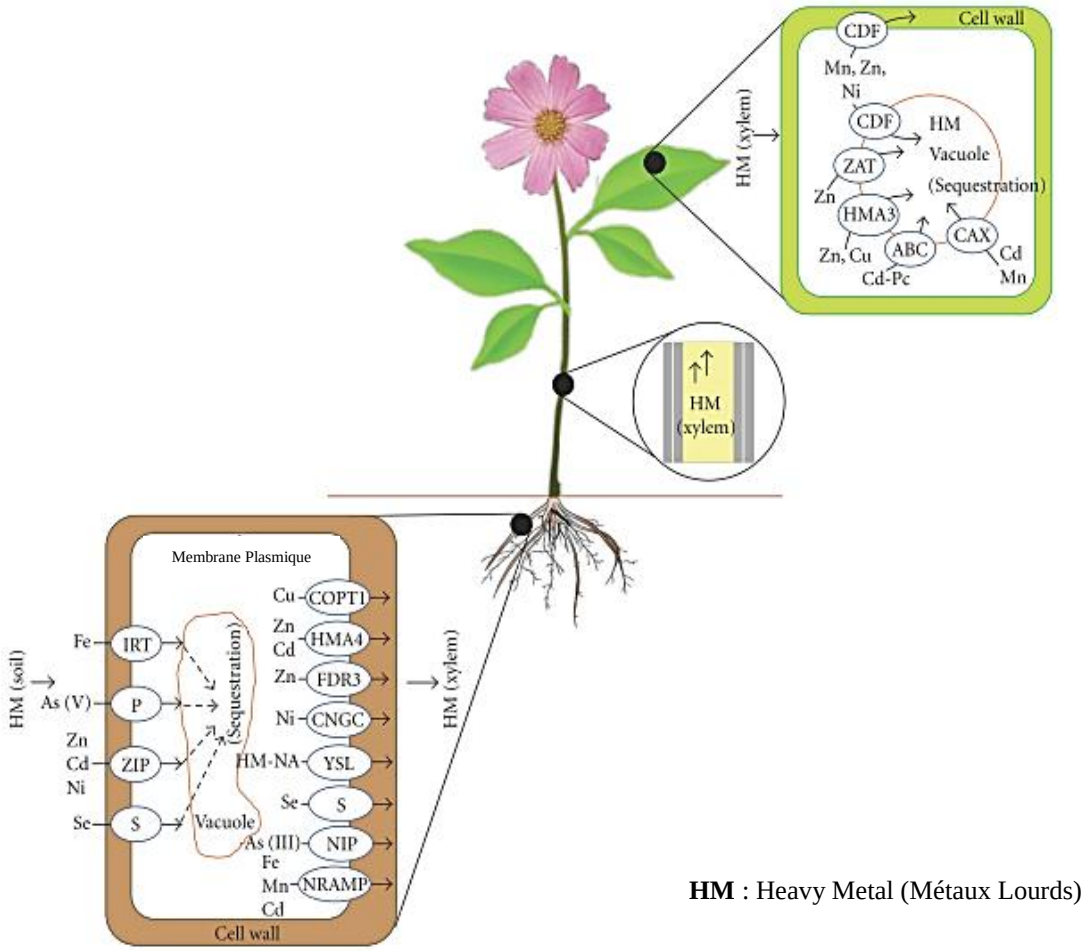

Figure I-10. Mécanismes impliqués dans l'accumulation et l'hypertolérance du métal chez les plantes (Figure tirée deHossain et al., 2012)

Les protéines NRAMP

Les membres de la famille des NRAMP (Natural Resistance Associated Macrophage Protein) sont impliqués dans le transport des ions métalliques divalents (Matsuzawa-Nagata et al., 2008). Des gènes *Nramp* ont été identifiés chez plusieurs espèces présentant une haute conservation durant l'évolution. Des études faites sur *Thlaspi caerulescens* ont montré que le gène *Nramp* était induit par le Cd et le Ni (DalCorso et al., 2008; Ouziad et al., 2005). Un transporteur membranaire NRAMP5 serait impliqué dans la translocation du Cd de la racine vers la feuille chez le riz (Sasaki et al., 2012). De plus, dans un milieu carencé en Fe, le gène *AtNramp3* chez *A. thaliana* induit l'accumulation de métal dans les cellules en mobilisant les ions métalliques de la vacuole au cytosol (Thomine et al., 2003). Inversement, la surexpression du gène *Nramp1* chez *A. thaliana* transgénique provoque une augmentation de

la résistance de la plante à un excès de Fe (Curie et al., 2000).

b/ Translocation

Le potentiel de transport des métaux lourds vers les feuilles caractérise les espèces accumulatrices des espèces tolérantes « excluder ». Des protéines transmembranaires de transport métallique ont été identifiées.

Les ATPases de types P

Les pompes ATPases de type P également désignés par HMAs (heavy metal transporting ATPases) sont des protéines membranaires impliquées dans le transport à longue distance du Cd, du Pb, du Zn et du Cu à travers les membranes biologiques contre leur gradient électrochimique (Wong and Cobbett, 2009) en utilisant l'énergie libérée par l'hydrolyse de l'ATP. Elles se caractérisent par la formation d'un intermédiaire phosphorylé durant le cycle réactionnel (Hall and Williams, 2003). Il existe cinq familles d'ATPases de type P, elles même divisées en sous familles dont la P1B ou CPx-ATPases (Hall et Williams, 2003).

Le gène *hma4* a été identifié chez *A. thaliana*, *A. halleri*, *A. lyrata* et *N. caerulescens* (Verbruggen et al., 2009) et révélé avoir un rôle dans la tolérance, la translocation et l'accumulation voire même l'hyperaccumulation de Zn et Cd par pompage vers le xylème (Mills et al., 2005; Figure I-10).

Le transporteur HMA2 a montré être impliqué dans le transport et l'homéostasie du Zn et aurait un rôle redondant à celui de HMA4 (Hussain et al., 2004). La surexpression du gène codant pour le transporteur HMA2 contribue également à la translocation du Cd, ainsi que cela a été mis en évidence chez *A. thaliana* (Wong and Cobbett, 2009). Plus récemment, il a été proposé que la protéine *Oshma2* soit impliquée dans le déversement du Cd dans le xylème du riz (Nocito et al., 2011, Figure I-10). En outre, le gène AtHMA5 a montré son implication dans la compartimentation du Cu par Andrés-Colás et al. (2006, Figure I-11).

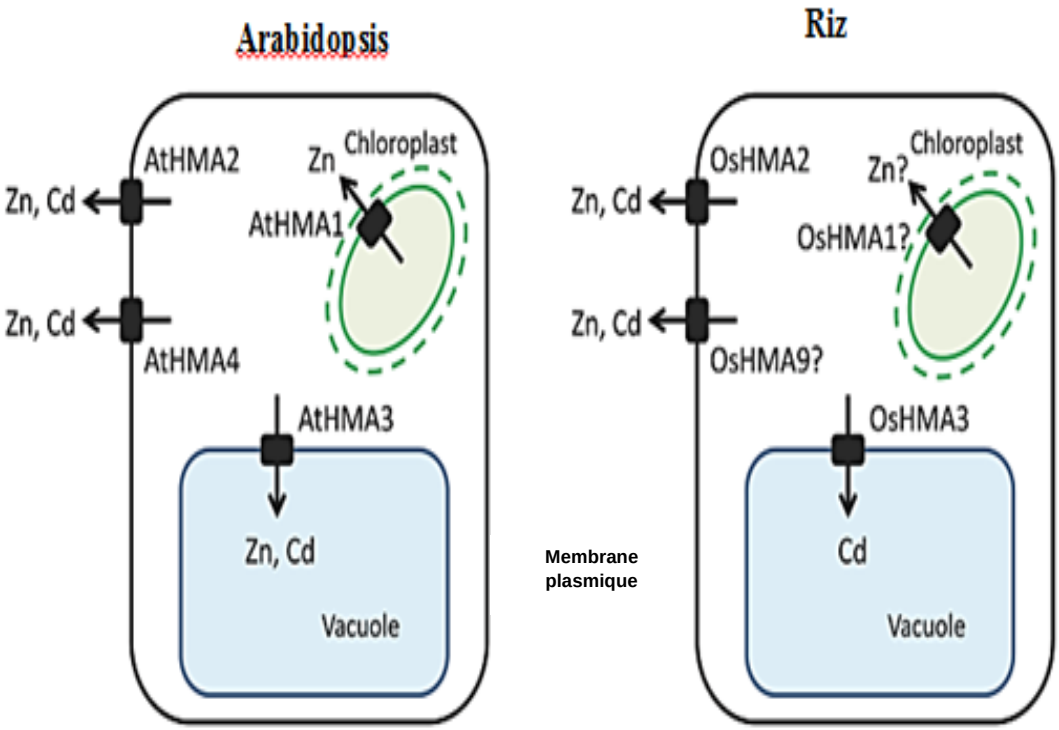

Figure I-11. Prédiction de la localisation et des fonctions des HMAs chez Arabidopsis et le riz (d'après CUN, 2013)

La famille des MATEs

La famille MATE (Multidrug and toxin efflux family) est une famille de protéines membranaires impliquées dans l'élimination des métaux toxiques de la cellule. FRD3 (Ferric Reductase Defective 3) est une protéine MATE susceptible de déverser des ions Fe^{2+} et de citrate dans les tissus. Les complexes ferrique-citrate sont nécessaire pour la translocation du Fe vers les feuilles car Fe se déplace à travers les xylèmes sous forme chélaté (Durrett et al., 2007). Les exsudats de xylème des plantes mutantes en *FRD3* contiennent moins de citrate et Fe que les plantes de type sauvage, tandis que celles des plantes transgéniques sur-exprimant *FRD3* produisent plus de citrate dans les exsudats des racines. Toutefois, FRD3, a été identifiée comme pouvant être impliquée dans la translocation du Zn chez *A. halleri* (Talke, 2006) et *N. caerulescens* (van de Mortel et al., 2006).

La famille des ABC

Les différentes composantes (taille) de la famille des ABC (ATP-binding cassette) partagent le même domaine de liaison nucléotidique (NBD). Ces protéines sont connues pour transporter une large variété de substances, notamment des ions, des hydrates de carbones, des lipides, des xénobiotiques, des médicaments et des métaux toxiques (Kim et al., 2007). Chez

les plantes supérieures, les protéines ABC sont organisées en huit sous-familles dont les membres PDR (Pleiotropic Drug Resistance) et la sous-famille ABCG sont les plus abondants. Cette famille de gènes a été bien étudiée chez plusieurs espèces végétales comme Arabidopsis (Figure I-12b) et le riz.

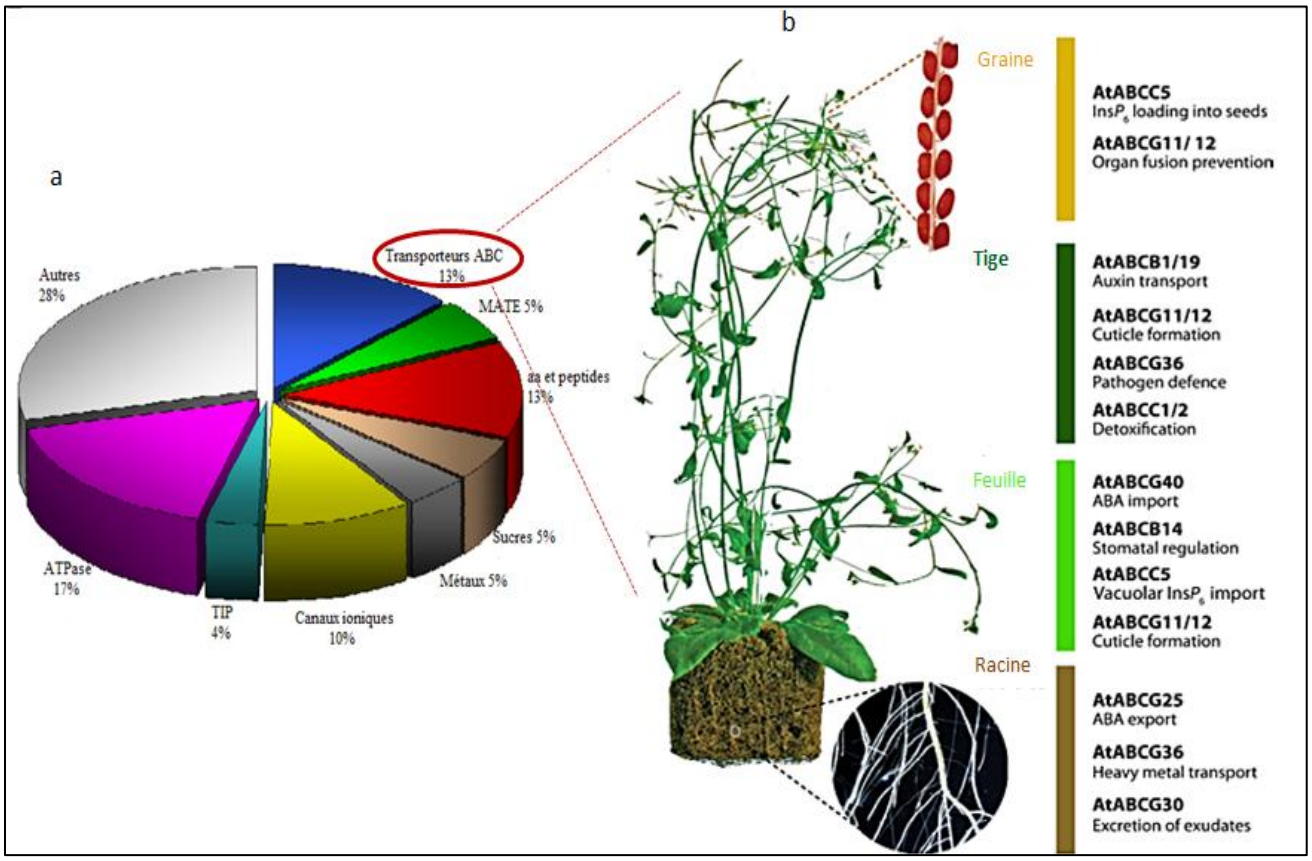

Figure I-12. (a) Principales classes de transporteurs identifiées dans le tonoplaste de la vacuole (b) et les différents types de transporteurs ABC chez Arabidopsis avec leur substrat et/ou fonction (Figure adaptée de Kretzschmar et al., 2011).

Les transporteurs ABC jouent un rôle important dans le mécanisme général de détoxication. Les membres ABCG ont été identifiés comme candidats pour les exportations de Cd et de Pb (Kim et al., 2007). Des composés potentiellement toxiques intrinsèques sont d'abord incorporés et conjugués avec des chélateurs, qui sont alors reconnus par des transporteurs ABC tonoplastiques et séquestrés dans la vacuole (Frelet and Klein, 2006). Par ailleurs, 13 % des transporteurs identifiés dans le tonoplaste des vacuoles appartiennent à la famille des transporteurs ABC (Figure I-12a).

Le transporteur membranaire de type ABC comme PDR8 (Pleitropic drug resistance), exprimé dans toutes les cellules d'*A. thaliana*, mais plus intensément dans les poils et

l'épiderme racinaire, a été désigné comme transporteur d'efflux de Cd chez Arabidopsis (Kim et al., 2007) tandis que le PDR12 est impliqué dans l'exclusion du Cd et du Pb à la fois (Lee et al., 2004).

V.4.1.2. Identification et caractérisation de gènes codant pour des chélateurs de métaux lourds

Il existe deux stratégies de base pour diminuer la toxicité des métaux : la chélation ou l'efflux du cytosol, soit dans l'apoplasme, soit par séquestration intracellulaire. Les principaux et uniques chélateurs végétaux sont les phytochélatines (PCs) et les métallothionéines (MTs). La synthèse des PCs est induite par de nombreux métaux dont le Cd et le Pb (Piechalak et al., 2002).

Les phytochélatines

Les phytochélatines (PCs) sont des polypeptides intracellulaires issus d'une synthèse non ribosomale, de faibles masses moléculaires (2 à 10 kDa) et à forte affinité pour les métaux et métalloïdes. Les PCs sont composés de 3 acides aminés : Glu, Cys et Gly et leur formule générale est $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ où n est compris en général entre 2 et 11 (Rauser, 1995). Les PCs interagissent avec les ETMs à travers le groupement thiol (-SH) de la cystéine (Pal, 2010). Il a été démontré que la synthèse des phytochélatines dans les algues est activée fortement par la présence de Cd, Cu, Pb, Hg et Zn (Morelli and Scarano, 2004). Les ions métalliques libres tels que Cd^{2+} sont retenus par les PCs formant des complexes de faible masse moléculaire (Low Molecular Weight (LMW)) qui n'affectent pas le métabolisme au niveau cytoplasmique (Figure I-13). Des transporteurs membranaires de type ABC reconnaissent les complexes PC-métal et les transferts vers la vacuole où ils s'agrègent les uns aux autres par des ponts disulfures pour former des complexes de masse moléculaire élevée (High Molecular Weight (HMW)) (Clemens, 2006 ; DalCorso et al., 2008 ; Figure I-13).

A cause du pH vacuolaire, ces complexes finissent par se dissocier libérant les ions métalliques qui par la suite, seront pris en charge par les acides organiques présents, tels que le malate, le citrate ou encore l'oxalate. Les PCs sont ensuite recyclées et réexportées dans le cytoplasme pour capter d'autres ions et les réacheminer dans la vacuole (Sanità di Toppi et Gabbrielli, 1999 ; Figure I-13).

Les PCs sont synthétisées par la phytochélatine synthase (PCS) à partir du glutathion. La PCS, généralement active sous forme oligomérique, est strictement dépendante de la présence

de métaux en excès dans le cytosol (Vatamaniuk et al., 2004).

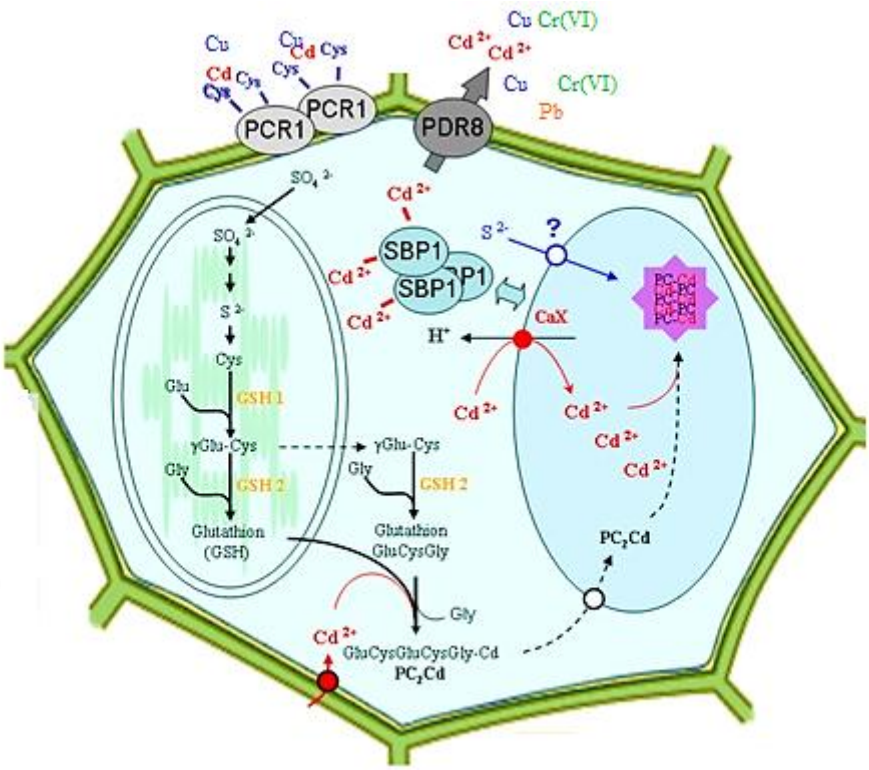

Figure I-13. Mécanismes de séquestration des métaux lourds dans la vacuole cellulaire (d'après Hernández et al., 2015)

Les principaux activateurs de l'enzyme sont organisés par potentiel d'activation décroissant : Cd> Ag> Pb> Zn> Cu> Mg>Or (Cobbett, 2000). Afin d'identifier les gènes *pcs*, des séquences nucléotidiques de *PCS* ont été clonés à partir du riz (*Ospcs1*), du blé (*Tapcs1*), d'*Arabidopsis thaliana* (*Atpcs1*, 2, 3, 4, 5), et *Brassica juncea* (*BjPCS1*, 2, 3) (Clemens et al., 1999; Heiss et al., 2003). Chez Arabidopsis, la surexpression de *AtPCS1* a contribué à une amélioration de la tolérance de la plante, mais aussi à une hypersensibilité au Cd (Li and Godzik, 2006). En revanche, l'expression de *Atpcs1* chez la moutarde indienne transgénique a augmenté la synthèse des PCs, ce qui améliore la tolérance de l'espèce au Cd et As. Toutefois, il a été proposé que la PCS soit une enzyme dont l'expression génique est constitutive c'est à dire non induite en réponse à l'exposition aux métaux lourds. Cette hypothèse a été démentie par Clemens (2006) qui a montré qu'au cours des premiers stades de développement, le niveau de l'expression génique de *Atpcs1* a doublé chez des graines d'*A. thaliana* exposées au Cd pendant 5 jours par rapport aux graines non traitées. De plus, Clemens et al. (2003) ont trouvé que le taux de *Tapcs1* dans les racines de blé traitées par 100 µM Cd étaient 5 à 10 fois

plus élevées que celles des racines non traitées. Ces études indiquent que l'expression des gènes *pcs* répond aux métaux lourds.

Les métallothionéines

Les métallothionéines (MTs) sont des protéines riches en cystéine (Cys) de faible masse moléculaire qui peuvent lier des métaux et peuvent jouer un rôle dans leur séquestration intracellulaire. Sur la base de leurs résidus de cystéines conservés, les MTs sont divisées en quatre types (Cobbett and Goldsbrough, 2002b). Elles jouent un rôle important dans la détoxication des métaux lourds et le maintien de l'homéostasie des ions métalliques intracellulaires (Cobbett et Goldsbrough, 2002). Elles peuvent conférer une tolérance aux métaux une fois exprimée chez la levure, et quelques-unes d'entre elles sont régulées positivement dans des conditions d'excès métallique (Cobbett and Goldsbrough, 2002a). Cependant, leur fonction exacte n'est toujours pas complètement comprise et les données existantes sur les effets des ions métalliques et le rôle des MTs de végétaux dans la détoxication des métaux et l'homéostasie sont parcellaires voire incompatibles.

Le cuivre, par exemple, induit l'expression des *mt1* chez Arabidopsis (Zhou and Goldsbrough, 1994), le riz (Hsieh et al., 1995) et le tabac (Borderouge and Nantes, 1999), mais diminue leur expression chez *Mimulus guttatus* (de Miranda et al., 1990). D'une manière générale, l'expression des *mt1* est généralement plus élevée dans les racines que dans les jeunes pousses, alors que l'inverse est observé pour les *mt2*. Les *mt3* sont surexprimées dans les feuilles et les fruits de mûrissement, tandis que l'expression des *mt4* reste limitée aux semences en développement (Cobbett & Goldsbrough, 2002). Toutefois, plusieurs autres facteurs influencent l'expression des *mts* tels que les blessures, l'infection pathogène, l'interaction symbiotique et la sénescence foliaire, ce qui suggère que les *mt*s peuvent être exprimées comme faisant partie d'une réponse générale au stress (Cobbett & Goldsbrough, 2002). Elles jouent également un rôle dans la réparation de la membrane plasmique (Hall, 2002).

V.4.2. Biomarqueurs biochimiques

Les biomarqueurs ne sont pas tous de la même nature. Les biomarqueurs moléculaires et biochimiques ne sont pas toujours spécifiques d'une pollution mais évaluent un stress général. Différents biomarqueurs biochimiques d'exposition et d'effet en relation avec la caractérisation des composés absorbés ont été identifiés.

V.4.2.1. Génération de stress oxydant

Les Espèces Réactives de l'Oxygène (ERO), produites avec un taux de production faible dans les conditions optimales de croissance des végétaux par de nombreux processus métaboliques principalement photochimique, représentent une réponse commune à toutes les contraintes. Les ETMs, comme toutes les autres contraintes environnementales, peuvent accroitre la formation d'ERO, dont l'accumulation peut occasionner des dommages oxydatifs qui sont à l'origine de leur sensibilité à cette contrainte. Ce stress oxydant généré est défini comme un déséquilibre entre les systèmes pro-oxydants et les systèmes de défense antioxydants qui est rompu en faveur de l'état oxydant. Dans les cellules végétales, la formation des radicaux libres peut se produire dans différents compartiments et organites : chloroplastes, mitochondries, réticulum, peroxysomes, membrane plasmique et parois cellulaires (De Laat et al., 2011). Ces radicaux incluent l'anion superoxyde ($O_2^{\cdot -}$), le radical hydroxyle (OH^-) ou encore le peroxyde d'hydrogène H_2O_2 (Figure I-14). Ils sont capables de diffuser loin de leur site de formation et d'occasionner des dégâts multiples tels que :

- la perturbation de nombreux processus physiologiques comme la photosynthèse (Arora et al., 2002; Langridge and Graner, 2002) causée principalement par la réactivité des ERO ($OH^{\cdot}$) envers les macromolécules biologiques,

- l'altération des structures protéiques surtout celles comportant un groupement sulphydryle (-SH) facilement oxydable par H_2O_2 et OH^-, ce qui conduit à l'inactivation de certains enzymes (Favier, 2003; Stadtman and LEVINE, 2000). La formation de groupements carbonyles sur la chaîne latérale de certains acides aminés en présence de radicaux libres conduit à la dégradation irréversible des enzymes (Wong and Cobbett, 2009),

- l'altération de la structure des lipides, et principalement les acides gras polyinsaturés (AGPI) membranaires qui sont la cible privilégiée des ERO,

- les altérations oxydatives sont considérées comme la source majeure de dommages à l'ADN.

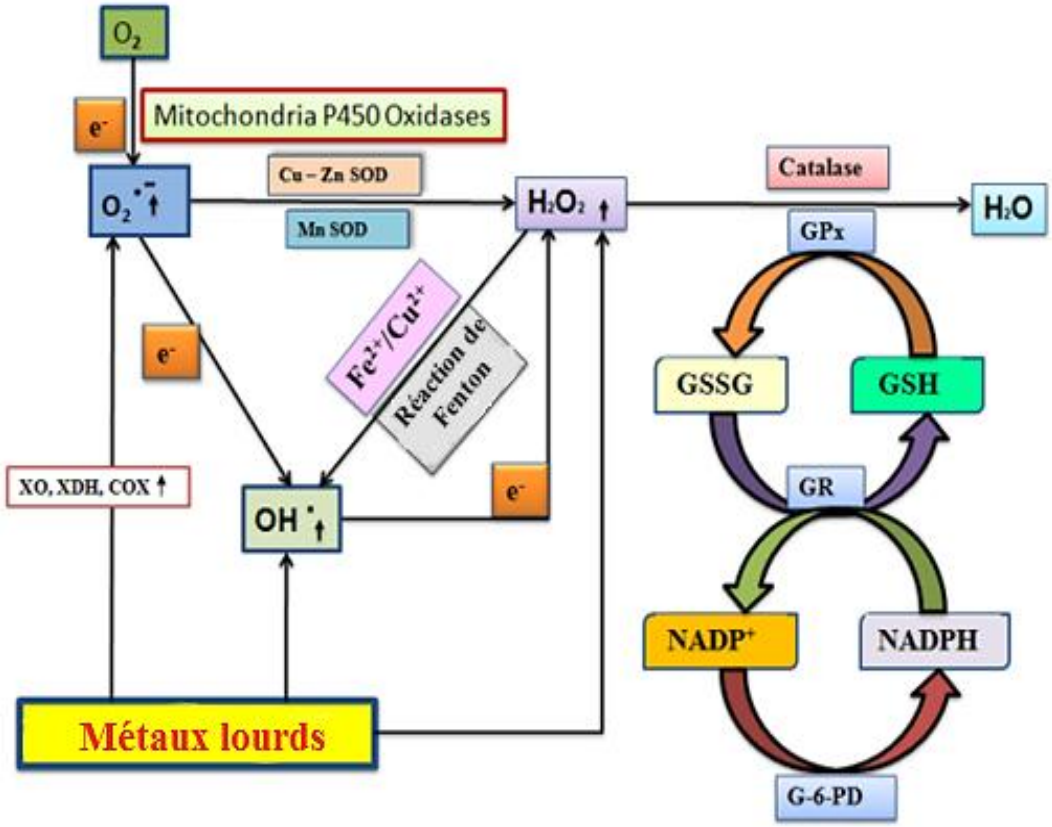

Figure I-14. Les métaux lourds induisant la formation de radicaux libres (d'après Bandyopadhyay et al., 2016)

Dans ce système complexe, il existe un grand nombre d'indicateurs potentiels permettant d'apprécier l'importance du stress oxydant ou les capacités de défense de l'organisme :

- Quantification des radicaux libres.
- Evaluation des effets du stress oxydant : oxydation des protéines, dommages à l'ADN, peroxydation des lipides. Le stade de peroxydation des lipides membranaires est révélé par le taux des malonaldehydes libres nommés TBARS qui présentent les marqueurs de stress oxydatif les plus étudiés (Amiard et al., 2008).
- Détermination de la capacité anti-oxydante: présence des anti-oxydants (vitamines E et C, glutathion, caroténoïdes…) et activité des enzymes antioxydantes.

V.4.2.2. Système de défense anti-oxydant : marqueur du stress oxydatif

Le diagnostic du stress oxydatif fait appel à plusieurs méthodes d'analyse se basant entre autre par la mesure des teneurs en anti-oxydants qui montrent de véritables changements lors d'un stress, ce qui permet de suggérer leur implication dans la lutte contre ce type d'agression (Artetxe et al., 2002).

Les principaux mécanismes d'activité anti-oxydante sont :

- le piégeage direct des ERO ;
- l'inhibition des enzymes impliquées dans le stress oxydant et la chélation des traces métalliques responsables de la production des ERO ;
- la protection des systèmes de défense anti-oxydant.

Ainsi, pour combattre les effets délétères des radicaux libres, les cellules végétales disposent de deux grands types de défense :

Système anti-oxydant enzymatique

Au niveau du métabolisme aérobie, la conséquence d'une production d'ERO est nécessairement corrélée avec la synthèse d'enzymes anti-oxydantes afin d'établir un état d'équilibre intracellulaire. Les organismes photosynthétiques développent une large gamme de mécanismes de protection contre les radicaux libres avant que ces derniers ne causent des dommages importants au niveau de membranes lipidiques (Pinto et al., 2004). Ils possèdent de nombreuses voies de dégradation enzymatique des ERO incluant :

-Les superoxydes dismutases (SOD) : sont des métallo-enzymes de trois types ; FeSOD, MnSOD et CuZnSOD qui peuvent éliminer l'anion superoxyde par dismutation, pour former de l'oxygène et de l'eau oxygéné.

-Les catalases (CAT) : sont des enzymes majoritairement peroxysomales, qui détruisent le peroxyde d'hydrogène, en aboutissant également à la formation d'oxygène et d'eau.

-Les ascorbates peroxydases (APX) : sont des métallo-enzymes qui existent dans tous les compartiments cellulaires, dans le cytosol et dans l'apoplaste, sous forme solubles ou liées aux membranes et qui catalysent la réduction du peroxyde d'hydrogène en utilisant l'ascorbate comme co-substrat.

-Les peroxydases (POX) : sont une large famille multigénique d'enzymes hémiques qui détruisent le peroxyde d'hydrogène en formant de l'eau.

-Les peroxyredoxines (PRX) : sont des peroxydases non hémiques qui possèdent généralement les mêmes capacités peroxydasiques que les POX, mais avec une efficience moindre (Navrot et al., 2006).

Système anti-oxydant non-enzymatique

Les composés phénoliques (CP), sont des molécules du métabolisme secondaire spécifiques du règne végétal. Plus de 8000 molécules ont été isolées et identifiées (Mi et al., 2003). Elles sont très hétérogènes tant par leur composition que par leur structure. Toutefois, Elles ont en

commun la présence d'un ou plusieurs cycles benzéniques portant une ou plusieurs fonctions hydroxyles qui peuvent être libres ou engagées (Patrick et al., 2004). La dynamique biochimique de ces composés intervenant dans la biosynthèse de divers métabolites de l'organisme réfute de les considérer comme des molécules secondaires et métaboliquement inactives.

Les polyphénols, particulièrement abondants dans les fruits, peuvent agir selon divers mécanismes. Ils peuvent être des éliminateurs de radicaux libres, des piégeurs d'oxygènes dans des systèmes fermés ou encore des chélateurs d'ions métalliques (Chew et al., 2009). Les polyphénols de type flavonoïdes (Fl-OH), largement diffusés sur la surface des feuilles et dans les cellules de l'épiderme, sont thermodynamiquement capables de neutraliser dans les milieux aqueux et organiques, les radicaux superoxydes, peroxyles, hydroxyles et alcoxyles grâce à leurs faibles potentiels redox (Hodek et al., 2002). Ces flavonoïdes peuvent chélater directement des métaux lourds, inhiber des enzymes qui génèrent des radicaux libres et empêcher la propagation de la réaction de lipoxygénation (Ross and Kasum, 2002). Selon les variations autour du squelette chimique de base en C_{15}, on distingue différents classes de flavonoïdes :

- les 4-oxoflavonoïdes : flavones, isoflavones, flavonols et flavanones ;
- les chalcones et dihydrochalcones ;
- les antocyanes
- les catéchines et proantocyanidines (tanins condensés)

Les tanins qui regroupent les substances phénoliques polymériques, sont caractérisés par une saveur astringente et sont trouvés dans toutes les parties de la plante. Ils interviennent entre autre dans la précipitation des alkaloïdes, la gélatine et d'autres protéines (Cowan, 1999).

Également, les anti-oxydants non-enzymatiques comme le glutathion (GSH ; le plus abondant chez les êtres vivants), sont nombreux. Plusieurs études ont montré que le glutathion, possédant une fonction anti-oxydante grâce au groupement thiol, joue un rôle très important dans la défense contre le stress métallique chez les plantes supérieures (Li et al., 2006, 2007; Dubey et al., 2010). Le GSH élimine le H_2O_2 et réagit de façon non-enzymatique avec les autres espèces radicalaires de l'oxygène : oxygène singulet, le radical superoxyde et le radical d'hydroxyle. Une autre fonction importante du GSH est de permettre la régénération de l'ascorbate via le cycle ascorbate-glutathion.

V.5. Évaluation des performances analytiques d'un biomarqueur

Les applications des biomarqueurs dépendent de l'objectif fixé qui peut être :

- Le dépistage ou « screening » : une détection et une identification rapide pour signaler la présence ou les effets de xénobiotiques ;
- Un diagnostic et une bioévaluation réalisés sur le terrain grâce à une batterie de biomarqueurs indiquant une réponse précoce ;
- Une biosurveillance et biomonitoring spatio-temporelle de tendances, avec une traduction des objectifs de qualité à atteindre en gammes de réponses des biomarqueurs indicatrices d'un environnement non pollué ;
- Une détermination des procédures prédictives de caractérisation du risque.

Le développement de l'écotoxicologie autour des biomarqueurs a conduit à la mise en place de nombreux programmes de recherche nationaux et internationaux visant à valider ces outils, dont certains très ambitieux. Dans une moindre mesure, ils cherchent à développer des réseaux de surveillance intégrant ces outils. Ces différents programmes de recherche à savoir BIOMAR, BEEP et MODELKEY ont contribué à la proposition d'outils et de stratégies de surveillance intégrant les biomarqueurs.

V.6. Vers une approche multi-biomarqueurs

De nombreux facteurs intrinsèques (espèce, âge, taille, caractéristiques génétiques,…) et extrinsèques (température, salinité, sol,…) sont susceptibles d'influencer la physiologie d'un organisme et donc la réponse d'un biomarqueur. Pour ces raisons, ainsi que pour la diversité importante des types de manifestations possibles des atteintes toxiques, les approches intégrées impliquant l'étude conjointe de plusieurs biomarqueurs sont fortement recommandés (Brown et al., 2004b; Galloway et al., 2006). A cette approche sont associées les notions de « multi-(bio)marqueurs », de « suite de biomarqueurs » ou de « batterie de biomarqueurs ». Avec l'apparition de ces termes scientifiques, la recherche de la standardisation d'un biomarqueur pour évaluer les effets toxiques intégrés d'un contaminant devient secondaire (Brown et al., 2004). L'étude combinée de plusieurs biomarqueurs permet d'éviter le risque d'avoir un faux diagnostic sur la qualité du milieu pour le biota.

Pour ne pas considérer un seul biomarqueur isolément dans la biosurveillance des écosystèmes, le recours à l'approche multi-biomarqueurs a rendu nécessaire le développement de stratégies intégratives de traitement et de présentation des données. En effet, il s'avérait

nécessaire de réduire la complexité de l'analyse des résultats (parfois générés en grand nombre) en les appréhendant de façon synthétique, et d'augmenter encore la pertinence de l'approche multi-biomarqueur par rapport à une analyse individuelle de chaque réponse biologique étudiée (Baumelle et al., 2017). Ainsi, l'usage de certains outils tend à se généraliser, comme par exemple :

L'application de méthodes d'analyses multi-variées (Galloway et al., 2006) ;
La proposition d'indices intégratifs (Broeg et al., 2005) ;
et récemment, un système expert d'aide à la décision, développé par Dagnino et al. (2007) qui se révèle extrêmement prometteur pour une réelle intégration des approches biomarqueurs dans les procédures d'évaluation des risques (Dondero et al., 2006). Son utilisation s'avère envisageable à l'échelle des gestionnaires de l'environnement, et ne s'adresse plus seulement à un petit nombre d'experts dans le milieu de la recherche.

VI. Site pilote et espèce végétale d'intérêt

V.1. Zone d'étude : la zone côtière Sud du Grand Sfax; aux alentours de la SIAPE

La zone d'étude est située à Sfax, dans le sud de la Tunisie. La région est caractérisée par un climat semi-aride à aride. Les précipitations annuelles enregistrées, sur une période de 60 ans, sont en moyenne de 237,8 mm et les vents dominants, généralement moyens à faibles, sont de sud ou au sud-est. Les sols se Sfax sont sableux ou encore formés de sable-calcaire. Les cultures dominantes sont les oliviers et les vergers qui occupent 80 % des terres agricoles, suivis des céréales avec 4,5 %.

Malgré un fort potentiel de développement durable, la zone côtière Sud du Grand Sfax souffre d'une dégradation de la qualité de son environnement. Elle subit, depuis des décennies, une pression industrielle accrue où l'industrie occupe plus de 50 % de la superficie totale. L'activité de transformation de phosphate de la Société Industrielle d'Acide Phosphorique et d'Engrais de Sfax (SIAPE) par ces trois unités, génèrent des quantités considérables de rejets liquides, solides et gazeux. Les émissions de poussières de la centrale (Figure I-15) sont estimées annuellement à 1610 tonnes (Béjaoui and Hadj, 2016a).

Figure I-15. Localisation du site de la SIAPE et des terrils de phosphogypse

Selon Azri et al. (2000), l'unité TSP (Triple Super Phosphate) de la centrale déverse dans l'atmosphère des flux de métaux lourds très élevés (653 kg Cd/an) et des particules d'oxyde de cadmium (Ø <1 µm) sont trouvées jusqu'à 5 km de l'industrie des engrais. De plus, les terrils de phosphogypse stockés qui dépassent les 15 millions de tonnes (Figure I.15) contiennent des impuretés et des particules solides qui sont transportées par le vent, ainsi que par les acides et les particules lavées (Choura, 2007). La pollution qui en a découlé, a provoqué une réaction qui a suscité des accords internationaux interdisant cette forme de rejet. Ainsi, les producteurs de ce sous-produit sont obligés de le stocker sur des sites terrestres qui entraînent, étant donnée l'importance de ce stock, des problèmes écologiques, notamment au niveau des nappes phréatiques et au niveau des régions côtières. A cela, s'ajoute l'impact visuel négatif notamment pour les zones côtières à caractère urbain et touristique.

Des études de base pour évaluer l'influence de la pollution émise par les usines de la SIAPE sur les sols et les plantes ont été réalisées dans la zone située à plus de 2 km de la centrale dans différentes directions (Béjaoui and Hadj, 2016a). La caractérisation du sol et les teneurs en cadmium dans le sol et les végétaux ont été effectuées. Il s'agit de sols alcalins présentant de faibles niveaux de matière organique. Les concentrations totales de Cd dans le sol varient de 1,2 à 6,7 mg/kg et sont supérieures aux limites permises pour l'utilisation agricole. Le

diagramme de répartition spatiale montre que ses zones, caractérisées comme des points chauds, sont situés à environ 500 m de l'usine. Le facteur de contamination a montré une contamination modérée ou élevée des sols tandis que les facteurs d'enrichissement ont révélé que l'enrichissement est dû à des activités anthropiques. Les végétaux collectés dans la zone d'étude ont montré diverses accumulations de Cd. Dans les plantes cultivées indigènes, les concentrations de Cd varient de 0 à 5,3 mg/kg respectivement chez *Bassia miricata* et *Aelurapus littoralis*. Pour les arbres fruitiers, les amandes ont accumulé 1,5 fois plus que les limites permises pour les fruits. La majorité des espèces étudiées présentaient des facteurs de bioconcentration inférieurs à 1.

VI.2. Espèce d'intérêt : Palmier dattier (*Phoenix dactylifera* L.)

VI.2.1. Description

Le palmier dattier : *Phoenix dactylifera L.*, provient du mot « *Phoenix* » qui signifie dattier chez les phéniciens, et dactylifera dérive du terme grec « *dactulos* » signifiant doigt, allusion faite à la forme du fruit (Djerbi, 1994). Le dattier est un MONOCOTYLEDONE de type DIOIQUE à reproduction allogame ayant des inflorescences mâles et des inflorescences femelles séparées : DOKKAR (palmier mâle) et NAKHLA (palmier femelle). Sa durée de vie peut dépasser 100 ans. C'est une espèce pérenne, thermophile qui exige un climat chaud, sec et ensoleillé. Il s'adapte à tous les sols grâce à sa grande variabilité. Il est sensible à l'humidité pendant la période de pollinisation et au cours de la maturation (Munier et al., 1973).

Le palmier est abondamment cultivé au Golfe Persique, où il forme la végétation caractéristique des oasis. Il est cultivé en outre aux Canaries, dans la Méditerranée septentrionale et dans la partie méridionale des Etats-Unis. Le secteur palmier dattier joue un rôle très important dans le sud Tunisien tant sur le plan socio-économique que sur le plan écologique. II est un pilier de l'économie des régions du Djérid et de Nefzaoua. Le palmier occupe une place de choix dans l'économie nationale. Les palmeraies tunisiennes couvrent une superficie de 22 500 ha, comptent environ 3 000 000 de pieds et assurent une production assez irrégulière mais en nette évolution. La part de la variété 'Deglet Nour' dans la production est très importante: de l'ordre de 60 % (d'après CRDA, 2000).

La production de dattes ne cesse d'augmenter. Elle est estimée annuellement à 105 mille tonnes dont 70 mille tonnes de Deglet Nour (doigt de lumière), variété considérée comme la

meilleure. Ces dattes renferment des substances dites composés phénoliques (Mansouri et al., 2005) dont l'analyse qualitative révèle la présence des acides cinnamiques, des flavones, des flavonones et des flavonols (Mansouri et al.,2005). Ces derniers jouent un rôle important dans le corps humain, ils ont des effets anti-inflammatoires, antioxydants, abaissent la tension artérielle et renforce le système immunitaire (García-Lafuente et al., 2009; Hämäläinen et al., 2007).

En Tunisie, il existe plusieurs sources de pollution par les métaux, liées notamment aux activités minières au centre du pays (industrie des engrais phosphatés) ou sont implantés des milliers de pieds de palmier dattier. Plusieurs études ont été réalisées sur le palmier dattier au moyen d'apporches protéomiques (Al-senaidy and Ismael, 2011 ; Daoud et al., 2015) et moléculaires-génomiques (Al-senaidy and Ismael, 2011; Daoud et al., 2015; Zhao et al., 2012). Cependant relativement peu d'informations sont disponibles sur les réponses de la plante au stress métallique.

VI.2.2. Classification

La place du palmier dattier dans le règne végétal est rappelée ci-dessous :

Groupe : Spadiciflores
Ordre : Palmale
Famille : Palmacées
Sous famille : Coriphoidées
Tribu : Phoenicées
Genre : ***Phoenix***
Espèce : ***dactylifera* L.**

Le genre *Phoenix* comporte au moins 12 espèces, la plus connue est le *dactylifera*, dont les fruits ‘‘dattes’’ font l'objet d'un commerce international.

Deuxième Partie

RESULTATS ET DISCUSSION

Chapitre I

La bioinformatique en écotoxicologie

-Avant-propos-

Le développement de réponses transcriptomiques aide les plantes à survivre et à résister aux stress abiotiques. Une première phase d'activation sensorielle, suivie d'une réponse physiologique, est habituellement induite par des stress abiotiques chez les plantes. Cependant, la littérature concernant la réponse transcriptomique des plantes non-modèles au stress métallique est rare, et quasi inexistante pour le palmier dattier *Phoenix dactylifera*. Pour cela, nous avons cherché à dévoiler le profil transcriptomique de la plante utilisée dans ces stratégies de résistance aux ions métalliques.

Dans le but de générer des données transcriptomiques, une librairie d'ADNc de feuilles de palmier dattier cv Deglet Nour collectées dans une zone soumise à une forte pollution métallique située au sud de la Tunisie a été construite. Pour cela, nous avons recours aux méthodes de séquençage de la nouvelle génération RNA-Seq, une technologie précieuse dans la recherche sur la génomique fonctionnelle. Toutefois, pour exploiter le potentiel de ces données issues du RNA-seq, il est nécessaire d'utiliser des outils *in silico* pour exploiter les nombreuses données. Le grand jeu de données EST généré nous permet d'élargir nos connaissances des ressources génomiques de la plante et d'étudier les gènes qui répondent au stress métallique. L'annotation de la banque, la détermination des voies métaboliques principales associées aux réseaux de régulation des gènes et l'identification de gènes candidats potentiellement impliqués dans la détoxication des métaux ont été réalisées à l'aide de différents logiciels et programmes de traitement des données bioinformatiques. Par ailleurs, la réponse des plantes aux divers stress abiotiques est une caractéristique sous contrôle génétique complexe. Un des objectifs est aussi de faire des progrès dans la compréhension des mécanismes intervenants dans la tolérance aux stress métalliques. Cette analyse *in silico* s'est accompagnée d'expérimentations faisant appel à des techniques de biotechnologie végétale impliquant notamment la culture *in vitro* de vitroplant de palmier dattier afin de valider certaines hypothèses.

Au cours de ce chapitre nous présentons i) la première analyse du transcriptome de *P. dactylifera* L. cv Deglet Nour en utilisant la plate-forme Illumina GA IIx, ii) la prédiction des voies métaboliques impliquées dans la réponse aux stress par le Cd ou le Cu, iii) le suivi du niveau d'expression de six gènes candidats ; iv) l'étude de l'histoire évolutive du gène codant pour la phytochélatine synthase et le suivi de son niveau d'expression dans des vitroplants

exposés à différents contextes métalliques.

-Publications-

A/ Zayneb Chaabene, Imen Rekik Hakim, Walid Kriaa, Vandenbulcke Franck, Mejdoub Hafedh, Amine Elleuch. ***De novo* constructed cDNA library of *Phoenix dactylifera* L. cv Deglet Nour reveals candidate genes involved in response to cadmium stress**. En cours

B/ Zayneb Chaâbene, Imen Rekik Hakim, Walid Kriaa, Amine Elleuch, Franck Vandenbulcke, Hafedh Mejdoub. ***In silico* prediction of copper-responsive genes regulatory network and qPCR genes expression of vitro-plants of date palm**. En cours

C/ Chaâbene Zayneb, Rekik Hakim Imen, Kriaa Walid, C. Douglas Grubb, Khemakhem Bassem, Vandenbulcke Franck, Mejdoub Hafedh, Elleuch Amine. **The phytochelatin synthase gene in date palm (*Phoenix dactylifera* L.): Phylogeny, evolution and expression**. *Ecotoxicology and Environmental Safety* 140 (2017) 7–17.

A-Transcriptome assembly and abiotic related gene expression analysis of *Phoenix dactylifera* L. cv Deglet Nour reveal candidate genes involved in response to cadmium stress

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I- Synthèse

La réponse au stress cadmique des plantes non-modèles est peu connue. Ce travail représente la première analyse du transcriptome de *Phoenix dactylifera* de la variété Deglet Nour. L'utilisation de la plate-forme Illumina GA IIx a permis de développer une approche puissante et efficace pour explorer la réponse au Cd à partir de l'information transcriptomique.

A partir de feuilles de palmier dattier collectées sur un site pollué du sud de la Tunisie, les ARNs ont été isolés puis séquencés massivement.37 049 lectures Illumina RNA-seq d'ARN (d'une longueur moyenne de 1.021 bp) ont été produites. Ce grand ensemble de données EST généré a été assemblé par le programme CLC Genomics Workbench (Table II-2). L'assemblage du transcriptome a permis l'obtention de 6789 contigs et 17 285 singletons respectivement d'une longueur moyenne de 858 pb et 1 042 (Table II-2).

L'annotation fonctionnelle du transcriptome généré a eu recours à des bases de données non redondantes (nr) et d'autres d'*Arabidosis thaliana* publiées dans NCBI. Utilisant le programme BLASTx, plus que 98 % des transcrits obtenus ont présenté une homologie avec des nr surtout de la variété khalas. De plus, 12 354 peptides ont été identifiés chez Deglet Nour présentant au moins 60 % de similarité et 30 % de couverture avec les gènes correspondants d'Arabidopsis (Figure II-1). La création d'un consortium génétique à l'aide du programme Blast2GO a permis de prédire une classification fonctionnelle des gènes du

transcriptome assemblé (Figure II-2). Parmis les 24 074 séquences obtenues, 16179 interviendraient dans le métabolisme de la plante et 17 753 séquences seraient associées à des processus cellulaires, ont été prédites. Davantage de détails sur les fonctions des gènes obtenus ont été prédits par le programme KEGG. 96 % du total des gènes identifiés semblent impliqués dans le métabolisme cellulaire de Deglet Nour mais une partie importante des gènes identifiés seraient des gènes responsables du traitement de l'information environnementale (Figure II-4). Par exemple, des gènes des voies de détoxications du Cd telle que **la voie du glutathion** a été prédite en tant que marqueur génétique capable de faciliter la détection des variations fonctionnelles de l'espèce sous stress (Figure II-5).

La construction d'une base solide basée sur les profils transcriptionnels a aidé à entamer une étude moléculaire de la réponse du végétal au Cd. Des gènes mis en lumière par l'analyse bioinformatique ont été spécifiquement étudiés au moyen d'une amplification quantitative en temps réel (qPCR). Le suivi de l'expression des gènes codant pour des chélateurs ; phytochélatine synthase (*Pdpcs1*) et métallothionéine (*Pdmt3*), et des transporteurs ; ATP binding cassette (*Pdabcc*), ATPase (*Pdhma2*), MATE (*Pdmate5*) et Natural resistance-associated macrophage proteins (*Nramp6*), a été effectué sur des cultures *in vitro* de Deglet Nour exposés pendant deux mois à des concentrations croissantes de $CdCl_2$ dans les milieux de culture (Table II-5). Des variations d'expression génique spécifiques pour chaque gène candidat ont été obtenues au cours de différentes phases de développement des explants. Ces gènes n'étaient donc pas seulement présents mais se sont avérés opérationnels chez *P. dactylifera*. Les différentes concentrations de Cd et le temps d'exposition ont significativement ($p < 0{,}000$) influencé l'expression des gènes dans les tissus. Notons, le très informatif profil d'expression obtenu pour *Pdpcs1* qui reste fonctionnel même à haute concentration cadmique et après une longue durée d'exposition (Table II-5).

Cette première caractérisation du transcriptome a permis : 1- de mettre en évidence des réseaux de gènes réactifs au Cd ; 2- a soulignée l'activation de nombreux gènes impliqués dans la réponse au stress et 3- a fourni une ressource précieuse pour l'analyse fonctionnelle future des gènes candidats dans la réponse à différents xénobiotiques.

II-Article

Transcriptome assembly and abiotic related gene expression analysis of *Phoenix dactylifera* L. cv Deglet Nour reveal candidate genes involved in response to cadmium stress

Abstract

Background: In North Africa, as in several tropical countries, oasis cultures consist of date-palm groves (*Phoenix dactylifera* L., 2n = 36), which are major factors of social, environmental and economic stability in these regions. *P. dactylifera* extensively occupies south of Tunisia habitats representing a wide-range abiotic tolerance and biotic resistance and thus harbors valuable genetic resources that may greatly benefit genetic improvement of cultivated date palm.

Results: In the present study, we report an annotated transcriptome assembly for *P. dactylifera v* Deglet Nour. RNA was isolated from representative plant tissues, and 37,049 unique Illumina RNA-seq reads were produced and used in the transcriptome assembly. The draft transcriptome assembly consists of 6789 contigs and 17.285 singletons with a means length of 858 bp and 1.042 respectively. The final assembly was functionally annotated using Blast2GO software, allowing the identification of putative genes controlling important agronomic traits such as cadmium transporter genes and Glutathione Metabolism. The annotated transcriptome data sets were used to query all known Kyoto Encyclopedia of Genes and Genomes (KEGG) biosynthetic pathways. The most represented molecular functions and biological processes were nucleotide binding and transcription, transport and response to stress and abiotic and biotic stimuli.

We predicted the genes interaction network by selecting corresponding functionally similar genes from *Arabidopsis* datasets, downloaded by GeneMANIA version 2.1. Several putative proteins were found to participate in cadmium transport and transduction of signal pathway in the network. We also identified 1297 transcription factors and 733 protein kinases that may be involved in the resistance to abiotic stress. Thus several Cd-responsive genes expression was monitored. Chelators encoding genes *Pdpcs1* and *Pdmt3* were upregulated and confirmed *in silico* findings. Genes encoding HMs transporters (*Pdabcc*, *Pdhm2*, *Pdnramp* and *Pdmate*) in date palm showed expression enhancement more pronuced after 20 days of exposure.

Conclusion: The *P. dactylifera* transcriptome highlights the activation of a large set of metal resistance genes in this species and provides a valuable resource for future functional analysis of candidate genes in metal stress response.

Keywords: Date palm, *Phoenix dactylifera*, transcriptome, cadmium stress, Illumina RNA-seq, gene expression

1. Introduction

The date palm (*Phoenix dactylifera* L.) is a woody plant belongs to the Arecaceae family, cultivated in the Middle East and North Africa (El-juhany, 2017).There are several palm species including African palm oil (*Elaeis guineensis*), coconut (*Cocos nucifera*) and the date palm (FAO, http://www.fao.org). The other two species of date palm are peach palm (*Bactris gasipaes*) and betel palm (*Areca catechu*). *Phoenix dactylifera* is a valuable tree because very high sugar content (50 % of the total weight of mesocarp) of the fruit (dates) (Baize et al., 2002).This species plays an important role in locations where it is planted. Its fruits are considered food, their trunks were used as architectural materials and palm trees are used as ornamental plants. The arid desert climate is very beneficial for *P. dactylifera*. We assume that this species contains genes for resistance to biotic and abiotic stresses.

One of the major problems of agriculture is the accumulation of heavy metal ions that are very toxic to cells. According to their effect on plant growth they can be divided in two categories. 1- Essential minerals; trace amounts of which are necessary for growth. The excess of these ions causes toxicity. 2- Non essential ions with no biological function in higher organisms cause toxicity such as cadmium (Cd) and lead (Pb). Cd is absorbed by the roots and transported to the shoot, thereby adversely affecting the absorption of nutrients and perturbing homeostasis in plants even at low concentrations. Cd^{2+} ions have adverse impacts on various biochemical and physiological processes (Krämer et al., 2007), including changes in the transcriptome and proteome of plants, resulting in the limitation of the process of seed germination and development (Thapa et al., 2012), the inhibition of the growth of shoots and roots, and ultimately the overall performance reduction of the plant (Clemens et al., 2002; Roth et al., 2006; Zhang et al., 2015). Because of its harmful effects, the use of Cd has been banned strictly in variable materials such as plastic material since December 2011 (the Regulation of the European Commission REACH No 1907/2006 of 18 December 2006). Despite these restrictions and prohibitions, another 96 tons of Cd were released into the atmosphere (EEA, 2013) in Europe. The metal continues to exist in arid countries soils such as Tunisia soil due to the emergence of heavy metals (HMs) contamination in mining wastes, the use of phosphate fertilizers and (Cd)-rich irrigation water. With a long biological half-life of 17 to 30 years (Tanase et al., 2012), the metal persistent in soil is a matter of concern for

soil fertility. It increases the danger for plants, seed grains and human health. Therefore, it is important to study the mechanisms of plant defenses to cope with Cd^{2+} exposure and toxicity. The development of transcriptomic responses helps plants survive and resist to abiotic stresses. An initial sensory activation phase, followed by a physiological response, is usually induced by abiotic stresses in plants (Mahajan and Tuteja, 2005; Zhu et al., 2003). Many stress response studies have been performed in *Arabidopsis thaliana* (Matsui et al., 2008; Zeller et al., 2009). However, little is known about stress responses to cadmium of non-model plants especially *Phoenix dactylifera* which showed a great potential for Cd accumulation (Zayneb et al., 2015).

In recent years, next generation sequencing has provided a rapid and effective approach to generate transcriptomic and genomic data from several non-model organisms (Chew et al., 2009; Tanase et al., 2012). RNA sequencing (RNA-Seq) is an effective and economic approach compared to whole genome sequencing in plants with relatively big genomes. This technology is valuable in functional genomics research (Alsford et al., 2011)and in the studies of the level of expression of new genes (Halvardson et al., 2013; Xiang et al., 2014).

In this study, we present the first analysis of the transcriptome of *P. dactylifera* L. cv Deglet Nour using the Illumina GA IIx platform. The generated large EST dataset enables us to broaden our knowledge of the genomic resources of the plant and to investigate the gene activities in diverse tissues and different stages under Cd-stressful conditions. Here, we used RNA-seq technology to explore the transcriptional profiles as well as to identify putative candidate genes and functional elements which determine the main metabolic pathways associated with resistance to Cd stress. A proportion of differentially regulated genes was identified and validated by quantitative real time polymerase chain reaction (qPCR). Based on the monitoring of gene expression variation along time exposure to different Cd concentrations, we represent a first comprehensive transcriptome-based characterization of Cd responsive gene regulatory networks in date palm. These genes are crucial to strength efforts for the genetic improvement of date palm.

2. Materials and methods

2.1. Plant material

Leaves of *Phoenix dactylifera cv* Deglet Nour have been used in this study. Sampled trees are located in Gabes in south of Tunisia. This area is submitted to strong heavy metals pollution.

After thorough washing with double distilled water, samples were immediately frozen in liquid nitrogen and transported to the laboratory on dry ice. The samples were stored at −80 °C until use.

2.2. Preparation of cDNA library for transcriptome sequencing

The CTAB method [15] was used for the extraction of total RNA from leaf tissues of *Phoenix dactylifera* CV DegletNour. RNA integrity was analyzed by capillary electrophoresis on an Agilent BioAnalyzer 2100. PolyadenylatedRNA was selected using oligo(dT) purification and reverse-transcribed to cDNA. These cDNAs were fragmented, blunt-ended, andligated to the Illumina TruSeq Adaptor Index 3 (Illumina Inc., http://www.illumina.com). The library was size-selected for an insert size of 250bp, and quantified using the Invitrogen Pico Green dsDNA assay (Life Technologies).

2.3. Illumina sequencing

From both 5' and 3' ends, the cDNA library was sequenced on the Illumina GA IIxplatform according to the manufacturer's instructions. The fluorescent images processing to sequences, base-calling and quality values calculation were performed by the Illumina data processing pipeline (version 1.4), in which 75 bp paired-end reads were generated.

2.4. Bioinformatics and Data mining

The CLC Genomics Workbench 8.1 de novo assembly tool was used to assemble the reads into contigs. Functional annotations and gene ontologies were predicted for each contig using Blast2GO software (Conesa et al., 2005) with the following parameters: BLASTx against the NCBI non redundant protein database and TAIR10 release of the Arabidopsis transcriptome and proteome (http://www.arabidopsis.org) (Lamesch et al., 2012), BLAST E-value = 0.001 and reporting the top 20 hits. After getting GO annotations for each transcript, WEGO software (Ye et al., 2006) was used to conduct GO functional classification. Furthermore, for all date palm sequences, the KEGG Automatic Annotation Server (http://www.genome.jp/tools/kaas/) was used to identify KEGG orthologs with default parameters. Domain-based comparisons with KOG (Eukaryotic Orthologous Groups) were carried out using RPS-BLAST (Reverse PSI-BLAST) tool from NCBI BLAST+ v2.2.18 software run locally. Using Geneious V5.6.2 (Biomatters; http://www.geneious.com), the underlying sequences for each contigs and singletons were trimmed and mapped to the

respective mRNA of *Phoenix dactylifera* cv khalas. SNPs were identified and counted for sites with ≥4× read coverage, where at least two of the reads contained a mutation at that site. Transcription factors (TF) and protein kinases in the cDNA library were identified and classified into different groups using the iTAK pipeline (http://bioinfo.bti.cornell.edu/tool/itak/) (Khachatryan et al., 2010). The presence of Simple Sequence Repeats (SSRs) in the *P. dactylifera* transcriptome was identified *in silico* using GDR server (http://www.rosaceae.org/search/markers).

2.5. Interaction Network Exploration

The genes interaction network was predicted by using selected corresponding functionally similar genes from *Arabidopsis* datasets, downloaded by GeneMANIA version 2.1 (Warde-farley et al., 2010; Zuberi et al., 2013) (http://www.genemania.org/) and visualized in Cytoscape (Montojo et al., 2010).

2.6. *In vitro* experimentation

Isolated explants of date palm cultivar Deglet Nour were obtained from the laboratory of vegetable biotechnology applied on the amelioration of culture, university of Sfax, Tunisia. Explants were transferred under sterile conditions into the Murashige and Skoog (MS) (1962) medium supplemented with different concentrations of $CdCl_2$ (0.02, 0.2 and 1 mM) and incubated in 16h photoperiod at 24 ° C during 60 days of exposure. Date shoot clusters of 20, 40 and 60 days of development were used for total RNAs extraction.

2.7. Total RNA isolation, cDNA synthesis and gene identification

RNAs extraction and cDNA synthesis were performed as per Chaâbene et al. (2017). From the previously constructed Deglet Nour date palm cDNA bank, we looked for ABC transporter C family membre 2 like (*abc*), *Atpase*, Multi-antimicrobial extrusion protein *mate*, natural resistance-associated macrophage protein (*Nramp*) coding genes. Homologous sequences of genes from various organisms recognized in NCBI (http://www.ncbi.nlm.nih.gov/) using BLASTn search has been aligned against clones nucleotide sequences from our date palm cDNA bank using BioEdit version 7.0.5.3. Some clones revealed partial homology with selected genes. Flowing methods described by (Chaâbene et al., 2017b) enabling retrieval of gene sequences coding for phytochelatin synthase (*Pdpcs*) and metallothionein (*Pdmt*), we have identified *Pdabcc*, *Pdhma*, *Pdmate* and *PdNramp*.

2.8. Real time qPCR amplification

Using candidate gene specific primers designed by Primer3Plus on line software (Rozen and Skaletsky, 2000; http://frodo.wi.mit.edu/) and verified using Net Primer and Beacon Designer programs (Table II-1), we performed quantitative real-time polymerase chain reaction (qPCR) amplification method according to Brulle et al. (2014). The expression levels and the relative fold expression (RFE) were determined according to previously described procedures (Brulle et al., 2006). The geometric mean of the 3 most stable reference genes in control and Cd-stressful condition identified by Chaâbene et al. (2017b) was used to calculate expression of target gene levels according to Brulle et al. (2014). Absolute quantification of genes expression levels are in log2.

Table II-1. qPCR Primer sequences of candidate genes used in this study

Name	Amplification length (bp)	qPCR specific Primer sequence (5' → 3')	Length (bp)	Tm	GC %	PCR Efficiency ±SD
Pdabcc	104	F : GATTTGCTTCCAGGAGGTGA	20	60.2	55.0	1.97±0.01
		R : TCCGAATATACTGCCCTTGC	20	60.1	50.0	
Pdhma2	162	F : CATGCAACCACAAGCAAGAC	20	60.3	55.0	2±0.01
		R : TCGACGTTCAAACATAGAGGAG	20	59.4	54.5	
Pdmate5	103	F : CTTGCTCTCAAAGCGAAAGG	20	60.3	50.0	1.98±0.05
		R : TTCCGTCGCCAACACATAG	19	60.7	52.6	
PdNramp6	154	F : CGGAAGCTCTGGTCTCACA	19	60.1	57.9	1.95±0.04
		R : CGACAGCAACTGGATCAGAA	20	60	50.0	

2.8. Statistical analysis

The relative expression ratios between control and treated plants were transformed and subjected to a one-way ANOVA. Student test has been used to analyze significance were $p<0.05$ was considered to be significant.

3. Results and discussion

3.1. Sequencing and Data analysis

We performed three out of four Illumina GA IIxrun on one normalized cDNA library constructed from *Phoenix dactylifera* cv Deglet Nour leaves generating approximately 37.84 Mbp of sequence data from 37,049 reads with an average length of 1,021 bp. *De novo* transcriptome assembly was realized using CLC Genomics Workbench 8 program, which has been shown to perform better than a number of other commonly used assemblers. Table II-2

shows the transcriptome sequencing and assembly statistics: 19.54 % of the reads were assembled into 6,789 contigs, with an average assembled length of 858 bp and 17,285 singletons with an average length of 1,042 bp. The size distribution of transcripts was shown in Additional file 1: Figure II-S1.

Table II-2. Illumina RNA-seq reads and *de novo* assembly statistics

Statistics	**Singletons**	**All Contigs**
Number of sequences	17.285	6789
Min Length (bp)	130	105
Average Length (bp)	1.042	858
Max Length (bp)	7.410	5505
N50 size (bp)	2376	1254

3.2. Functional annotation of the *P. dactylifera* transcriptome

To comprehensively annotate the transcriptome of *P. dactylifera*, several complementary approaches were adopted. First, the 24,074 transcripts (contigs and singletons) were blasted against the NCBI non-redundant (nr) and *Arabidopsis* databases. A total of 23,717 (98, 52%) transcripts matched a homolog in the nr database using BLASTx program (E-value threshold of $1e^{-5}$). The top-hit species distribution of nr BLAST matches is shown in Figure II-S2A. Approximately 90.3 % of the sequences had significant matches with genes from *P. dactylifera* cv Khalas, followed by *Musa acuminate* (2.29 %) and *Vitis vinifera* (0.74 %). E-value distribution of the top hits in the databases had shown 12,3 % of matched sequences with strong homology (< $1\ e^{-180}$; (Fig. II-S2B).

To study the level of global sequence conservation between *P. dactylifera* and *A. thaliana*, we realized a BLASTx comparison of the *P. dactylifera* transcriptome assembly to the Arabidopsis TAIR10 peptide database (Table II-S1). The homology of each predicted peptide to the most similar Arabidopsis protein was measured by the percentage of similarity (Fig. II-1a) and percentage coverage (Fig. II-1b). A smooth scatter plot representing the percentage similarity and percentage coverage for each *P. dactylifera* sequence compared to the closest *Arabidopsis* peptide sequence is shown in Fig. 1c. A large proportion of transcripts (>85 %) show at least 60 % similarity to an Arabidopsis proteins. A total of 12,354 *P. dactylifera*

predicted peptides had at least one match to an *Arabidopsis* gene with >60 % similarity >30 % coverage (Fig. II-1c, boxes).

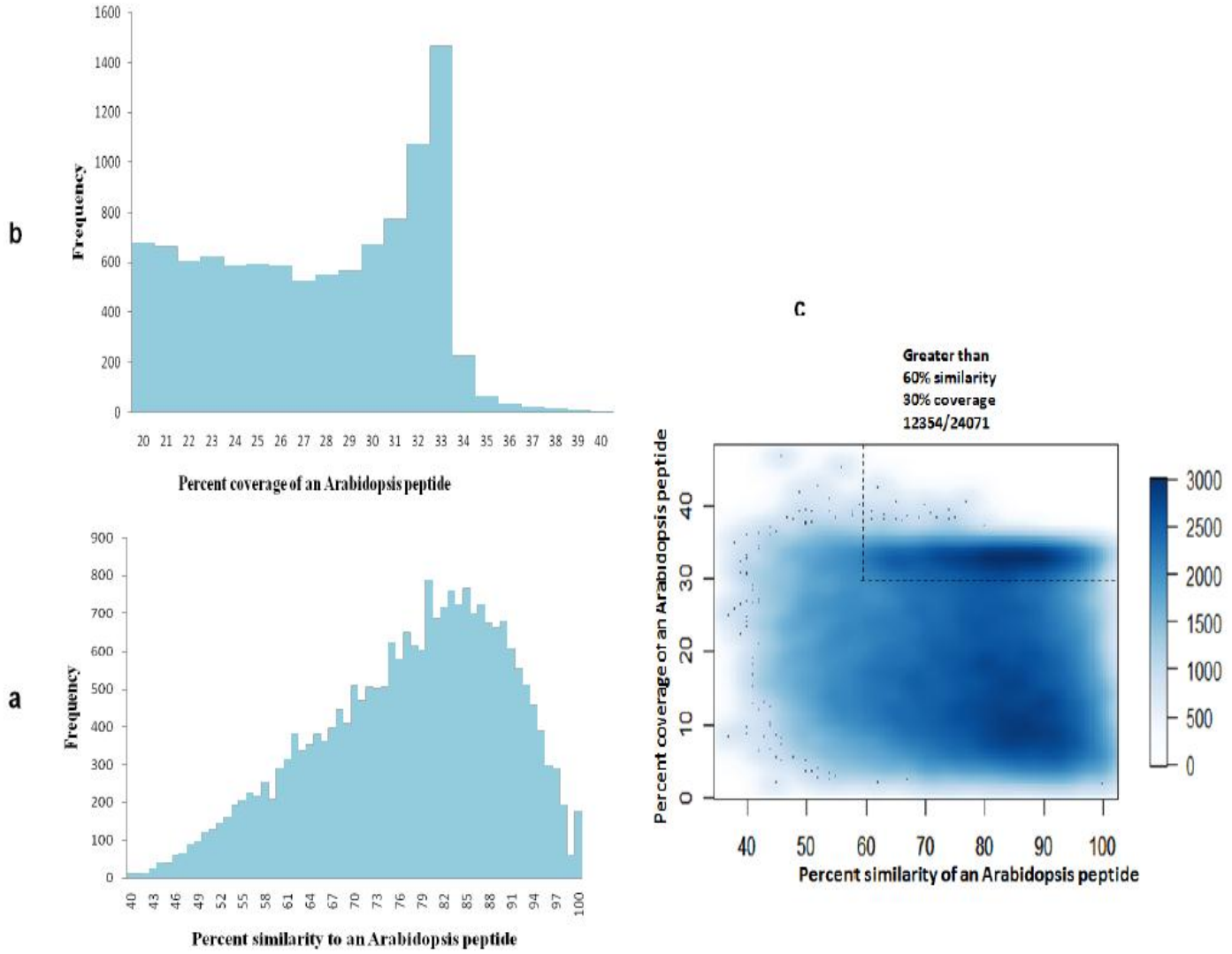

Figure II-1. Similarity and coverage of *Phoenix dactylifera* transcripts versus *Arabidopsis* genes. (a) Histogram showing frequency versus percentage similarity (positive amino acid identity) of *P. dactylifera* contigs versus an *Arabidopsis* peptide. (b) Histogram showing frequency versus percentage coverage (longest positive hit/peptide length) of *P. dactylifera* contigs versus an *Arabidopsis* peptide. (c) Smoothed color density representation of the percentage similarity (x axis) of each *P. dactylifera* transcript plotted against the percent coverage of the *Arabidopsis* protein similarity (y axis). The plot was produced using the 'smooth Scatter' function in R (R Development Core Team, 2008), which produces a smoothed density representation of the scatter plot using a kernel density estimate (nbin= 100). Darker color indicates a higher density of transcripts in a given position, with the darkest 'bin' containing over 3000 transcripts. Boxes encompassing transcripts encoding peptides with 60 % sequence similarity and 30 % coverage are shown in the upper right corner.

The creation of the consortium GO (gene ontology) is a joint project of many databases from different organizations with a structured and controlled vocabulary to describe gene functions and gene products in specific organizations (Harris et al., 2000). Therefore, we used the Gene

Ontology (GO) classification system to annotate the possible functions of our genes. Briefly, the sequences with a best match from *Arabidopsis* database were further assigned with GO terms and Enzyme Commission (EC) numbers using the Blast2GO platform. A total of 38,379 GO terms were assigned to 21,655 cDNAs (including contigs and singletons) (Table II-3).

Table II-3. Summary of annotation of *P.dactylifera* transcripts

Category	**Number of transcripts**	**Percentage**
TAIR annotated transcripts	21655	89.95%
KOG classified	15020	62.39%
KEGG classified	10632	44.16%

Among all the GO terms extracted, 2391 GO term (6.22 %) belong to the Molecular Function class, 4,272 (11,13 %) to Biological Process class and 773 (2 %) to Cellular Component class. There are 12,519 cDNAs assigned to multiple GO terms (Fig. II-2).

The GO terms of Biological Process (BP) category comprise different types of metabolic processes (the most representative categories). We found 16,179 sequences associated with metabolic processes and 17,753 sequences associated with Cellular Process (GO level 2) (Fig. II-S3a). This result is in agreement with the metabolic network in plants which is more extensive compared to other organisms (Aharoni, 2010).

We also identified GO terms of primary metabolites (sugars, amino acids, nucleotides, lipids) and energy sources that are essential for plant survival. Another category worthy to mention is "response to stimulus" (BP category, level 2). We found 13,216 sequences associated with this category, which include candidate genes for resistance to biotic and abiotic stress. We found GO terms associated with secondary metabolites that play key roles in maintaining plant wellness and the protection of plants against metallic stress.

In the molecular function (MF) category, 11,768 transcripts haven high similarity to proteins with binding activity (nucleotide binding, ion binding and oxidoreductase binding ; Fig. II-S3b) and 9,815 transcripts for catalytic activity.

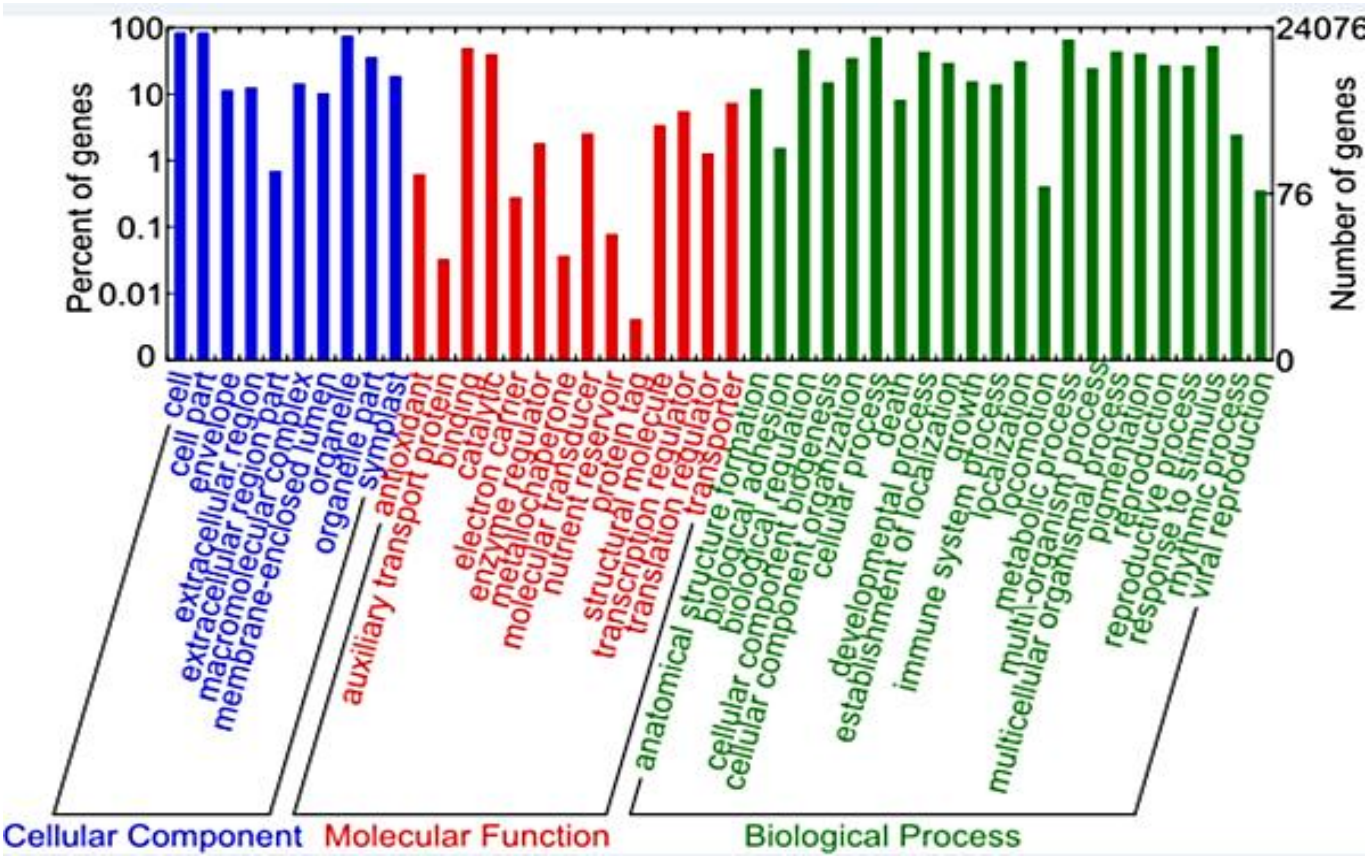

Figure II-2. Gene ontology classification of assembled transcripts

Among the 21,655 sequences annotated with GO terms, 6,692 sequences were assigned with EC numbers. In detail, transferase activity (39 %), hydrolase activity (29 %) and oxidoreductase activity (16 %) were the most represented enzyme activities (Fig. II-3).

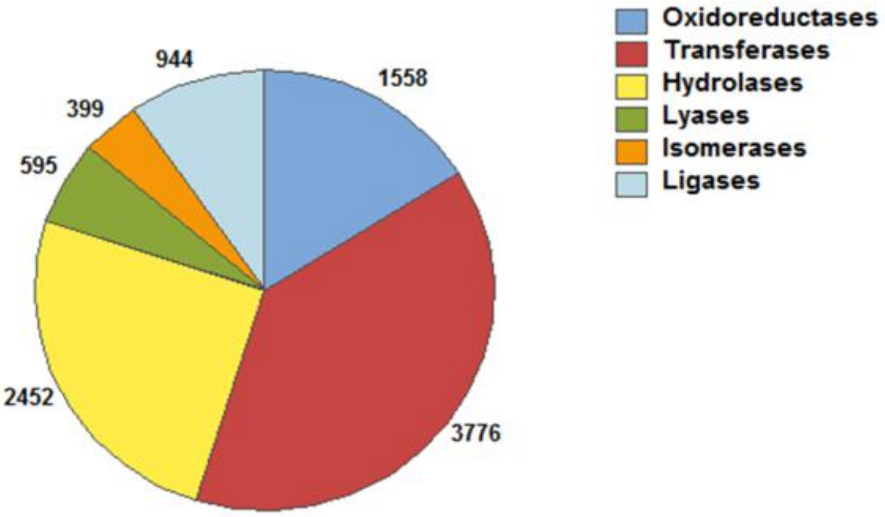

Figure II-3. Catalytic activity distribution in annotated *P. dactylifera* transcripts

The genes associated with pathways of secondary metabolite biosynthesis (Torales et al., 2013) represent a large number of annotated enzymes within these three groups.

3.3. Functional classification by Kyoto encyclopedia of genes and genomes (KEGG)

The KEGG (Kyoto Encyclopedia of Genes and Genomes) could be an info linking genomic data with higher order, purposeful data. It represents a collection of manually drawn pathway maps representing current data on cellular processes and standardized factor annotations (Matsuzawa-Nagata et al., 2008).

By mapping EC numbers to the referral canonical pathways, 10,632 transcripts (44.16 %) had important matches and were appointed to 140 KEGG pathways (Table II -S2, Table II-4, Fig. II-S4).

Table II-4. KEGG pathway mapping results

Kegg pathways	sequences number
Metabolism pathway	9341
Carbohydrate metabolism	2419
Energy metabolism	424
Lipid metabolism	1098
Nucleotide metabolism	714
Amino acid metabolism	712
Metabolism of other amino acids	1850
Metabolism of cofactors and vitamins	508
Metabolism of terpenoids and polyketides	272
Biosynthesis of other secondary metabolites	584
Xenobiotics biodegradation and metabolism	500
Glycan biosynthesis and metabolism	260
Environmental Information Processing	**338**
Genetic Information Processing	**77**

‘‘Metabolism’’ is the biggest class (9,341 sequences, 96.5%), followed by Environmental Information Processing (338, 2.11%) containing solely a pair of sub-units (‘‘mTOR signaling pathway’’ (51 sequences) and a second set of ‘‘mTOR signaling pathway’’ (154 sequences)). ‘‘Genetic Information Processing’’ (77, 0.8 %) was the smallest class. The phosphatidyl inositol signaling system comprised kinase activity (99 sequences) and phosphtase activity (55 sequences) and alternative enzymes (Table II-S3). Many studies illustrated that the phosphatidyl inositol signaling system provide a vital role in resistance to abiotic stresses (Levine, 2002; Zhu et al., 2003; Fig. II -S5).

Figure II-4 shows the eleven KEGG metabolic pathways largely matched by transcripts of *P. dactylifera*.

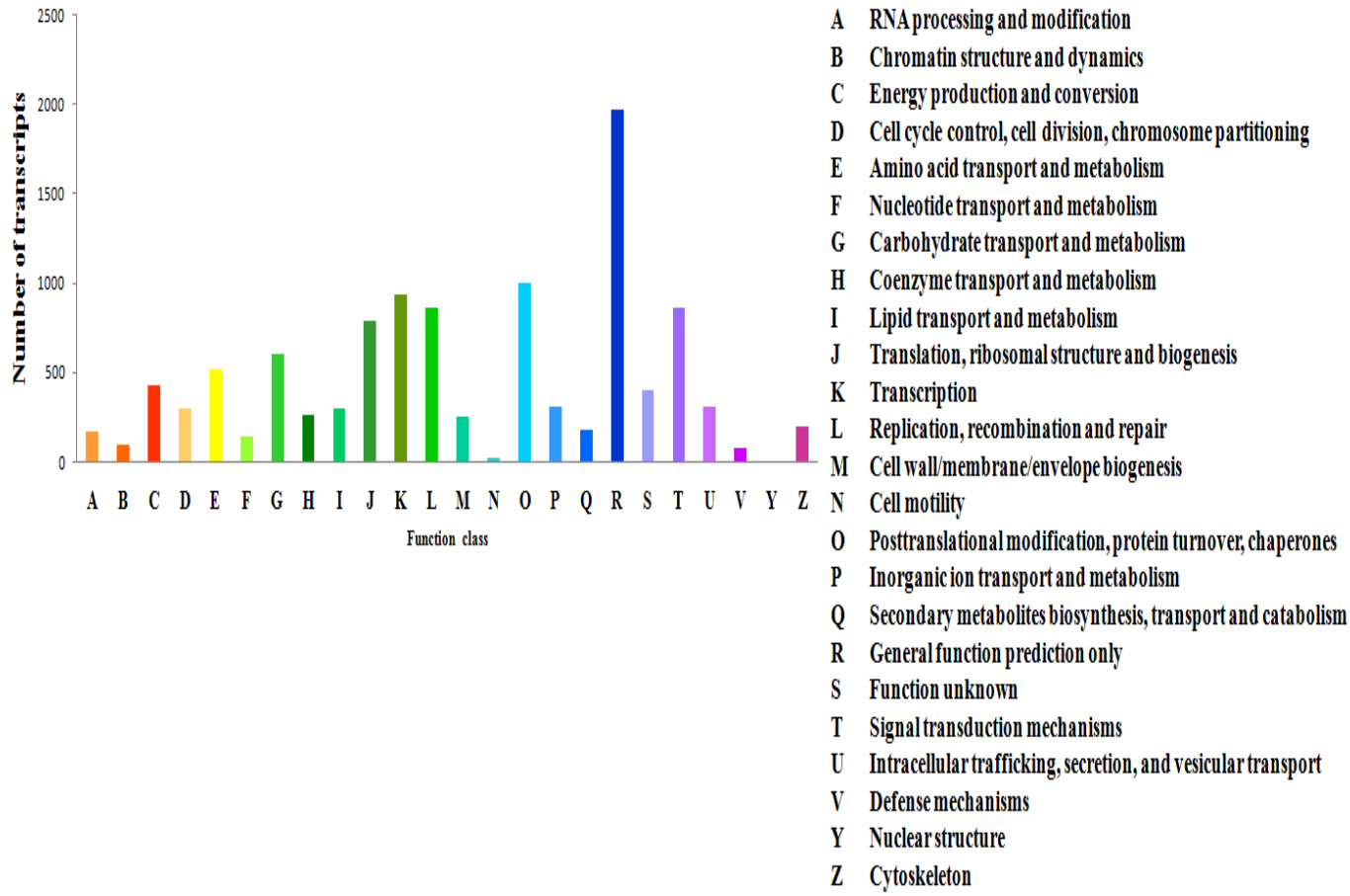

Figure II-4. KOG function classification of the *P. dactylifera* transcriptome. A total of 15020 with significant homologies to the KOG database (E-values≤$1.0E-6$) were classified into 25 KOG categories.

In metabolism pathways, we found that polypeptides involved in amino acids, carbohydrates and lipid metabolism consisted of about 65% of the whole sequences. The relative quantity of proteins belonging to metabolism of cofactors and vitamins (MCV), xenobiotics biodegradation and secondary metabolites (BGSM), and nucleotide metabolism are conjointly represented by a sizable amount of sequences (Table II-S2).

We additionally found 221 transcripts involved in phenyl propanoid biosynthesis, 152 sequences related to glutathione metabolism and 128 sequences assigned to TCA cycle. These pathways, better-known to induce defense response in plants through the salicylic acid and Ca^{2+}- related signaling pathways (Ahn et al., 2005; Goyer, 2010), might play roles in biotic or abiotic stresses (Korkina, 2007).

Moreover, secondary metabolites like phenyl propanoid derivatives play vital roles in resistance to pathogens and are recently used in health issues given their antioxidants, anti tumor and anti-inflammatory activities (Amiard et al., 1998; Korkina, 2007).

3.4. Candidate genes for cadmium transporters, Signal Sensing and Transduction Proteins

The evolutionary relationship between *Phoenix dactylifera* and Arabidopsis enabled identification of potential date palm orthologs responsible for controlling metal transport and stress response. For each *P. dactylifera* transcript, the top 20 BLASTx hits against the Arabidopsis peptide database were mined for hits to Arabidopsis genes known to control these traits. Metal carriers could play a very important role in reducing the intracellular concentration of heavy metals through the transport of metal ions out of the cell or sequestering them in the vacuole. There are several metal transporters families that can contribute to the resistance of plants to heavy metals (Hall, 2002) including ATP binding cassette (ABC), Natural resistance-associated macrophage proteins (Nramp), Zrt- and IRT-related proteins (ZIP), Cation Exchanger (CAX) and Pleiotropic Drug Resistance protein family (PDR). To investigate the conservation of the metal transport pathway, we attempted to reconstruct the transport and signal transduction pathways in date palm using predicted peptides from the transcriptome assembly. Using keywords "cadmium ion Transport: GO: 0015691", 53 genes were identified as candidate genes involved as members of different metal transporter families. We predicted using the program GeneMania interactions between these proteins which were mainly related to ABC (PDR and ABCG43), ZIP families, CAX and Nramp proteins (Fig. II-4, Table II-S3).

Plants are often exposed to changing environmental stresses to which they must resist to survive. Many biotic or abiotic extracellular stimuli can change the intracellular concentration of Ca^{2+} (Knight et al., 2014). These variations are responsible for the over expression of the various calcium-binding proteins and protein kinases (Myouga et al., 2008).

Cellular Responses to Ca^{2+} signals are mediated by different families of genes in plants, such as Ca^{2+} dependent protein kinases (CDPKs) and CDPK-related kinases (CRKs), by calmodulins (CAMs) and calmodulin-dependent protein kinases (CaMKs), by calcineurin B-like proteins (CBLs) and CBL interacting protein kinases (CIPKs, also called SnRK3s), and by Ca^{2+} and calmodulin-dependent protein kinases (CCaMKs). CCaMKs are present as single gene in a number of plant species having symbiotic interactions, but not in Arabidopsis [40].

Using keywords "regulation of metal ion Transport: GO: 0010959" for our search in the annotation results, we found 9 cDNAs calcineurin B-like protein (CBL), 30 cDNAs CBL-interacting protein kinase (CIPK), 19 cDNAs were identified that were highly homologous to genes encoding calcium-dependent protein kinase (CPK or CDPK) and 13 cDNAs histidine kinase (AHK) have a very important role in the regulation of transport of metal ions and transduction signals. The interactions between the proteins were predicted using the program GeneMania (Fig. II-4 and Table II-S4).

3.5. Identification of Candidate Genes Involved in Biosynthesis of Chelating Compounds and Glutathione Metabolism

Another mechanism used by plants for resistance against heavy metals is the synthesis of chelating metal compounds (Aarts, 2012). Metallothionein (MTs) are bonding metal rich peptides low molecular weight cysteine, which are generally classified into four groups (MT1-4). Currently, the MT genes have been identified in a number of higher plants such as Arabidopsis and *Brassica juncea* (Cobbett and Goldsbrough, 2002b).

In this study, we found six transcripts corresponding to homologous genes encoding MT2 and MT3 (Table II-S5). Phytochelatins (PC) is another important class of heavy metal-binding ligands, which can bind metal ions via thiolate coordination (Cobbett, 2000). Many studies have confirmed that glutathione (GSH) is the substrate for the biosynthesis of PC (Anjum et al.).The conversion of GSH to PC can be catalyzed by the enzyme phytochelatin sythase (PCS ; Yadav, 2010).

In the present study, we found four sequences that code for the enzyme glutathione synthase (EC 6.3.2.3) (Table II-S6). Based on the KEGG pathway assignment, 152 transcript

sequences were found to encode 16 putative enzymes involved in glutathione metabolism from the present *de novo* assembled transcriptome (Fig. II-5).

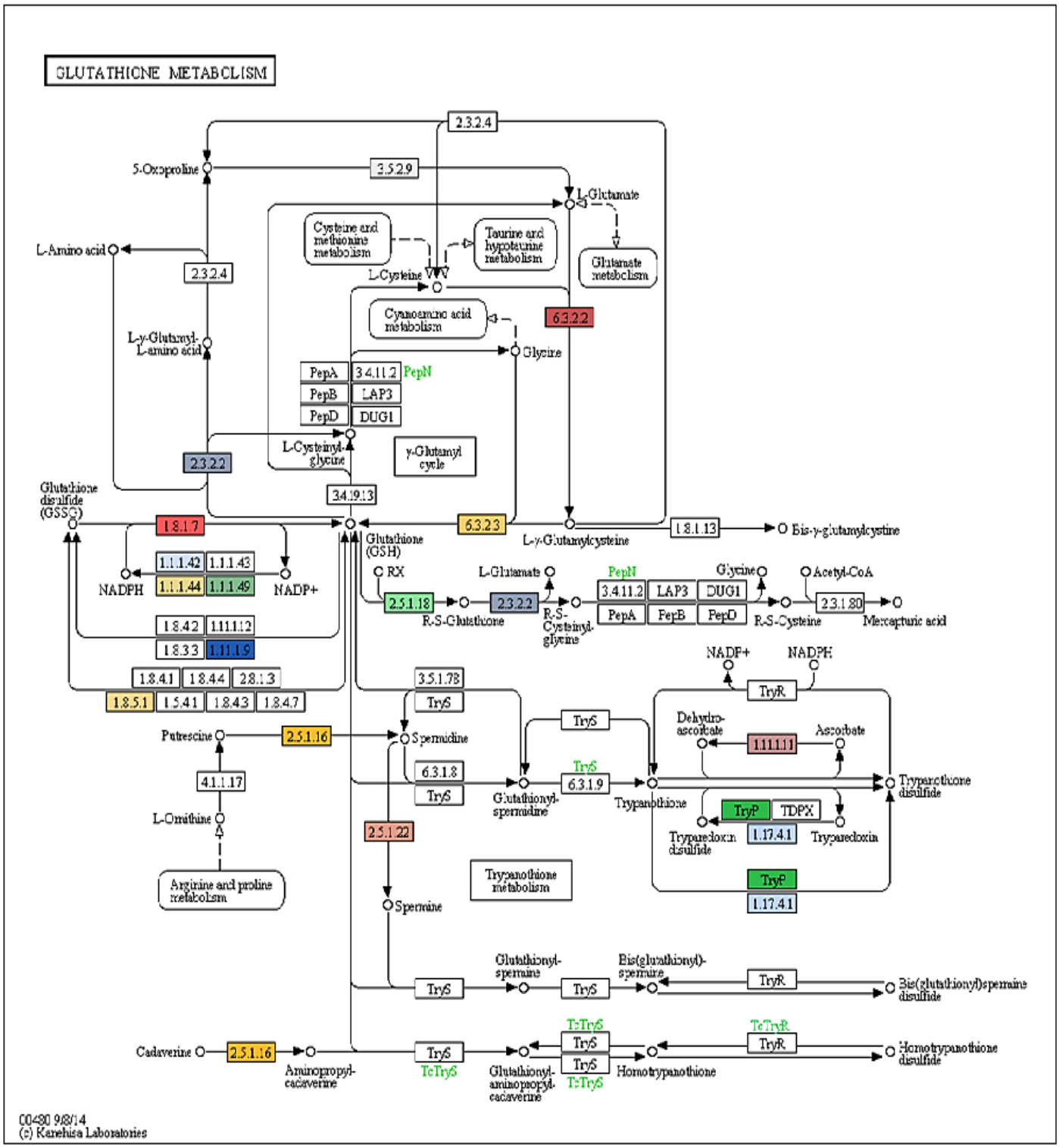

Figure II-5. Interaction network of cadmium transporters and regulation proteins in *P. dactylifera*. Protein interactions of metal-responsive genes were predicted by protein homologs in *Arabidopsis* by GeneMANIA analysis.

3.6. Response of Cd-related candidate gene expression variation

Explant *in vitro* generated leaves were selected to uncover the molecular basis involved in Cd^{2+} storage and tolerance capabilities of date palm cultivar Deglet Nour genotypes. The expression of orthologous genes known to respond to elevated metal concentrations in other plants was monitored by qPCR expression analysis allowing to study multiple durations of exposure. Each Cd-responsive gene was expressed in a specific manner along metal concentration variations during two months of treatment. Results obtained (Table II-5) proved that candidate genes are not only present but fully operational in *P. dactylifera*. Their expressions were altered during abiotic stress. Both Cd concentrations and time exposure influences significantly ($p < 0.000$) gene expression in vitroplant tissues (Table II-5).The mean relative expression values of biomarkers were presented after scaling with values of non-treated samples. The relative fold expression of candidate genes appeared differentially expressed (Table II-5). The Cd treatment induced the expression of target genes since the first 20 days of exposure (Table II-5).

Table II-5. Overview of relative expression pattern (log2) results in explant of *P. dactylifera* exposed to different Cd concentrations for 60 days. Data presented are means ± standard error of three independent experiments. Two-way ANOVA main effects shown as *p* values. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test. where small letters show differences between concentrations (in columns) and major letters show differences in time exposure (in rows). Means not showing the same letter are statically different ($p<0.05$). Genes relative fold expression (RFE, gene expression in exposed samples compared to gene expression in control samples) is presented by arrows; ↗ non-significant upregulation ($1.25<RFE<2.00$), ↑ significant upregulation ($RFE>2.00$), ↘ non-significant downregulation ($-2.00<RFE<-1.25$), ↓ significant downregulation ($RFE<-2.00$).

		20 d	**40 d**	**60 d**
Pdpcs1	0 mM	0.42 ± 0.04 aA	0.54 ± 0.02 aA	0.71 ± 0.06 aA
	0.02 mM	6.57 ± 0.10 ↑ bA	4.67 ± 0.10 ↑ bB	2.47 ± 0.04 ↑ bC
	0.2 mM	4.18 ± 0.05 ↑ cA	4.33 ± 0.12 ↑ bA	2.00 ± 0.00 ↑ cB
	1 mM	4.44 ± 0.20 ↑ cA	2.01± 0.10 ↑ cB	1.19 ± 0.04 ↗ cC

Pdmt3	0 mM	2.58 ± 0.00 aA	2.67 ± 0.04 aB	2.55 ± 0.00 aC
	0.02 mM	1.71 ± 0.01 bA	3.57 ± 0.09 bB	3.29 ± 0.01 bC
	0.2 mM	2.89 ± 0.06 cA	5.23 ± 0.71 cB	-3.53 ± 0.63 cC
	1 mM	3.28 ± 0.30 cA	-0.44 ± 0.07 aB	-1.76 ± 0.66 cB
Pdabcc	0 mM	0.94 ± 0.05 aA	1.09 ± 0.01 aB	1.30 ± 0.00 aC
	0.02 mM	2.8 ± 0.12 bA	9.07 ± 0.1 bB	-0.36 ± 0.15 bC
	0.2 mM	1.43 ± 0.04 cA	0.33 ± 0.01 aB	0.72 ± 0.03 cC
	1 mM	2.07 ± 0.1 dA	-1.64 ± 0.21 cB	2.24 ± 0.14 dA
Pdhma2	0 mM	4.34 ± 0.07 aA	2.71 ± 0.12 aB	3.34 ± 0.07 aC
	0.02 mM	6.1 ± 0.15 bA	4.93 ± 0.09 bB	1.93 ± 0.08 bC
	0.2 mM	4.63 ± 0.19 aA	3.43 ± 0.09 cB	1.64 ± 0.36 bC
	1 mM	6.72 ± 0.24 bA	3.85 ± 0.06 cB	0.82 ± 0.1 cC
Pdmate5	0 mM	4.27 ± 0.1 aA	3.45 ± 0.17 aB	4.8 ± 0.14 aA
	0.02 mM	6.87 ± 0.04 bA	8.05 ± 0.07 bB	6.3 ± 0.12 bC
	0.2 mM	5.92 ± 0.1 cA	6.55 ± 0.08 cB	4.8 ± 0.13 aC
	1 mM	6.01 ± 0.02 cA	2.02 ± 0.03 dB	1.59 ± 0.13 cC
PdNramp6	0 mM	5.26 ± 0.09 aA	5.78 ±0.01 aB	5.57 ± 0.11 aAB
	0.02 mM	6.6 ± 0.15 bA	9.29 ± 0.13 bB	6.01 ± 0.02 aB
	0.2 mM	7.92 ± 0.09 cA	6.79 ±0.13 cB	-0.16 ± 0.04 bB
	1 mM	9.09 ± 0.14 dA	4.07 ± 0.11 dB	2.65 ± 0.92 cC

However, *Pdpcs1* induction factor (more than 15 folds) in date palm explant exposed to 0.02 Cd during 20 days of exposure is more than in date palm hypocotyl tissues (more than 4 folds) (Chaâbene et al., 2017b). This may be to genetic diversity date palm seeds which may produce less tolerante varieties than Deglet Nour cultivar. *Pdmt3* was found to be the less

sensitive gene. This latter showed interesting non-significant induction during explant development in standard conditions (Table II-5).

Metallothionein are cysteine (Cys)-rich proteins that detoxify heavy metals, participate in the regulation of the homeostasis of essential metals like zinc and copper (Dabrowska et al., 2012) containing in MS medium and in controlling the concentration of ROS by activating defenses, e.g. via the mitogen-activated protein kinase MAPK cascade (Finatto et al., 2015). Increasing metal time exposure further induced genes expression under 0.2 mM Cd. Significantly highly induction of *Pdabcc* has been obtained under 0.02 and 0.2 mM Cd during 40 days of treatment. *PdABC* expression reached a maximum (9.07 ± 0.1) at 0.02 mM. However, the expression level of the other HMs transporters (*Pdhma2*, *PdNramp* and *Pdmate*) expressed a significant slightly increase. High enhancement of ABC encoding genes by Cd stress in wheat; *Taabcc3*, *Taabcc4*, *Taabcc11*was showed (Bhati et al., 2015). Indirectly, *abcc* may be induced by the accumulation of H_2O_2 in Cd-stressed cells. ABCC are known to be involved in transport and detoxification of glutathione-conjugates (Bhati et al., 2015). Whereas, at high Cd amount (1 mM Cd), negative values of absolute quantification of *Pdabcc* was obtained after 40 days of metal stress (Table II-5). Similarly, some of the wheat ABCC genes showed suppression in the expression under Cd stress (Bhati et al., 2015). Whereas, decrease in *Pdmate5* and *PdNramp6* has been shown. Although they still induced, *Pdmate5* and *PdNramp6* remained lower than the control at high metal concentration after 40 days of stress. In contrast under these conditions, *Pdhma2* showed more resistant capability to metal stress (Table II-5). This later has important Cd transport activity in cells. However, it lack high specificity because it is involved in Zn transport in addition to Cd (Ueno et al., 2010). Likewise, Nramp family lack Cd transport specificity and acts as an efflux pump of elements in the membrane controlling metal availability (Bereczky et al., 2003). Furthermore, comprising the multidrug transporter superfamily (MTS), MATEs are responsible for secondary metabolite transport (Thompson et al., 2010). It has been shown that MATE transporters mediate the citrate efflux to confer plant tolerance to Al toxicity (Magalhaes, 2010). Further protein functions maintained related genes expression even at high Cd amount. However, increasing metal exposure further decreased gene expression indicating the progression of Cd stress severity. Table 5 showed that higher Cd amount suppressed target genes expression in date palm explant especially *Pdmt*, *Pdabcc* and *PdNramp6*. This linear regression analysis was in correlation with phenotypic disturbances, toxicity signs occurrence

and *Phoenix* explant growth and development inhibition showed during experiment (data not shown).

5. Conclusions

The sequencing of the transcriptome is a powerful and efficient approach to discover genomic information in non-model organisms (Leng et al., 2015). The assembly and annotation of transcriptome of *P. dactylifera* stressed by cadmium have provided complete information on date palm resistance to heavy metals. Our data are an important tool for the identification of genes of interest and genetic markers, allowing the study of the functional diversity in natural populations. Our study generated (i) 24,074 transcripts from *P. dactylifera*, (ii) identified putative function in 21,655 transcripts for the species and several genes related to numerous metabolic and biochemical pathways and (iii) predicted pathways to detoxify Cd such glutathione pathway. These markers will facilitate the detection of functional variations and the signature of selection in genome scans or association genetic studies. Multiple candidate genes involved in defense and detoxification were successfully predicted in response to Cd stress. The molecular basis of the response to Cd stress in *P. dactylifera* was first comprehensively characterized in this study, which resulted in useful information and provided a solid foundation for the investigation of the molecular regulation mechanisms of heavy metal accumulation and tolerance in leaves and roots. The expression pattern of gene involved in date palm response to Cd showed gene expression variation to different metal amount during different phase of explant development. Candidate genes expression regulation proved their involvement in *P. dactylifera* resistance to lower Cd concentration and sensibility to higher amount.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Amine Elleuch, Franck Vandenbulcke and HafedhMejdoub, designed the research; Imen Rekik Hakim and Zayneb Chaâbene performed research and analyzed the data; Imen Rekik Hakim, Zayneb Chaâbene and lassad belbahri wrote the paper. All authors read and approved the final manuscript.

B- *In silico* prediction of copper-responsive genes regulatory network and qPCR genes expression of vitro-plants of date palm

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I- Synthèse

La construction d'une base solide de trancriptome de *Phoenix dactylifera* cv Deglet Nour a permis d'identifier des voies métaboliques impliquées dans le stress cuivrique. L'alignement de la banque ADNc construite contre des gènes d'*Arabidopsis* réactifs au cuivre disponibles dans NCBI a aidé à identifier des gènes potentiellement impliqués dans la tolérance au Cu chez le palmier dattier. Parmi les gènes identifiés, on trouve des gènes codant : des facteurs de transcription (TF), des gènes codant « SQUAMOSA promoter binding protein-like » (SPL) ainsi que la famille des gènes d'Ubiquitin C (*ubc*) (Figure II-6). Toutefois, à l'aide du programme GeneMANIA, des liens fonctionnels entre les différents réseaux de régulation des gènes réactifs au cuivre ont été prédits. Le *spl7* exprimé a interagi avec un nombre élevé de séquences de gènes. Les gènes de la famille de l'ubiquitine (UBC2/13/32) liés entre eux et avec *mmz3* ont été impliqués dans la mobilisation du cuivre chez le palmier dattier (Figure II-6). De plus, des relations de co-régulation entre gènes de la même famille comme *fsd* (codant pour FeSOD) et de gènes de différentes familles comme *fsd* et *csd* (codant pour Cu/ZnSOD) ont été montrées (Figure II-6). De plus, pour mieux dévoiler les voies de régulation du cuivre adoptées par les cellules de *P. dactylifera*, des analyses spécifiques sur les gènes codant pour des transporteurs de Cu ont été réalisées. Des liens de co-expression entre des transporteurs de Cu responsables de l'absorption des métaux tels que *copt* (pour le transporteur de particules) ou des transporteurs de métaux entre la vacuole et le cytoplasme comme *Nramp* (pour les protéines de macrophages associées à la résistance naturelle) ou ceux responsables de l'efflux de Cu de la vacuole comme *abcc* (pour ATP-Binding Cassette) ont été prédites par GeneMANIA (Figure II-7). Par ailleurs, des membres de la famille *hma* codant pour *hma2*/*4*/*5*/*6* (PAA1) et *8* (PAA2) ont été identifiés et sont fortement exprimés. Des liaisons fonctionnelles entre les gènes *hma5* et *Nramp2* ainsi qu'une co-expression entre les gènes

abcc et *Nramp3* ont été prédites (Figure II-7).

Afin d'étudier les mécanismes moléculaires sous-jacents aux gènes réactifs au Cu, une proportion des gènes sensibles au métal, identifiés au moyen de la bioinformatique ont été suivi en PCR temps réel. Le suivi de l'expression de gènes codant des chélateurs ; phytochélatine synthase (*Pdpcs1*) et métallothionéine (*Pdmt3*), et des transporteurs ; ATP binding cassette (*Pdabcc*), ATPase (*Pdhma2*), MATE (*Pdmate5*) et Natural resistance-associated macrophage proteins (*Nramp6*), a été effectué sur des cultures *in vitro* de Deglet Nour exposés pendant deux mois à des concentrations croissantes de $CuSO_4$. *Pdpcs1*, a présenté le meilleur facteur d'induction en réponse au stress cuivrique (Figure II-8). Toutefois, une induction plus importante du gène (facteur d'induction > 15) a été obtenue chez des explants après 20 jours d'exposition à 0.02 mM Cd. *Pdmt3* répond très bien aux ions cuivriques même lors d'une exposition à de fortes concentrations. *Pdmt3* répond aussi au Cd mais une chute considérable a été observée après 60 jours de stress pour les doses supérieures à 0.2 mM Cd. De même, les niveaux d'expression des gènes codant pour des transporteurs cellulaires étaient plus importants en cas de stress par le Cu (Figure II-8) que dans des conditions de stress par le Cd. Le gène *Pdhma2* a présenté le profil d'expression le plus clair lors d'une exposition aux ions Cu^{2+} comparé aux autres gènes de transporteurs étudiés. Son niveau d'expression a augmenté parallèlement à l'augmentation de la concentration en Cu et du temps d'exposition. *Pdmate5* était le moins sensible aux ions Cu^{2+} surtout après une longue exposition alors qu'au cas de stress par le Cd, *PdNramp6* était le moins sensible au stress.

A l'exception de *Pdpcs1* et *Pdhma2*, le niveau d'expression des gènes candidats testés a diminué considérablement lors de l'augmentation de la concentration de cuivre et du temps d'exposition (Figure II-8). Toutefois, malgré cette diminution, les gènes sont restés surexprimés ce qui est très différent de ce qui a été observé lors de l'exposition des explants au stress cadmique. Cependant, même si ces gènes sont restés actifs à des conditions de stress accentué, leur activité n'était pas suffisante pour protéger complètement les explants d'un excès de Cu puisque ceux-ci présentaient une nécrose et une altération de la croissance.

II-Article

In silico prediction of copper-responsive genes regulatory network and qPCR genes expression of vitro-plants of date palm

Abstract

As essential micronutrient, minimal amount of Cu is needed to plant cellular functions, while excess Cu induced stress. To investigate date palm cv Deglet Nour Cu-response at transcriptional level, bioinformatique tools were used to analysis a cDNA banq of the plant exposed to metal stress. A gene regulatory network links of copper-responsive genes identified in *P. dactylifera* cDNA library was predicted using GeneMANIA program. The program examined a subset of the inferred network related to Cu-tolerance genes of *A. thaliana* available in NCBI. Among expressed genes, Cu-transcription factors (TFs) were well-characterized including SQUAMOSA promoter binding protein-like (SPL). The expressed *spl7* interacted with high number of gene sequences. Co-regulation between genes of the same family like *fsd* (coding for FeSOD) and of different families *fsd* and *csd* (Cu/Zn SOD) were predicted in the present cDNA library. Other Co-expression links between Cu-transporters responsible for metal uptake such as *copt* (for COPper Transporter) or for metal transport between vacuole and cytoplasm like *Nramp* (for natural resistance associated macrophage proteins) or for Cu-efflux to the vacuole like *abcc* (ATP-Binding Cassette) were predicted by GeneMANIA. In order to study the molecular mechanism underlying responding genes to Cu-stress, monitoring of gene's expression in *in vitro* generated explant of *P. dactylifera* cv Deglet Nour (genetically homogeneous) has been made along two months of metal exposure. Tested genes *Pdpcs1*, *Pdmt3*, *Pdabcc*, *Pdhma2*, *Pdmate5* and *PdNramp6*, were unregulated and were influenced significantly by metal concentrations and time exposure except for *Pdpcs1*. This later genes did not decrease by increasing metal stress during experience. However, for the other genes amounts of transcript levels significantly decreased by increasing metal and exposure time which induced signs of alteration in explants.

Keywords

Cu-transporters, Cu-responsive genes, GeneMANIA, *P. dactylifera*, qPCR, Transcriptome analysis.

1. Introduction

In addition to the socio-economic importance of date palm, *Phoenix dactylifera* L., in arid and semi-arid regions, the plant showed different ecological activities. It exhibit high capacity to survive under extremophile environment such as drought and salinity (Patankar et al., 2016). Furthermore, with a long taproot of the Arecaceae family, date palm is used as a potential absorbent of unwanted materials (dyes, and phenolic compounds,…) from wastewater. It may also, tolerate and accumulate heavy metals (HMs; Chaâbene et al. 2017a). Thus, the plant has been of great interest for ecological and ecotoxicological studies on metabolomics and molecular mechanisms developed to cope with and survive such harsh conditions (Chaâbene et al. 2017a, b). Date palm seeds of Deglet Nour variety, exhibit high levels of tolerance and accumulation of cadmium (Cd), copper (Cu) and chromium (Cr) (Chaâbene et al. 2017 b). However, being a cross-pollinating species, date palm maintains genetic variability, differences in phenotypic characters between cultivars, and high seeds heterogeneity (Naqvi et al., 2015). This genetic diversity has important ecological effects on the long-term viability of populations and functioning of ecosystems, while it is not able to identify underlie and predict genetic mechanisms involved in detoxification of HMs.

The use of *in vitro* culture of plant explant for underling molecular genetics has opened new avenues in plant improvement. Tissue culture is a powerful tool that gives physiological information about plant cells behavior under stress conditions (Ibrahim and Yousir, 2009). It consists of a collection of experimental procedures for aseptic culture of isolated plant callus on nutrient media under controlled environmental conditions. Growth and morphogenesis of explant are greatly influenced by the composition of the culture medium. It has often been modified for defined purposes. Cell lines tolerant to elevated levels of salt and elements concentrations such as aluminum have been studied (Ibrahim and Yousir, 2009) (Jain et al., 1991).

Being a redox-active transition microelement, copper (Cu) has many functions for plant growth and development (Prazak and Molas, 2015). As a cofactor for many enzymes in the processes of respiration, electrons transport and photosynthesis and a precursor of signaling of hormones and transcription, optimum Cu concentrations in the medium enhance morphogenetic potential of explants of plants. High concentrations of Cu may further have positive effects on development of explants of many *in vitro* cultivated species (Amarasinghe, 2009) (Haratym and Weryszko-chmielewska, 2012). However, as a redox metal, copper could

be toxic and may lead to various disorders in metal uptake from the medium and then in explant growth of other species (Bojarczuk, 2004; Kowalska et al., 2012).

Understanding the transcriptomic basis of date palm cv Deglet Nour under Cu stress is a fundamental challenge. Thus, with the rapid progress of transcriptome RNA sequencing (RNA-seq), the new generation sequencing technologies produce large amounts of sequence data. It made new possibilities for creating genomic resources and identifying (SNP) markers with reduced cost and without reference genome information. A first analysis of the transcriptome of *P. dactylifera* L. cv Deglet Nour using the Illumina GA IIx platform as well as the main transcriptional pathways associated with resistance to Cd stress have been very recently publicized (Chaâbene et al., 2017b). However, there are still no investigations on transcriptional profiles of the species under Cu and Cr stress have been shown.

To fully exploit the potential of RNA-seq data, tools for *in silico* transcriptome analysis is necessary. In this study we aim to i) research on key regulatory network of transcriptome of the plant subjected to Cu stress, ii) characterize and investigate functional categories of responsive genes to Cu stress with mean of current Gene Ontology annotations, and iiii) improve *in silico* finding with monitoring genes expression via qPCR methods of *in vitro* cells exposed to metal stress.

2. Material and Methods

2.1. Copper related genes interaction Network

The first cDNA library construction and transcriptome sequencing of date palm cv Deglet Nour was occurred by Zayneb et al. (2017). In summary, authors used highly advanced techniques for library sequencing (Illumina GA IIxplatform), *De novo* transcriptome assembly using RNA-seq program and annotation based on similarity with known genes from NCBI using BLASTx and BLASTn algorithms. The unigenes were aligned by BLASTx search against NCBI protein databases including non-redundant sequences database (Nr), KEGG and Swiss-Prot. The best alignment results were used to predict the coding DNA sequences. The complex biological behavior of the gene was analyzed by pathways annotation based on the KEGG database. Functional annotation was conducted using GO terms that were analyzed using the Blast2GO software. Thus, based on this previously constructed cDNA library of the Deglet Nour variety, the interaction network of genes involved in copper detoxification

mechanisms was predicted by using selected corresponding functionally similar genes from Arabidopsis datasets, downloaded by GeneMANIA version 2.1 (Warde-farley et al., 2010; Zuberi et al., 2013) (http://www.genemania.org/) and visualized in Cytoscape (Montojo et al., 2010). Interest was given to Cu-transporter genes identified and functionally predicted as described above.

2.2. *In vitro* experimentation, genes identification and qPCR measurement

Isolated explants of date palm cultivar Deglet Nour were obtained from the laboratory of vegetable biotechnology applied on the amelioration of culture, university of Sfax, Tunisia. Explants were transferred under sterile conditions into the Murashige and Skoog (MS) (1962) medium supplemented with different concentrations of $CuSO_4$ (0.02, 0.2 and 2 mM) and incubated in 16h photoperiod at 24 ° C during 60 days of exposure. Date shoot clusters of 20, 40 and 60 days of development were used for total RNAs extraction. Interest was given to genes encoding phytochelatin synthase type-1 (*Pdpcs1*), metallothionein type-3 (*Pdmt3*) ABC transporter C family membre 2 like (*Pdabcc*), ATPase (*Pdhma2*), Multi-antimicrobial extrusion protein MATE type 5 (*Pdmate5*), and natural resistance-associated macrophage protein 6-like (*PdNramp6*). Using candidate gene specific primers designed by Primer3Plus on line software (Rozen and Skaletsky 2000; http://frodo.wi.mit.edu/) and verified using Net Primer and Beacon Designer programs (Table II-6), we performed quantitative real-time polymerase chain reaction (qPCR) amplification method according to Brulle et al. (2014).

Table II-6. qPCR Primer sequences of candidate genes used in this study

Name	Amplification length (bp)	qPCR specific Primer sequence (5' → 3')	Length (bp)	Tm	GC %	PCR Efficiency ±SD
Pdabcc	104	F : GATTTGCTTCCAGGAGGTGA	20	60.2	55.0	1.97±0.01
		R : TCCGAATATACTGCCCTTGC	20	60.1	50.0	
Pdhma2	162	F : CATGCAACCACAAGCAAGAC	20	60.3	55.0	2±0.01
		R : TCGACGTTCAAACATAGAGGAG	20	59.4	54.5	
Pdmate5	103	F : CTTGCTCTCAAAGCGAAAGG	20	60.3	50.0	1.98±0.05
		R : TTCCGTCGCCAACACATAG	19	60.7	52.6	
PdNramp6	154	F : CGGAAGCTCTGGTCTCACA	19	60.1	57.9	1.95±0.04
		R : CGACAGCAACTGGATCAGAA	20	60	50.0	

The expression levels and the relative fold expression (RFE) were determined according to previously described procedures (Brulle et al. 2006). The geometric mean of the 3 most stable reference genes in control and Cd-stressful condition identified by Chaâbene et al. (2017b) was used to calculate expression of target gene levels according to Brulle et al. (2014).

Absolute quantification of genes expression levels are in log2. The results were expressed as "Induction factor" were obtained by scaling the absolute quantification of genes in stressful conditions with those in control conditions.

2.3. Statistical analysis

The relative expression ratios between control and treated plants were transformed and subjected to a one-way and two-ways ANOVA. Student test has been used to analyze significance were $p<0.05$ was considered to be significant.

3. Results and Discussion

3.1. Cu-signaling genes functional partners' prediction

To further disclose Cu-signaling gene's link predictions, we examined a subset of the inferred network related to *A. thaliana* copper-responsive genes available in NCBI. The high degree of conservation among organisms of most of the gene sequences helps for the draw of the diagram of the inferred copper regulatory network showed in figure II-6.

GeneMANIA recovered well-characterized Cu-transcription factors (TFs). They describe large classes of regulators that control gene expression at the transcriptional level (Sun and Oberley, 1996). Among TFs, the SQUAMOSA promoter binding protein-like (SPL) is encoded by a large gene family in plants (Mao et al., 2016). In the present cDNA library of *P. dactylifera* we found 12 classes of *spl*. The *spl7* interacted with high number of gene sequences and is expressed. With an SBP (SQUAMOSA promoter binding protein) domain, SPL7 is proved to be required for the expression of multiple microRNAs, including miR397, miR398, miR408 and miR857. It may, also, activates the transcription of multiple copper-responsive genes such as copper transporters (Yamasaki et al., 2009), and copper chaperone (CCH; the first metallochaperone described in plant). In *Arabidopsis*, Yamasaki et al. (2009) proposed that SPL7 is a master regulator involved in copper-sensing and homeostasis. It was found to mediate the copper-dependent switching of SOD between Cu/Zn SOD and FeSOD controlled by *fsd1* (FeSOD) which disturbed in *spl7*. In the figure III-6, co-regulation between *fsd2* and *fsd3* was shown. They respond to oxidative stress signals especially to ROS (reactive oxygen species) accumulation (Apel and Hirt, 2004). The formation of hetero-complexes between FSD2 and FSD3 in chloroplast nucleosides enhanced transgenic Arabidopsis tolerance to oxidative stress induced by Cu^{2+} ions (Myouga et al., 2008). The regulation of

genes encoding chloroplast antioxidant enzymes is further improved by the expression of CuZnSOD encoding gene (*csd*; Figure II-6).

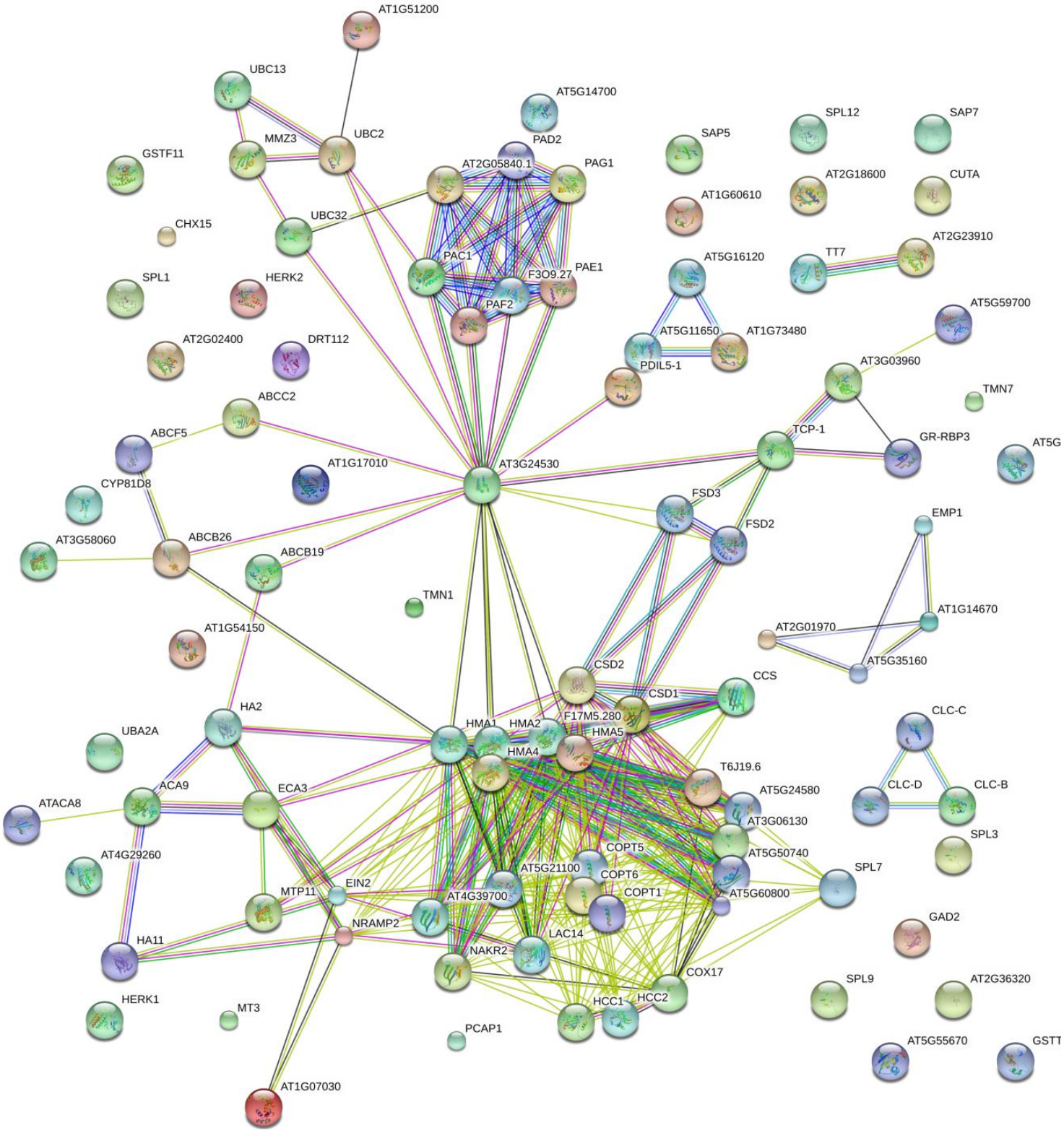

Figure II-6. Copper-controlled regulatory network in *P. dactylifera* cells inferred by the GeneMANIA

Gene's link predictions between *fsd* and *csd* were shown in figure II-6. The overexpression of *fsd2* and *fsd3* resulted in high *csd2* transcription level in Arabidopsis (Myouga et al., 2008). Thereby, a strong copper mediated link between *csd1*, *csd2* and plastocyanin expression was found (Abdel-ghany and Pilon, 2008). As the plastocyanin is the most prominent Cu-binding protein in plants, that controlled the expression of *csd1* and *csd2*, evidence may be concluded of copper regulation pathways adopted by *P. dactylifera* plant cells. Additionally, Cu-insertion into CuZnSOD was controlled by copper chaperone for SOD (CCS). Direct link between *ccs* and *csd* genes was predicted (Figure II-6). These results proved previous funding which suggest that *csd1-3* were activated by *ccs* in Arabidopsis (Chu et al., 2005). On the other hand, intracellular copper distribution and its uptake through the plasma membrane are performed by Cu-transporters that contain GTAC binding sites for the transcription factor SPL7 in their promoter (Gielen et al., 2016). With high affinity copper uptake, six members of COPT (COPper Transporter) encoding genes has been identified in date palm similarly to *Arabidopsis* genome (Aguirre and Pilon, 2016). This CTR-like sequences of Cu transporters helps Cu^{2+} enters eukaryotic cells (Aguirre and Pilon, 2016). Co-expression of *copt1*, *copt5* and *copt6* has been predicted in *P. dactylifera* (Figure II-6). However, only, COPT1 and COPT5, have been characterized for their functions in Cu transport in rice (Yuan et al., 2011). The transcript coding for COX (mitochondrial Cytochrome c Oxidase) have been validated as miR398 targets (Beauclair et al., 2010). *Cox17* has been identified in *P. dactylifera* to be involved in copper tolerance. Since COX17 is a highly conserved protein, enzyme may mediate the delivery of Cu to mitochondria (Printz et al., 2016). Besides, date palm copper metabolism requires other Cu^{2+} chelaters. 32 Members of UBC genes including *ubc13* have been identified in the plant. Phytochelatin synthase type 1 (*Pdpcs1*) and Metallothioneins type 3 (*Pdmt3*) have been present in figure 1 and have been previously identified in date palm cv Deglet Nour (Chaâbene et al., 2017a). Gene's up-regulation under Cu-stress proved its involvement in copper detoxification mechanisms. Described as the third major copper homeostasis proteins, MTs may sequester until 12 copper ions and are involved in copper transport in the phloem (Guo et al., 2008). Likewise, (Gonzalez-mendoza et al., 2007) suggested that *pcs* was the most active gene involved in copper regulation in *A. germinans* leaves.

From more than 30 members of *ubc* family identified in *P. davtylifera*, gene coding UBC2/13/32 were predicted to be linked to each other and to *mmz3* and are involved in copper mobilization in the plant. The UBC 13 catalyze non-canonical Lys63-linked ubiquitin

chains and plays important roles in signal transduction among eukaryotes (Li and Schmidt, 2010). However, MMZ3 play a role in DNA damage responses and error-free post-replicative DNA repair by participating in lysine-63-based polyubiquitination reactions (Callis, 2014). The MMZ3 gene is known to interact with UBC35/36 (Callis, 2014), while, in our plant model, *mmz3* were showed to be linked to *ubc2*/*13*/*32*. It was demonstrated that MMZ3 can form diubiquitin and triubiquitin chains in combination with UBC13A/UBC35. This interaction was predicted to be controlled at transcriptional level.

3.2. Cu-transporter genes regulatory network

Because Cu-transporters have primary importance in maintenance of physiological limits of Cu homeostasis in plant cells, figure II-7 put the accent on the transporter gene's links in date palm exposed to metal stress. Different categories of transporters are identified in organisms. Important process of Cu-transport between chloroplast and cytoplasm is controlled by Heavy Metal ATPase (HMA) family of transporters (Hanikenne and Baurain, 2014). There are necessary to maintain a concentration gradient for free Cu ions over the plasma membrane with a relatively low free Cu concentration in the cytosol (Aguirre and Pilon, 2016). Although its transmembrane metal binding site is different from that of Cu-transporting ATPases (Argüello et al., 2007), HMA1 have been implicated in the reverse process. It can efflux nutrient out of the chloroplast. Gene coding for the third ATP-driven metal transporter in the chloroplast; *Pdhma1* (Aguirre and Pilon, 2016), has been identified in the present cDNA library. Besides, other members of *hma* family encoding *hma2*/*4*/*5*/*6* (*PAA1*) and *8* (*PAA2*) have been identified and are highly expressed (Figure III-6). HMA2 to 4 were shown to be also involved in tolerance to excess zinc in *Arabidopsis* (Morel et al., 2008). Whereas, HMA5-8 described Cu-ATPases. The HMA5, primary expressed in *A. thaliana* in the plasma membrane, removes Cu from the cell to allow xylem loading in the roots and prevent cellular Cu overload (Andrés-Colás et al., 2006). Functional links between *hma5* and *Nramp2* (for natural resistance associated macrophage proteins) were predicted describing co-expression relationship (Figure II-7). The Nramp family described a group of divalent metal cation transporters. They regulates nutrient transport between vacuole and cytoplasm (Pearce et al., 2014). With EIN2 (Ethylene Insensitive 2) domain shared between Nramp2/3 and 5 (Figure II-7), Nramps play a central role in ethylene signaling (Blaby-Haas and Merchant, 2012) and may be involved in sensing metal, which could be related with copper requirement of the ethylene receptor (Hirayama and Alonso, 2000). The Nramp proteins have been isolated and

characterized from plant-metal hyperaccumulator like *A. halleri* and *T. japonicum*. In yeast, NRAMP1 was involved in iron uptake and was further participated in manganese transport in leaves of *Arabidopsis* (Blaby-Haas and Merchant, 2012).

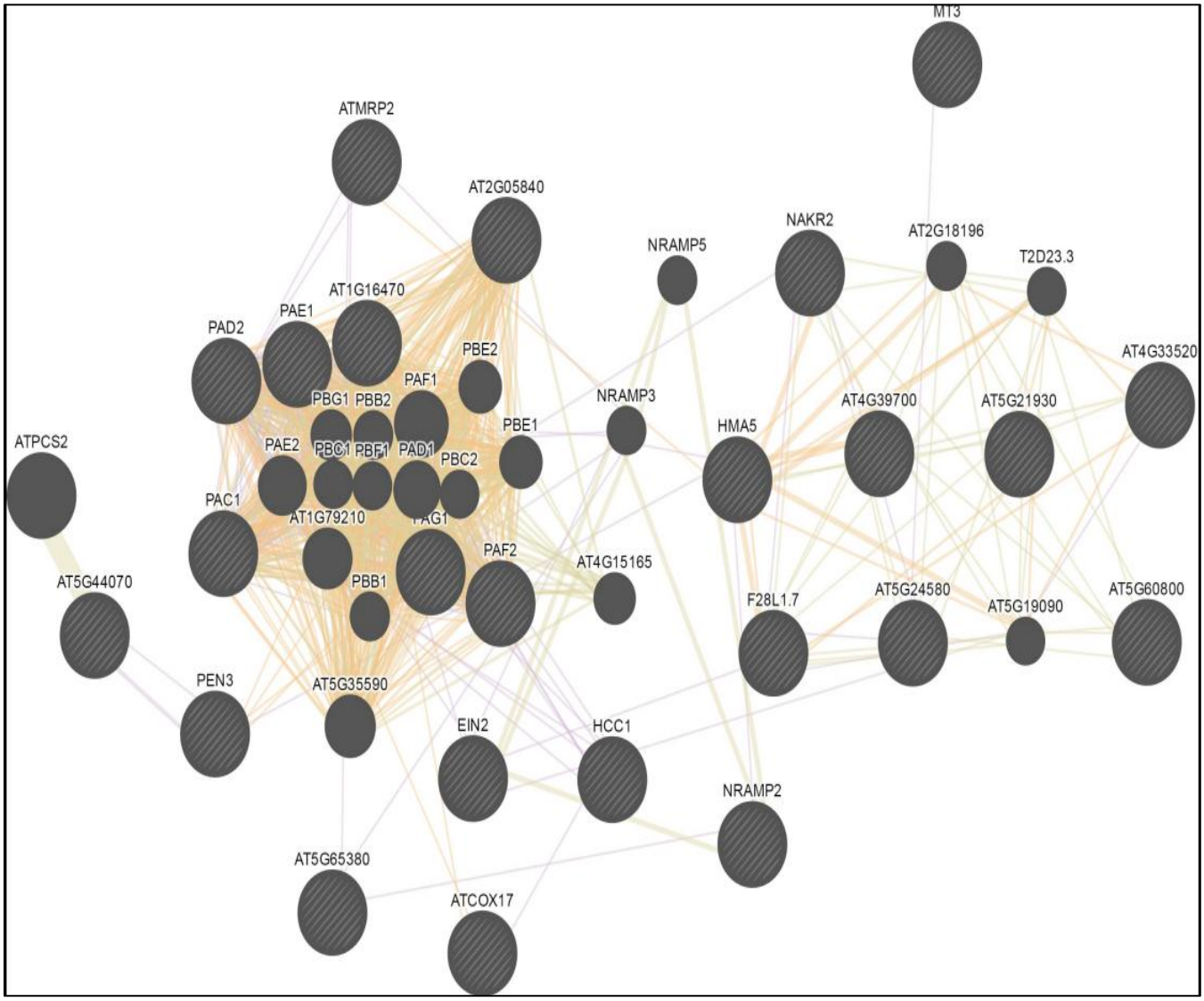

Figure II-7. Copper-transporters genes links in P. dactylifera by the GeneMANIA

Across membranes, membrane ATP-Binding Cassette (ABC) transporters are divided into two subfamilies (MRP and PDR) and are responsible for transporting various solutes such as heavy metal including Cu^{2+} ions. As multidrug resistance-related proteins, the ABC, especially the subgroup ABCC, efflux heavy metal into the vacuole (Singh et al., 2016) and may have key roles to sequester or exclude chelated forms of Cu or other toxic adducts (Wanke and Üner Kolukisaoglu, 2010). In date palm cDNA library, co-expression between *abcc* and *Nramp3* was predicted (Figure II-7).

No genes functional links have been shown before. However, co-expression has been shown only between *abcc* of *A. Thaliana* which exhibit exhibit overlapping expression patterns due to duplicated regulatory DNA motifs in their promoters indicating its involvement in similar process (Haberer et al., 2006). Also, AtPCS1 and AtABCC1 are shown to be vacuolar PC transporters exhibiting an increase in arsenic resistance in *A. thaliana* (Song et al., 2010). Like ABC, MATE family of transporters are important multidrug transporters superfamily. However, although its importance to reduce ROS production in mitochondria (Finatto et al., 2015), GeneMANIA program did not found MATE (Multi antimicrobial extrusion protein) encoding gene involved in HMs tolerance such as Cu in brown algae; *E. siliculosus* (Ritter et al., 2014a) and aluminuim (Al) in rice (Yokosho et al., 2011). According to Ritter et al, *mate* up-regulation is related to the secretion of Cu-conjugates. While, these secondary active transporters, function as proton-dependent efflux transporters (Eckardt, 2001) and were up-regulated under Cu stress playing important role in Cu-detoxification in grapevine (Leng et al., 2015).

3.3. Gene's expression responding to Cu stress

In order to study the molecular mechanism underlying responding genes to Cu-stress, monitoring of gene's expression in *in vitro* generated explant of *P. dactylifera* cv Deglet Nour has been made along two months of metal exposure. *In vitro* generated Deglet Nour tissues allowed as avoiding the genetic diversity of *P. dactylifera* that may influence genes expression. qPCR expression analysis allowing to study multiple durations of the treatment of selected two chelates and four transporters genes sensitive to Cu stress (Figure II-8). Their expressions were altered during Cu-stress.

Both metal concentrations and time exposure influenced significantly ($p < 0.000$) gene expression in the vitroplant tissues except for *Pdpcs1* which showed the best mRNA production (Table II-7). The gene induction factor continued to increase with increasing metal stress and time exposure. *Pdpcs1* reached its maximum (10.03) after 60d of containing 0.2 mM Cu in the MS medium (Figure III-8a). The gene overexpression in *E. coli* cells transformed with pGEX-5X-pcs ameliorated its growth under high temperature, NaCl (6% w/v), $CdCl_2$ (4mM), $CuCl_2$ (1 mM), and UV-B (10 min) exposure. It revealed its role in tolerance against different abiotic stressers (Chaurasia et al., 2008). Important non-significant enhancement of *Pdpcs1* has been observed since the first 20d (Figure II-8).

Table II-7. Significant differences between gene's expressions were tested using Tukey's test HSD test after one-way and two-ways ANOVA with exposure time and metal concentrations as the two factors.

Two way ANOVA main effects

		Pdpcs1	*Pdmt3*	*Pdabcc*	*Pdhma2*	*PdNramp*	*Pdmate5*
Cu	**Concentration**	0.023*	0.000*	0.000*	0.000*	0.353	0.024*
	Exposure Time	0.000*	0.000*	0.028*	0.000*	0.000*	0.000*
	Concentration x Exposure time	0.313	0.000*	0.000*	0.000*	0.000*	0.000*

However, PdPCS1 mRNA production decreased from the first 40d of exposure with 1 mM Cd-stress of explant of Deglet Nour (Chaâbene et al., 2017). Long exposure stress influenced gene expression event at low Cd amount (0.02 mM). Similarly, long time exposure reduced *Pdmt3* expression although it is still up-regulated by 0.2 and 2 mM Cu (Figure II-8a). *Pdmt3* transcription factor described a biphasic curve at 2 mM Cu, attending a maximum by 5.5 after 40d before it declined by a half at the end of the experience. In *Silene vulgaris*, both copies of *Svmt3* have been identified and were functional. While, high Cu and Cd concentrations down-regulated gene expression in *S. vulgaris* ecotypes and Deglet Nour explants respectively (Nevrtalova et al. 2014; Chaâbene et al., 2017). *pcs1* and *mt3* offers a key non-translationally phytochelatins (PCs) and translationally metallothionein (MTs) chelaters respectively scavenging free Cu^{2+} and controlling copper homeostasis in plants (Chaurasia et al., 2008; Nevrtalova et al., 2014). However, overexpressing AtPCS1 alone did not result in an increased metal tolerance (Song et al., 2010). The Cu-conjugates are transported and compartmentalized in vacuoles. ABCC and MATE proteins are particularly active in the sequestration of chelated Cu^{2+} (Ritter et al., 2014a). Thereby, *abcc* expression increased in parallel to *pcs1* and *mt3* expression (Figure II-8b). It maintained linear increase throw time exposure by 0.02 and 0.2 mM Cu. Members of *abcc* family has been identified in *A. thaliana* (Wanke and Üner Kolukisaoglu, 2010). ABCC1 and ABCC2 was identified in *A. thaliana* exhibiting redundant function in the translocation of plant vacuolar phytochelatin (Song et al., 2010).

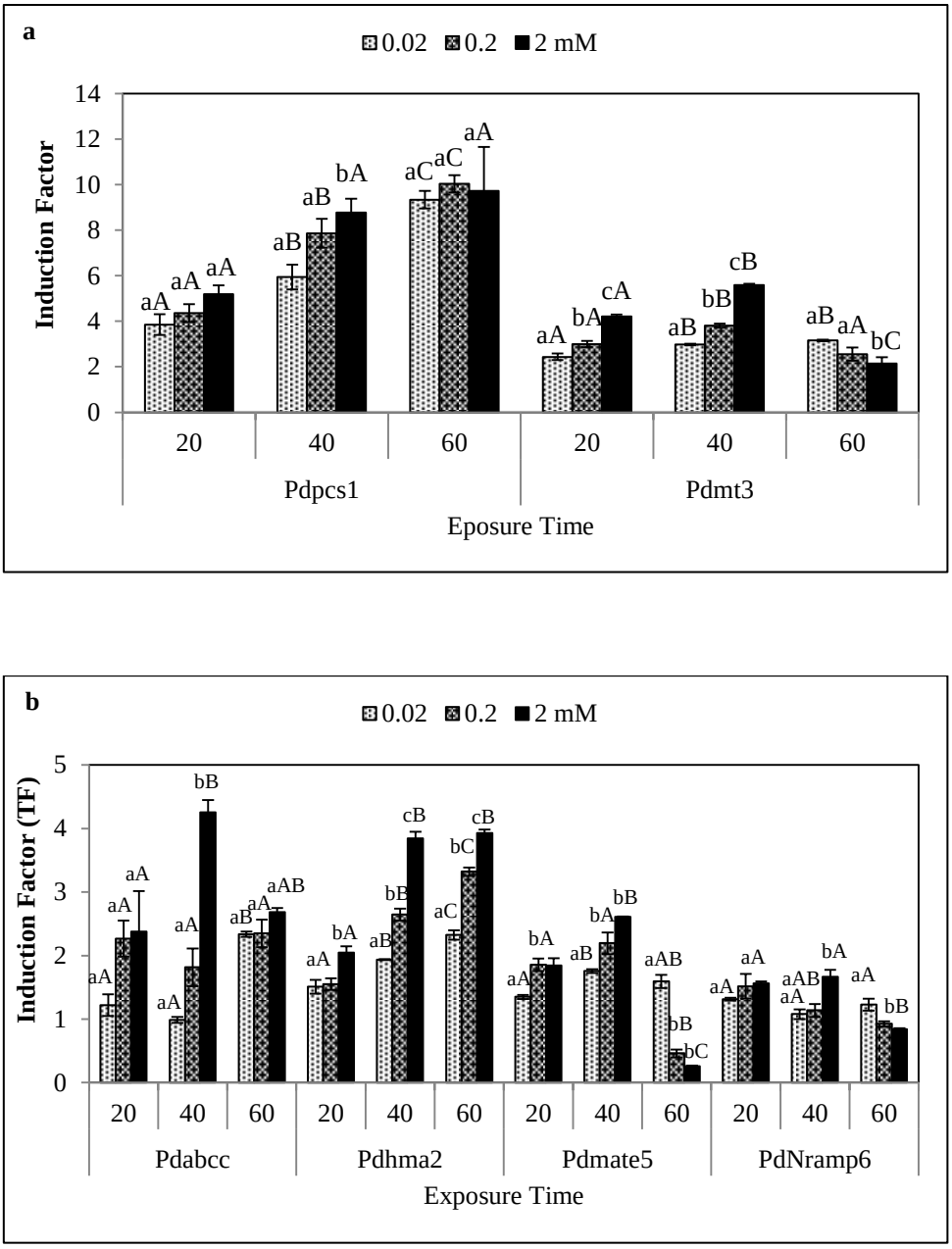

Figure II-8. Transcription factor of a-Cu chelators (Pdpcs1 and Pdmt3) and b-Cu-transporters (Pdabbc, Pdhma2, Pdmate5 and PdNramp6). Data presented are means ± standard error of three independent experiments. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test; where small letters show differences between concentrations. Means not showing the same letter are statically different.

Beside, *Atpcs1* and *Atabcc1* exhibited a consistent increase as result in plants with enhanced arsenic tolerance (Song et al., 2010). AtABCC1 and AtABCC2 are not synthesized de novo but constitutively present in a plant cell to rapidly respond to toxic metals and xenobiotic stresses in a way similar to PCS1. Yet, similarly to *mt3, abcc* expression has fallen by more than 50 % after 60d of exposure with 2 mM (Figure II-8b). However, Cd stress badly influenced ABCC mRNA production more than Cu since *abcc* down-expression has been

shown from the first 40d of exposure with 1 mM (Chaâbene et al., 2017). Although *pcs1* was highly expressed under copper stress especially with 0.2 mM concentration in the medium, *mate5* transcript levels were significantly deceased after 2 months of treatment by more than 75 % and 90 % with respectively 0.2 and 2 mM Cu. Long exposure time affected gene expression (Figure II-8b). Yet, in brown algae, MATE stress-related transporters were specifically up-regulated by Cu stress (Ritter et al., 2014a). Also, *mate* was described as the candidate gene for the export of toxic cations such as Al in soybean and rice respectively (Liu et al., 2016; Yokosho et al., 2011). It may be involved in the transport of secondary metabolites and the detoxification of xenobiotics (Chen et al., 2015b). Other transporters involved in copper tolerance in date palm have been monitored (Figure II-8b). In the same way to *mt3*, *abcc* and *mate5*, *Nramp6* transcript levels, with the lowest mRNA production, described a biphasic curve (Figure II-8b). Maximum transcript factor exceeding 1.6 was accrued by 2 mM Cu after 40d of exposure. After that period, only 0.02 mM Cu continued to improve gene expression, while it decreased for 0.2 mM Cu and especially 2 mM Cu (Figure III-8b). Member 6 of Nramp family encoding gene is not much studied before. Based on this finding, we suggest a novel gene expressed in date palm in response to Cu stress; *PdNramp6.* It was also involved in Cd tolerance in the plant (Chaâbene et al., 2017). Also, (Ishimaru et al., 2012) highlighted the importance of OsNramp5 for Cd phytoremediation were OsNramp5 may contributes to manganese (Mn), Cd, and Fe transport in rice. *AtNramp1* pays a pivotal role in Fe uptake and transport in the plant (Castaings et al., 2016). However, the overexpression of *nramp3* in *A. Thaliana* increased Cd sensitivity (Thomine et al., 2000). On the other hand, HMA2 is a Zn^{2+}-dependent ATPase that is activated especially by Cd^{2+} but also by other divalent heavy metals like Cu^{2+}, Pb^{2+}, Ni^{2+} and cobalt (Co^{2+} ; Eren and Argüello, 2004). In our experience, *Pdhma2* is the unique gene, from the tested genes that expressed significant increase even at high copper level and after long time of treatment (Figure III-8b). However, the gene may act differently against metal stress and between plant types. In fact, at the same conditions, *P. dactylifera* explants decreased *hma2* after 2 months of Cd-containing medium (Chaâbene et al. 2017). In addition, *hma2*, characterized as efflux transporter genes responsible for the transport of Cd^{2+} from pericycle parenchyma cells to xylem, was up-regulated in Baiyewuyueman and down-regulated in Kuishan'aijiaoheiye in Cd treatments compared with the controls (Yu et al., 2017).

4. Conclusions

GeneMania analysis showed abundance of members of *spl* and *ubc* family in date palm cv Deglet Nour cDNA library. Genes of UBC2/13/32 were predicted to be linked to each other and to *mmz3* and were involved in copper mobilization in the plant. Beside, co-regulation relationships between *fsd2* and *fsd3* followed by gene functional link predictions between *fsd* and *csd* as well as between *fsd* and *csd* were shown. To further disclose copper regulation pathways adopted by *P. dactylifera* cells, specific research on transporter genes was made. Members of *hma* family encoding *hma2*/*4*/*5*/*6* (*PAA1*) and *8* (*PAA2*) have been identified and are highly expressed. Functional links between *hma5* and *Nramp2* as well as co-expression between *abcc* and *Nramp3* was predicted. Among the high number of Cu-responsive genes, qPCR amplification of six genes in *in vitro* culture of explant of date palm cv Deglet Nour exposed to increasing Cu concentration was used. In general, all tested genes, *Pdpcs1*, *Pdmt3*, *Pdabcc*, *Pdhma2*, *Pdmate5* and *PdNramp6*, were up-regulated. However, except for *Pdpcs1*, the best induced gene, amounts of transcript levels significantly decreased by increasing metal and exposure time. Thus, event genes still overexpressed, there were not strong enough to confer increased Cu-tolerance in which explants showed necrosis and growth alteration.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Amine Elleuch designed the research; Imen Rekik Hakim and Zayneb Chaâbene performed research and analyzed the data; Zayneb Chaâbene wrote the paper. All authors read and approved the final manuscript.

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C-The Phytochelatin-synthase gene in date palm (*Phoenix dactilyfera* L.): *in silico and in vivo* studies of its involvement in metal detoxification mechanisms

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I. Synthèse

Parmi les gènes les plus exprimés en cas de stress métallique chez le palmier dattier cv Deglet Nour, le gène codant pour la phytochélatine synthase type 1 (*Pdpcs1*). PdPCS1 catalyse la synthèse cytosolique des phytochelatines (PCs) qui sont capables de fixer des métaux lourds dans les cellules végétales. Les données EST obtenues ont clairement confirmé la notion que les gènes *pcs* sont présents au moins chez toutes les plantes supérieures. *Pdpcs1* a été identifié chez *P. dactylifera* (Table II-S7). Il est composé de 7 introns et 8 exons codant pour une protéine de 528 acides aminés (Figure II-9). Toutefois, la construction de l'arbre phylogénétique de *pcs* n'a pas pu refléter les relations phylogénétiques actuellement acceptées entre espèces pour ce gène, car les gènes des lignées majeures n'étaient pas regroupés dans un même clade. L'histoire évolutive du *pcs* a été étudiée utilisant le programme Notung. Au cours de son évolution, l'évènement de perte de gènes chez plusieurs lignées a été prédit, y compris chez des Proteobactéries, des Bilateria et des Brassicaceae (Figure II-11). De plus, onze événements de duplication de gènes retrouvés dans des nœuds intérieurs de l'arbre réconcilié et 4 événements de duplication de gènes obtenus dans des nœuds externes ont été prédits. Des séquences ultérieures appartenant à des espèces ayant plus qu'une copie de *pcs* ont suggéré que ce gène évolue par la sous-fonctionnalisation (Figure II-10). Le niveau d'expression de *Pdpcs1* a été mesuré par qPCR dans des hypocotyles de palmier dattier cv Deglet Nour exposées à 0.02 mM de Cd ou de Cu ou de Cr. Dans des conditions contrôles, le gène était légèrement exprimé (Figure II-14). Son niveau d'expression augmente sous stress

métallique suggérant que le gène est fonctionnel. De plus, le niveau d'expression de *Pdpcs1* a montré une variation considérable en fonction des éléments métalliques utilisés suggérant que le produit du gène *Pdpcs1* est impliqué dans la tolérance aux métaux (Figure II-14). Ainsi, les ions Cu^{2+} ont montré la plus faible induction du gène par rapport aux ions Cd^{2+} et en particulier aux ions Cr^{6+} même si des études antérieures (Shanker et al., 2004) ont montré l'absence de phytochélatines en réponse au stress par le chrome (Figure II-14).

En outre, la structure de la protéine PdPCS1 a été prédite par les logiciels Discovery Studio et PyMol en utilisant son homologue de Nostoc (cyanobactérie, NsPCS, Figure II-13). Sur la base de l'identification des acides aminés consensus autour du site actif, nous avons émis l'hypothèse d'un mécanisme catalytique pour une seconde cavité de liaison du substrat chez PdPCS1 (Figure II-13). Ceci a permis d'identifier la structure 3D de l'enzyme PdPCS de type I avec ses différents acides aminés consensus impliqués dans son site actif. De plus, d'autres acides aminés très conservés entres espèces ont été identifiés dans une deuxième cavité de liaison du substrat qui peut pourrait constituer un deuxième site catalytique fonctionnel de l'enzyme.

II- Article

The Phytochelatin-synthase gene in date palm (*Phoenix dactilyfera* L.): *in silico and in vivo* studies of its involvement in metal detoxification mechanisms

Abstract

In the present work, a focus is done on date palm phytochelatin synthase type I (PdPCS1). PdPCS1 catalyzes the cytosolic synthesis of phytochelatins (PCs), one of the best heavy metal binding candidates in plant cells. The gene encoding PdPCS1 (pcs) was identified as composed by 8 exons and 7 introns and encodes a protein of 528 aa. *pcs* gene history has been studied using Notung phylogeny. During evolution, gene loss from several lineages has been predicted including proteobacteria, bilateria and brassicaceae. Beside, eleven gene duplication events appear toward interior nodes of the reconciled tree and 4 gene duplication events appear toward the external nodes. These later sequences belong to species with a second copy of *pcs* suggesting that this gene evolves through subfunctionalization. *Pdpcs1* gene expression level was measured in seeds hypocotyl exposed to Cd, Cu and Cr using quantitative real-time polymerase chain reaction (qPCR) amplification technique. A *Pdpcs1* overexpression was evidenced in *P. dactylifera* seedlings exposed to metals suggesting that 1-the *Pdpcs1* gene is functional, 2-there is an implication of the enzyme in metals detoxification mechanisms. Besides, PdPCS1 protein structure was predicted using its homologue from *Nostoc* (cyanobacterium, NsPCS) as a template in Discovery studio and PyMol software. These analysis allowed us to identify phytochelatin synthase type I enzyme, first discovered in palm date (PdPCS1) with its different consensus amino acids involved in the catalytic mechanism and to propose a hypothetical binding and catalytic site for an additional substrate binding cavity.

Keywords*:* Ancestral sequence, duplication events, gene loss, *Pdpcs1* gene expression, phylogenetic tree, substrate binding site

1. Introduction

Heavy metals are typically toxic at submicro- to micromolar concentrations. They displace endogenous metal cofactors from their cellular binding sites, undergo aberrant capping reactions with the thiol groups of proteins and promote the formation of reactive oxygen species (Rea et al., 2004).

The interaction of organisms with toxic metal and metalloid elements has been used as a productive model for physiological, ecological, genetic and evolutionary research for over half a century. In eukaryotic cells, toxic ions appear to be removed from the cytosol mainly by chelating and sequestration (Clemens et al., 1999). Plants resort to a series of defense mechanisms that control uptake, accumulation and translocation of toxic elements. One recurrent general tool for heavy metal detoxification is complexion with amino acids, organic acids, metal-binding peptides and/or sequestration into vacuoles (Hall 2002). A number of metal-binding ligands and their roles have been reviewed by Rauser (1999). Bacteria, yeasts, worms as well as plants synthesize phytochelatins (PCs) which chelate Cd^{2+}, Cu^{2+} and other heavy metals ions. PCs are $(\gamma Glu\text{-}Cys)_n\text{-}Gly$ (n = 2–11) polymers, discovered about 30 years ago as non-protein cysteine-rich oligopeptides entirely different from metallothioneins (MTs) in structure and biosynthetic pathways (Masahiro 2005). These cysteine (Cys)-rich polypeptides act as high-affinity metal chelators and facilitate their transport (Cobbett 2000a). Amount of PCs has been proposed as a biomarker to assess metal stress (Keltjens and Beusichem 1998).

Phytochelatin synthase (PCS; EC 2.3.2.15) is a γ-glutamylcysteine dipeptidyl transpeptidase which catalyzes the synthesis of PCs using glutathione (GSH, γGlu-Cys-Gly) or previously synthesized PC as substrates (Grill 1989). In 1999, three groups simultaneously and independently cloned and characterized a small family of genes encoding this enzyme in *Arabidopsis thaliana*, *Schizosaccharomyces pombe* and *Triticum aestivum*, designated *Atpcs1*, *Sppcs1* and *Tapcs1*, respectively (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999). However, PCS polypeptides are much more widely distributed than was suspected in 1999. Systematic sequence database searches disclose PCS-like genes in some diatoms, some downy mildews, some ciliates, chordates, echinoderms, annelids and flatworms (Clemens and Persoh 2009) as well as in prokaryotes (Rea et al., 2004). In contrast, in the limited number of species investigated, it so far appears that the earliest diverged extant land plants, like bryophytes, mosses and hornworts, do not synthesize PCs under metal stress (Leinenweber et al., 2009; Bleuel et al., 2011). Yet, the presence of metal chelating activity particularly in the lineages originated during the Devonian period succeeding the palaeoenvironments rich in PC-inducing metal and extended with bryophytes and lycophytes might represent a remnant of that time (Petraglia et al., 2013).

Furthermore, a large number of protein sequences homologous to PCS have been found over the last decade. The eukaryotic PCS proteins are 50–55 kDa polypeptides that display 40 %–

50 % sequence similarity, and contain a highly conserved N-terminal domain, with a papain-like catalytic triad (Vivares et al., 2005; Romanyuk et al., 2006; Rea 2012) and a variable C-terminal domain (Cobbett 2000b).

A range of metal ions can induce PCS activity, although with different efficiency. The enzyme is activated by copper, cadmium, arsenic, silver, mercury, zinc and lead ions (Oven et al., 2002). However, the dependence of PCS on the provision of heavy metal ions for activity in media containing GSH and other thiol peptides reflects its requirement for GSH-like peptides containing blocked thiol groups. A possibility of synthesizing PCs in plastids by overexpressing a plastid-targeted phytochelatin synthase has been shown (Picault et al., 2006). All of the former experiments aimed at phytoremediation and tried to enhance heavy metal accumulation in plants. Beside from metal detoxification, new functions have been suggested for PCS and PCs alike. With respect to metals, PCs have been implicated in long distance transport of Cd (Gong et al., 2003), predominantly in the phloem (Mendoza-Cozalt et al., 2008). They may also serve additional functions in glutathione metabolism. PCS catalyzes the first step in the degradation of GSH conjugates (Blum et al., 2010). The participation of AtPCS1 in glucosinolate metabolism and consequently in plant innate immunity has been reported (Clay et al., 2009).

To draw attention to diversity and growth of plants that routinely experience multiple major stresses (aridity + heavy metal contamination), this work addresses one of a very few trees that can tolerate the desert environment; date palm (*Phoenix dactylifera* L.). Around the world, it accounts for more than 1500 cultivars (FAO 2002). It has a minimal water demand, tolerates harsh weather and high levels of salinity (Diallo 2005). In fact, it is more salt tolerant than any other fruit crop (FAO 1982). A recent research demonstrated a Cd-hyperaccumulation phenotype of the plant (Zayneb et al., 2013). The recent completion of the date palm cv. Deglet Nour genome sequencing project (Rekik et al., 2013), together with its automated annotation process, enabled us for the first time to examine the *Pdpcs* gene *in silico* and *in vivo*. For that, it is necessary to accomplish our work with model methods to unravel the molecular mechanisms for its function under metallic stress.

The aim of the present study was to: 1-perform *in silico* characterization of the *Pdpcs* gene, 2- predict the protein structure and 3- test the hypothesis of an implication of the enzyme in metals detoxification mechanisms in date palm.

2. Materials and methods

2.1. Plant material and cDNA library construction (mRNA isolation, cDNA synthesis and cloning)

The date palm, *Phoenix dactylifera* cultivar "Deglet Nour", was used as the RNA source. Leaves were collected from a large number of plant samples and ground to a fine powder in liquid nitrogen, and homogenized in total RNA extraction buffer, according to Cathala et al., (1983). The total mRNA was selected by oligo (dT) cellulose chromatography, using the mRNA Purification Kit (QIAGEN) according to the manufacturer's specifications. Double stranded cDNA was synthesized using a cDNA Synthesis Kit (QIAGEN), following the manufacturer's instructions. cDNAs were ligated to EcoRI adaptators, cloned into the EcoRI restriction site of pUC 18 and used to transform JM 105 cells. Bacteria were plated on LB agarose medium containing 50 mg/ml ampicillin, 0.1 µM IPTG and 0.1% X-Gal. Fifty white colonies were randomly selected out of 1500 and screened for the presence of recombinant plasmids with inserts greater than 400 bp. Plasmid DNA from these colonies was prepared by a large scale procedure, as described by Lebrun et al., (1988).

2.2. Isolation, cloning and sequencing of PCR products of *PCS* cDNA

BLASTn searches in NCBI (http://www.ncbi. nlm.nih.gov/) with default parameters allowed retrieval of homologous sequences of *pcs* from various organisms. Multiple alignment using BioEdit version 7.0.5.3 of some clones revealed partial homology with a region of *pcs* cDNA. We searched *pcs* domains (Pfam with E value <1e-5) against the recovered clone nucleotide sequences and other sequenced plant genes/genomes using HMMER v3.0 and validated the candidate *Pdpcs1* genes using BLASTp against plant PCS protein sequences of UniProt (http://www.uniprot.org/). Degenerate primers were synthesized from the conserved domain found (Table II-8). Completion of the other domains and 5'untranslated region (UTR) of putative *pcs* was done using the rapid amplification of 5'cDNA ends (5' RACE) kit (Invitrogen) according to the manufacturer's instructions. The first strand cDNAs were synthesized from mRNAs using gene-specific primers and gene specific tested primers designed according to the *Pdpcs1* gene sequence found using PerlPrimer v 1.1.21. The sequences of the primers were 5' GTCTCTATCGGCGAGCTCTC 3' and 5'TAGACTCTCAGAATAGGAGTCCA 3'. In brief, the first strand cDNA synthesis was done with Superscript TM II RT, RNA template removed by RNAse mix, purified in the

SNAP columns, followed by homopolymeric tailing of cDNA and then amplification of the target cDNA. The PCR product was cloned in the pGEMTeasy vector.

Table II-8. Primer sequenced used in 5' RACE PCR

PdPCS3: 5'GTCTCTATCGGCGAGCTCTC 3'
PdPCS4: 5'ACCTCCTGTTCAAGAACATCC 3'
PdPCS5: 5'AAAGTGGAACCATGGAAGGA 3'
PdPCS6: 5'GGTACAAGCATATCAACTCCT 3'
PdPCS7: 5'AGGAAGAAAGTGGAAAGGA 3'
PdPCS8: 5'TGTCTCCTAACTTCTGCAATCC 3'
PdPCS9: 5'ATATTGGCATGTTTCTTCGTGACC 3'
PdPCS10: 5'GAAGGAAACTCAAACCTACCCGA 3'
PdPCS11: 5'CTTAAGCTTGACCCATCTTCCT 3'

2.3. Estimation of duplication history

Using the information on species relationships available at the Angiosperm Phylogeny Site (www.mobot.org/mobot/research/apweb/) a cladogram representing the species tree was constructed. Estimation of the gene duplication and loss history of the *pcs* gene were performed, using NOTUNG v2.6 (Yang 1998), through reconciliation of the species tree with the *pcs* gene trees. In order to obtain the most parsimonious estimation of duplication and loss events, the reconciliation of the species and gene trees was followed by rooting, and rearranging branches with more than 75 % bootstrap support.

2.4. Phylogenetic analysis

The phylogeny of PCS proteins was obtained from 84 sequences, representing all major phylogenetic lineages. *Homo sapiens* syntaxin 1B (STX1B), protein (NM_052874.3) was used as an outgroup. We selected the Jones-Taylor-Thornton model to obtain the phylogenies using the Maximum Likelihood (ML) method. We ran the ML analysis with 100 bootstrap replicates using PHYLIP 3.69 (http://evolution.genetics. washington.edu/phylip.html).

2.5. Prediction of Ancestral PCS Proteins

Ancestral sequence reconstruction (ASR) was used to reconstruct ancestral proteins at specific nodes of the PCS phylogenetic tree. Several different methods of ASR were compared to

determine which method resulted in minimal apparent biases for the PCS dataset. The ancestral sequences were predicted using Ancescon (Cai et al., 2004) with a maximum likelihood (ML) tree calculated using PhyML as reference for the reconstruction.

2.6. Determination of 3D PCS model

Basic components of amino acids and molecular weight of PdPCS1 were analyzed via the ProtParam tool (http://www.expasy.ch/tools/protparam.html). The server Phyre2 (protein homology/analogy recognition engine), which is an improved version of Phyre, was used. After creating the profile of non-redundant sequence, the secondary structure was predicted. Using the profile and model of secondary structure a full length 3D model was generated. The program threads the protein sequence through a PDB file and searches for the possible alignments.

The molecular model of PdPCS1 was generated using the coordinates of the *Nostoc* NsPCS structure as the template in Discovery Studio 4.0 (Accelrys) as described previously (Wang et al., 2009). The resulting docking conformation was further visualized using PyMOL.

2.7. RNA isolation and cDNA synthesis for quantitative PCR

Thirty days old Deglet Nour date hypocotyls have been used for total RNA extractions. Seeds then seedlings have been exposed from day 0 to control or contaminated growth medium (containing 0.02 mM of $CdCl_2$, or 0.02mM $CuSO_4$ or 0.02 mM of K_2CrO_4). RNAs were isolated with the Plant RNeasy mini kit (Qiagen, Courtaboeuf, France), including the on-column DNase digestion step. Concentration and purity of the RNA samples was determined using a Spectrophotometer (SPECTROstar Nano Microplate Reader). RNA quality was visualized using a method adapted from Masek et al., (2005). Reverse transcription was performed on 1 µg of total RNA from each sample using the Random hexamer primers and the Thermo Scientific Maxima H Minus First strand cDNA Synthesis Kit according to manufacturer's instructions.

2.8. Reference genes identification and qPCR amplification

From the previous constructed Deglet Nour date palm cDNA bank we have identified 6 reference gene candidates using HMMER v3.0 program. Primer sequences were designed using Primer 3 plus on line software (Rozen and Skaletsky 2000) and verified using Net

Primer and Beacon Designer programs (Table II-S7). PCR products were cleaned with the EZ-10 spin column DNA gel extraction kit (Biobasic, Markham, Canada) and amplicons were directly sequenced. We identified and examined the expression patterns of elongation factor α1 (*Pdefα1*), aldehyde dehydrogenase type 1 (*Pdaldh1*), actine (*Pdact*), SAND family protein (*Pdsand*), Tubiline β6 (*Pdtub 6*) and TATA-box-binding protein (*Pdtbp*) genes in response to Cd, Cu or Cr stress conditions. Quantitative real-time PCR (qPCR) analysis of gene expression was performed on reverse transcripted RNAs extracted from *Phoenix dactylifera* using MESA Blue qPCR Master Mix reagent kit (Eurogentec, Seraing, Belgium). qPCR reactions were performed with a LightCycler 480 Real Time PCR system (Roche Diagnostics, Mannheim, Germany). The expression levels and amplification efficiencies (E) of each gene, reference (Rg), and target genes (Tg) were determined according to previously described procedures (Brulle et al. 2006). Reference genes were selected using BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004), and geNorm (Vandesompele et al. 2002) algorithms according to the procedure fully described by Brulle et al. (2014).

2.9. Relative quantification and expression level

Real-time PCR efficiencies (E) were calculated from the given slope of the standard curve according to the equation $E = 10^{(-1/slope)}$. E values ranged from 1.91 to 2 (with100 % = 2) and calculated from standard curve. Moreover, *PdPCS* expression level was calculated compared to those of the reference genes selected after gene expression analysis according to previously described procedures (Brulle et al., 2006). The average of the 3 most stable reference genes in each stress condition was used to calculate expression of candidate gene levels according to Brulle et al. (2014). Expression ratio (R) was calculated according to the formula $R= (ETg)^{CPTg} / (Eref)^{CPref}$.

3. Results

3.1. Gene identification and genomic structure

The screening of a cDNA bank of *P. dactylifera* leaves obtained previously in my lab was performed by sequencing some cDNA clones. A full length of 5764 bp was found including 8 exons and 7 introns determined with means of Eukaryotic GeneMark.hmm and GSDS 2.0 software (Lomsadze et al., 2005) (Figure II-9). The codon sequences were collected and verified using the FGENESH 2.6 software and BLASTx. The PCR amplification conditions

were successfully optimized using the degenerate primers indicated in material and methods. Its product was sequenced and a full length *Pdpcs1* mRNA sequence of 1587 bp was obtained.

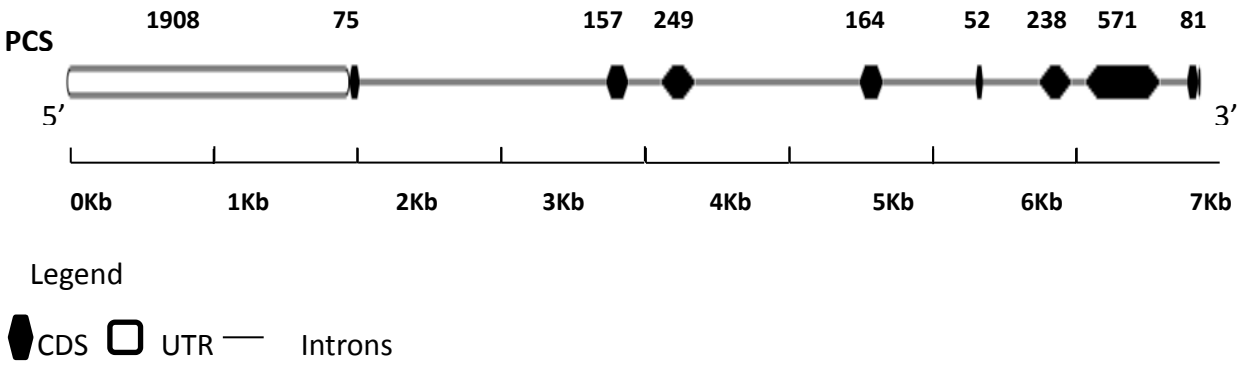

Figure II-9. Genomic organization of the *Pdpcs1* gene. Black boxes: exons, lines: introns, empty boxes: untranslated region (UTR) and Numbers: nucleotide number.

BLASTx searches for additional *pcs* cDNA sequences were performed using the date palm phytochelatin synthase mRNA as a query sequence. From the NCBI and PlantGDB databases we selected 84 sequences of the PCS family from different taxa. *pcs* homologous sequences were found in basically all monocots and dicots for which an appreciable amount of sequence data is available. Sequences from 45 taxa in 8 families of angiosperms have been collected (Table II-S7). Also, *pcs* genes were found in bilateria, bacteria, fungi and ferns. We identified 2 sequences from two different algae species (*Nannochloropsis gaditana*, *Thalassiosira pseudonana*) and from amoeba species (*Dictyostelium fasciculatum*, *Dictyostelium discoideum*). Nucleotide blast sequences alignment showed homology between *Pdpcs1* and other *pcs* sequences higher than 70 %, and largely confined to highly conserved regions in the 5' terminal sequence in which nucleotide identity was as high as 84–95 %. It presented 75 % identity to *pcs* sequence from *Typha latifolia*, and 73 % identity to that of *Nelumbo nucifera*.

3.2. *pcs* gene duplication events

Numerous studies have revealed different fates for duplicate genes in plant development (Kramer et al., 2004; Causier et al., 2005; Yamaguchi et al., 2006). The estimation of *pcs* gene duplication and loss history was performed, using the NOTUNG v2.6 program (Vernot et al., 2008). Based on the reconciled phylogeny (Figure II-10), we have estimated 30 independent gene duplication and 109 gene loss events during its diversification.

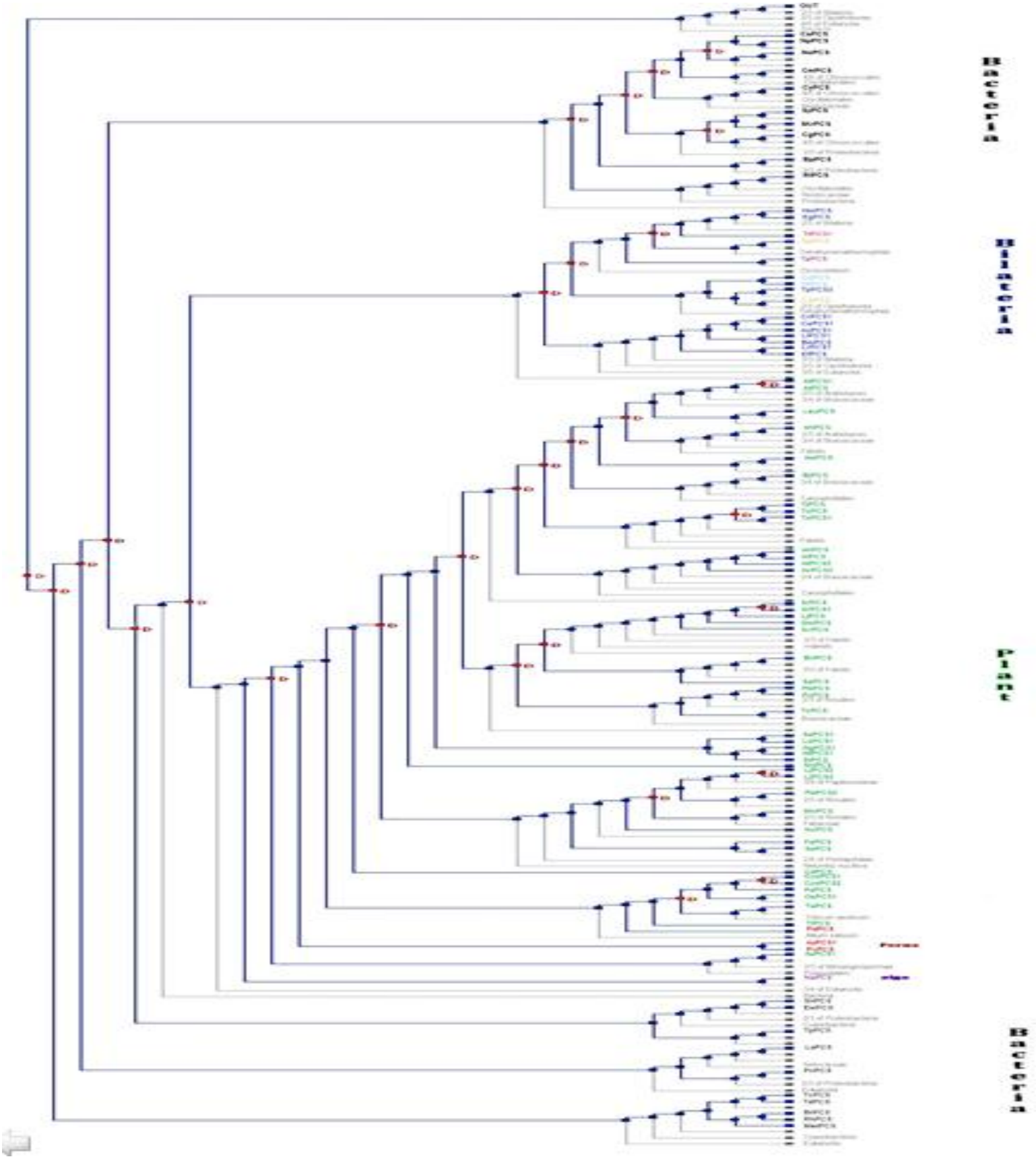

Figure II-10. The phylogeny of *pcs* indicates multiple independent duplication events throughout its evolutionary history. The reconciled *pcs* tree obtained using NOTUNG v2.6, has a duplication/loss score of 27.5. Node "D" for duplication event and grey writing for loss event.

Sequences were duplicated first into two different groups; proteobacteria and cyanobacteria. Dissimilar to bacteria groups, bilateria group contain members of algae, fungi and protozoa *pcs* genes (Figure II-10). Moreover, members of *pcs* plant clade are relatively large compared to the three first clades. Eleven gene duplication events in *pcs* appear toward interior nodes of the reconciled tree and 4 gene duplication events appear toward the terminal nodes.

3.3. PCS protein phylogeny and ancestral sequence prediction

In order to assess PCS distribution in nature, a phylogenetic tree based on the full length of 84 protein sequences has been built (Fig. II-11).

The tree was made using PhyML v3.6 software based on a matrix using Maximum Likelihood method with 100 bootstrap replications. The sequences were grouped into 4 main clades that reflect the phylum of origin of the PCS sequences (Fig. II-11). About 62 % of branches are strongly supported (>75 % bootstrap). In addition, protein-tree analysis showed a remarkably wide and scattered distribution of PCS within the eukaryotic branches. Amoeba, fungi, algae and two bilateria species (HmPCS and EgPCS) were all strongly supported as monophyletic groups and were sisters to all other bilateria with 78 % bootstrap support (Figure II-11, Clade II). In addition, the tree showed that PdPCS1 branches (79 % bootstrap support) in a common sub-clade together with other monocots, including *Typha latifolia*, *Triticum aestivum*, *Oryza sativa*, *Phragmite australis* and *Cynodon dactylon*, and separated from the sub-clade that clusters ferns (79 % bootstrap support).

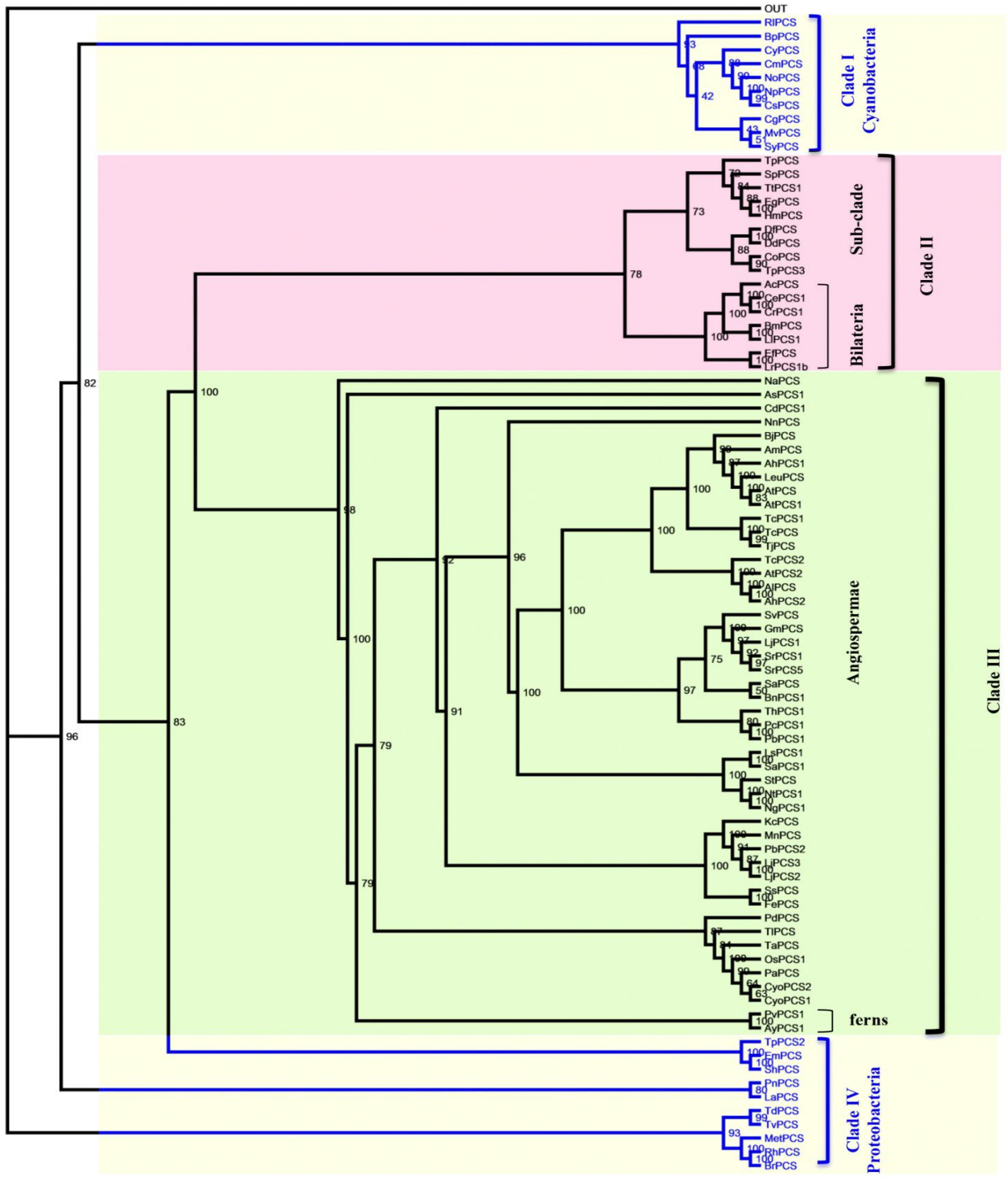

Figure II-11. Phylogenetic relationships of the phytochelatin synthase proteins from NCBI and PlantGDB database. The tree was constructed from the alignment using Clustal W and the Maximum Likelihood method with PhyML v3.6 software. The root was placed using the Human syntaxin 1B protein as outgroup. Numbers on branches indicate the percentage of 100 bootstrap replicates that support the adjacent node; low bootstrap support (<50%) was not reported.

To provide more direct observation of PCS protein evolutionary history, the sequence of ancient protein has been predicted using the ANCESCON program with default parameters (Fig. II-12).

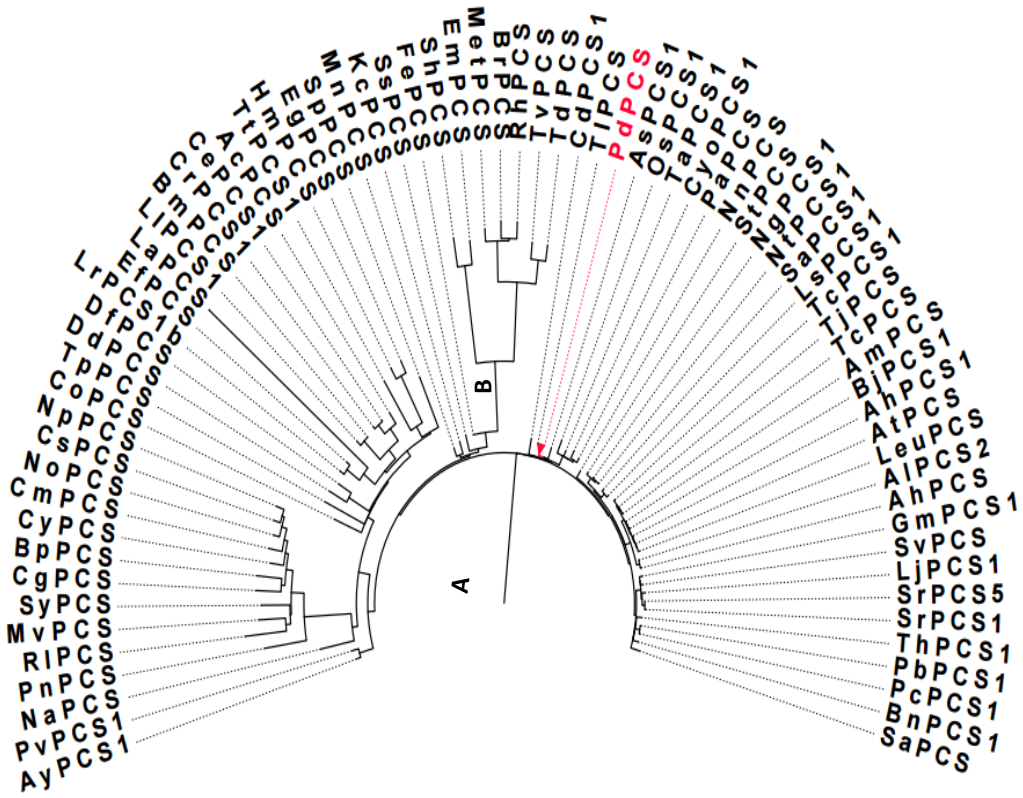

Figure II-12. The tree representing ancestral sequence reconstruction constructed using the ANCESCON program. Evolutionary distances are shown to scale. Nodes A (the ancestor of all PCS amino acid sequences) and B (most recent common ancestor to PdPCS1) are indicated.

In our study, the common ancestor of all PCS amino acid sequences was presented in node **A** (Figure II-12), were branches join, and the most recent common ancestor (MRCA) to PdPCS1 was represented in node **B** (Figure II-12). Surprisingly, protein sequences of bacteria (cyanobacteria, proteobacteria) and of bilateria have diverged significantly, relative to the ancestral protein (node A) compared to mesangiospermae amino acid sequences, suggesting that these sequences have diverged so far that evolutionary relationships are no longer readily apparent. For truly related proteins, even those which are quite divergent; it is often possible to identify locally conserved regions that are important for structure and/or function. Therefore, our next approach was to search for such motifs using Block Maker (Henikoff et al., 1995). Major blocks of conserved sequences have been identified. Thirteen distinct motifs were found (Figure II-S5) and were highly conserved especially in the N-terminal region

(residues 12-218) compared to the C-terminus. The aligned sequences containing the first 7 motifs which constitute the conserved N-ter domain confirmed previous results and represented 95 % of the homology between PCS proteins (Figure II-S5).

3.4. PdPCS1 molecular model

The putative PdPCS1 protein sequence contains 528 amino acids, has a predicted relative molecular weight of 58.39 kDa and theoretical isoelectric point of 6.13. The high instability index (II) (43.91) classifies the protein as unstable.

The protein structural model of PdPCS1 was predicted *in silico* by using the *Nostoc* sp phytochelatin synthase NsPCS partial structure as a template (Fig. II-13). Kinetic studies indicated a substituted enzyme mechanism with the creation of two substrate binding sites in PCS, a high and a low-affinity binding site for GSH to provide the energy for the formation of the new peptide bond in the presence of Cd^{2+} (Vatamaniuk et al., 2002). Five amino acid residues that are highly conserved in plant PCS, namely the cysteine residues at positions 56, 90, 91, 109 and 113 has been found in our PdPCS1 sequence. The first catalytic site is presented by Cys 56 His 162 and Asp 180 (Fig. II-13a). At the same time, three adjacent Cys-Cys components (331 – 332, 351 – 352 and 417 – 418 amino acids) are presented at the C-terminal variable domain of the PdPCS1 protein and suggested to be involved in heavy metal binding sites (Vatamaniuk et al., 2002). Furthermore, Ju-Chen et al., (2013) proposed that a cavity in proximity to the active site of AtPCS might represent the second substrate binding site in which PC molecules were generated from a γGlu-Cys unit and a Cd-GS2 molecule as second substrate. Our PdPCS1 model showed a similar cavity (Fig. II-13b). The second substrate binding cavity is shaped and surrounded by several key amino acids strictly conserved among the PCS sequences; two basic (Arg 152 and Lys 185), one acidic (Glu 52), three aromatic (Tyr 55, Phe 184 and Tyr 186), and two amide residue-containing amino acids (Gln 50 and Gln 157 ; Fig. II-13b).

3.5. Reference gene validation and *Pdpcs1* gene expression

In order to analyze qPCR measurements, we have used an internal control (reference genes) that should be expressed with minimal changes of its expression variation in our experimental conditions. Gene's expression measurement on hypocotyls of date palm exposed during 30 days to metallic stress showed different stability pattern reflecting some genes sensibility to metal.

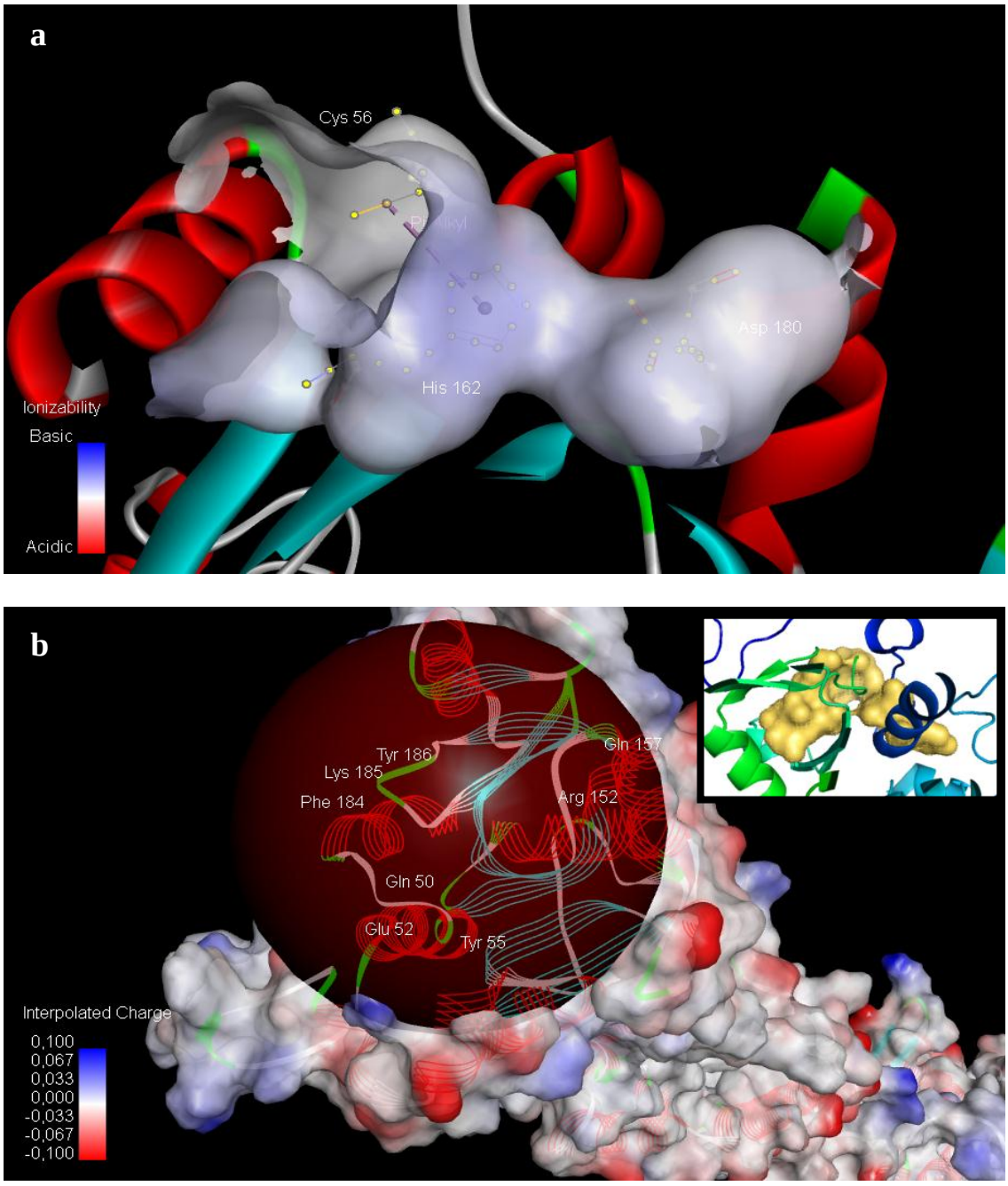

Figure II-13. An overview of the molecular model of PdPCS1, with the putative second substrate binding cavity. (a) The catalytic triad (Cys56, His162, and Asp180) is also shown. (b) The eight conserved residues involved in the formation of the cavity (Gln50, Arg152, Gln157, Phe184, Lys185, and Tyr186, Glu52 and Tyr55). The structures were predicted using the coordinates of *Nostoc sp* phytochelatin synthase NsPCS as a template in Discovery Studio 3.0 (Accelrys) .The resulting docking conformation was further visualized using PyMOL.

The most three stable genes of each condition determined with means of BestKeeper, NormFinder and geNorm algorithms were used to normalize candidate gene expression. Under control conditions, *Pdact*, *Pdtbp* and *Pdsand* have been proved to be the most

appropriate reference genes for normalizing transcript level in palm date tissues. Otherwise, the algorithms ranked the 3 best stably HKs for each stressful condition as follow; for Cd exposure, *Pdtbp*, *Pdact* and *Pdefα1* were the best stable reference genes, for Cu exposure, *Pdefα1*, *Pdaldh1* and *Pdtub6* showed no significant expression variation and under Cr stress, *Pdaldh1*, *Pdact* and *Pdefα1* expressed insensibility even at metal higher amount.

To assess the reliability of previously selected control genes, we determined the expression profile of Pdpcs1 in date palm normalized against its geometric mean. Figure II-14 demonstrated gene expression for control and stressful condition (2.27 units) of hypocotyls tissues generated after 30 days of seeds germination. It showed gene expression enhancement to HMs exposure. Gene transcript level was more expressed under Cd conditions than Cu one but the highest expression level 3.87 folds more than the control was obtained with germs treated with K2CrO4 (Figure II-14).

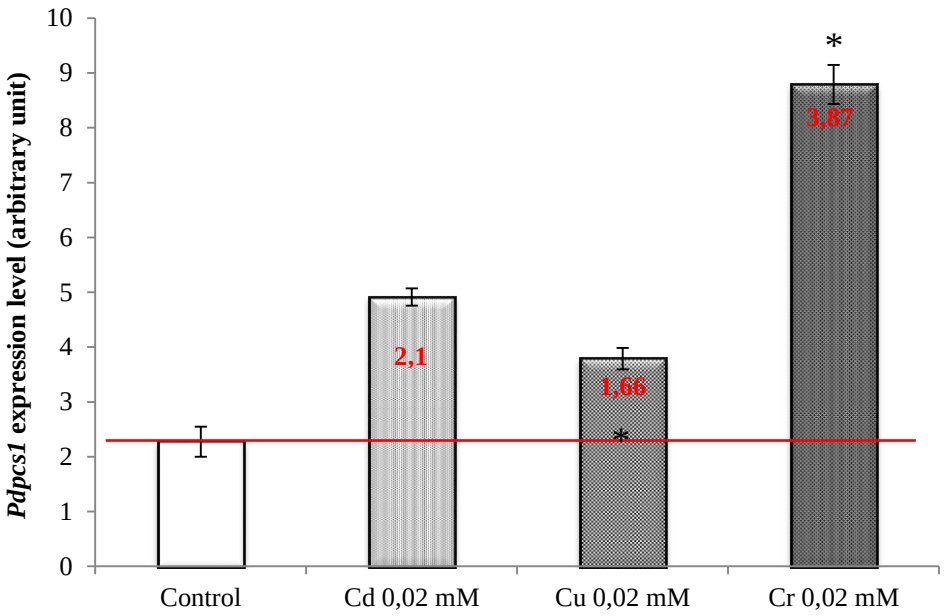

Figure II-14. *Pdpcs1* gene expression levels in *phoenix dactylifera* seeds hypocotyl of 30 days of germination under Cd, Cu or Cr stress conditions. Data presented are means ± standard error of three independent experiments. * P<0.05 level of significant mean difference from control (Student's t test).

4. Discussion

The date palm genome has 36 chromosomes (2n=2x=36), and its size was estimated between 550 Mb (Malek 2010) and ~658 Mb (Al-Dous et al., 2011). Compared to many other crop species, there has been relatively little investment in date palm molecular genetics research,

resulting in serious constraint of an under-developed infrastructure of genetic and genomic tools (Zhao et al., 2013). Interestingly, molecular mechanisms involve in palm resistance to heavy metals remain not explained. For these reasons, cDNA extraction was performed in order to get an idea of date palm gene diversification looking essentially for biomarkers of metallic stress in *Phoenix dactylifera*.

4.1. *PdPCS1* gene isolation and evolution

The assembling of nucleotide fragments provide a full length *Pdpcs1* gene covering 3495 nucleotides of a 1587 bp ORF, and 1908 nucleotides of 5′ untranslated region (5′ UTR) containing TATA box region (Fig. III-9). The DNA sequence consists of 5764 bp, 8 exons and 7 introns, is longer than other plant *pcs* DNA such as pear (*Pyrus betulaefolia*) (You et al., 2012), however, it have the same introns and exons number compared to genes structures of *Pbpcs1* and *Ljpcs1*. Yet, *Atpcs1* consists of nine exons. Interestingly, exons 1, 2, 4 and 5 of *Pdpcs1* gene have identical sizes with those in *Atpcs1*, *Ljpcs1* and *Pbpcs1* (75, 157, 164 and 52 bp) (You et al., 2012).

A search in the NCBI database using the basic local alignment search tool (BLAST) was performed (Altschul et al., 1990). *pcs* gene family from different taxa has been identified. Data clearly support the notion that *pcs* genes are present at least in all higher plants; angiosperm. However, even in the PlantGDB database, no *pcs* from gymnosperms has been found. Nevertheless, much still remains to be learned about the evolutionary outcome of duplicated genes regarding their impact on genetic pathway and trait evolution (Preston and Hileman 2013). Gene duplication is common in plants and plays a key role in trait evolution (Airoldi and Davies 2012). The resulting Notung tree (Figure II-10) showed multiple independent duplication events which occurred throughout the evolutionary history of the *pcs* gene. During its diversification, *pcs* gene describes 109 gene loss and 30 gene duplication events. Loses were predicted from several lineages during evolution including bacteria, fungi, bilateria and angiospermae which may simply represent unidentified genes. However, duplication of *pcs* genes appears likely in some species such *Arabidopsis*, *Triticum* and *Allium*. The first duplication evolves into proteobacteria and cyanobacteria describing a sub-functionalization. In fact, species development and especially photosynthetic potential acquired by cyanobacteria may be the cause of the functional evolution of *pcs* gene through the partitioning of ancestral functions. Moreover, the heterogeneity of bilateria clade deduce by the notoung tree may be due to animal *pcs* genes which are sparsely found across the tree

because they are not found in all animal phyla; and major sub-groups within phyla may be lacking *pcs* homologues. In the other hand, *pcs* plant clade represent eleven gene duplication events appear toward interior nodes of the reconciled tree and 4 gene duplication events appear toward the terminal nodes indicating that this later events were more recent in evolutionary time (Fig. II-10). All four duplications appear in species that have a second copy of *pcs* suggesting that this gene evolves through subfunctionalization. Moreover, one of the strongest evolutionary pressures for land colonization by plants (and by other phototrophic organisms, such as lichens) arose from their potential access to much greater amounts of nutritive ions from surface rocks, compared with water environments (Ligrone et al., 2012). As a consequence, land colonization may have resulted in a need to carefully regulate trace element homeostasis and to minimize the risk of accumulation of toxic concentrations of certain metal(loid)s, thus leading to the evolution of response mechanisms among which PC synthesis plays a central role (Sanità diToppi and Gabbrielli, 1999). In fact, climate change and the increase of heavy metals widespread in ecosystems may require plants to modify their defense machinery. Furthermore, dimensional increases of the enzyme length were found in angiosperms as demonstrated in Supplemented material S1. Similar findings were presented by Rea (2012) and further explored by Petraglia et al., (2013), who found that improve of enzyme functionality can be mainly ascribed to increases in the length of the C-terminal domain.

Besides, looking at the phylogenetic tree (Fig. II-10), monophyletic groups appear to be far-scattered which may suggest, according to Clemens report (Clemens 2006), that the *pcs* coding-gene was likely acquired via horizontal gene transfer (HGT) from other eukaryote lineage. However, fern *pcs* genes (*Athyrium yokoscense Aypcs1* and *Pteris vittata Pvpcs1*) as well as *Pdpcs1* showed less duplication events and highly conserved domain (Supplementary Figure II-S5). We inconclusively hypothesized that ferns might be an example of an intermediate evolutionary forms supporting previous founding that angiosperms may be an example for the former and the date palm one for the latter case regarding to its perennial character, classified as one of the world's first cultivated fruit trees representing an ancient group of fruit trees (Zhao et al., 2013).

4.2. PCS protein phylogeny

PHYLIP generating protein tree showed a distribution of the PCS across many phyla including bilateria (Fig. II-11). These findings contradict previous reports (Clemens 2006)

suggesting that PCs have not been detected in any organisms carrying PCS other than plants, fungi and algae. However, to date, PCS homologues have been identified in only a very small number of animal species (and biochemical characterization of recombinant PCS so far restricted to three: *C. elegans*, *S. mansoni*, and *A. ceylanicum*). In fact, proteins are found across the deepest divisions of the Metazoa (Bundy and Kille 2014). Still within the phylum Nematoda, Rigouin et al., (2013) looked at the parasitic species *A. ceylanicum*, and showed that the AcPCS also produced PCs when expressed in *S. cerevisiae*. Furthermore, they reported close homologues of PCS in other parasitic nematode species, including *Brugia malayi*, *Loa loa*, and *Ascaris suum*. Unlike the situation for *C. elegans*, though, exposing the flukes to Cd did not result in any detectable PCs in tissue extracts (Cui et al., 2007). Nonetheless, this distribution argues that it is likely to be found in more animal species in the future.

PCS clade III (Fig. II-11) proteins are characterized by their large size including seed plants and ferns which formed a sub-clade. Ferns and angiosperm strongly supported monophyletic groups, but their basal relationships were not fully resolved. They shared a common ancestor suggesting a probability that too many reversals of nucleotide substitutions disable a reliable phylogenetic reconstruction (Figure II-12). Yet, ancestral sequence reconstruction (ASR) has proved a useful experimental tool for studying the diverse structure and function of proteins (Harms and Thornton 2010). To date, experimental molecular archeology using ASR has been applied to several enzymes (Perez-jimenez et al., 2011), including photo-reactive proteins (Field and Matz 2010), viral proteins (Kaiser and Malik 2007) and a number of peptides (Hult et al., 2008).

PHYLIP tree (Fig. II-11) showed first divergence of bacteria PCS protein witch may hypothesize that evolutionary relationships are obscured due to the shorter PCS length sequences (Table II-S6). In fact, from prokaryotes, some cyanobacterial (including two Nostoc species) and five of proteobacterial origin, are approximately one-half the length of their cognates from eukaryotes because they lack the variable C-ter domain (starting from approximately amino acid residue 220) (Rea et al., 2012). This domain is generally less well conserved between PCS proteins and is present only in eukaryotic, but not prokaryotic species (Rigouin et al., 2013). The occurrence of the half-size PCS proteins in prokaryotes is extremely scattered. Out of more than 2000 analyzed bacterial genomes only 38 have *pcs* genes (Clemens and Persoh 2009). Remarkably, even sister strains often differ in the possession of PCS-like genes. Information on metal tolerance of these strains or their ability

to synthesize phytochelatins is to our knowledge not available.

Therefore, we are interested on major blocks of conserved sequences. We have been identified thirteen distinct motifs highly conserved especially in the N-terminal region (residues 12-218) compared to the C-terminus (Figure II-S5). The N-ter represents the catalytic active domain of PCS (Cys 56, His 162 and Asp 180) (Figure II-S5). Whereas, C-ter PCS function is not completely understood. It was suggested to function in metal regulation of protein activity in the *Arabidopsis thaliana* PCS (Romanyuk et al., 2006) and recently, Rigouin et al., (2013) shown that the C-ter domain of *Schistosoma mansoni* PCS is neither involved in metal sensitivity nor in the modulation of protein activity. However, the lack of significant overall sequence identity between the members of these groups could indicate that these proteins arrived at a common function by convergent evolution from different progenitors.

4.3. PdPCS1 structure

In AtPCS1, Cys 56, His 162 and Asp 180 were demonstrated to be able to catalyze a transpeptidation (Rea *et al.,* 2004) and so confirmed to be the catalytic triad, explaining the mechanism of γ-glutamylcysteine synthesis (Ju-Chen et al., 2013). Indeed, in our model (Figure II-12a), the same residues have been found describing the first site of PdPCS1 acylation. Residue Cys 56 (which is 100 % conserved in all known PCS) was subsequently proven to be indispensable for PC synthesis activity and to represent the first site of (Cd^{2+} independent) acylation (Clemens 2006). It catalyzes the removal of Gly on the first GSH, resulting in the formation of the γGlu-Cys acyl-enzyme intermediate.

For the second binding cavity (Fig. II-12b), the positive charge of Lys185 might attract the carboxyl group, leaving the free amino group of the second substrate available to attack the acylated γGlu-Cys on the active site and produce PC (Ju-Chen et al., 2013). The carboxyl group on Glu 52 might couple with the cationic Cd through ionic interaction, whereas the aromatic group on Tyr 55 might produce a cation-π complex with Cd (Singh et al., 2009). Arg 152 and Lys 185 are 2 basic amino acids that might play roles in interaction with the second GSH. However, Lys185 might directly interact with the second substrate, whereas Arg 152 stabilizes the binding site structure through noncovalent bonds with residues His 162, Asp 180, and Tyr 186 (Ju-Chen et al., 2013). Gln50 may stabilize the tetrahedral transition state assumed by the first substrate and the modifications of the chain length at this residue completely abolish catalytic activity (Rea 2012). Our hypothesized interaction between The

49 and Arg 183 may act to connect B-loop 1 and B-loop 4. This interaction is triggered by protein phosphorylation and might be critical for the formation of a functional catalytic site (Ju-Chen et al., 2013).

Based on the model of the N-ter structure of AtPCS1, we gained some information on the tentative second substrate binding site on PdPCS1. Differences between bacterial proteins, with lower synthetic activity and lack of the C-ter, and eukaryotic PCS proteins in structure and activity likely reflects the bi-functionality of eukaryotic phytochelatin synthases as peptidases and transpeptidases (Clemens and Persoh 2009). Recent observations indicate that PCS serves physiological functions aside from metal detoxification. They are hypothesized to be involved in Zn homeostasis, plant defense responses relied surveillance systems for nonself recognition and may act as a peptidase in indole glucosinolate metabolism (Clemens and Persoh 2009).

4.4. *Pdpcs1* gene expression pattern

Detoxification mechanism developed by *Phoenix dactylifera* with PCS was not investigated yet. We used an *in silico* approach to identify gene exhibiting and molecular expression technique to investigate whether the gene is active. Based on the present study, we provide evidence that *Pdpcs* type 1 gene is part of the tolerance mechanism to metallic stress in seeds hypocotyl. The expression levels of *Pdpcs1* gene were analyzed by quantitative real-time PCR (Figure II-13). This technique has been adopted in a wide range of scientific areas and research works (Pfaffl et al., 2004) because of its advantages in sensitivity, specificity and of its extensive range of detection (Bustin, 2000). However, to evaluate qPCR data, identification of internal control genes; stable reference genes whatever the studied condition, is required. In this study we identified the three best stably housekeeping for each tested condition for normalizing qPCR assays in date palm plant model. Although several studies have used the qPCR for date palm gene expression, none has been validated reference genes before. At control conditions, gene expression was 2.27 arbitrary units after 30 days of seeds germination. The transcript levels of candidate gene showed that low Cu^{2+} concentration (0.02 mM) has the lowest gene induction compared to Cd^{2+} and especially Cr^{6+} (Fig. II-13). Similarly, the transcript levels of phytochelatin synthase type 1 of *Laetuca sativa* (*Lspcs1*) on leaf and root were all much higher under cadmium stress (Zhenyan et al., 2005). Yet, *Avicennia germinans* phytochelatin synthase gene *Avpcs* expression showed a significant increase under copper conditions from the first hours of stress application (Gonzalez-

Mendoza et al., 2007). Authors suggested that this rapid increase in *Avpcs* expression confirm that it was the most active gene involved in the regulation of copper in *A. germinans* leaves. Authors demonstrated that the time was different for the transcript levels of *Lspcs1* to reach peak level. It is needed 12 h for the leaf, and only 2 h for the root in the Cd treatment (Zhenyan et al., 2005). However, *Phoenix dactylifera* seeds germination was completed within 40 – 45 days after generating a cotyledon leave developed from hypocotyl used for RNAs extractions. In this way, the exposure to Cu^{2+} induces *Avicennia germinans Avpcs* gene expression in leaves. Indeed, previous researches reported absence of metals sequestering phytochelatins under Cr^{6+} stress (Shanker et al., 2004). Manipulation of enzymes involved in the PC biosynthesis pathway (*GS*, *Tapcs1*, *Atpcs1*) leads to higher PC production, and consequently higher heavy metal tolerance and accumulation in plants (Gasic and Korban 2007) and bacteria (Sauge-Merle et al., 2003).

5. Conclusions

Phytochelatins are essential for toxic element detoxification in plants, some fungi, and animals. The fact that the responsible enzymes, phytochelatin synthases (PCS) are in most species constitutively expressed and so widespread in nature, raises questions. The *pcs* gene trees barely reflected the currently accepted phylogenetic relationships, as the genes of major lineages did not cluster together. Yet, enzyme function was proven for three model systems from three different kingdoms (prokaryote, plant and fungi, nematodes). EST data clearly support the notion that *pcs* genes are present at least in all higher plants. In addition, based on the identification of the consensus amino acids surrounding the active site, we hypothesized a possible binding and catalytic mechanism for the second substrate binding cavity on PdPCS1. Data mining and *in silico* approach led to the identification and description of a *Pdpcs* gene in date palm and to a prediction of its protein structure. The results reported by qPCR showed that *Pdpcs1* gene is not only present but fully operational in *P. dactylifera*. Its level of expression may vary in seedlings exposed to metals suggesting that *Pdpcs1* gene product is involved in tolerance to metals.

Conflict of interest

The authors declare that they have no conflict of interest and there are responsible for the content and writing of the article

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Chapitre II

Effets de la variabilité génétique sur la réponse métallique

-Avant-propos-

La germination des graines est considérée comme l'un des meilleurs indicateurs pour le développement d'une plante dans certaines conditions. À ce stade, les semis sont faibles, tendres et équipés de mécanismes qui lui permettent d'assurer une germination lorsque les facteurs environnementaux sont favorables. Toutefois, ils peuvent être affectés par des conditions défavorables. Le test de tolérance aux éléments traces métalliques (ETMs) au stade de la germination est une étape clé.

De plus, étant une espèce à pollinisation croisée, le palmier dattier maintient une importante variabilité génétique et des différences de caractères phénotypiques entre cultivars ainsi qu'une importante hétérogénéité des graines. Cette diversité génétique a des effets écologiques importants sur la viabilité à long terme des populations et le fonctionnement des écosystèmes. Cependant, on sait très peu de choses sur les réponses des graines de palmier dattier aux stress métalliques dans les stades de germination et de croissance précoces. Nous avons donc réalisé des essais de germination des graines à différentes concentrations métalliques afin de mieux comprendre les stratégies de défenses adoptées par le palmier dattier et dans le but d'identifier des biomarqueurs individuels et infra-individuels..

Au cours de ce chapitre nous présentons i) la réponse morphologique, physiologique et biochimique des graines de palmier dattier face au stress par le Cd, le Cu ou le Cr ii) la différence d'expression génique de biomarqueurs de stress métallique, ainsi que iii) les méthodes de validation des gènes de référence, décrites pour la première fois chez le palmier dattier en conditions de stress par les métaux lourds, une étape cruciale au suivi de l'expression de gènes candidats au fil du temps.

-Publications-

A/ Chaâbene Zayneb, Rekik Hakim Imen, Rorat Agnieszka, Fki Lotfi, Amine Elleuch, Mejdoub Hafedh, Vandenbulcke Franck. **Copper toxicity and date palm seedling tolerance: monitoring of related biomarkers.** *Environmental Toxicology and Chemistry*. Article accepté le 11-10-2017.

B/ Zayneb Chaâbene, Rorat Agnieszka, Rekik Hakim Imen, Bernard Fabien, C. Douglas

Grubb, Elleuch Amine, Vandenbulcke Franck, Mejdoub Hafedh. **Insight into the expression variation of metal-responsive genes in the seedling of date palm (*Phoenix dactylifera*)**. *Chemosphere*. In press

A-Copper toxicity and date palm seedling tolerance: monitoring of related biomarkers

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I. Synthèse

Des graines de palmier dattier cv Deglet Nour ont été mises à germer dans différentes concentration de $CuSO_4$ afin d'examiner leurs réponses au stress métallique. A des teneurs métalliques faibles de 0,02 mM de Cu, seuls des changements dans l'expression des gènes *Pdpcs* et *Pdmt* ont été significatifs (Figure II-18). Comme chez les vitroplants de Deglet Nour, le gène *Pdpcs* était plus sensible aux ions Cu^{2+} que celui de *Pdmt* pour lequel le niveau d'expression était plus faible. Toutefois, le contact direct des graines de *P. dactylifera* dans des solutions de $CuSO_4$ sans milieu de culture, a diminué le niveau d'expression de *Pdpcs* dès 45 jours de stress par 2 mM de Cu. Par ailleurs, *Pdmt* a été sous-exprimé à fortes doses métalliques (Figure II-18) chez les graines contrairement à ce qui est observé chez les vitroplants.

A de faibles doses métalliques, les mécanismes oxydatifs (H_2O_2, TBARS) et antioxydatifs (CAT, SOD) ont augmenté linéairement avec l'augmentation du temps d'exposition (Tableau II-10). Les maximums des teneurs en H_2O_2 et en TBARS ont été observés après 2 mois d'exposition à 2 mM de Cu. A ce niveau élevé de contamination, des perturbations de l'équilibre redox dérégulant le système de défense antioxydant des graines ont été montrés provoquant des dommages oxydatifs surtout après une longue exposition. L'activité CAT et l'activité SOD ont diminué de presque 80 % sous l'effet de 2 mM Cu après deux mois d'exposition (Tableau II-10). De plus, l'étude a montré que l'activité amylolytique était dépendante de la dose du métal et du temps d'exposition (Figure II-16). Une amélioration de l'activité hydrolytique lors des expositions à faible doses a été obtenue. Cependant, l'effet de 2 mM Cu était défavorable pour l'enzyme qui a diminué son activité de plus de 70 % (Figure

II-16).

Les perturbations biochimiques et moléculaires mises en évidence à des concentrations élevées en Cu ont probablement inhibé significativement la germination des graines (Figure II-15a), ont retardé l'allongement des hypocotyles (Figure II-15b), et ont induit la mortalité des semis réduisant ainsi, l'indice de germination de plus de 90 % (Figure II-15a). Ainsi, des symptômes de toxicité liés au stress cuivrique sont apparus dès les premiers 15 jours d'exposition à 2 mM de Cu. Ces observations ont fourni un nouvel aperçu de la réponse des cellules de *P. dactylifera* aux ions cuivriques qui peuvent être toxiques même s'il s'agit d'un métal essentiel.

II. Article

Copper toxicity and date palm seedling tolerance: monitoring of related biomarkers

Abstract

Date palm seeds were exposed to different copper solutions to examine plant stress responses. Low Cu concentrations (0.02 and 0.2 mM) caused an increase of seed germination, whereas, higher Cu amount (2 mM) significantly inhibited seeds germination, delayed hypocotyl elongation, increased seedling mortality and reduced germination index by more than 90 %. Metal-related toxicity symptoms appeared after 15 days of 2 mM Cu exposure. Biochemical activities, like amylase activity and redox balance elements were examined in order to study the relationship between external Cu amount and internal plant response. The study showed that amylolytic activity was dose and time dependent. Likewise, H_2O_2 production increased after exposure to Cu, which was correlated with thiobarbituric acid reactive substance accumulation. Furthermore, at low Cu concentrations, superoxide dismutase and catalase activities increased, suggesting that date palm seed stimulated its metal homeostasis networks. However, highest cupric ions amount increased cell oxidant accumulation and reduced enzymes production. Gene expression level measures of *Phoenix dactylifera* phytochelatin synthase (Pd*pcs*) and metallothionin (Pd*mt*) encoding genes have been carried out to investigate the implication of PdPCS and PdMT proteins in Cu homeostasis and/or its sequestration. *Pdmt* induction reached a peak after 30 days of exposure on 0.2 mM Cu. However, it was down-regulated in plants exposed to higher Cu concentrations. In the same conditions, *Pdpcs* was overexpressed during a month of exposure before it decreased thereafter. These observations provide a new insight into date palm cell response to copper, a metal which can be toxic but which is also an essential element.

Keywords: date palms, seed germination, amylase activity, antioxidative responses, biomarkers gene expression.

1. Introduction

Metals are natural components of terrestrial systems occurring in soil, rock, air, water, and organisms. Several transition metals, including zinc (Zn), manganese (Mn) and copper (Cu), are essential constituents of physiological processes in living organisms including plants. However, their presence in higher amounts makes them not only dangerous poisons for plants' life but also toxic pollutants for the environment (Zimeri et al., 2005). The potential

toxicity of trace metals in soil depends on their mobility and bioavailability. Naturally, the most radical change in the availability of some transition metal ions has been shown when the Earth's atmosphere became oxygenic. It dramatically reduced the bioavailability of some transition metals, like iron, and made others more available, primarily copper (Ute Krämer, 2005).

Cu is an essential redox-active transition metal involved in many biochemical processes. However, exposure to excess Cu has a detrimental effects on plant growth. Among the others, it may reduce biomass, affect root growth and morphology, induces chlorotic symptoms on older leaves and dark-red margin necrosis. Moreover, a decrease of the photochemical activity was shown due to a decrease in the electron transfer rates as a result of cupric ions occupation to several sites of photosystem II (PSII), particularly susceptible to these ions (Quartacci et al., 2001). Redox properties of Cu^{2+} might increase the production of toxic oxygen free radicals (ROS), particularly the highly reactive hydroxyl radical, via the Haber-Weiss reaction by the biodegradation of membranes and biotransformation of metallic moieties (Azqueta et al., 2009; Soundararajan et al., 2009). Mechanisms involved in the management of ROS have been extensively studied since they can avoid oxidative damages (Drążkiewicz et al., 2014; Wang et al., 2004). Among these homeostatic mechanisms, plants promoted the Halliwell-Asada pathway, for instanceglutathione reductase (GR), and ascorbate peroxidase (APX) responsible for the scavenging of ROS produced in part by superoxide dismutase (SOD). The enzymes, which react with superoxide radicals to convert them to oxygen (O_2) and hydrogen peroxide (H_2O_2) represent an early line of defense. Activities of enzymes implied in ROS production and degradation have been extensively studied in numerous plant and animal species in order to estimate the impact of contaminants and especially metals and/or to identify biomarkers of exposure (Bernard et al., 2016).

Exhibiting high affinities to ions, phytochelatins (PCs) and metallothioneins (MTs) were characterized as the two most efficient heavy metal binding ligands in plant cells (Hall, 2002). It has been shown that amounts of PCs increase in response to many heavy metals including copper which constitute a strong activator of enzymes' biosynthesis, both *in vivo* and *in vitro* (Morelli and Scarano, 2004). A clear role in Cu detoxification has been shown (Cobbett and Goldsbrough, 2002). During the exposure of plants to metals, PCs are synthesized from glutathione (GSH) by phytochelatin synthase (PCS) activities (Cobbett, 2000). PCS have been well described in plants, which makes the characterization of the genes encoding these

enzymes attractive (Vatamaniuk et al., 2000). These dumbbell-type enzymes contain two metal-binding and cysteine-rich domains. In addition, MTs are small cysteine-rich proteins produced directly by mRNA translation. Heavy metals such as Cu and arsenic (As) greatly induced the expression of *mt* in *Festuca rubra* (Mi et al., 2003) and *Fucus Vesiculosus* (Merrifield et al., 2004) providing evidence to heavy metal detoxification of MT proteins in these plant species.

Previously cited molecules (ROS, antioxidant enzymes, chelators, …) offer a battery of potential biomarkers. Nevertheless, further identification of specific molecular, biochemical, physiological and morphological changes in plants following cupric stress is necessary. Biomarkers constitute a powerful tool for measuring the extent of exposure and predicting the impacts of metal contaminants in biota (Bielen et al., 2013). For instance, gene expression variations may be "early warning" signals, enabling a more vigorous environmental monitoring (Snape et al., 2004) and constitutes a suitable and well-established approach for analyzing the effects of heavy metals (HMs) exposures (Bonassi et al., 2005).

However, very little is known about date palm responses to metal-related stress during germination and in the early growing stages. At this stage, seedlings are weak, tender and equipped with sensing mechanisms that allow it to assure germination when environmental factors are favorable. Hence, they can be affected by adverse conditions making heavy metal tolerance test at crop germination stage a key step for the plants establishment under any prevailing environment (Wang and Zhou, 2005). In this paper, copper toxicity and seedling tolerance was assessed in date palm using individual and infra-infra-individual biomarkers.

2. Material and methods

2.1. Experimental layout, treatment and seedling growth parameters measurement

Seeds from the Deglet Nour date palm cultivar were used. Mature seeds were washed extensively in water then surface-sterilized with sodium hypochlorite solution. Under sterile conditions, 100 % of commercial sodium hypochlorite was added to seeds for 10 min followed by 50 % of diluted solution for other 10 min and completed with more diluted sodium hypochlorite (25 %) during the same time. Seeds were then rinsed 3 times for 10 min with distilled water. Formerly, seeds were exposed in direct contact with 0, 0.02, 0.2 or 2 mM Copper solutions prepared in distilled water using Cupric sulfate ($CuSO_4 \cdot 5H_2O$, Sigma) and

kept in the dark at 26°C during two months. Seedlings (10 per replicate; three biological replicates per concentration) were excised from seeds at 12, 15, 30, 45 and 60 days after germination.

The inhibition rate on seed germination and seedling growth were calculated using the formula of Ahn and Chung (2000) (Ahn et al., 2000)=[(Treated samples results)/(Control results) * 100].

Germination associate parameters were calculated by using following formulas described by Chaâbene et al., (2015) (Chaâbene et al., 2015):
Germination percentage represents the number of germinated seeds / total number of seeds × 100;
Speed of Germination= n1/d1+n2/d2+n3/d3+---------- Where, n = number of germinated seeds, d= number of days;
Mean Daily germination (MDG) = Total number of germinated seeds/ Total number of days;
Mean Germination Time (MGT) = MGT = Σdn/ Σn, where n: Number of seeds which were germinated on day d, d: Number of days counted from the beginning of germination;
Peak Value (PV) = Highest seed germinated/ Number of days;
Seedling Mortality (SLM) = number of germinated seeds / total number of seeds × 100;
Germination Index (GI) were measured following the formula $GI\% = \frac{NE \times LE}{NC \times LC} \times 100$.

2.2. Estimation of H_2O_2 content and Lipid peroxidation

Hydrogen peroxide concentration was determined as previously described by Elleuch et al. (2013) (Elleuch et al., 2013). The levels of lipid peroxidation products were evaluated as thiobarbituric acid reactive substances (TBARS) aldehydes according to Rustérucci et al. (2016) (Rustérucci et al., 1996). The concentrations of TBARS were calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.3. Enzyme analysis

Hypocotyl fresh tissue (0.5 g) was homogenized in 1.5 mL of potassium phosphate buffer 0.1 M, pH 7 containing 4% polyvinylpyrrolidone (PVP) and centrifuged at 10 000 g for 30 min and the supernatant was used as enzyme extract. Total Protein content was determined spectrophotometrically according to the method of Bradford (1976) (Bradford, 1976), using bovine serum albumin as a standard.

2.4. Amylase activity, antioxidative enzyme assays and protein content measurement

Amylase activity was measured as described by Elleuch et al., (2013) (Elleuch et al., 2013). The reaction was performed at 60 °C and pH 7 for10 min. The reaction mixture contained 0.5 % (w/v) starch in 25 mM phosphate buffer pH 7 and the enzyme solution in a final volume of 1 mL. The activity of total amylase enzymes was measured colorimetrically by estimating the concentration of reducing sugar formed (maltose) determined by the 3, 5 dinitrosalicyclic acid (DNS) method (Miller, 1959).

Assays of antioxidant enzyme activities in hypocotyls samples were prepared for catalase (CAT) and superoxide dismutase (SOD) analyses by homogenizing the frozen tissue with a mortar and pestle in a potassium phosphate buffer 0.1 M, pH 7 (1.5 ml: 0.5 g fresh weight) to extract soluble protein. The homogenate was centrifuged at 13.000 g for 15 min. All operations were performed at 4 °C.

SOD activity was assayed using the photochemical nitrobluetetrazolium (NBT) method and measured according to Beyer and Fridovich (2016) (Beyer and Fridovich, 1987). Catalase was determined as described by Aebi (1984) (Aebi, 1984).

2.5. RNA isolation, cDNA synthesis, HKGs validation for PdPCS and PdMT gene expression measurement

Frozen hypocotyl tissues (approximately 100 mg) were ground in liquid nitrogen using a tissue homogenizer. RNAs extraction and cDNA synthesis were performed as per Chaâbene et al. (2017). A mixture of frozen seedling tissues of all experimental time point of each experimental condition has been used for RNAs extraction and cDNA synthesis to clone candidate reference gene. Using the previously designed specific primers for; elongation factor α1 (*PdEFα1*), aldehyde dehydrogenase type 1 (*Pdaldh1*), actine (*Pdact*), SAND family protein (*Pdsand*), Tubiline β6 (*Pdtub 6*) and TATA-box-binding protein (*Pdtbp*) and candidate gene *Pdpcs* and *Pdmt* (Chaâbene et al., 2017a) we performed quantitative real-time polymerase chain reaction (qPCR) amplification method according to Brulle et al., (2006) (Brulle et al., 2006). The expression levels and amplification efficiencies (E) of each gene, reference (Rg), and target genes (Tg) were determined according to previously described procedures (Brulle et al., 2006). To estimate the expression stability of reference genes, we measured the relative quantities of Cq values of genes obtained using the formula of (Hellemans et al., 2007). As per Martins et al., (2016) (Martins et al., 2016a) different

algorithms and statistical analysis were applied to evaluate the expression stability of the reference genes of *P. datylifera* plants in seedling under control and copper conditions. The analysis procedures strictly followed the manuals of BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004) and geNorm (Vandesompele et al., 2002) algorithms algorithms. The geometric mean of the 3 most stable reference genes in cupric condition was used to calculate expression of target gene levels according to Brulle et al., (2014) (Brulle et al., 2014). Absolute quantification of genes expression levels are in log2. Expression level of control samples vs expression level of exposed samples have to be normalized to calculate « relative fold expression » as detailed by Bernard et al., (2016) (Bernard et al., 2016).

2.6. Statistical analysis

The data in all experiments are the averages of three replicates ± S.E statistically analyzed for the calculation of standard error (S.E.). Student's t-test and ANOVA analyses were performed for testing hypotheses using SPSS Statistic 17.0. A probability of $p<0.05$ was considered statistically significant.

3. Results

3.1. Copper effects on date palm seeds germinability and its related physiological perturbations

Date palm hypocotyl appeared always after 12 days in darkness. Germination percentage (GP) varied widely (Fig. II-15a). Low copper amounts (0.02 and 0.2 mM $CuSO_4$) did not affect long term seed germination, whereas, high copper concentration (2 mM) significantly reduced seed germination by approximately 20 %. The highest germination rate (GP = 100 %) was observed after 12 days for seeds exposed to 0.2 mM Cu and was slightly higher than in control (97 %) and in 0.02 mM Cu (97 %) exposed groups. Seed soaking in 2 mM Cu reached its maximum GP (80.5 %) after 20 days of exposure (Fig. II-15a). The hypocotyl length of treated and non-treated date palm seeds has been monitored and is showed in figure 1b. It was found that, except of the highest copper concentration (2 mM), there were no important differences between treatments. After 60 days of exposure, the best growth (i.e., corresponding to negative inhibitory rate of hypocotyl length) was observed for seeds exposed to 0.02 mM Cu. Hypocotyl growth was significantly affected by excess copper. Indeed, a slight inhibitory effect was reported for seeds exposed to 0.2 mM and a strong inhibition of seedling growth (i.e., highest inhibitory rate of hypocotyl growth) was observed when seeds

were exposed to 2 mM Cu.

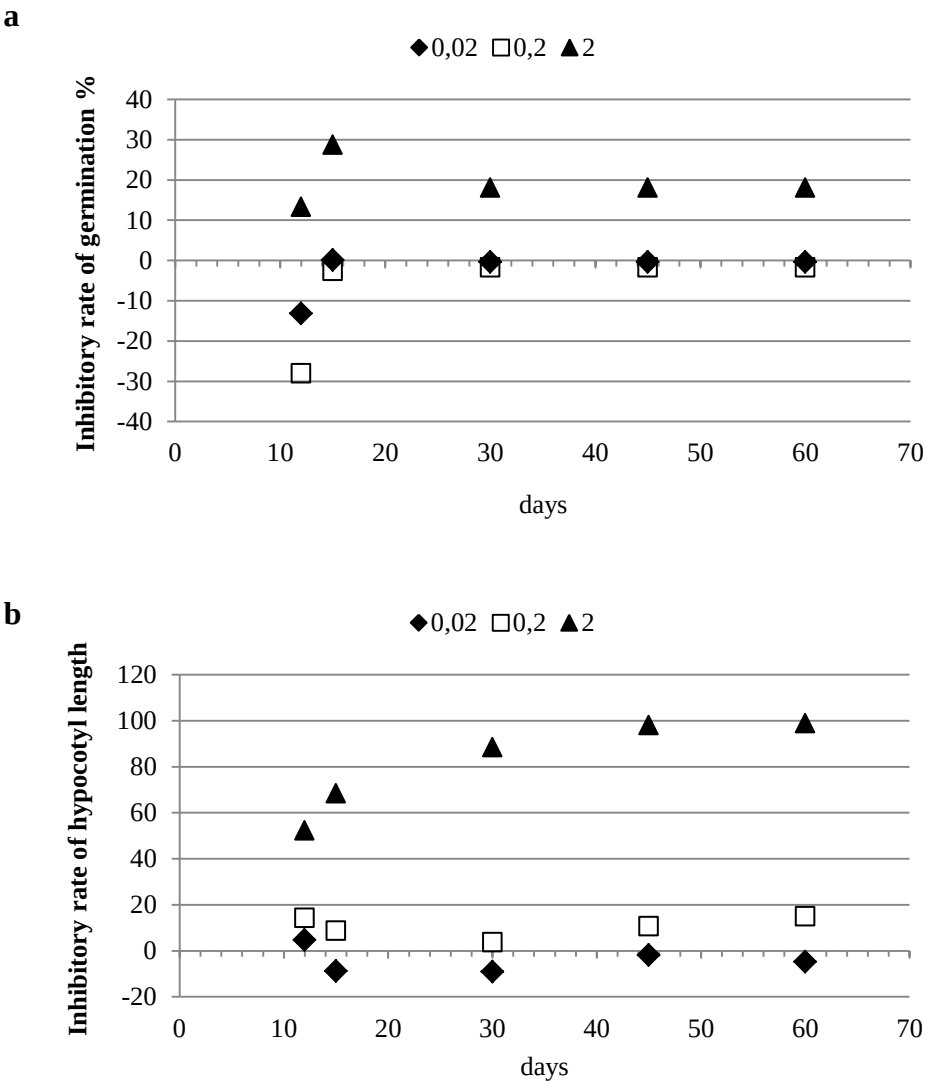

Figure II-15. Copper effect on the inhibitory rate of germination of date palm seeds (a) and of hypocotyl length (b).

More precisely, after exposure to 2 mM Cu, hypocotyl elongation stopped after 15 days (Fig. II-15b). For instance, hypocotyl length was 28.6, 30, 24.3 and 0.3 cm in 0, 0.02, 0.2 and 2 mM Cu contaminated media, respectively. Moreover, hypocotyl became considerably or completely necrotic at the end of the treatment with 0.2 and 2 mM Cu respectively. Thus, it was found that hypocotyl growth was more sensitive to metallic stress than germination percentage. Additional physiological parameters have been measured to investigate what happens during date palm seeds germination (table II-9). With a slight enhancement in the speed of germination (SG), seeds exposed to 0.02 mM Cu behaved similarly to untreated seeds and presented almost the same values in mean daily germination (MDG), mean

germination time (MGT), peak value (PV), seedling mortality (SLM) and event in germination index (GI) (Table II-9). MGT was found to be similar (287, 289 and 294 d) for seeds exposed to the lowest concentrations of Cu (0, 0.02 and 0.2 mM respectively) but significantly lower (234 d) for seeds exposed to 2 mM Cu. This indicates that date palm seeds requested a long time to accomplish their germination and that addition of copper may slightly accelerate seed emergence. Moreover, at 0.2 mM Cu, SLM was nil compared to 2.78 in control conditions, suggesting also that this metal may stimulate seeds germination. Maximum speed of germination (SG; 172.32 s/d) has been recorded in seeds exposed to 0.2 mM, and it declined by 15 % in response to 2 mM Cu.

Table II-9. Copper effect on different physiological parameters of date palm seed germination

Cu (mM)	SG (s/d)	MDG (s/d)	MGT (d)	PV (s/d)	SLM (%)	GI (%)
0	159.36 ± 5.6	0.163 ± 0.01	287.77 ± 6.4	0.49 ± 0.16	2.78 ± 0.33	100
0.02	167.2 ± 4.3	0.164 ± 0.04	289.28 ± 4.6	0.49 ± 0.03	2.78 ± 0.33	98.04
0.2	172.32 ± 5.3	0.166 ± 0.00	293.93 ± 2.4	0.66 ± 0.00	0 ± 1	84.36
2 mM	138.09 ± 5.9	0.134 ± 0.01	234.11 ± 3.3	0.40 ± 0.02	3.05 ± 2.5	8.37

SG speed of germination. MGT mean germination time. MDG mean daily germination. SLM seedling mortality. PV peak value. GI germination index

All the considered parameters improved at 0.2 mM Cu and declined at higher concentration except GI. The latter parameter showed a harmful decline by more than 90 % after treatment with 2 mM Cu (Table II-8). The small value of GI obtained confirmed the slow rate of germination obtained at high copper stress. It also significantly decreased MDG, MGT and PV.

3.2. Copper effects on amylase activity

Date palm (*Phoenix dactylifera* L.) hypocotyls were used as starting material for monitoring protein amylolytic activities. During the long phase of imbibition or the first 15 days of germination in darkness, amylase levels in seeds were similar for all copper treatments and comparable to the control (Fig. II-16). Amylase level presented a steep rise up to 60 days post-germination in seeds exposed to 0, 0.02 and 0.2 mM Cu and the maximum (38.14 units) was found in hypocotyls exposed to 0.2 mM Cu.

However, at higher copper concentration (2 mM), total amylolytic activity was severely

affected (Fig. II-16); this was observed clearly after 60 days of exposure (amylase activity = 6.18 units compared to 25.09 units in control conditions).

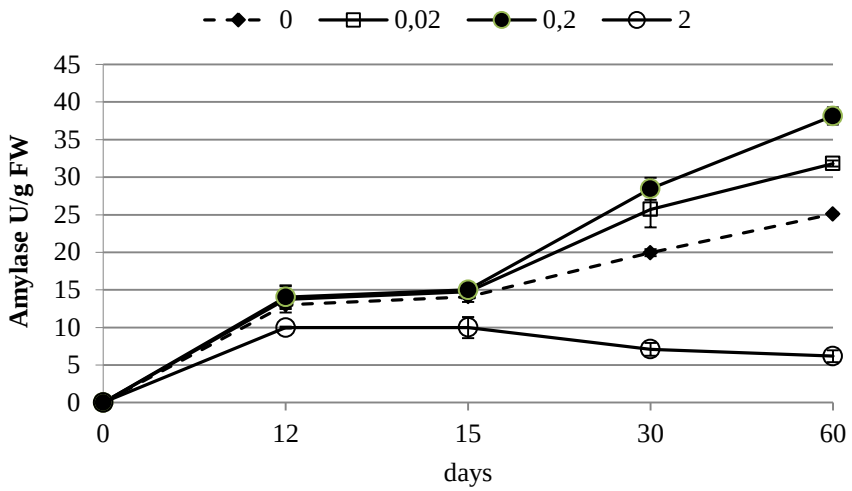

Figure II-16. Amylase activity in date palm seeds exposed to copper treatment during 60 days. Data presented are means ± standard error of three independent experiments.

3.3. Redox balance perturbation under copper stress

In standard conditions, hydrogen peroxide content in germinating date palm seeds increased gradually over time (Table II-10). In the samples treated with 0.02 or 0.2 mM copper, the pattern was similar but H_2O_2 contents were always higher than in control conditions. For instance, H_2O_2 concentration reached a maximum of 291.08 µmol/g FW after 60 days of exposure on 0.2 mM Cu. At the same time, H_2O_2 concentration was 182.28 µmol/g FW in control, while, H_2O_2 content was strongly enhanced in samples treated with 2 mM copper.

A significant boost of 2.5, 3.6, 2.8 and 3.07 times after 15, 30, 45 and 60 days (respectively) was noted (Table II-10). This accumulation of H_2O_2 resulted in deterioration of membrane lipids illustrated by the significant increase in TBARS accumulation observed in cells.

Catalase, the main H_2O_2-scavenging enzyme in plants, exhibited an enhancement of its activity in all cupric conditions compared to control during the first 15 days of exposure. For instance, CAT activity was 2 times higher in seedlings exposed to 0.2 mM Cu and 3 times higher in seedlings exposed to 2 mM Cu than in control (Table II-10). CAT activity continued to increase significantly over time in control, 0.02 and 0.2 mM Cu conditions until the end of the exposure period. In cupric treatment, it was always above the corresponding control. The

highest enzyme activity level (7.47±0.09 µmole H_2O_2/mg Protein) was measured after 60 days of exposure on 0.2 mM Cu, when H_2O_2 level in hypocotyl cells was 1.5 times higher than the control. The initial increase in CAT activity was also reported in seedlings exposed to 2 mM Cu during 12 and 15 days but was followed by a significant drop. CAT activity remained much lower than in control until the end of the exposure period (Table II-10), maintaining redox balance heavier in its oxidative side. Moreover, some necrotic signs in tissue were observed in hypocotyls grown in 2 mM Cu.

The activity of SOD, an enzyme implied in the elimination of superoxide radicals in cells, presented a pattern almost similar to CAT activity. In controls, SOD activity increased in a linear manner over time. Enzyme activity in hypocotyls exposed to 0.02 and 0.2 mM Cu was increased significantly by 65 % and 80 % respectively (Table II-10) at the end of the treatment. Yet, higher copper treatment (2 mM) induced critical reduction in enzyme activity by almost 80 % after long time of exposure inducing TBARS accumulation and growth blockage. These results showed that date palm seeds developed effective protection strategy by expressing CAT enzyme more than SOD activity.

Table II-10. Monitoring of elements of oxidative and antioxidative state of date palm seeds subjected to different copper concentrations Results shown as mean ± SD. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test. where small letters show differences between concentrations (in columns) and major letters show differences in time exposure (in rows). Means not showing the same letter are statically different.

	H_2O_2 µmole/g FW					TBARS nmole/g FW				
Cu (mM)	12	15	30	45	60 d	12	15	30	45	60 d
0	145.24±4.9a A	158.67±2.3a A	153.56±1.4a AB	195.01±2.9a C	182.28±0.7a BC	33.67±1.8a A	38.52±2.0a AC	42.43±0.7a BC	52.56±0.6a D	53.08±1.5a D
0.02	149.77±5.9a A	149.14±6.8a A	208.78±1.7b BC	227.55±3.6b CD	249.60±0.5a D	39.75±3.1a A	42.87±1.5a A	66.57±0.8b B	55.56±0.6a C	59.07±1.3a C
0.2	174.42±2.2a A	190.99±14.1b AB	275.85±7.2c BC	294.18±5.4c C	291.08±4.1a C	59.35±0.9b A	56.10±1.2b AB	56.73±3.8c AB	61.10±1.5b AC	71.57±0.8b C
2	279.21±11b A	403.23±9.5c B	552.71±5.2d C	555.68±7.5d C	559.01±7.09b C	75.06±1.4c A	81.27±2.4c AB	84.2±0.2d BC	90.51±0.7c C	97.09±2.7c C
	CAT activity µmole H_2O_2/mg Protein					**SOD activity U/mg Protein**				
0	1.66±0.23a A	2.09±0.00a A	3.71±0.3a BC	4.20±0.14a CD	4.88±0.04a D	3.00±0.01a A	4.04±0.06a B	9.81±0.16a C	9.95±0.21a C	10.04±0.06a C
0.02	1.97±0.31a A	2.7±0.03b B	5.85±0.19b B	5.6±0.21b B	5.77±0.05b B	3.87±0.17b A	4.82±0.1b A	14.35±1.91bc B	15.05±0.21b B	16.65±0.22b B
0.2	2.52±0.67a A	4.5±0.7c B	6.36±1.56b C	6.24±0.34b C	7.47±0.09c C	4.07±0.03b A	5.10±0.14cb A	13.23±1.08ac B	16.35±0.63b C	18.17±0.09c C
2	2.89±0.12b A	4.99±0.00d B	2.09±0.28c C	1.95±0.07c C	0.99±0.26d D	4.99±0.01c A	3.99±0.0a B	3.04±0.06d C	2.75±0.35c CD	2.14±0.05d D

3.4. Internal reference gene validation for qPCR analysis

Six House Keeping Genes (HKGs) candidates (*Pdefα1*, *Pdact*, *Pdsand*, *Pdaldh1*, *Pdtub6*, *Pdtbp*) were selected for stability analyses in *P. dactylifera* subjected to cupric stress. The average of cycle threshold (Ct) values, expression levels, of each candidate reference gene in 10 different date palm hypocotyl stressed samples was transferred to software as fully described by Brulle et al., 2014. To ensure the unchanged expression of reference genes under copper conditions, BestKeeper, geNorm and NormFinder algorithms were used to select the best stably internal control genes for qPCR normalization. The transcription levels of *Pdefα1* and *Pdaldh1* were higher than the four other genes indicated by lower average Ct values of 24.32 and 24.43, respectively (Fig. II-17). Whereas, *Pdtbp* displayed the lowest expression Cp value of 28.68. Ranking expression levels of six genes 10 samples as *Pdefα1* > *Pdaldh1* > *Pdact* > *Pdsand* > *Pdtub6* > *Pdtbp* (Fig. II-17).

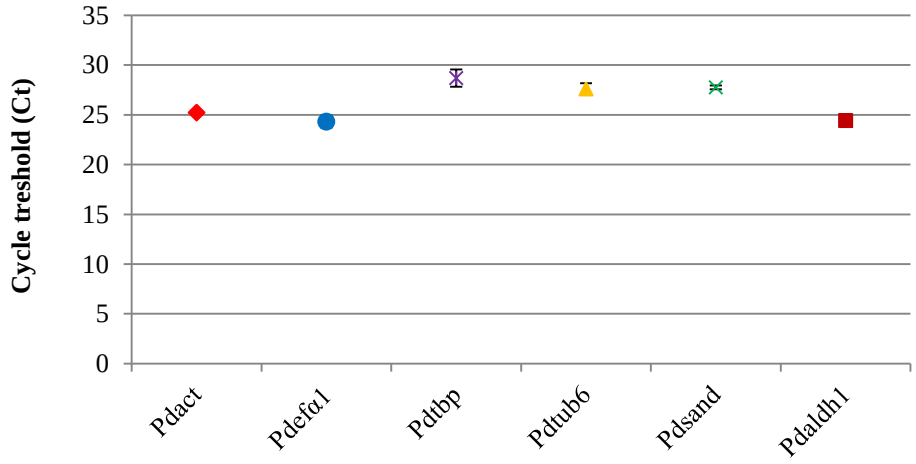

Figure II-17. Cycle treshold (Ct) of candidate reference genes for each experimental condition. Data presented are means ± standard error of three independent experiments.

BestKeeper algorithm generated a Pearson's correlation coefficient (r). In fact, the most stably expressed gene is the one with the highest BestKeeper correlation coefficient. Algorithm ranked candidate reference genes as follows *Pdefα1* > *Pdtub6* > *Pdaldh1* > *PdACT* > *Pdtbp* > *Pdsand* (Table II-10). Yet, geNorm software with different approach, provided *M*-value, mean pairwise variation between a genes compared to the others. It considered gene with the lowest *M*-value, the most stable one. Thus, it recommended the use of *Pdtub6*, *Pdtbp* and *Pdact*. Regarding the variations between algorithm results we also tested the NormFinder

software (Demidenko et al., 2011). According to Expósito-rodríguez et al. (2008), the top ranked gene is the gene with the lowest SD value. Indeed, NormFinder ranked the four best stable genes similarly to geNorm software. Consequently, in the present work, the average Ct value of *Pdtub6*, *Pdtbp* and *Pdact* was used for qPCR normalization of date palm tissues exposed to cupric stress.

3.5. *Pdpcs* and *Pdmt* expression under copper stress

In order to understand better the molecular basis of date palm seeds tolerance to Cu treatments, the levels of expression of two selected genes *(Pdpcs* and *Pdmt)* were measured after scaling with values of non-treated samples. Both genes were induced since the first days of germination with 0.02 and 0.2 mM Cu (Fig. II-18). *Pdmt* expression was described by a biphasic curve. First 45 days of exposure on 0.02mM Cu caused linear induction of *Pdmt*, while further it decreased up to the level 1.4 fold higher than in control conditions. These gene inductions observed in presence of copper could not be correlated to significant morphological alterations in palm date seeds germination after 60 days of exposure. A maximum transcription level of *Pdmt* was observed in hypocotyls exposed to 0.2 mM Cu (Fig. II-18). *Pdpcs* transcription level increased with increasing exposure time reaching its maximum at the end of the treatment with 0.02 and 0.2 mM Cu. Similarly to *Pdmt* relative expression under 0.2 mM Cu-stress, *Pdpcs* was described by a strong induction (5.25) compared to corresponding control. At lower copper amount, *Pdpcs* up-regulation reached a plateau at long exposure time. A coordinated expression of *Pdpcs* and *Pdmt* can be suggested to explain at least partially the Cu-resistance of *P. dactylifera* seeds at low concentrations. At high Cu concentration (2mM), destructive effects and gene expression alterations were observed. For instance, *Pdmt* and *Pdpcs* were down-regulated from the beginning of the treatment and continued to depress over time.

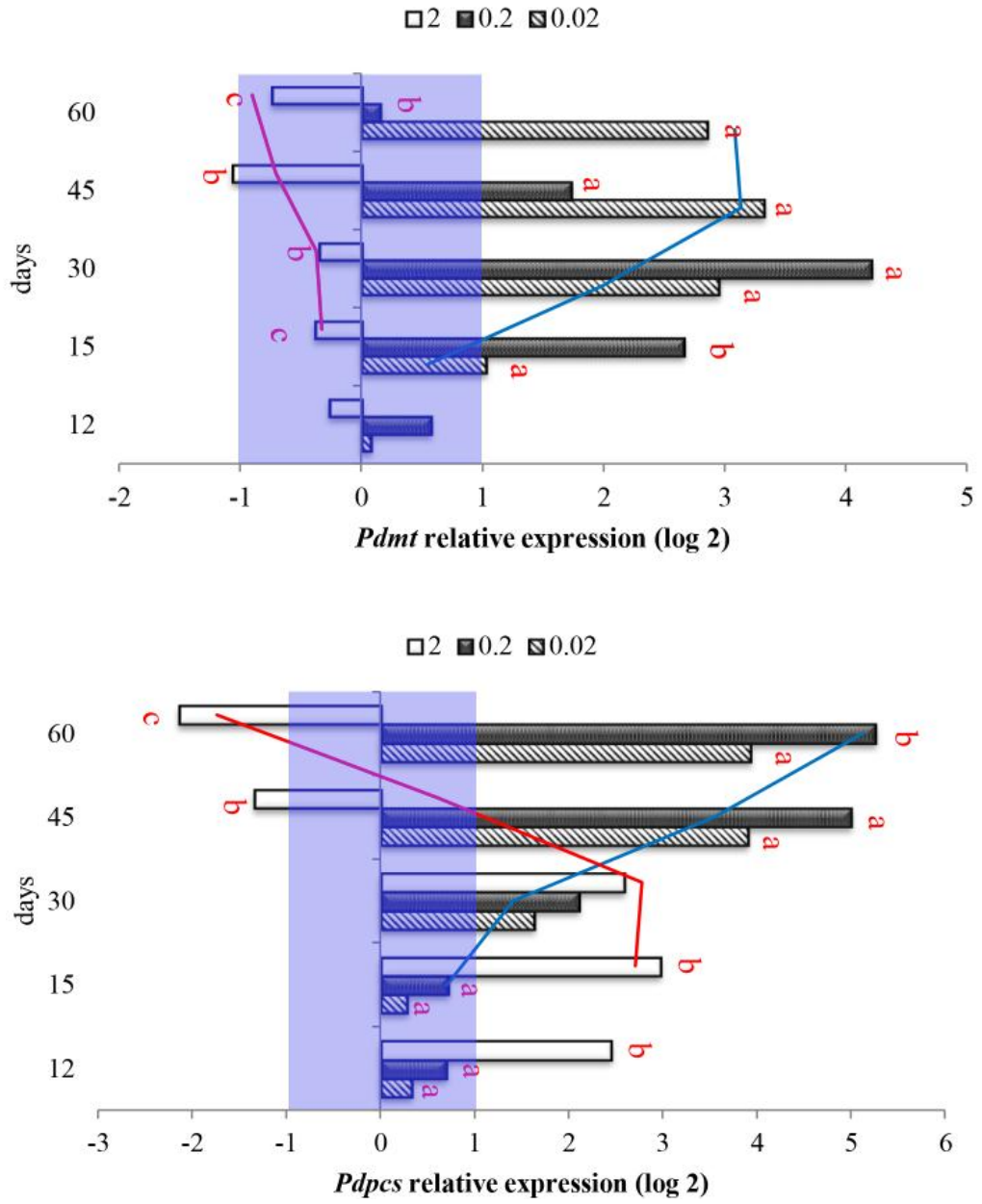

Figure II-18. Relative expression pattern of *Pdmt* (a) and *Pdpcs* (b) exposed to Cu treatment. Blue area shows basal genes variations. Data presented are means ± standard error of three independent experiments. Differences between groups are shown as results of post-hoc Tukey's test. where small letters show differences between concentrations among the time point. Means not showing the same letter are statically different.

5. Discussion

The increasing time of exposure, suggesting better tolerance of the plant in Cu-induced toxicity when exposed to the lower concentration. Furthermore, measured indices under high metal concentration showed an evident concentration-dependent inhibition of germinability. The GI declined significantly by 91.63 % showing a large decrease in SG (Table II-9). In fact,

the initiation of radical emergence necessitates high seed water content so that the metabolic activity necessary for germination can occur. Still, the response of seeds to this hydration varies depending on its capacity to control interior water level, the alteration of seed germination can be explained by a modification in water absorption. Actually, cupric ions have a toxic effect by creating an external osmotic potential preventing water uptake (Ahsan et al., 2007) similarly to Na^+ and Cl^- ions (Khajeh-hosseini et al., 2003). Lack of water absorption may be added to reduction of meristematic cells by accelerating break down of stored food materials in seeds exposed to iron (Fe) and Cd (Kabir et al., 2008). Such important phenomenon is correlated to amylase activity in seeds which deceased in seedlings exposed to 2 mM copper. Amylase activity was increased in seeds exposed at lower concentration (Fig. II-16). Amylase, member of hydrolyzing enzymes group, becomes extensively activated during germination in order to hydrolyze starch reserve stored in the endosperm. This step is necessary to release glucose and fructose which will be used by the embryo for respiration and other anabolic reactions. This parameter is a key molecular marker for monitoring of early events in plant development (Chaâbene et al., 2015). In the present work, and in the line with the findings of Robledo et al. (2014) and Fendri et al. (2013), we showed that the amylolytic activity was dose and time dependent. Indeed, enzyme activity increased linearly up to 60 days post-germination in 0.02 and 0.2 mM Cu (Fig. II-16). Yet, excess supply of Cu affected the mobilization of reserve materials affecting seedling size (Kabir et al., 2008). Similarly, suppressed amylase activity has been observed in pea seeds exposed to Cu (Lee and Kim, 2007), garlic seeds to As (Liu et al., 2003) and sunflower seeds to Cd (Sadiq and Maqbool, 2016). Interestingly, amylase activity is an indicator of the gibberellic acid (GA) level in germinating seeds (Beri and Gupta, 2007); GA being a classical plant hormone that mediates cell elongation and seed germination (Richards et al., 2001).

During cupric stress exposure, seed activates its metabolic machinery depending on the redox properties of the metal and catalyzes the formation of free radicals through the Fenton-type reactions (Hegedu, 2001). Because this metabolic response is highly dependent on the plant species, ROS content constitutes a common physiological biomarker of multiple abiotic stressors. Although, the half-lives of ROS are usually extremely short (Orhan et al., 2004), H_2O_2 is relatively stable and able to penetrate the plasma membrane as an uncharged molecule. In the present investigation, a marked increase in H_2O_2 has been noted in treated date palm seeds (Table II-10). Under physiological steady-state conditions, it slightly

increased over time in accordance to hypocotyl elongation and development causing surface and water limitation, which may stimulate ROS production. In addition, excessive cupric ion (2 mM) significantly multiplied H_2O_2 level by 2.5 folds within only 15 days of exposure (Table II-10) which can directly inhibit CO_2 fixation (Yamazaki et al., 2003). With regard to oxidative level in cells, close correlation with a moderate degree of production of TBARS, a biomarker of lipid peroxidation used as an indicator of membrane damage (Jarošová and Kundu, 2010; Reddy et al., 2005), to H_2O_2 content have been shown. In fact, small increase of TBARS contents at low copper treatment suggest that date palm was well protected against oxidative damage under 0.02 mM Cu. However, 0.2 mM Cu affected cell membrane only after extension of copper exposure time enhancing TBARS accumulation by more than 30 % after 60 days. This situation promotes necrosis, which may be partially due to Cu-mediated Fe^{2+} deficiency. Decrease of free Fe ions released by hydrolyzing enzyme from its organic component may also decrease seed germination and radical growth. In accordance with the above-mentioned physiological parameters, the amount of TBARS production was not directly correlated with H_2O_2 concentrations. It described a steady state levels more pronounced in samples treated with 2 mM Cu than 0.2 mM Cu (Table II-10). Yet, 0.02 mM Cu did not exhibit a significant reduction comparing to untreated samples. More destructive situation has been presented by samples exposed to the highest metal concentration. ROS and TBARS accumulation from the first days of germination under 2 mM Cu suggested plant sensitivity to high copper amount which ultimately resulted in hypocotyl elongation inhibition and finally in cell death since such metal amount was found to be critical for plant defensive biomolecules. The latter includes scavenging enzyme that often presented to limit free radicals availability. ROS, in the form of superoxide anion radicals ($O_2^{\cdot-}$), was dismutated by SOD activity to prevent the oxidative damage. SOD constitutes the first defense line in plants, exhibited by plant metal homeostasis networks. Seeds exposed to low metal concentrations stimulated SOD activity in 0.2 mM Cu. Promoted enzyme amount were necessary to control free radicals availability and equilibrate redox balance. However, SOD activity should not be considered alone in metal homeostasis system. Additional implication of CAT has been shown. With a low affinity compared to ascorbate peroxidase (APX), CAT catalyzes the destruction of H_2O_2 molecules only in peroxisomes (Vaseva et al., 2012). It increased at low Cu level and decreased significantly after 30 days of exposure to 2 mM Cu. Inhibited CAT activities could decrease membranes stability and CO_2 fixation by blocking several enzymes of the Calvin cycle in chloroplasts (Bhutta, 2005). Conversely, increase in both enzymes in

stressed plants with lesser concentrations of the metal indicated a constant detoxification of ROS and enhanced oxidative stress tolerance.

NormFinder and geNorm software algorithms showed that *Pdtub6*, *Pdtbp* and *Pdact* were overall the most stable reference genes under our experimental conditions. *Pdtbp* showed insensibility to Cu, Cd or Cr stress and ranked among the best three stable reference genes (Chaâbene et al., 2017). In the present investigation we confirmed the potential regulation of such traditionally used reference genes in various physiological states (Jarošová and Kundu, 2010; Reddy et al., 2005). For example, *EFα1* gene which was reported to be stably expressed in different plant during abiotic stress such as date palm (Chaâbene et al., 2017) and potato (Shao et al., 2010), has shown sensibility to cupric ions.

Two successive normalizations, (1) against the geometric means of the top 3 best identified HKGs and (2) against target gene expression level in control conditions, allowed description of the expression profiles of two HMs specific biomarkers (PCS and MT) catalyzing the uptake of essential elements above the threshold-level which they become toxic in date palm seeds. Increased resistance to HMs stress by overexpressing PCS and MT gene has been extensively reported (Bernard et al., 2016; Chaâbene et al., 2017), but not in *P. dactylifera* seeds subjected to copper treatment. Candidate genes showed discriminative expression profile between $CuSO_4$-concentrations (Fig. II-18). qPCR results indicated that *Pdpcs* and *Pdmt* responded strongly to low copper concentrations. Gene expression levels were significantly induced suggesting their prominent involvement in Cu-tolerance either by increasing production of copper-sequestering protein or by maintaining the local redox balance as a preventive action to the deleterious Fenton chemistry reactions (Zhang et al., 1999). Our results are in agreement with the hypothesis of (Zhang et al., 2005) who reported that PCS is controlled at the transcriptional level. Also, *Avicennia germinans* phytochelatin synthase gene *Avpcs* expression showed a significant increase under copper conditions from the first hours of stress application (Gonzalez-mendoza et al., 2007). Authors suggested that this rapid increase in *Avpcs* expression confirm that it was the most active gene involved in the regulation of copper in *A. germinans* leaves.

Likewise, Bernard et al. (2016) and Huguet et al. (2004) showed MT gene stimulation in response to oxidative stress induction in other species. Yet, *Pdmt* mRNA level was lower compared to *Pdpcs* level at the end of treatment with 0.02 and 0.2 mM Cu. Our findings

disagreed with (Foley and Singh, 1994; Ouziad et al., 2005; Zhou and Goldsbrough, 1994) others who suggested that MT gene is organ-specifically expressed only in roots. Evident symptoms of Cu-toxicity have been shown at germination stage of date palm seeds.

5. Conclusions

Regarding the biological significance of Cu response in germinating date palm seeds under increasing metal stress, it was demonstrated that, at low Cu concentration (0.02 mM), only changes in *Pdpcs* and *Pdmt* gene expression were significant. Under increasing copper amount (0.2 mM), both oxidative (H_2O_2, TBARS) and antioxidative mechanisms (CAT, SOD) became informative. The induction of genes involved in the synthesis of metal-chelating peptides is not only involved in metal homeostasis but constitutes also a defense against excess of Cu. However, synchronous fluctuation in the redox balance at high Cu concentration (2 mM) deregulates seeds antioxidative defense system causing oxidative damage especially after a long exposure period leading to necrosis and blockage in hypocotyl elongation. Molecular mechanisms underlying Cu response in palm tree deserve further attention to elucidate those by which Cu is distinguished from other ions.

Conflict of interest: Authors disclose that there are not relationships or interests that could have direct or potential influence or impart bias on the work. The authors declare that they have no conflict of interest and there are responsible for the content and writing of the article

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B-Insight into the expression variation of metal-responsive genes in the seedling of date palm (*Phoenix dactylifera*)

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Highlights

Reference genes were selected in date palm hypocotyls exposed to Cd and Cr

Phytochelatin synthase gene was upregulated under Cd and Cr stress

Metallothionein less responsive to heavy metal stress than phytochelatin synthase

Identification of potential biomarkers of HMs contamination

I. Synthèse

Parallèlement à l'étude de l'expression des gènes *Pdpcs* et *Pdmt* dans des hypocotyles de palmier dattier cv Deglet Nour exposés au stress cuivrique, une étude comparative de l'expression génique des gènes candidats a été poursuivie dans des conditions de stress par le Cd ou le Cr. Il s'agit de premières études qui ont cherché à comprendre les mécanismes moléculaires impliqués dans la réponse du palmier dattier au stress par le Cd, le Cu ou le Cr pendant la germination. C'est au cours de ce travail qu'une procédure spécifique de validation de gènes de référence utilisant les programmes BestKeeper, NormFinder et GeNorm a été adoptée (Figure II-20, Tableau II-13). Cette approche a été adoptée afin de sélectionner les trois gènes de référence les plus stables dans un contexte de contamination par le Cd, le Cu ou le Cr. Six gènes de référence candidats sélectionnés, à savoir le facteur d'élongation α1 (*Pdefα1*), l'actine (*Pdact*), l'aldéhyde déshydrogénase (*Pdalhd1*), la famille SAND (*Pdsand*), la tubuline 6 (*Pdtub6*) et TaTa box binding protein (*Pdtbp*) ont montré des différences d'expression entre les différentes conditions expérimentales. Ainsi, les classifications de stabilité pour les gènes de référence (de ménage) candidats obtenus étaient distinctes d'un algorithme à un autre (Figure II-19). Toutefois, l'étude a montré que *Pdact*, *Pdtbp* et *Pdsand* étaient des gènes de référence les plus appropriés pour normaliser le niveau de d'expression

des gènes étudiés dans les tissus de palmier dattier. De plus, les gènes de référence normalisés les plus appropriés pour la plante en condition de stress par le Cd ou le Cr ont été *Pdtbp*, *Pdefα1*, *Pdact* et *Pdaldh1*, *Pdefα1*, *Pdtbp*, respectivement (Figure II-20, Tableau II-13).

Après une identification soigneuse des gènes de référence pour chaque condition expérimentale, une mesure fiable de l'expression de *Pdpcs* et *Pdmt* a été réalisée. Les résultats obtenus ont montré que *Pdpcs* et *Pdmt* étaient régulés au niveau transcriptionel (Figure II-22). À faibles doses de Cd (0,02 et 0,2 mM), les gènes ont été surexprimés jusqu'au 60ème jour d'exposition. Toutefois, à fortes concentrations (1 et 2 mM Cd), *Pdpcs* est réprimé (Figure II-22a) après une longue exposition métallique. Cette sous-expression du gène trouvée chez les hypocotyles au stade germinatif n'a pas été visualisée dans les vitroplants. Encore, l'exposition aux ions Cr^{6+} a induit significativement l'expression génique de *Pdpcs* mais le gène s'est avéré moins sensible aux concentrations croissantes de Cr que le gène codant pour la métallothionéine (Figure II-22 c et d). L'expression de ce dernier a montré la même évolution chez les hypocotyles issus des graines et chez les explants lors d'une exposition au Cd (Figure II-22b). L'exposition au Cd ou au Cr a influencé clairement la germination des graines et l'allongement des hypocotyles.

Sur la base de cette étude, la surexpression des gènes responsables de l'absorption, la translocation et la séquestration de Cd et de Cr pourront expliquer en partie la tolérance métallique exceptionnelle du palmier dattier tout en constituant des biomarqueurs potentiels pour surveiller l'effet de la contamination métallique chez *P. dactylifera*.

II. Article

Insight into the expression variation of metal-responsive genes in the seedling of date palm (*Phoenix dactylifera* L)

Abstract

Phytochelatin synthase and metallothionein gene expressions were monitored via qPCR in order to investigate the molecular mechanisms involved in Cd and Cr detoxification in date palm (*Phoenix dactylifera*). A specific reference gene validation procedure using BestKeeper, NormFinder and geNorm programs allowed selection of the three most stable reference genes in a context of Cd or Cr contamination among six reference gene candidates, namely elongation factor α1, actin, aldehyde dehydrogenase, SAND family, tubulin 6 and TaTa box binding protein. Phytochelatin synthase and metallothionein encoding gene expression were induced from the first days of exposure. At low Cd stress (0.02 mM), genes still up-regulated until 60th day of exposure. At higher metal amount, however, mRNA concentrations decreased. Phytochelatin synthase encoding gene was significantly up-regulated under Cr exposure, and was more responsive to increasing Cr concentration than metallothionein encoding gene. Moreover, exposure to Cd or Cr influenced clearly seed germination and hypocotyls elongation. Expression levels of genes encoding phytochelatin synthase and metallothionein constitute potential biomarkers to monitor the effect of Cd or Cr contamination in date palm.

Keywords: Biomarkers - Date palm hypocotyls - Gene normalization - Reference gene validation - Gene expression – Detoxification mechanisms.

1. Introduction

Although informative, physicochemical analyses reach their limits as tools in environmental management assessment since speciation and/or bioavailability may strongly influence the impacts on organisms (Amiard et al., 1998). Therefore, it is useful to associate them with measurements of biological parameters in organisms in order to assess sub-lethal and sometimes discrete effects. Plants frequently respond in some measurable and somewhat predictable ways through mechanisms at various levels of biological organization (Bickham, 2000) starting from the community to individual and infra-individual levels. Particular factors involved in those mechanisms (bioindicators, biomarkers) can serve as tools for documenting

the biological response to environmental menace and the extent of organism's exposure to chemical contaminants so that preventive measures can be taken. Biomarkers may indicate exposure to specific classes of pollutants such as metals (Marigomez et al., 2002).

Of all non-essential metals, cadmium (Cd) is probably the most common toxicant to plants, animals and humans (Demidenko et al., 2011). Environmental effects of this element have been extensively reviewed (Benavides et al., 2005; McBride, 2003). Briefly, in plants, Cd has major detrimental impacts on physiology and biochemistry inducing growth reduction, on chloroplast ultrastructure causing alteration of photochemical reactions, on mineral nutrition, on carbohydrate metabolism and on oxidative stress enhancement (Expósito-rodríguez et al., 2008; Gill et al., 2011; Shanker and Venkateswarlu, 2009). Because of its great mobility in the plant–soil system, Cd has the potential to accumulate in some crops with a significant potential to contaminate the diets of animals and humans (Gabbrielli, 1999).

Likewise, with its high affinity for living organisms (Campbell, 2005), chromium, especially its hexavalent chemical form (Cr^{6+}), is known to be a very toxic contaminant steadily increasing in the environment due to the development of industry, especially metal processing, chromium plating and leather tanning (Huguet et al., 2004b; Wyszkowska, 2002). Coal and Area (2013) reported that Cr^{6+} accumulation was important in the shoots of exposed rice plants, causing chlorosis and compromised photosynthesis. Thus, Cr may interfere with several metabolic processes and poses a threat to the health of organisms. As a result of their accumulation in crop plants, Cr can contaminate the food web. The United States Environment Protection Agency has established a standard limit of 0.01 ppm Cr in foods (Agency, 2009). However, both the Food and Agriculture Organization (FAO) and World Health Organization (WHO) allow 0.05 ppm Cd in food products (Solidum, 2011).

Controlling metal pollution sources is important but insufficient for protecting soil health. Metal specific biomarkers could act as early warning signals allowing a better exposure assessment. The enzymes that synthesize molecules capable of chelating metals are potentially good biomarkers. The main species of chelators in plants are phytochelatins (PCs). PCs are synthesized by phytochelatin synthase (PCS), an enzyme requiring glutathione (GSH) as a precursor (Oa et al., 2008). PCS is encoded by a gene (*pcs*), the expression of which might be influenced by metals (Hall, 2002). The enzyme activation in cells depends on the presence of heavy metal ions such as Cd^{2+}, one of the strongest inducers of *pcs* expression (Vatamaniuk et al., 2000). Over the last two decades, many PCS coding genes have been isolated from a wide range of organisms such as plants (Clemens et al., 1999; Heiss et al.,

2003; Oa et al., 2008) and animals (Brulle et al., 2010) and have been documented to contribute in HMs detoxification processes. However, *pcs* is not found in any vertebrate species (Pal and Rai, 2010). Essential metal homeostasis and detoxification also relies on other metal-binding ligands, such as metallothioneins (MTs) (Huguet et al., 2004a; Zimeri et al., 2005). Most cells can induce the synthesis of MT in response to HM challenge. Thus, MT content and/or *mt* gene expression are specific biomarker candidates for HMs risk assessment. Although MT and PCS coding genes have been both described in many plant species, few authors studied the control of their expression (Gonzalez-mendoza et al., 2007).

Molecular biology techniques applied to ecotoxicology help to better understand the detoxification and defense mechanisms of organisms. Quantitative real-time PCR (qPCR) is a standard method to study gene expression variation under different experimental conditions. It has been adopted in a wide range of scientific areas and research work (Pfaffl, 2001) because of its advantages in sensitivity, specificity and its extensive range of detection (Bustin et al., 2009). To further improve the reproducibility and reliability of qPCR experiments, Bustin et al. (2009) described approaches to normalize mRNA. It requires the use of at least one endogenous reference gene transcription as internal control for relative quantification (Czechowski et al., 2005). These reference genes are typically constitutive genes with housekeeping (HKG) functions required for the maintenance of basic cellular function. The expression levels of these genes should be stable among cells of different tissues across the developmental stages under normal and adverse conditions (Bustin, 2000; Crismani et al., 2006; Villaseñor et al., 2011). Recently, a number of commonly accepted reference genes presented expression variability in response to some experimental conditions (Chandna et al., 2012; Lilly et al., 2011; Morgante et al., 2011). Consequently, validation of appropriate reference genes is required prior to any quantification (Guénin et al., 2009; Gutierrez et al., 2008; Martins et al., 2016b). Several statistical algorithms such as geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) have been developed and are now commonly used in HKG validation. Such a validation procedure has not been performed in date palm in a Cd or Cr contamination.

Thus, in this paper, we evaluated, for the first time, the expression stability of six traditionally used reference genes in date palm in order to measure the gene encoding phytochelatin synthase (*Pdpcs*) and metallothionein (*Pdmt*) in date palm, *Phoenix dactylifera* L., subjected to various and environmentally relevant Cd or Cr concentrations.

2. Methods

2.1. Seeds germination and treatments

Mature seeds of the date palm cultivar Deglet Nour were washed with distilled water extensively. Then, under sterile conditions, 100 % of commercial sodium hypochlorite was added to seeds for 10 min followed by 50 % of diluted solution for another 10 min and completed with more diluted sodium hypochlorite (25 %) during the same time. Seeds were then rinsed 3 times with distilled water for 10 min. They were germinated in direct contact with 0, 0.02, 0.2, 1 and 2 mM cadmium solutions prepared in distilled water using cadmium chloride ($CdCl_2$) or with 0, 0.02, 0.2 and 2 mM of chromium solution prepared in distilled water using potassium chromate (K_2CrO_4) and kept in the dark at 26 °C in order to induce germination. Hypocotyls (10 per replicate; three biological replicates per sample) were excised from seeds at 15, 30, 45 and 60 days after germination and used for total RNA extraction.

2.2. Seedling growth parameters measurement

Germination Percentage (GP), Speed of Germination (SG), Mean Daily Germination (MDG), Mean Germination Time (MGT), Peak Value (PV), Seedling Mortality (SLM) and Germination Index (GI) were measured according to previous studies of Chaâbene et al. (2015).

2.3. Gene sequences identification in date palm

From previously constructed Deglet Nour date palm cDNA library (Dhieb et al., 2012), we isolated *mt* and reference encoding genes. Homologous sequences of genes of metallothionin (*mt*), elongation factor alpha 1 (*Efα1*), aldehyde dehydrogenase (*aldh1*), actine (*act*), SAND family protein (*sand*), Tubulin beta-6 (*tub6*) and TATA-box-binding protein (*tbp*) from various organisms recognized in NCBI (http://www.ncbi. nlm.nih.gov/) using BLASTn were aligned against nucleotide sequences from our date palm cDNA bank using BioEdit version 7.0.5.3. Some clones revealed partial homology with selected genes. Following methods described by Chaâbene et al. (2017), we have identified *Pdmt* amplified using the primer pair (5’ CGTGATCACCGAGACTGAAA 3’ and 5’ TTGGTTGTGGAGGAGTGTCA 3’) and the six candidate Housekeeping genes (HKGs) using Pfam (with E value <1e-5), HMMER v3.0 and UniProt (http://www.uniprot.org/) programs.

2.4. Total RNA isolation and cDNA synthesis

Deglet Nour date seedling which experienced 0.5, 1, 1.5 or 2 months growth in stressful conditions with $CdCl_2$ or K_2CrO_4 was used for total RNA extraction. RNAs were isolated with the Plant RNeasy mini kit (Qiagen, Courtaboeuf, France), including the on-column DNase digestion step. Concentration and purity of the RNA samples was determined using a Spectrophotometer (SPECTROstar Nano Microplate Reader). RNA integrity was checked using a method adapted from (Masek et al., 2005). Reverse transcription was performed on 1 µg of total RNA from each sample using the random hexamer primers and the Maxima H Minus First strand (Thermo Scientific, USA) cDNA Synthesis Kit according to manufacturer's instructions.

2.5. Gene amplification and sequencing

Primer sequences of candidate genes were designed from the conserved domain found using Primer3Plus (Rozen and Skaletsky, 2000); http://frodo.wi.mit.edu/) and verified using Net Primer and Beacon Designer. Table II-11 lists the primer pairs used.

Table II-11. Primer sequences used for cloning of candidate and reference genes.

Name	Functional description	Amplification length (bp)	qPCR specific Primer sequence (5' → 3')	Length (bp)	Tm	GC %	Contig Length (bp)
Pdefα1	Elongation factor 1-alpha	235	F : CTTGACTGCCACACCTCTCA	20	60.02	55.0	237
			R : TGATGACACCAACAGCCACT	20	60.16	50.0	
Pdaldh1	Aldehyde dehydrogenase	239	F : CAACCGTGGATGTGTCTGTC	20	60.00	55.0	241
			R : GTCGTACCAAGCCACCAACT	20	60.04	55.0	
Pdact2	Actine 2	265	F : AGGTGCCCAGAGGTTCTTTT	20	60.11	50.0	165
			R : AGCAATACCAGGGAACATGG	20	59.81	50.0	
Pdsand	SAND family protein	218	F : TGTGAGGCATTGAGGGAACAA	20	57.7	47.6	129
			R : TTCCATAAGCAAGAGGAAGGCA	20	57.6	45.5	
Pdtub6	Tubulin beta-6	236	F : GCCTCAGTGAATTCCATCTCAT	20	60.5	45.5	138
			R : TGCAGAACAAGAACTCCTCCTAC	20	59.9	47.8	
Pdtbp	TATA-box-binding protein	251	F : TGATCCGAGCGTACTTCCTT	20	59.84	50.0	152
			R : CCCTTCTGGCATTGTTCCTA	20	60.1	50.0	
*Pdmt**	Metallothionin	107	F : GTGCGTGAAGAAGGGAAATG	20	60.4	50.0	107
			R : ACTTGCAGTCAGGCTCCGTA	20	60	55	
*Pdpcs**	Phytochelatin synthase	171	F : GCTGCGAACCTTTGGATAGA	20	60.4	50.0	113
			R : TCAGATGGCAGTCCTCAGTG	20	60	55.0	

***** Primer sequences from Chaâbene et al. (2017)

PCR products were cleaned with the EZ-10 spin column DNA gel extraction kit (Biobasic, Markham, Canada). Amplification products were sequenced in both directions by Genoscreen Company (Pasteur Institute, Lille, France). Using CodonCode Aligner and Sequencher, a

contig was assembled and used to design qPCR primers (Table II-12).

Table II-12. qPCR Primer sequences of the candidate and reference genes used for relative gene expression analysis.

Name	Amplification length (bp)	qPCR specific Primer sequence (5' → 3')	Length (bp)	Tm	GC%	PCR Efficiency ±SD
Pdefα 1	153	F : TGACTGCCACACCTCTCACATTGC	24	59.4	54.2	2±0.01
		R : ACAACCATGGGCTTGGTGGGAA	22	59.1	54.5	
Pdaldh 1	149	F : ACCGTGGATGTGTCTGTCGTGGAT	24	59.9	54.2	2±0.01
		R : AAGTCGGTGGACACCAAGTCCTCT	24	59.3	54.2	
Pdact 2	129	F : TTTCCAGCCGTCCCTCATTGGAA	23	58.2	52.2	1.95±0.03
		R : GTTGATCCTCCACTGAGCACAACG	24	58.0	54.2	
Pdsand	120	F : CTGCTTATCTTGACAAAGTCAGTTC	25	58.7	40.0	1.98±0.02
		R : CCAGCTGAAGGAATGAATCAG	21	59.8	47.6	
Pdtub 6	183	F : ATCCATTCCCTCCCCTGTAT	20	59.5	50.0	2.02±0.04
		R : GGATCCCCAACAATGTGAAG	20	60.2	50.0	
Pdtbp	152	F : TTGCTCCTGTACAAACCATTTT	22	58.7	36.4	1.99±0.01
		R : GGACTGCAAGTTGGACCTTA	20	57.8	50.0	
*Pdmt**	107	F : GTGCGTGAAGAAGGGAAATG	20	60.4	50.0	1.99±0.02
		R : ACTTGCAGTCAGGCTCCGTA	20	60	55	
*Pdpcs**	113	F : GGCAGTCCTCAGTGGAAGTA	20	57.9	55	2±0.00
		R : TGCTTGCTTGGCTTACTGTG	20	60.2	50.0	

2.6. Real time qPCR amplification

Quantitative real-time PCR (qPCR) analysis of gene expression was performed on reverse transcribed RNAs extracted from *Phoenix dactylifera* using MESA Blue qPCR Master Mix reagent kit (Eurogentec, Seraing, Belgium). Gene-specific qPCR primers were designed with Primer3Plus with special settings selection. Primers verified with Net Primer and Beacon Designer, are listed in Table II-12. qPCR reactions were performed with a LightCycler 480 Real Time PCR system (Roche Diagnostics, Mannheim, Germany) according to previously described procedures (Brulle et al., 2014). Real-time PCR efficiencies (E) were calculated from the given slope of the standard curve according to the equation $E = 10^{(-1/slope)}$. E values ranged from 1.91 to 2 (with 100 % = 2) and calculated from a standard curve. *Pdpcs* and *Pdmt* relative expression levels were normalized to those of the reference genes selected after gene expression validation according to previously described procedures (Pfaffl, 2001). Absolute quantification of genes expression levels are in log2. Relative expression level of control samples vs relative expression level of exposed samples was normalized to calculate relative fold expression or transcription factor according to Bernard et al. (2016).

2.7. Expression stability analysis

Performance of the six genes as potential reference genes for date palm was assessed in samples divided into three experimental sets of two treatments; including samples not subjected to stress conditions. To estimate the expression stability of reference genes, the relative quantities of Cq values of genes obtained using the formula of (Hellemans et al., 2007) were used. As per Martins et al. (2016b) different algorithms and statistical analysis were applied to evaluate the expression stability of the reference genes of *P. datylifera* plants in seedling under stress conditions. The analysis procedures strictly followed the manuals of used algorithms. Reference genes were validated using BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004) and geNorm (Vandesompele et al., 2002) following (Brulle et al., 2014) procedure.

2.8. Statistical analysis

The relative expression ratios between control and treated plants were transformed and subjected to a one-way and two-ways ANOVA.

3. Results

3.3. Target gene identification and HKGs selection

In addition to previously identified full length *Pdpcs* in date palm (Chaâbene et al., 2017), a second heavy metal biomarker candidate has been identified: a metallothionein mRNA sequence of 387pb (*Pdmt*) was identified with via Eukaryotic GeneMark.hmm, FGENESH 2.6 and BLASTx. Following the same procedure, six candidate HKGs were selected. They were named *Pdefα1*, *Pdaldh1*, *Pdact*, *Pdsand*, *Pdtub6* and *Pdtbp*, following the nomenclature of *P. dactylifera* (Table II-12). The latter genes exhibit highly conserved domains which allowed NCBI database exploration using Blastn and design of primers for PCR amplification. Generated sequences for candidates and HKGs from date palm var. Deglet Nour after PCR product sequencing and re-amplification were used to evaluate gene expression in qPCR.

3.2. Reference gene amplification efficiency and expression levels

qPCR analysis of gene expression was performed on reverse transcribed mRNA extracted from hypocotyl samples of *Phoenix dactylifera* seedlings exposed in various metallic

conditions. Transcript abundance was evaluated by qPCR. All products showed a single peak in the melting curve analysis using the LC480. The calculated amplification efficiency for primers ranged from 97.5 % to 100 %, $r^2 \geq 0.95$ (Table II-12) and were considered appropriate for qPCR (Zhao, 2005).

Mean values showed that they were moderately abundant and they differed between stress conditions (Fig. II-19). The gene showing the highest expression was *Pdefα1*. *Pdtub6* exhibited the lowest level of expression and was the most stable reference gene showing no expression variability between samples. Indeed, the expression variation of all HKGs, represented by different cycle threshold (Ct) values, in control and chromium conditions was almost the same (Fig. II-19). A Ct dispersal was observed under Cd stress for *Pdefα1* having mean Ct value of 24.7 cycles and revealing sensitivity to heavy metal treatment. *Actin*, frequently used as reference gene, showed expression induction in Cd treated simples. As a consequence of this considerable variability among candidate HKGs, a simple comparison of the raw Ct values was inadequate, requiring further analysis in order to identify a suitable combination of reference genes for normalizing expression under specific experimental conditions.

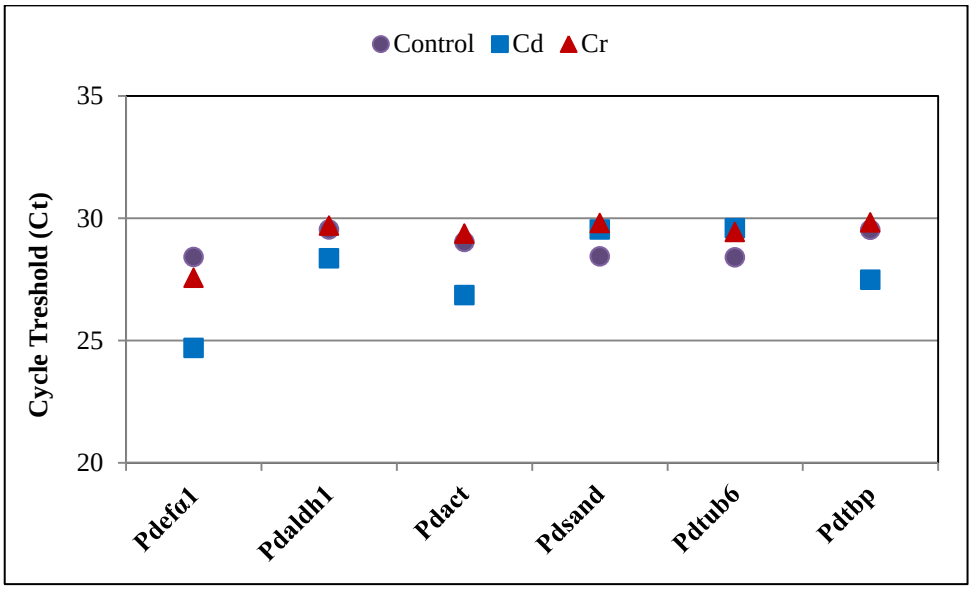


Figure II-19. Cycle treshold (Ct) of candidate reference genes for each experimental condition. Data presented are means ± standard error of three independent experiments.

3.3. Reference gene expression stability across different experimental conditions

Expression stability has been initially evaluated by BestKeeper based on the pairwise

correlation coefficient and the BestKeeper index (geometric mean of Ct value for each candidate gene (Pfaffl, 2001) Genes with high BestKeeper coefficient of correlation indicates that their expression correlates well with the expression patterns of other reference genes and so were considered as the most stably expressed. The algorithm calculated the stability of the six candidates and present different expression behavior between experimental conditions (Fig. II-20). Hypocotyls of non-treated date palm seeds expressed HKGs ranking in order of most stable to least stable: *Pdact*, *Pdefα1*, *Pdsand*, *Pdtbp*, *Pdaldh1* and *Pdtub6* (Fig. II-20a). However under HMs stress, *Pdsand* showed an unstable expression pattern, indicated by a low Pearson's Coefficient value under Cr exposure (Fig. II-20c) and a negative value obtained with Cd treatment (Fig. II-20b) suggesting gene sensitivity to abiotic stress caused by these toxic elements. Although gene expression variations occur, the same four genes exhibited the highest stability in different samples across different exposure treatments (Fig. II-20).

Expression stability was also evaluated using another approach relying on the geNorm algorithm. The stability measure calculated with the geNorm applet (*M* value) is the mean pairwise variation between a gene and the other candidate HKGs. $M < 0.5$ indicates good stability and HKGs with lowest *M* values are the most stably expressed. *Actin* was one of the most stable genes in samples under standard conditions (Table II-13). Surprisingly, *Pdtbp* was the least stable HKG in the chromium treatment according to BestKeeper, while geNorm ranked it as the most stable. We cannot explain this contradiction. Also, *Pdtub6*, the third best stable gene in control hypocotyls with *M* value equal to 0.16 has been established as the lowest stable expressed gene by Bestkeeper. This latter gene remained unstable after Cd exposure and the best three stable genes according to geNorm were *Pdtbp*, *Pdact* and *Pdefα1*).

Lastly, NormFinder software was used to re-analyze and confirm results since it is less sensitive to co-regulation (Expósito-rodríguez et al., 2008). This algorithm uses a model-based approach ranking the best candidate reference genes by comparing inter- and intra-group expression variation (Demidenko et al., 2011).

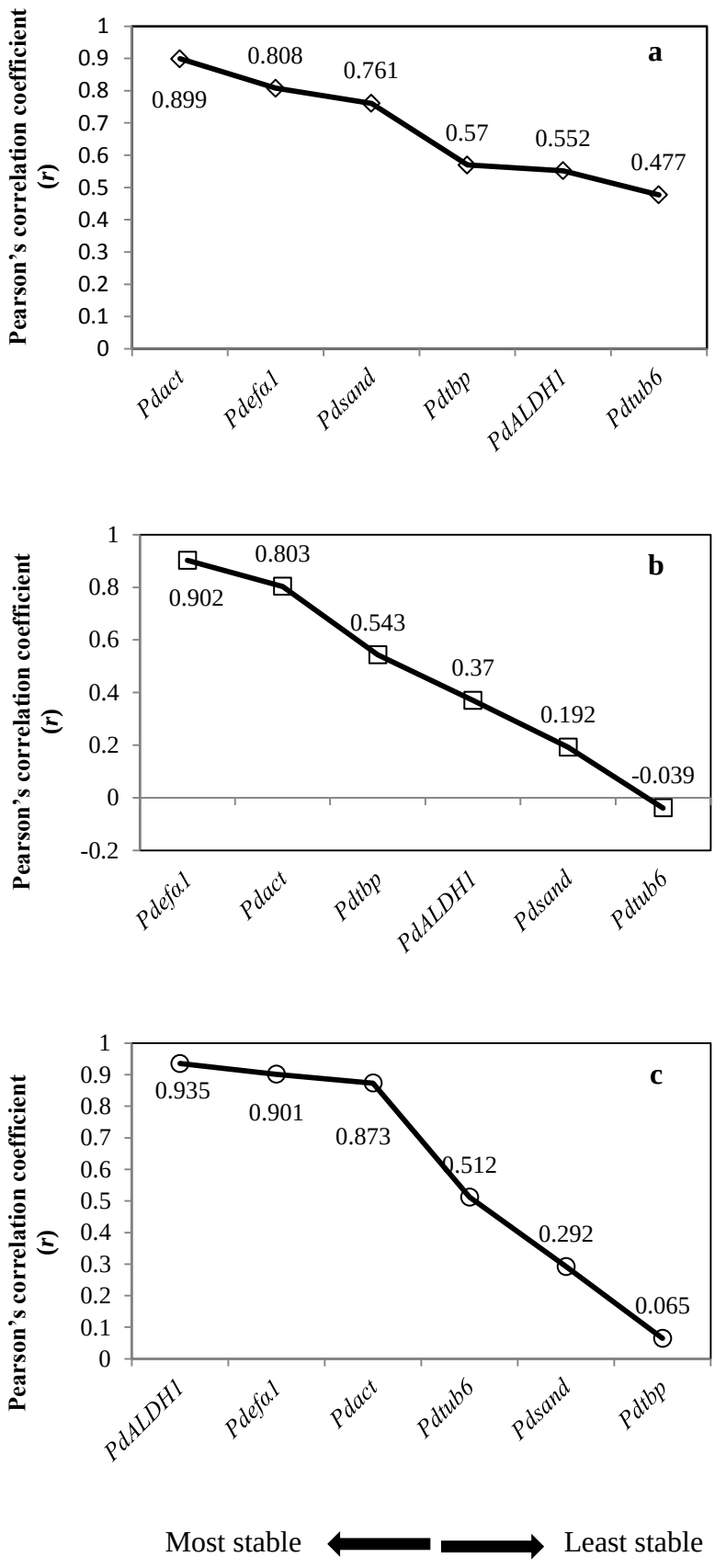


Figure II-20. Ranking and expression stability of candidate reference genes measured by BestKeeper for samples exposed to control (a), cadmium (b) and chromium (c) treatment.

For NormFinder, top ranked genes will present the lowest standard deviation values (Demidenko et al., 2011). A gene ranked as stable by geNorm should receive atop four ranking in NormFinder (Simon et al., 2013). As shown in Table II-13, geNorm and NormFinder produced very similar results suggesting the reliability of analysis. NormFinder confirmed that *Pdtbp* could be used as reference gene across abiotic stress treatments.

Table II-13. geNorm *M values* and NormFinder SD values of six date palm HKGs. The most stably expressed genes values are presented in fold.

		Pdefα1	*Pdaldh1*	*Pdact*	*Pdsand*	*Pdtub6*	*Pdtbp*
GeNorm *M* values	Control	0.20	0.30	**0.01**	0.19	**0.16**	**0.12**
	Cd	**0.15**	0.20	**0.19**	0.19	0.21	**0.02**
	Cr	**0.10**	**0.09**	0.12	0.18	0.40	**0.07**
NormFinder SD values	Control	0.450	0.608	**0.021**	**0.395**	0.432	**0.247**
	Cd	**0.367**	0.502	**0.41**	0.473	0.523	**0.071**
	Cr	**0.127**	**0.113**	0.155	0.247	0.502	**0.198**

To conclude, the described selection procedure allowed identification of suitable reference genes for the present experimental conditions. Yet, the obtained results indicated that *Pdact*, *Pdtbp* and *Pdsand* could be the most appropriate reference genes for normalizing transcription level in non-stressed palm date tissues. Otherwise, the most suitable normalized HKGs for Cd and Cr stressed plant were *Pdtbp*, *Pdefα1*, *Pdact* and *Pdaldh1*, *Pdefα1*, *Pdtbp*, respectively.

3.4. Determination of the optimal number of reference genes

Two or more HKGs were required for the planned qPCR experiments. The geNorm algorithm was used further to determine the optimal number of HKGs required for reliable normalization. Different combinations of reference genes were used. According to Vandesompele et al. (2002), Pairwise Variation (V*n*/V*n*+1) between normalization factors (NF) allowed estimation of the best number of reference genes for normalization. The NFs

were calculated initially for control genes with the lowest *M* value (most stable gene) and then, in a stepwise manner, other reference genes with lower *M* values were added (Fig. II-21).

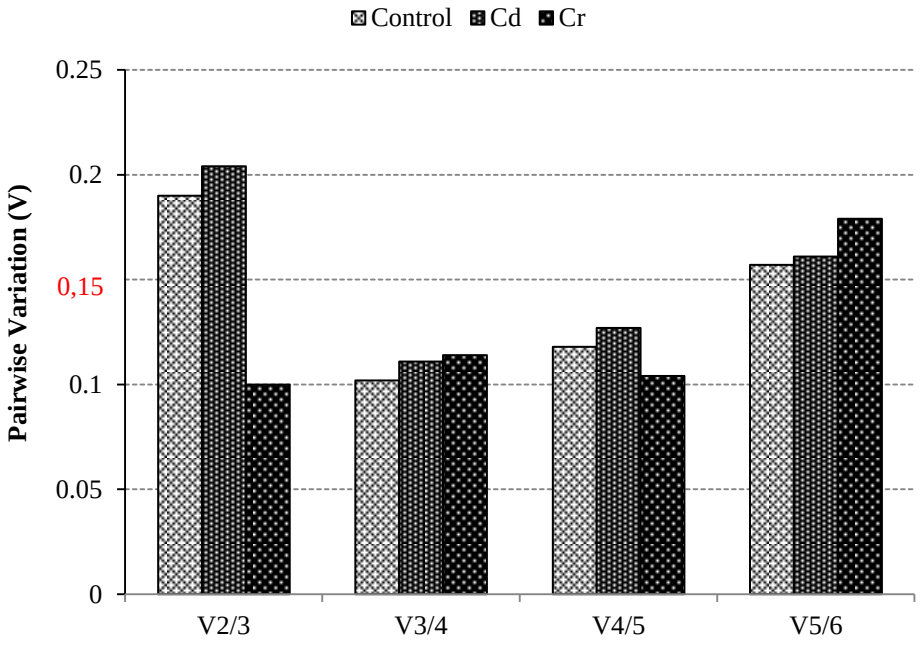


Figure II-21. Predicted geNorm pairwise variation of the six candidate reference genes to determine the optimal number of HKGs required for qPCR data normalization.

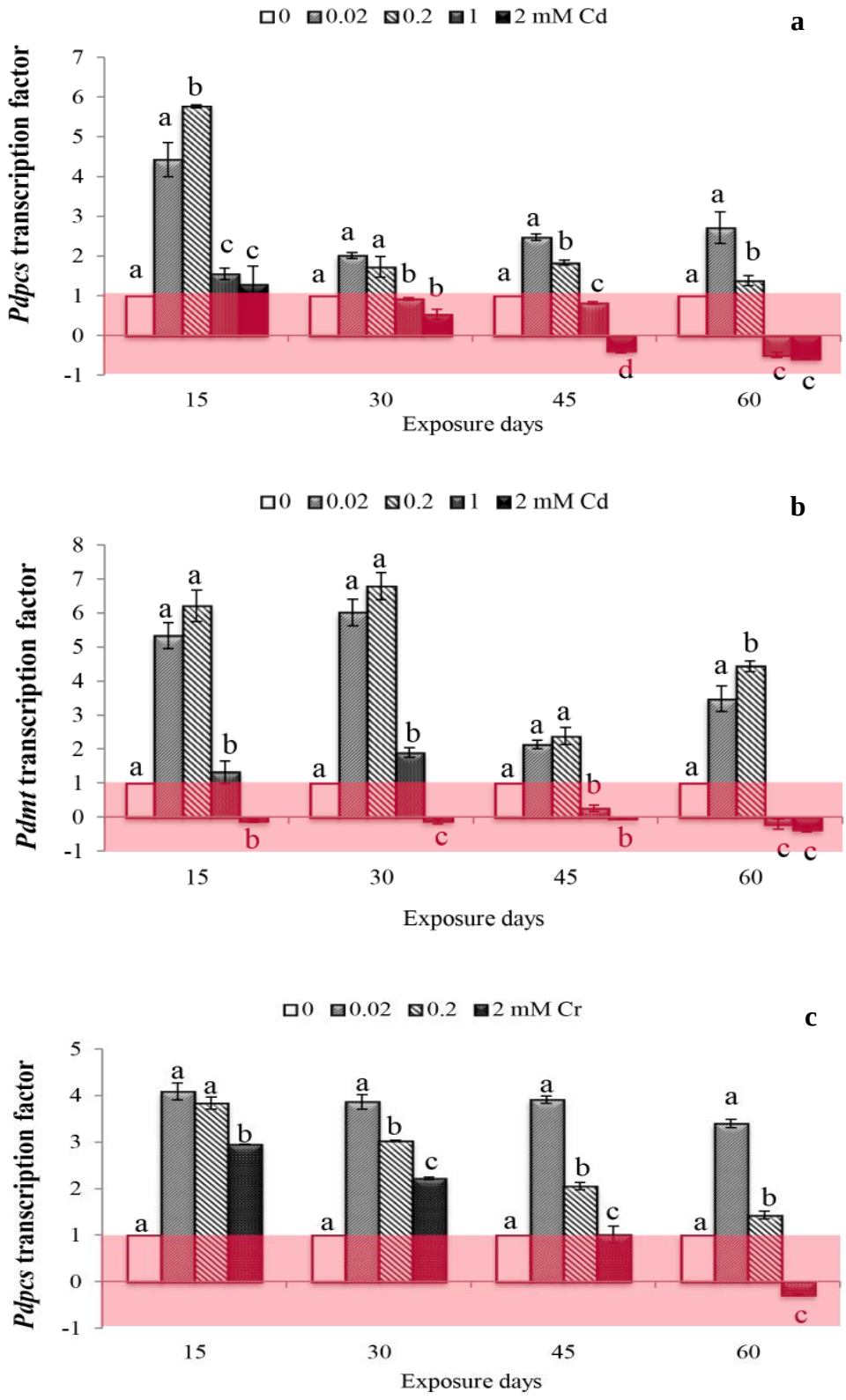
These analyses indicated that two or three genes with the most stable expression would be optimal for reliable normalization. For samples subjected to chromium stress, *Pdaldh1*, *Pdefα1* and *Pdtbp* would be sufficient for qPCR analysis (V2/3 value = 0.100 < 0.15, the recommended cutoff calculated by Vandesompele et al.(Vandesompele et al., 2002) (Fig. II-21). However, for the control and Cd treated samples, the pairwise variation V2/3 value (0.19 and 0.204 respectively) was higher than 0.15, indicating that two reference genes were not sufficient for normalization, and a third gene is recommended. Indeed, V3/4 values of 0.102 and of 0.111 were below the threshold, suggesting that the optimal number of reference genes for normalization in different conditions was three (Fig. II-21).

This crucial step was necessary to identify the reference genes allowing quantification of *pcs* and *mt* gene expression levels in *P. dactylifera*.

3.5. *Pdpcs* and *Pdmt* expression variation in response to heavy metal stress

The expression profiles of the two selected target genes have been normalized using the

geometric means of the three best HKGs for each treatment concentration at various time points during a 60 days development period (Fig. II-22).



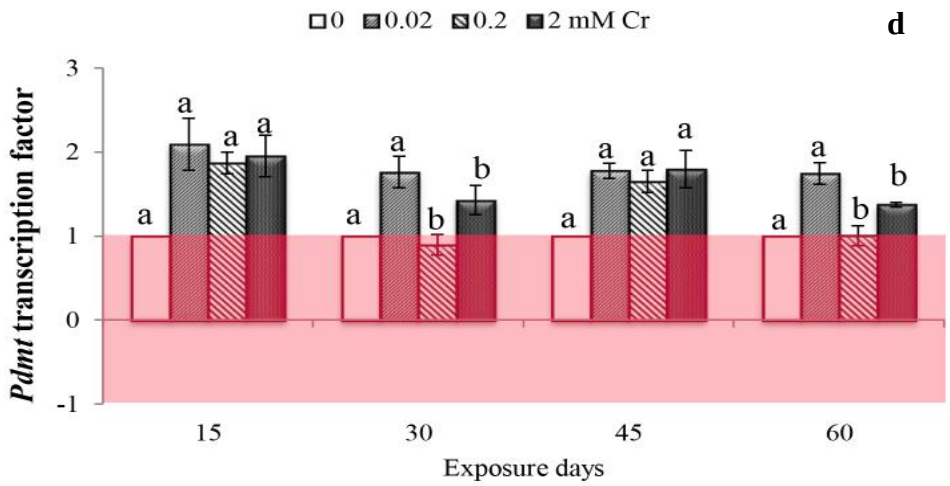


Figure II-22. Transcription factor of *Pdpcs* and *Pdmt* exposed to Cd (a,b) and Cr (c, d) stress respectively. The red area shows basal genes variations. Data presented are means ± standard error of three independent experiments. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test; where small letters show differences between concentrations. Means not showing the same letter are statically different.

Obtained results proved that candidate genes are not only present but fully operational in *P. dactylifera*. The effects of stressors (Cd or Cr) were tested with a two-way ANOVA, with metal concentration as factor one and time exposure as factor two. In control conditions, both gene expressions enhanced slightly over time (Table II-13). Results presented in Table II-13 indicated that Cd and Cr concentrations and stress exposure time had a significant overall effect on *Pdpcs* and *Pdmt* expression (p =0.000 < 0.05).

Major differences were observed at high Cd and Cr concentration for the longest exposure time periods. Thus, after 15 days of exposure, the expression levels of *Pdpcs* and *Pdmt* increased in all tested conditions (Table II-13) except a slight and not significant decrease of *Pdmt* at 2 mM of Cd (Table II-13).

Interestingly, low Cd^{2+} and Cr^{6+} concentrations (0.02, and 0.2 mM) strongly induced expression of both target genes at various time points. At high metal concentrations, expression was suppressed. This was more pronounced under Cd stress and can be linked to the severity of toxicity symptoms reported in *P. dactylifera* seedlings, including inhibition of germination.

Data can be presented as transcription factors after a second normalization and scaling with values of non-treated samples. It varied significantly (p<0.05) between treatments (Fig. II-22).

Table II-14. Log2 relative expression pattern of *Pdpcs* and *Pdmt* exposed to Cd and Cr stress. Results shown as mean ± SD. Two-way ANOVA main effects shown as *p* values. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test. where small letters show differences between concentrations (in columns) and major letters show differences in time exposure (in rows). Means not showing the same letter are statically different.

		Pdpcs				***Pdmt***			
		15	**30**	**45**	**60**	**15**	**30**	**45**	**60**
Cd	0	1.03aA (0.05)	2.03aA (0.05)	2.13aA (0.01)	2.04aA (1.14)	0.45aA (0.36)	0.46aA (0.48)	1.57aB (0.16)	0.92aA (0.10)
	0.02	4.58bA (0.44)	4.11bA (0.15)	5.27bA (0.18)	5.54bA (0.81)	2.45bA (0.63)	2.77bA (0.18)	3.35bA (0.20)	3.21bA (0.34)
	0.2	5.97cA (0.03)	3.52bB (0.52)	3.90cB (0.13)	2.82aB (0.25)	2.85bA (0.21)	3.12bA (0.18)	3.74bA (0.40)	4.09cB (0.14)
	1	1.60dA (0.15)	1.87aA (0.06)	1.75aA (0.07)	-1.00cB (0.12)	0.60aA (0.15)	0.87cA (0.06)	0.40cA (0.14)	-0.19dB (0.14)
	2 mM	1.33dA (0.47)	1.08aA (0.26)	-0.80dB (0.11)	-1.19cB (0.02)	-0.05aA (0.01)	-0.05cA (0.03)	-0.07cA (0.00)	-0.34dB (0.05)

Two way ANOVA main effects

Concentration	0.000*	0.000*
Time	0.000*	0.000*
Concentration x Time	0.000*	0.000*

Cr	0	2.03aA (0.05)	2.27aA (0.27)	2.37aA (0.32)	2.91aA (0.07)	1.68aA (0.68)	2.460aA (0.48)	2.57aA (0.16)	3.25aA (0.07)
	0.02	8.32bA (0.37)	8.78bA (0.35)	9.26bA (0.18)	9.91bB (0.26)	3.53aA (0.52)	4.33bcA (0.46)	4.57bA (0.24)	5.66bB (0.42)
	0.2	7.81bA (0.26)	6.88cB (0.02)	4.86cC (0.18)	4.17cC (0.23)	3.15aA (0.21)	2.21aAB (0.29)	4.24bAC (0.34)	3.27aA (0.38)
	2 Mm	6.00cA (0.01)	5.04dB (0.05)	2.4aC (0.42)	-0.82dD (0.05)	3.29aA (0.41)	3.51acA (0.42)	4.62bA (0.56)	4.46cA (0.09)

Two way ANOVA main effects

Concentration	0.000*	0.000*
Time	0.000*	0.000*
Concentration x Time	0.000*	0.019*

		Pdpcs				***Pdmt***			
		15	**30**	**45**	**60**	**15**	**30**	**45**	**60**
Cd	0	1.03aA (0.05)	2.03aA (0.05)	2.13aA (0.01)	2.04aA (1.14)	0.45aA (0.36)	0.46aA (0.48)	1.57aB (0.16)	0.92aA (0.10)
	0.02	4.58bA (0.44)	4.11bA (0.15)	5.27bA (0.18)	5.54bA (0.81)	2.45bA (0.63)	2.77bA (0.18)	3.35bA (0.20)	3.21bA (0.34)
	0.2	5.97cA (0.03)	3.52bB (0.52)	3.90cB (0.13)	2.82aB (0.25)	2.85bA (0.21)	3.12bA (0.18)	3.74bA (0.40)	4.09cB (0.14)
	1	1.60dA (0.15)	1.87aA (0.06)	1.75aA (0.07)	-1.00cB (0.12)	0.60aA (0.15)	0.87cA (0.06)	0.40cA (0.14)	-0.19dB (0.14)
	2 mM	1.33dA (0.47)	1.08aA (0.26)	-0.80dB (0.11)	-1.19cB (0.02)	-0.05aA (0.01)	-0.05cA (0.03)	-0.07cA (0.00)	-0.34dB (0.05)

Two way ANOVA main effects

Concentration				0.000*			0.000*		
Time				0.000*			0.000*		
Concentration x Time				0.000*			0.000*		
	0	2.03aA (0.05)	2.27aA (0.27)	2.37aA (0.32)	2.91aA (0.07)	1.68aA (0.68)	2.460aA (0.48)	2.57aA (0.16)	3.25aA (0.07)
	0.02	8.32bA (0.37)	8.78bA (0.35)	9.26bA (0.18)	9.91bB (0.26)	3.53aA (0.52)	4.33bcA (0.46)	4.57bA (0.24)	5.66bB (0.42)
Cr	0.2	7.81bA (0.26)	6.88cB (0.02)	4.86cC (0.18)	4.17cC (0.23)	3.15aA (0.21)	2.21aAB (0.29)	4.24bAC (0.34)	3.27aA (0.38)
	2 Mm	6.00cA (0.01)	5.04dB (0.05)	2.4aC (0.42)	-0.82dD (0.05)	3.29aA (0.41)	3.51acA (0.42)	4.62bA (0.56)	4.46cA (0.09)

Two way ANOVA main effects

Concentration	0.000*	0.000*
Time	0.000*	0.000*
Concentration x Time	0.000*	0.019*

Both genes were down regulated or up-regulated in both metal treatments indifferent time points. Target genes induction began at the lowest Cd concentration showing remarkable sensitivity (Fig. II-22a et b).

Increasing metal amount adversely affected gene expressions, especially *Pdmt* (Fig. 4b). After 15 days of exposure, *Pdpcs* expression reached a maximum at 0.2 mM Cd and decreased to a stable, low level after that time (Fig. II-22a). In addition, at this concentration *Pdmt* relative expression was up-regulated (3-folds) after 30 days of treatment (Fig. II-22b). Yet, at high Cd levels (1 and 2 mM), a significant (p<0.05) down-regulation starting from the first days of exposure for *Pdmt* (Fig. II-22b) and after 45 days for *Pdpcs* (Fig. II-22a) was observed. Suppression of *Pdpcs* and *Pdmt* expressions in date palm seedlings under these conditions resulted in a severe Cd toxicity phenotype (Fig. II-23a). The influence of chromium exposure on gene expression suggests the existence of a regulation of *pcs* and *mt* at transcriptomic level. After 60 days of treatment with 2 mM Cr^{6+} a significant down-regulation in *Pdpcs* expression was observed (Fig. II-22c). *Pdmt* gene expression in response to Cr^{6+} ions increased linearly with exposure time but not in a dose-dependent manner (Fig. II-22d). A mild decrease in *Pdmt* gene expression was observed after two months of exposure to 2 mM Cr (relative to 0.02 mM ; Fig. II-22d). The highest *Pdmt* level of expression was seen at 2 mM Cr^{6+}. Thus, during chromium exposure, coordination in genes expression was more pronounced. Moreover, significant morphological alterations in date palm seedlings grown for 60 days at the highest concentration of Cr was not reported.

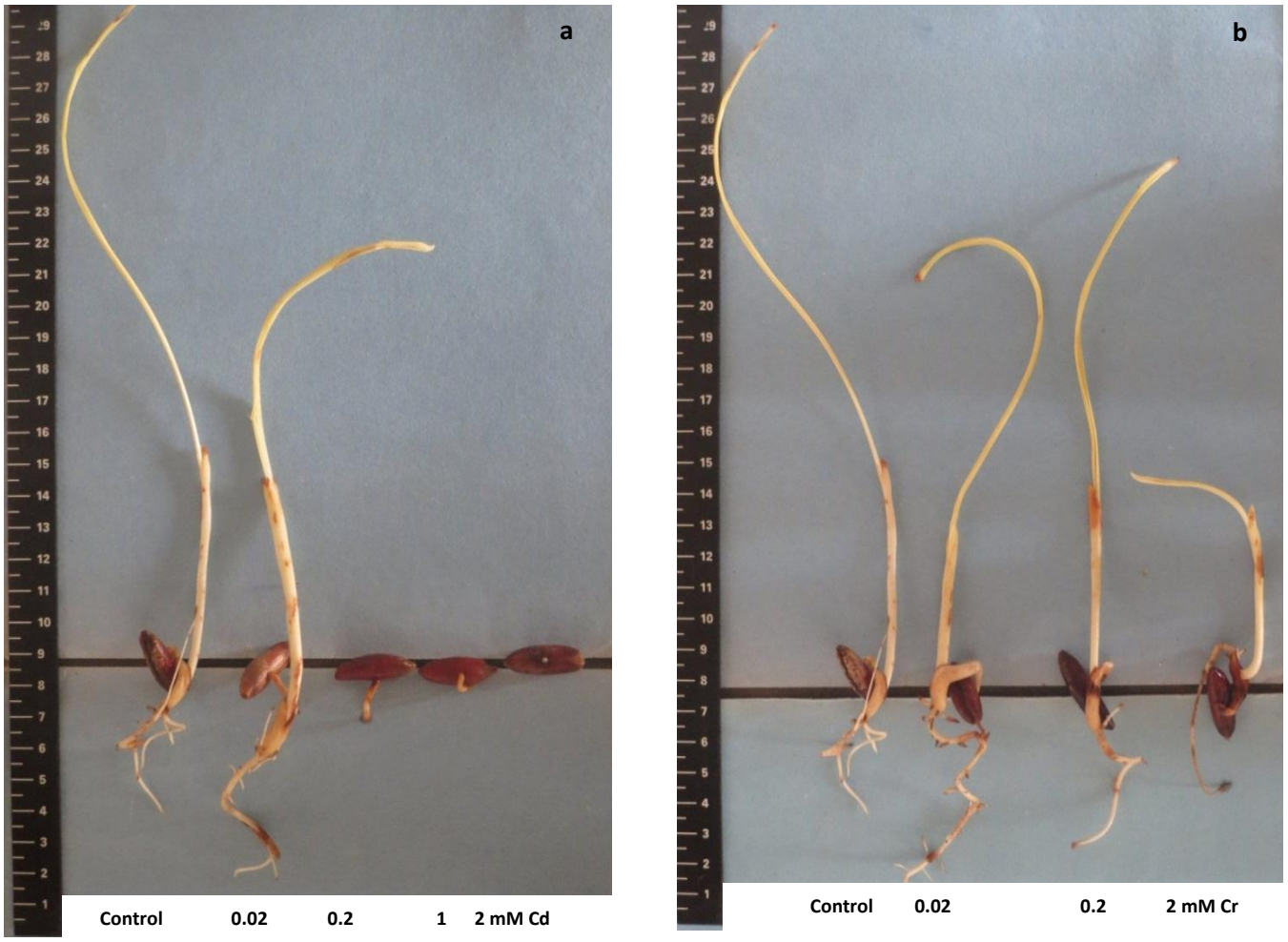
3.6. Metal effects on date palm seeds germinability and its related physiological perturbations

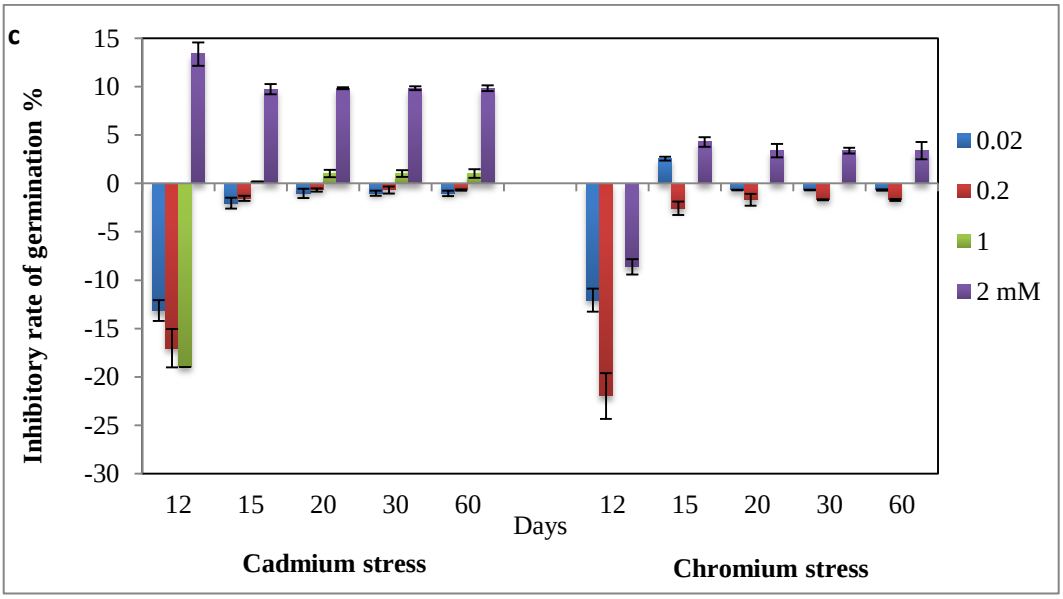
Because the ability of a plant to resist or tolerate toxicity to heavy metals depends on its ability to maintain germination in a polluted environment (Peralta et al., 2001), *P. dactylifera* seeds germination was monitored during 60 days of continual exposure to cadmium or chromium solutions. The seeds, which start to grow after 10 days of darkness, showed significant resistance to xenobiotics. Indeed, 92 % and 84 % of seed germination under 1 mM Cd and 2 mM Cr respectively was registered against 77.33 % in control suggesting that metal induced germination process (Fig. II-23b and e). This promoting effect on seed germination continued to improve along treatment, whereas, high cadmium concentration (2 mM) significantly inhibited seeds germination by more than 13 %. The highest promoting effect; GP = 99.5 % (Fig. II-23b) and 100 % (Fig. II-23e), was achieved by 0.02 mM Cd and 0.2 mM Cr respectively came up to 15 days. Hypocotyl length was more sensitive to metallic stress than germination percentage. It was found that, except at high Cd or Cr concentrations, there was no significant difference between treatments (Fig. II-23f). Hypocotyl growth was dramatically inhibited when exposed to Cd (Fig. II-23c). The lowest hypocotyl length with the highest inhibitory rate (98,97) was obtained after 60 days of exposure to 2 mM Cd. Hypocotyl elongation was stopped at 0.3 cm after 15 days of Cd treatment (Fig. II-23c) and hypocotyl also becomes considerably or completely necrotic at the end of the treatment with 0.2, 1 and 2 mM Cd respectively (Fig. II-23a). Some necrotic marking are also shown after a long treatment with 2 mM Cr (Fig. II-23d).

4. Discussion

The date palm was chosen because of its economic and social importance. For these reasons, countries such as Tunisia ranked it as a research priority for further development of crop production (Hammadi et al. 2015). Furthermore, this species has an additional ecological importance due to its use for biomonitoring of pollutants in many countries (Thomas 2002; Al-khlaifat and Al-khashman 2007). The emergence of heavy metal (HM) contamination in arid countries associated especially with re-use of wastewater (Lazarova and Bahri, 2004) or the presence of phosphate mining activity (Magni et al., 2005) is creating new environmental challenges for the plant. Moreover, date palm showed a great potential for Cr uptake (Enas et al., 2010), removing of HMs from wastewater and has been classified by (Zayneb et al., 2013) as a Cd hyperaccumulator. More recently, identification and involvement of a *pcs* type I gene

in HM chelation has been reported in date palm (Chaâbene et al., 2017).





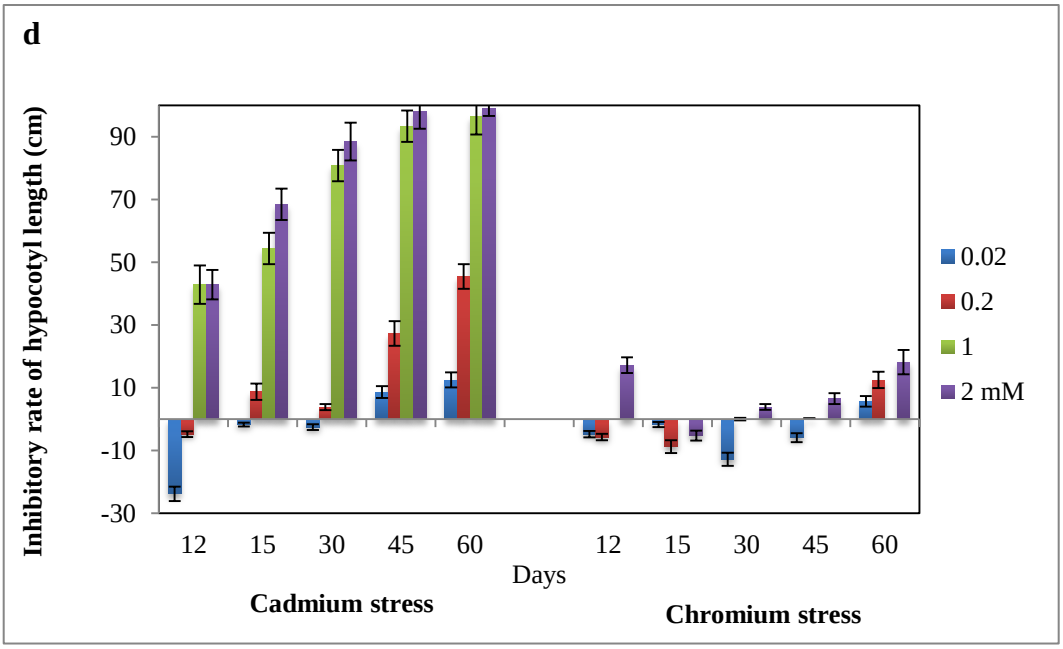


Figure II-23. Photograph of 2 months seedling of date palm exposed to Cd (a) and Cr stress (b). Effects of different Cd or Cr concentrations on germination percentage (c) and hypocotyl length inhibition (d) have been shown. Data presented are means ± standard error of three independent experiments.

Monitoring the existence of contamination in terrestrial environments typically involves frequent chemical analyses of environmental samples (soil, air, water). Biological data can be an effective supplement for these techniques, giving rise to the term "biomonitoring" (Bone et al., 2010). Biomonitoring consists not only of measuring contaminants in organisms, but also measuring physiological, biochemical or molecular parameters which can provide information on the performance of the organisms (Weeks, 1995a). In fact, biomarker analysis can constitute an effective monitoring network, sensitive to a wide range of xenobiotics, including heavy metals (Monteiro et al., 2009). It provides an early and specific response which will be a critical element for any restoration or protection project. Physiological responses to environmental stressors are accompanied by changes in specific gene expression profiles that can be used as biomarkers (Brulle et al., 2010). The regulation of gene expression represents the first level of integration between environmental stress and genome. The isolation of the genes encoding PCS and MT from the HM tolerant (Layla and Ahmed, 2011) and Cd hyperaccumulator *P. dactylifera* (Zayneb et al., 2013), enable us to explore their involvement in metal detoxification. It is not easy to draw general rules since the expression of target genes may vary within plant species, tissue and even cultivars. In *Arabidopsis* different isoforms for each *mt* type has been identified showing differential gene expression patterns suggesting that they have specialized functions in different tissues (Garcı et al.,

1998). *Pdmt* is similar to *MT3*-like mRNAs detected in developing embryos in *Arabidopsis* (Dong, and Dunstan, 1996) and to other *mt3* from other plant species.

Due to its sensitivity, accuracy and practical simplicity, qPCR technology is widely used in gene expression analysis (VanGuilder et al. 2008). Measuring target mRNA abundance with this method requires selection of suitable internal controls that have relatively stable expression irrespective of treatment to control for non-specific variation between testers and obtain biologically meaningful data (Czechowski et al. 2005; Crismani et al. 2006). Because HKG expression can vary considerably (Bustin 2001; Warrington et al. 2000), universal reference genes qPCR normalization which are stable under all experimental conditions do not exist (Gutierrez et al. 2008; Artico et al. 2010).

Therefore, validation of reference genes is required to avoid inaccurate data interpretation and false conclusions (Silveira et al., 2009). Despite the potential of date palm to take up and accumulate HMs (Layla and Ahmed, 2011), there was no information on the expression stability of reference genes in the plant during exposure to HMs stress. Previous study on date palm (Saidi et al., 2009) relied on classical reference genes without any prior validation. To our knowledge, the present investigation is the first to assess selection of potential reference genes suitable for gene expression quantification in *P. dactylifera* exposed to Cd or Cr. The candidate housekeeping genes that were evaluated in this study were identified with bioinformatics software detecting similarity to various genes expressed in plants. Stability of six candidate reference genes was evaluated in date palm subjected to abiotic stress caused by Cd and Cr, which are known to affect HKGs expression in numerous plant models (Morgante et al. 2011; Teng et al. 2012; Lee et al. 2013). Expression patterns have been analyzed with various mathematical methods relying on different algorithms (Bestkeeper, geNorm and NomFinder) in order to identify the most stable reference genes (Rapacz, 2013). Robledo et al. (2014) suggested that none of these programs is currently considered the best one. However, previous studies preferred geNorm which select similarly expressed reference genes by a pairwise approach (Andersen et al. 2004; Wood et al. 2008). Results presented in Table II-13 showed good stability of all investigated candidate genes with *M* value inferior to 0.5 (Hellemans et al., 2007). Although the algorithms of these software-programs are different, very similar results have been obtained with NormFinder software. Indeed, the most stable HKGs found by these two programs were the same in all samples but they ranked differently. This difference was expected and has been previously observed in other plants (Almeida et al. 2010; Demidenko et al. 2011). Under controlled environments, the classical reference gene

actin, was ranked first by geNorm and NormFinder (Table II-13) and further confirmed by BestKeeper algorithm (Fig. II-20a). Its validity as an internal standard has been previously questioned and changed by *PDA* in Northern blot analysis (Teste et al., 2009). Under the approach taken in this work, the three algorithms recommended the use of *Pdtbp*, *Pdact* and *Pdefα1* for Cd stress samples. These genes were insensitive to metal stress and stayed stable during the entire exposure period. Different outcomes have been produced in samples with chromium treatments. The ranking of reference gene stability identified by BestKeeper was different to that produced by geNorm and NormFinder. The Tata Box Protein encoding gene (*Pdtbp*) showed stable expression, according to both geNorm and NormFinder (Table II-13). While, with low correlation (r = 0.065), BestKeeper ranked it as the least stable gene (Fig. II-20c). The fact that expression of gene generated contradictory results among software programs discourages its use as a reference for qPCR normalization. Furthermore, a previous study proved that a conventional normalization based on a single HKG may lead to erroneous normalization (Vandesompele et al., 2002). Our approach determined a normalization factor indicating the number of HKGs required for reliable normalization. Figure II-21 clearly shows that three stable control genes allowed accurate normalization of samples.

In order to alleviate the stress, re-establish internal homeostasis and antioxidant capacity and minimize the detrimental effects of nonessential metals, plants have developed highly effective mechanisms to avoid or to regulate the introduction of HM ions, which employ metal transporters, chaperones and chelators such as PC(s) and MT(s). Identification of protein markers could serve as a good starting point for revealing new aspects of HM stress in plants and may complement the set of biological indicators traditionally used to evaluate the impact of metal contamination in natural ecosystems. Because it offers great potential for deployment in bioassays for evaluating environmental risk as well as in monitoring the effectiveness of remediation processes in contaminated environments with respect to biological site disturbances indications, toxicologists frequently study the effects heavy metals by monitoring the variation of the expression genes know to act as toxicity biomarkers (Benhamed et al., 2016). qPCR results normalization with a proper selection and evaluation of potential reference genes for each experimental condition and after scaling with values of non-treated samples revealed significant differential *Pdpcs* and *Pdmt* transcription levels in response to treatment with metals. The present study provides important evidence that *Pdpcs* and *Pdmt* genes are part of the tolerance and detoxification mechanisms for HMs in palm date hypocotyl tissues.

In fact, organism's deficient in PCS become unable to tolerate HMs-induced toxicity or display hypersensitivity to excess metals (Pal and Rai, 2010). Both time and metal concentration influenced target gene expression. In addition, according to two-way ANOVA (Table II-14), a significant metal stress and time exposure interaction for both genes was detected (p range from 0.000 and 0.019 < 0.05). Data showed that genes transcript levels increased significantly in hypocotyls of seeds treated with low Cd^{2+} or Cr^{6+} concentrations compared to controls. This is in accordance to previous research suggesting that PCS and MT were induced and regulated by several stimuli notably HMs at the transcriptional level (Clemens et al., 1999). Indeed, *Pdpcs* was rapidly induced in seedlings 15 days after germination (Fig. II-22a). Number of transcripts continued to increase significantly in tissues exposed to low Cr concentrations (Fig. II-22c) and was still up-regulated after exposure to 0.02 mM Cd (Fig. II-22a). This is in agreement with the hypothesis that *pcs* is controlled at the transcriptional level as proposed for garlic (Zhang et al., 2005). Increased tolerance to HM stress by *pcs* overexpression in *Tuberculous granuloma* has been reported (Zhu et al., 2003). Furthermore, the rapid reaction in the gene expression may play a pivotal role in immediate HM detoxification and may act as a part of the protection mechanism against oxidative stress caused by this xenobiotic in garlic as reported by (Zhang et al., 2005). However, gene expression was strongly dependent on metals ions concentration. At high Cd concentrations, both genes displayed decreased mRNA concentration compared to controls (Fig. II-22a et b). At these highest concentrations seed germination and hypocotyls' growth were inhibited (Fig. II-23a, b et c). This observation is similar to previous findings in other plant species (Kohler et al. 2002; Gonzalez-mendoza et al. 2007). A similar observation can in part be done where palm date seedlings exhibited toxicity symptoms only after 60 days of exposure to 2 mM Cr^{6+} (Fig. II-23d). The fact that *Pdpcs* was found to be more highly expressed under chromium stress contrasts previous results indicating that PCs were not involved in Cr^{6+} detoxification (Dubey et al. 2010; Shanker and Venkateswarlu 2009).

The coordinate expression of *Pdpcs* and *Pdmt* genes can give a part of the explanation of the high resistance of *P. dactylifera* seedlings to HMs. The transcription of *Pdpcs* in response to Cd reached a maximum after 15 days of exposure to 0.2 mM. It was significantly inhibited after 45 days of 2 mM Cd treatment and after 60 days of 1 mM Cd treatment which were highly inhibitory to hypocotyl elongation, most probably because the toxicity level has been reached. *Pdmt* expression level was less strongly induced (compared to *Pdpcs*) in seedlings exposed to Cd. In fact, it has been found in *Arabidopsis* that PCs were involved in HM

detoxification only under MTs deficiency (Zhou and Goldsbrough, 1994). Under chromium treatment, mRNA accumulation was more induced and *Pdpcs* was more sensitive to high Cr concentration than *Pdmt*. Under experimental conditions of 2 mM Cr, hypocotyls suffered necrosis and slight growth inhibition (Fig. II-23). The observed *mt* gene overexpression may play a pivotal role in Cr resistance. In addition to its high affinity for HMs, MT is commonly involved in a protective mechanism which appears to be the dominant metal detoxification pathway (Zhou and Goldsbrough 1994; Yu et al. 1994). It may maintain local redox balance, either by sequestering HMs or by otherwise preventing potentially deleterious Fenton chemistry reactions (Zhang et al., 1999). However, it was reported to be more specifically a response to metals, rather than oxidative stress (Ramesh et al., 2009). Similar findings have been described by Shanker and Venkateswarlu (Shanker and Venkateswarlu, 2009) who showed an increase of MT amounts as compared to the absence of metals sequestering PCs under Cr stress.

5. Conclusions

The present research gave an insight into the molecular basis of date palm heavy metals resistance. To the best of our knowledge, this is the first work investigating acclimation mechanisms involved in seed germination and in seedlings exposed to Cd or to Cr. Molecular mechanisms were studied through the quantification of the expression levels of two genes encoding proteins involved in metal detoxification, namely PCS and MT. Results displayed that *pcs* and *mt* genes were regulated at the transcriptional level in date palm subjected to heavy metal stress. Based on this study, the overexpression of genes responsible for Cd and Cr uptake, translocation, and sequestration can explain the exceptional metal tolerance of date palm, which can be observed in highly contaminated areas and which could be successfully exploited in phytoremediation.

Conflict of interest

Authors disclose that there are no relationships or interests that could have direct or potential influence or impart bias on the work. The authors declare that they have no conflict of interest and there are responsible for the content and writing of the article.

Authors' contributions

Amine Elleuch designed the research; Zayneb Chaâbene, Agnieszka Rorat and Imen Rekik

Hakim performed research and analyzed the data; Zayneb Chaâbene wrote the paper, C. Douglass Grubb proofread the paper and improved the grammar and the English. Pr. Mejdoub Hafedh and Pr. Franck Vandenbulcke followed the work and participate in article writing. All authors read and approved the final manuscript.

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Chapitre III

Biodisponibilité, bioindicateurs et biomarqueurs

-Avant-propos-

En Tunisie, la pollution des sols par les métaux lourds est essentiellement d'origine anthropique. Les rejets des activités d'extractions minières, riches en ETMs et qui sont stockés sous forme de digues sans mesure de protection des sols et des eaux souterraines provoquent l'accumulation des métaux traces dans les terres agricoles. Certains métaux représentent une menace aussi bien pour les plantes que pour la santé animale et humaine. Des dysfonctionnements de l'écosystème peuvent être observés en raison de leur transfert dans la chaîne alimentaire.

Le grand terril de phosphogypse (PG) de la région de Sfax, Sud-Est de la Tunisie, est une source de pollution atmosphérique intense. La détermination de la biodisponibilité des contaminants métalliques continus dans les aérosols est une étape nécessaire afin de déterminer les impacts sur les organismes vivants autour de la zone industrielle. Cette biodisponibilité a été évaluée via la mesure de paramètres biologiques permettant d'appréhender la fraction mobile et disponible en évaluant l'absorption, la bioaccumulation et la toxicité. De plus, afin de déterminer les risques additionnels d'exposition aux ions Cd, Cu ou Cr, des essais de croissance de plantules de palmier dattier cv Deglet Nour dans des sols dopés en conditions contrôlées *ex-situ* ont été réalisés.

Au fil de ce chapitre nous présentons i) l'intensité de pollution métallique de la zone industrielle de Sfax et son impact sur le palmier dattier ii) les capacités d'accumulation du Cd, du Cu ou du Cr chez le palmier dattier et iii) les mécanismes intervenant dans la réponse de la plante au stress métallique, essentiellement à l'échelle infra-individuelle.

-Publications-

a/ Chaâbene Zayneb, Rorat Agnieszka, Elleuch Amine, Vandenbulcke Franck*, Mejdoub Hafedh. **The effect of bioaccumulation of cadmium, copper and chromium on stress response biomarkers in date palm**. En cours

A-The effect of bioaccumulation of cadmium, copper and chromium on stress response biomarkers in date palm

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I. Synthèse

Les émissions atmosphériques des installations industrielles de la région de Sfax (usines de la SIAPE productrices d'engrais phosphatés notamment) semblent affecter les plantes (Figure II-24). Les métaux lourds contenus dans les poussières constituent une source majeure de contamination des écosystèmes. Une étude *in situ* des palmiers dattiers d'une zone contaminée proche de la ville de Sfax a été réalisée. Les teneurs métalliques, mesurées par ICP-AES, dans des échantillons de feuilles de palmiers cultivés depuis des années dans le site ont montré des niveaux élevés de Cd, de Cu et de Cr dépassant les limites admissibles de l'OMS (1996) (Tableau II-15). Au-delà du risque toxicologique engendré, l'étude de la capacité de l'espèce à résister à de telles conditions environnementales a été entreprise dans le présent projet. Ainsi, pour mieux comprendre la réponse de la plante aux stress métalliques, des plantules de palmier dattier cv Deglet Nour ont été cultivées en conditions expérimentales contrôlées dans des sols dopés par des concentrations croissantes de Cd, de Cu ou de Cr. Après deux mois d'exposition au métal, des valeurs du facteur de bioaccumulation (BCF) et du facteur de translocation (TF) supérieures à 1 ont été observées chez les plantules exposées à du sol contenant 10 mg/kg de Cd; Tableau II-15). Le palmier dattier semble donc présenter un assez grand potentiel pour absorber, transférer et accumuler le Cd. En revanche, les facteurs de translocation (TF) se sont avérés faibles chez les plantules traitées avec le Cr ou le Cu suggérant que le palmier dattier serait une espèce appropriée pour la phytostabilisation de ces métaux.

L'étude des stratégies de détoxication des métaux adoptée par la plante a permis d'identifier des biomarqueurs intégratifs pour chaque métal testé. Bien que le transfert des ions cadmiques soit significatif à de plus faibles concentration en Cd comparé aux ions Cu^{2+} et Cr^{6+}, le bilan

biochimique des paramètres testés (H_2O_2, TBARS, CAT, APX, SOD, polyphénols, flavonoïdes et tanins) s'est avéré plus propice pour des éléments métalliques essentiels. Une accumulation de H_2O_2 et de TBARS principalement dans les racines des plantules exposées au Cd a été observée (Tableau II-17). En contrepartie, les activités CAT et SOD des cellules stressées par le Cu ou le Cr ont été augmentées. L'activité APX a été davantage induite en réponse à l'excès de cuivre. En outre, le niveau de production de métabolites secondaires dans les cellules a été différemment influencé par le type et la concentration du métal. Par exemple, l'accumulation des polyphénols et des flavonoïdes a augmenté en réponse à de faible taux de stress avant de diminuer par la suite (Figure II-16). Cette diminution était plus importante sous l'effet des ions Cd^{2+}.

Toujours dans l'objectif d'étudier la capacité de la plante à survivre dans un contexte de contamination métallique, une étude des niveaux d'expression de certains gènes candidat a été réalisée.

Les profils d'expression des gènes *Pdpcs1* et de *Pdmt3* des plantules de palmier dattier exposées au Cd ou au Cu (Figure II-25a) étaient similaires aux niveaux mesurés dans les hypocotyles au stade germination. De faibles teneurs en Cd dans les sols ont stimulé l'expression des deux gènes candidats. Toutefois, une augmentation plus importante de l'expression de *Pdpcs1* a été obtenue sous l'effet des ions Cr^{6+}. Les gènes *Pdmt3*, *Pdabcc*, *Pdhma2* et surtout *Pdmate5* ont été plus sensibles au chrome qu'au cadmium ou au cuivre (Figure II-25). Sous l'effet du Cd, *Pdmate5* a été induit surtout au niveau racinaire (Figure II-25f). L'expression génique de *Pdmate5* était en corrélation avec l'accumulation des flavonoïdes dans les cellules. Les MATEs (Multidrug And Toxic compound Extrusion) produites peuvent mobiliser des métabolites secondaires comme les flavonoïdes et l'efflux de citrate dans les cellules stressées. Cela peut réduire l'absorption et la translocation de Mn (Figure II-15) ce qui peut influencer l'expression génique.

Les meilleurs gènes de transporteurs de métaux les plus sensibles au stress métalliques étaient *Pdhma2* et *PdNramp6* qui ont resté surexprimés même à fortes doses de Cd. De plus, les ions Cu^{2+} et Cr^{6+}, sans induire une sous-expression, ont stimulé l'expression de tous les gènes candidats même à fortes teneurs métalliques (500 mg/kg). Le comportement au niveau transcriptionnel des plantules au stress cuivrique a montré une augmentation marquée de l'expression de *Pdpcs1*, *Pdhma2* et *PdNramp6* (Figure II-25a, c et e). Cependant, le stress aux

ions Cr^{6+} a induit davantage l'expression de *Pdmt3*.

En plus de l'intérêt que représente la description fine des mécanismes, l'identification des mécanismes sous-jacents à l'accumulation de métal chez le *P. dactylifera* pourraient fournir des outils génétiques pour l'identification ou la sélection de la plante pour la phytoremédiation.

II. Article

The effect of bioaccumulation of cadmium, copper and chromium on stress response biomarkers in date palm

Abstract

Survival of date palm trees in contaminated area of Sfax city has drawn our attentions. Leaves samples of the plant grown in the study field showed high level of Cd, Cu and Cr exceeding standard limits. Under controlled conditions, we investigated date palm strategies to accumulate metal. After two months of treatment, high BCF and TF (>1) at low Cd amount has been found. They may confer to date palm great potential to uptake, translocate and accumulate Cd for phytoabsoption. However, low values of TF obtained for plantlets treated with Cr and Cu classified date palm as suitable for metal phytostabilisation. Thereby, significantly differences between tissues accumulation of oxidants showed enhancement of H_2O_2 and TBARS in roots of Cd and Cu-exposed plantlets in which Cd actions were more harmful. Moreover, coordination between CAT and APX activities has been found in plantlets treated with 50 mg/kg Cd. While, higher Cu and Cr amount in soils stimulate antioxidant enzyme activities more than Cd. Also, secondary metabolites level in cells was influenced by metal type and concentration. Polyphenol and flavonoid level enhanced at low stress amount and declined thereafter. Accumulation of flavonoid in cells was in correlation to gene encoding PdMATE5 responsible for secondary metabolites transport especially flavonoid. Other transporter genes responded positively to metal incorporation especially *Pdhma2* but also *Pdabcc* and *Pdnramp6*. This latter is suggested to be a novel gene expressed in plants in response to metal stress. As metal usually transported in complex, *Pdpcs1* enhanced to produce PCS metal chelator. It described the highest transcription factors of more 150 folds under 500 mg/kg Cr exposure. However, the highest expression level (14,61) was occurred with *Pdmt3* in leaves of Cr-stressed date palm.

Keywords: Gene expression, Bioaccumulation factor, detoxification mechanisms, Phosphogypsum, Phytoextraction, Phytostabilisation.

1. Introduction

Based on their physicochemical properties, bioactive-metals are divided into two groups: the redox-active metals such as chromium (Cr), copper (Cu) and iron (Fe) and redox-inactive metals like cadmium (Cd), nickel (Ni), aluminum (Al) and zinc (Zn). The metals belonging to

the first group are key elements of various biological processes and play a pivotal role in oxygen formation and enzymes' and proteins' structure (Valko et al., 2005). They can directly generate oxidative injury via undergoing Haber-Weiss and Fenton reactions, which leads to an uncontrolled formation of reactive oxygen species (ROS) in plants. At high levels, they may cause cell homeostasis disruption, defragmentation of biological macromolecules such as DNA (DNA strand breakage), proteins and lipids or cell membrane and damage of photosynthetic pigments, which may trigger cell death (Flora, 2009). Via inhibiting antioxidative enzymes, inducing ROS-producing enzymes (NADPH oxidases) and glutathione depletion (Valko et al., 2005), non-essential elements that belong to the second group can indirectly inflict oxidative stress (Bielen et al., 2013). Thereby, despite its redox state, the concentration of both essential and non-essential metals is crucial for environment.

A slightly elevated concentration of Cu can induce phytotoxicity (Guo et al., 2009), i.e. affect plant growth and alter cellular antioxidant system (Wang et al., 2004a). The pigment and protein components of photosynthetic membranes are the targets of Cu ions inducing perturbation of photosynthetic metabolisms (Aghaz and Bandehagh, 2013). Thereby, in the presence of high copper amount, leaves suffer chlorosis and necrosis caused by inhibiting chlorophyll and carotenoid biosynthesis and delaying the incorporation of these pigments into photosystem complexes. It may also reduce absorption of essential nutrients, especially Fe (Tanyolac et al., 2007). On the other hand, with unknown function as nutriment in plant, cadmium negatively affects plant growth and development and even causes plant death. Pinto et al. (Pinto et al., 2004) characterized Cd as an extremely significant pollutant due to its high toxicity and large solubility in water. It is also recognized as one of the most phytotoxic heavy metal (HM) contaminants (Baryla et al., 2001). Its toxicity is correlated to the alteration of the uptake and distribution of macro- and micro-nutriments, especially Fe and Magnesium (Mg) in plants (Garcia, 1999). Stomatal opening, transpiration and photosynthesis are also sensitive to cadmium. Chlorophyll is one of its targets and chlorosis may appear to be Fe deficiency caused by root Fe(III) reductase inhibition (Sandalio et al., 2001), substitution of central Mg ion in the chlorophyll molecules (Küpper et al., 1998) and/or by enhancement of chlorophyllase catabolic activity which may seriously affect photosynthesis (Baryla et al., 2001). Chromium, the seventh most abundant metal on earth, is considered as the most toxic HMs to living organisms and ecosystems due to its wide industrial applications (Xie et al., 2015). Being a strong oxidizer, Cr (VI), the second stable form of Cr, is highly toxic and more

mobile in soil/water systems even at low concentration (Xie et al., 2015). Cr phytotoxicity can result in root growth and biomass reduction, perturbation of nutrients balance and enzymes' activities, degradation of pigment status and induction of leaf chlorosis and oxidative stress in plants (Kumar Panda and Khan, 2003).

Plants, especially tree species with their long reproductive cycles, have evolved a complex network of highly effective homeostatic mechanisms. They serve to maintain physiological concentrations of essential metal ions like Cu and to minimize exposure to non-essential HMs like Cd and Cr. Some mechanisms, usually described by ubiquitous, are required to minimize the damage caused by high concentrations of elements. Plant cells contain a well-equipped antioxidative defense elements activated by sensing the heavy metal and aimed to attain a new balanced redox status. This signal transduction network involved stress-related proteins such as ROS-removing enzymes (superoxide dismutase (SOD), catalases (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR),…), and HMs scavengers like phytochelatins (PCs), metallothioneins (MTs) and non-enzymatic antioxidants include low molecular mass antioxidants scavengers (ascorbate (ASC), glutathione (GSH), vitamins, polyphenols, flavoids, tanins,…).

Other mechanisms target individual metal ions to control the accumulation, trafficking, and detoxification of metals. These later are metal specific occurred at transcriptional level and include the transcriptional expression of specific metal-responsive genes to counteract the stress stimuli. After sensing the HM, the plant cell activates specific genes such as *pcs* (Dalcorso et al., 2010). PCS is responsible for the biosynthesis of chelating compounds PCs containing glutathione (GSH) (Cobbett, 2000). It has been demonstrated that PCS is constitutively expressed, but its expression further enhanced after HM introduction and it is post-translationally activated by HMs (Cobbett and Goldsbrough, 2002b). Also, MTs are able to bind metal ions and are produced from mRNA translation. *mt* overexpression has been shown in different plants to enhance HMs tolerance (Hassinen et al., 2009). Also, the plasma membrane transporters in plants may contribute to heavy metal stress responses as there are involved in metal uptake and homeostasis. Numerous families of transporter genes have been identified. The plant ABC (ATP−binding cassette) gene family plays an important role in the general detoxification mechanism. Their expressions are enhanced by xenobiotic incorporation. Potentially toxic compounds are first incorporated into conjugates, which are then recognized by tonoplastic ABC transporters and sequestered into the vacuole for

detoxification purposes (Klein et al., 2006). PCs and PC-HM complexes are transported by ABCC−type transporters (Kretzschmar et al., 2011). P-type ATPase, have been reported to be involved in nutrients uptake and distribution (Palmer and Guerinot, 2009). Among them, the transporters belong to the heavy metal P_{1B}-ATPase subfamily including HM-transporting P-type ATPase (HMA) (Burkhead et al., 2009) and the NRAMP transporters (Lanquar et al., 2005). MATE is a family of membrane-localized efflux proteins involved in extrusion of multidrug and toxic compound from the cell. MATE genes are expressed especially in response to Fe ions presences in cells. Thereby, MATE proteins participate in iron-citrate efflux and are engaged to translocate Fe^{2+} from the roots to leaves (Durrett et al., 2007).

Although plant detoxification mechanisms are extensively reported in literature, little is known about date palm's; *Phoenix dactylifera*, responses to metals stress except it showed great capacities to remove HMs from wastewater (Al-Busaidi et al., 2015) and is able to accumulate Cd and Cr (Chaâbene et al., 2017a). The species is widely cultivated in the southern part of Tunisia country, where soils around opencast mines and near the industrial sites, especially the phosphate fertilizer industry exhibit very high contents of HMs (Galfati et al., 2011) (Wali et al., 2013a). In fact, the erosion of phosphogypsum (PG) piles, waste of the treatment of phosphate rock (PR) containing highly polluting hazardous element, can cause the contamination of the surrounding areas (Béjaoui and Hadj, 2016b). This is the case in the vicinity of PG stockpiles in Lebanon in which the environment was found to be contaminated with HMs attending toxic amount considerably above-threshold of Cd, Zn and radionuclide (U) (Nafeh Kassir et al., 2012).

The aims of the present work were to i) monitor the level of accumulation of HMs in date palm exposed to field contaminated soils, ii) estimate the accumulation of particular metals (Cd, Cu and Cr) in different plant compartments in controlled conditions, iii) disclose the plant metal detoxification mechanisms and iv) investigate the potential of biomarkers of HMs stress.

2. Material and Methods

2.1. Soils and plants sampling from the study site

The study field was located in Sfax, southern Tunisia (237 kilometers). It was about 0.3 km far away the PG stockpiles of SIAPE factory and lead melting industry (Fig. II-25), the main

source of HMs in Sfax (Azri et al., 2002). SIAPE emitted Dust estimated annually to 1610 tons (Béjaoui et al., 2016). With an extended period of time, since 1950s, and two piles of PG of more than 15 million tons (Azri et al., 2000), plant makes the assessment of its impact on the surrounding environment reliable.

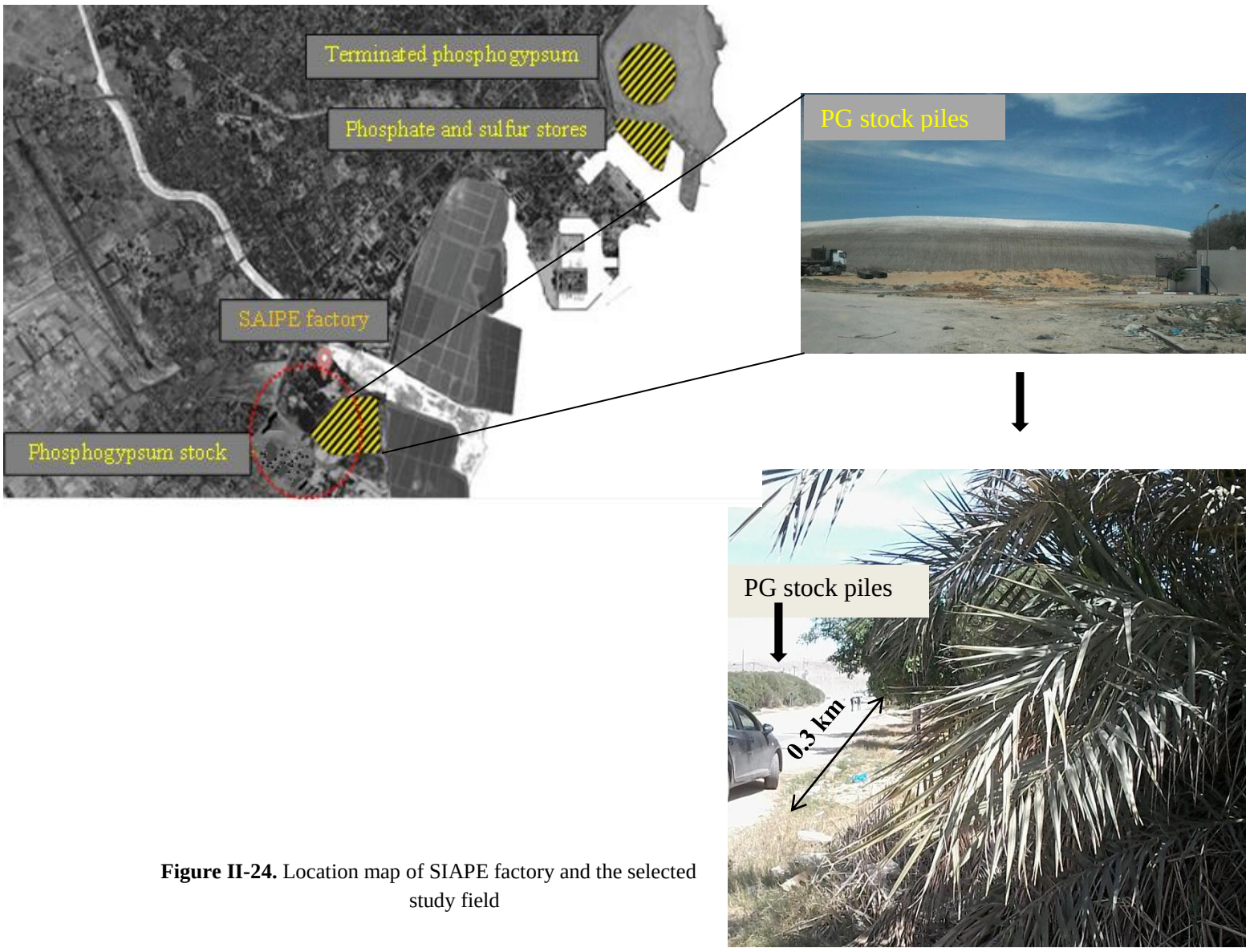


Figure II-24. Location map of SIAPE factory and the selected study field

Soil samples were collected from 5 different directions of 0.3 km distant from the PG piles. (Fig. II-253). The samples were collected in a depth of surface soils of all sites (0-20 cm). Soil samples were homogenized, collected in plastic bags, brought to laboratory, air-dried and stored for analysis. The date palm leaves samples were collected from the same sites from where the soil samples were collected. Samples were washed with distilled water to remove

soil particles, ground with liquid nitrogen and stored at -20 °C. Part of collected leaves samples were lyophilized and grounded in liquid nitrogen for biochemical analysis in three replicates.

2.2. Ex-situ soils contamination and plant transfer

A mixture of 2/3 peat and 1/3 potting soil has been prepared, dehydrated at 50 °C and distributed in pots in measured amounts (1 kg). Amount of 10, 50, and 100 mg of $CdCl_2$ and of 50, 100 and 500 of $CuSO_4$ and of K_2CrO_4 powder were homogenized in distilled water. After a complete dilution, dried soils were gradually soaked with the solution by long mixing.

Seedling of date palm of about 2 month of germination in control conditions produced roots reached 5 to 10 cm length. They were transferred to previously prepared pots at rate of 4 plantlets per pot. The seedlings were grown in a glasshouse at 25±3 °C and 16h of photoperiod. When the plants were growing, they were sprayed with distilled water. In order to avoid soils leaching, the infiltrated irrigated water was recovered and reused again for irrigation. The pots were prepared in triplicates. After 4 months of growth, the plants were harvested. Shoot and root length were separated and rinsed with distilled water. The fresh plant materials were ground in liquid nitrogen for chemical and molecular analysis. Plant tissues were oven-dried at 60 °C for 72 h, and used for biochemical analysis. The analyses were done in triplicates.

2.3. Soils and plant digestion for metal spectroscopic analysis

After four months of metal exposure, roots and shoots were separated for each plant, washed with water and rinsed with distillated water, to ensure that outside contamination has been removed. Soil and plant samples were lyophilized before plant material was ground to a fine powder with liquid nitrogen. One mL of HNO_3 (65 %, 108 m/V, trace pure) was added to 0.1 g of soil and plant tissue samples. The mixture was left at least 12h at room temperature under a hood. Then, the mixture was heated to reflux for 2h in a sand bath in 120 °C. Before a second digestion at 180°C, 1 mL of acid mixture (HNO_3: H_2SO_4: $HClO_4$: 10: 2: 3, v/v/v) was added. The digests prepared in this way were then analyzed for the elements contents spectroscopically using atomic emission spectrometry with inductively coupled plasma, ICP-AES (Varian 720-ES, USA). Five elements have been monitored in different compartments (soil, roots and leaves): Cd, Cu, Cr, manganese (Mn) and Zn.

2.4. Data processing

The bioaccumulation Factor (BAF) defined by (Yanqun et al., 2005), was calculated as the ratio of metal contents in the entire plant to that in the soil (Yanqun et al., 2005) and is given in equation 1.

BAF = [Metal] shoot / [Metal] soil ---------- Eq. 1

The Translocation factor (TF) was described as the ratio of concentration of heavy metal in plant shoot to that in plant root (Yoon et al., 2006) and is given in equation 2.

TF = [Metal] shoot / [Metal] root ---------- Eq.2

The Bioconcentration factor (BCF) was calculated as ratio plant roots HM to that of soil (Bu-Olayan and Thomas, 2009) and is given in equation 3.

BCF = [Metal] root / [Metal] soil ----------Eq.3

The enrichment factor (EF) was calculated as the ratio of metal content in above ground plant parts over metal content in soil and given in equation 4. The EF is considered to be ideal HM stabilizers.

EF= [Metal] plant roots+leaves / [Metal] soil ……..Eq.4.

2.5. Estimation of H_2O_2 content and Lipid peroxidation

Hydrogen peroxide concentration was determined as previously described by Elleuch et al. (2013). The levels of products of lipid peroxidation were measured as thiobarbituric acid reactive substances (TBARS) aldehydes according to Rustérucci et al. (1996). The concentrations of TBARS were calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.6. Enzyme analysis

Total protein extraction from date palm roots and leaves was made according to Chaâbene et al (2017). Total protein content was determined spectrophotometrically according to the method of Bradford (1976), using bovine serum albumin as a standard. Assays of antioxidant enzyme activities in date palm tissues were prepared for catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) as described previously Chaâbene et al (2017). SOD activity was assayed using the photochemical nitrobluetetrazolium (NBT) method and measured according to Beyer and Fridovich (2016). Catalase and ascorbate peroxidase

activities were determined as described by Aebi (1984) and Nakano and Asada (1981) (1981) methods respectively.

2.7. Analysis of plant secondary metabolites

The secondary metabolites are extracted by maceration of 50 mg of tissues powder in 2 mL of organic solvent (80 % acetone), under ultrasonic conditions for 45 minutes, at 4 °C. After centrifugation, the supernatant containing phenols was recovered. A second identical extraction was carried out to extract 30 % of additional phenols and thus to obtain a more complete dosage. The supernatants were combined before being concentrated to dryness under vacuum. The total phenols content in tissue was determined using the Folin Ciocalteu method described by (Pinelo et al., 2004). Total flavonoids were determined according to (Zhishen et al., 1999). Based on the condensation of polyphenolic compounds with vanillin in an acid medium (Price et al., 1979), the content of tannins in roots and leaves of date palm plantlets was measured

2.8. Real time qPCR amplification

RNAs extraction and cDNA synthesis were performed as previously (Chaâbene et al., 2017a). From previously published partial DNA gene sequences encoding genes, quantitative real-time polymerase chain reaction (qPCR) amplification method was performed according to Brulle et al. (2014). The specific phytochelatin synthase type 1 (*Pdpcs1*) and metallothionein type 3 (*Pdmt3*), *Pdabcc family*, *Pdhma2*, *Pdmate5* and *Pdnramp6* primers (Chaâbene et al., 2017), were used. The expression levels and the relative fold expression (RFE) were determined according to previously described procedures (Brulle et al. 2006). The geometric mean of the 3 most stable reference genes in control and Cd-stressful condition identified by Chaâbene et al. (2017a) was used to calculate expression of target gene levels according to Brulle et al. (2014). Absolute quantification of genes expression levels is shown as $\log_2$.

2.9. Statistical analysis

Results for each treatment were expressed as means ± SD. All analysis were conducted using STATISTICA 10. Significant differences between parameters were tested using Tukey's test HSD test after one-way and two-ways ANOVA with tissues and metal concentrations as the two factors. Differences at $p < 0.05$ were considered statistically significant.

3. Results

3.1. Field contamination and metal concentrations and bioaccumulation

Compared to control site, the soil being tested from contaminated area of Sfax region (Fig. II-23), showed higher chromium level of 28 mg/kg. This concentration as well as Cu level in field soil samples are within permissible limit recommended by Dutch standard, which are 100 mg/kg Cr and 36 mg/kg respectively. Cadmium detected concentration of 0.9 mg/kg (Table II-15) slightly exceed Dutch standard permissible limit (0.8 mg/kg). Moreover, Cd and Cu level in harvested leaves of date palm in contaminated field was almost 2 times higher than in soil (Table II-15). All tested metal in plant leaves exceed the metal common range according to (WHO, 1996). Cd, Cu and Cr concentrations in date palm leaves coming from Contaminate site of Sfax region were 1.6, 14.6, 17.9 mg/kg respectively. The standard limits of WHO (1996) are 0.02, 10 and 1.3 mg/kg for Cd, Cu and Cr respectively. They reveal high atmospheric contamination of HMs in this area which may be absorbed by the plant upper parts.

To better understand metal bioavailability and plant uptake and translocation, doped soils with increasing concentrations of Cd, Cu and Cr were used. Significant differences between metal levels in tested compartments (soil, roots and leaves) were shown in control plants (Table II-15). After four months of Cd-exposure, about half of metal content in soil was absorbed by date palm plantlets mainly in its roots part (Table II-15). They described a bioconcentration factor BCF >1 at 10 and 50 mg/kg of Cd stress (Table II-15). Moreover, important Cd-translocation reaction of date palm was deduced by TF >1 in samples treated with lowest metal amount (Table II-15).

Table II-15. Heavy metal accumulation in different compartments of site collected samples and *ex-situ* experiments. Results shown as mean ± SD. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test. where small letters show differences between compartments (in rows) and major letters show differences between concentrations (in columns). Means not showing the same letter are statically different.

		Uncontaminated Site			contaminated Site			Doped Soils											
								Cd (mg/kg)				Cu (mg/kg)				Cr (mg/kg)			
		Cd	Cu	Cr	Cd	Cu	Cr	0	10	50	100	0	50	100	500	0	50	100	500
Metal Content (mg/kg)	Soil	0.3 (0.1)	5.4 (0.4)	0.1 (0.1)	0.9 (0.1)	7.5 (0.3)	28.0 (0.8)	0.7aA (0.02)	4.4aB (0.2)	26.3aC (1.1)	63.9aD (0.9)	5.68aA (0.02)	19.3aB (1.4)	33.4aC (2.5)	64.7aD (0.9)	0.05aA (0.02)	26.5aB (2.0)	71.8aC (1.6)	29.2aD (3.9)
	Roots	-	-	-	-	-	-	0.2bA (0.02)	4.5aA B (0.0)	16.0aBC (5.04)	27.1bC (5.1)	4.85aA (0.02)	17.2ab B (2.9)	18.8bB (2.1)	19.6bB (1.9)	0.64bA (0.02)	9.8bB (0.6)	15.4bB (0.8)	25.8bC (0.2)
	Leaves	0.2 (0.0)	3.8 (0.7)	0.1 (0.8)	1.6 (0.7)	14.6 (2.8)	17.9 (1.9)	0.4cA (0.09)	2.1aA (1.5)	12.2bB (1.2)	15.3ab B (3.7)	3.82bA (0.09)	9.3bB (1.2)	12.7bB (1.9)	13.8bB (0.7)	0.02aA (0.09)	7.2bB (0.3)	8.7bB (0.2)	14.2cC (2.5)
	BAF							0.64 (0.0)	0.74 (0.2)	0.46 (0.1)	0.24 (0.1)	0.29 (0.0)	0.49 (0.0)	0.38 (0.1)	0.21 (0.1)	0.38 (0.0)	0.27 (0.0)	0.12 (0.0)	0.04 (0.0)
	EF							2.15 (0.4)	0.69 (0.0)	0.76 (0.3)	0.56 (0.2)	0.45 (0.04)	0.54 (0.0)	0.67 (0.1)	0.7 (0.0)	0.03 (0.4)	0.73 (0.3)	0.57 (0.2)	0.55 (0.0)
	TF							0.23 (0.)	1.02 (0.0)	0.60 (0.0)	0.42 (0.1)	1.77 (0.)	0.9 (0.2)	0.56 (0.0)	0.30 (0.0)	1.5 (0.0)	0.90 (0.1)	0.56 (0.0)	0.30 (0.0)
	BCF							0.93 (0.1)	1.72 (0.0)	1.07 (0.0)	0.66 (0.1)	0.19 (0.1)	1.37 (0.1)	0.98 (0.3)	0.51 (0.0)	1.89 (0.1)	0.37 (0.0)	0.21 (0.0)	0.08 (0.0)

Increasing Zn content in soil and plants parts increased its uptake, while no pairwise correlation between metal content in soil/plant has been found (Table II-16). On the other hand, Cd negatively influenced Mn uptake by date palm (Table II-16). Significantly negative correlation between Cd and Mn was approved by Pairwise correlation coefficient (r = -0,61), implying the antagonistic effect of Cd on Mn absorption and translocation. Similarly, Cr^{VI} altered Mn translocation to date palm young leaves although Cr content increased in roots by increasing metal in soils (Table II-16). These observations were further improved by low TF under chromium-stress. However, EF <1 may classify date palm as ideal Cr-stabilizer (Yoon et al., 2006). In addition, copper content in roots range from 17.2 to 19.6 mg/kg (Table II-17). It has shown important BCF exceeding 1.3 and TF of 0.9 at low metal stress.

3.2. Accumulation of oxidants in plant under HMs stress

Under natural physiological conditions, date palm plantlets generated ROS within plant cells. The accumulation of hydrogen peroxide (H_2O_2) and the product of lipid peroxidation, in the form of tiobarbituric acid reactive substances (TBARS) significantly differ between tissues (p<0.05). Leaves of the control plants accumulated oxidants more than roots (Table II-17). Two-way ANOVA indicated that except for chromium stress, plant tissues and metal concentrations (Cd and Cu), influence H_2O_2 production in cells. Increasing Cd amounts in soil further enhanced H_2O_2 and TBARS accumulation, especially in roots. However, no significant difference between roots and leaves exposed to Cu stress in H_2O_2 accumulation was shown (Table II-17). The highest level of oxidant (H_2O_2=709.56 µmol/gFW) was accrued in roots of young plantlets of date palm grown in 100 mg/kg of Cd amended soils whereas, the lowest one (H_2O_2=317.79 µmol/gFW) was accrued in roots of treated plants with 100 mg/kg Cr. Event at 500 mg/kg of Cu and Cr, H_2O_2 amount in cells never exceed 600 µmol/FW (Table II-17). Similarly, TBARS production, a biomarker of oxidative damage in cells, followed the same trend as H_2O_2 accumulation. It differed significantly between roots and leaves of Cd-stressed plants. However, no significance between tissues has been shown for exposed plants to Cu and Cr treatment (Table II-17).

Table II-16. Mn and Zn content in different compartments of site collected samples and *ex-situ* experiments. Results shown as mean ± SD. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test. where small letters show differences between compartments (in rows) and major letters show differences between concentrations (in columns). Means not showing the same letter are statically different. Pairwise correlation coefficient (*r*) has been calculated by Statistica 10. Red value showed correlation.

				Doped Soils											
		Uncontaminated Site	**contaminated Site**	Cd (mg/kg)				Cu (mg/kg)				Cr (mg/kg)			
				0	10	50	100	0	50	100	500	0	50	100	500
Mn Content (mg/kg)	Soil	9.0 (0.4)	14.1 (1.8)	40.7aA (0.3)	42.2aB (0.3)	44.9aC (0.0)	44.9aC (0.1)	40.7aA (0.3)	41.8aB (1.5)	44.0aAB (0.0)	44.3aB (0.3)	40.7aA (0.3)	46.8aA (2.6)	43.6aA (1.9)	46.4aA (2.0)
	Roots	-	-	4.2bA (0.4)	6.2bB (0.3)	4.8bAB (1.0)	2.2bA (0.3)	4.2bA (0.4)	6.1bB (0.1)	8.4bC (0.7)	4.0bA (0.5)	4.2bA (0.4)	8.4bB (0.5)	7.8bC (0.2)	6.8bBC (1.1)
	Leaves	4.5 (0.9)	16.5 (1.3)	18.3cA (0.5)	12.3cB (0.5)	7.1bC (0.2)	5.1cD (0.3)	18.3cA (0.5)	11.2bB (0.3)	8.6bC (0.5)	5.6bD (0.5)	18.3cA (0.5)	12.3bB (0.4)	8.9bC (0.0)	7.1bD (0.2)
Zn Content (mg/kg)	Soil	9.4 (0.2)	15.4 (0.7)	19.7aA (0.4)	24.9aB (0.0)	16.1aC (0.1)	11.2aD (0.3)	19.7aA (0.4)	19.6aA (1.8)	17.8aA (5.3)	22.0aA (2.8)	19.7aA (0.4)	23.1aB (0.1)	14.1aC (0.2)	12.4aC (0.6)
	Roots	-	-	7.5bA (0.6)	15.47bB (0.6)	13.4aAB (2.0)	11.6aAB (2.2)	7.5bA (0.6)	11.1bB (0.2)	18.2aC (0.3)	20.2aD (0.0)	7.5bA (0.6)	13.3bB (0.4)	24.1bC (3.0)	22.8bC (4.0)
	Leaves	14.0 (1.1)	36.0 (1.5)	29.3cA (0.9)	28.9aA (1.4)	25.5bA (0.7)	16.3bB (0.5)	29.3cA (0.9)	15.1abB (2.8)	20.0aC (0.2)	21.0aC (0.0)	29.3cA (0.9)	14.7bB (0.3)	10.2aC (4.0)	9.9aC (0.1)

	Pairwise Correlation coefficient (*r*)							
Metal content-Mn content	0,914191	-0,611946	0,914191	-0,48363	0,914191	-0,33352		
Metal content-Zn content	0,944324	-0,327384	0,944324	-0,03393	0,944324	-0,277297		

3.3. Enzymatic antioxidant and Secondary metabolites potential developed in date palm under HMs stress

High levels of antioxidant enzyme activities were obtained for all tested HMs (Table II-17). In fact, except for date palm plantlets treated with chromium, CAT, APX and SOD enzymes amounts were significantly much higher in leaves than in roots. CAT activity enhanced under Cd stress and reached its maximum at 10 mg/kg with 2.76 and 3.5 µmole H_2O_2/mg protein in roots and leaves respectively. Important amounts of Cu and Cr in soils (100 mg/kg) stimulate CAT activity more than Cd (Table II-17). CAT activity continued to increase even with 500 mg/kg of metal stress, while it decreased under enhancing Cd stress. APX activity showed coordination to CAT activity. It was upregulated when CAT activity was reduced under 50 mg/kg Cd (Table II-17). Indeed, it was more induced in Cd and Cr treated plantlets describing no significant differences between plants tissues treated with Cr (Table II-17). Positive correlations were found between SOD activity and Cu and Cr concentrations in the roots and leaves of date palm young plants. Yet, enzyme activity induction highly decreased with stress by 500 mg/kg Cu. The only case where a significant decrease in SOD activity was obtained for samples treated with higher Cd amounts. High Cd level inhibited SOD activity by more than 30 % in leaves (Table1).

The secondary plant metabolism was influenced by metal stress and plant tissues ($p< 0.05$) especially for samples treated with Cd (Table II-17). Leaves showed higher accumulation of polyphenol, flavonoid and tannins than roots. At low Cd stress (10 mg/kg), flavonoids were more significantly induced compared to polyphenol and tannins (Table II-17), while, no significant difference between tissues has been found. Increasing Cd stress decreased production of non-enzymatic antioxidant metabolites. A harmful decline of tannins by more than 40 % has been observed in roots of plants treated with 100 mg/kg Cd. However, it was still higher than the control even at high concentrations of Cr. Flavonoid percentage was more induced in roots under cupric stress (Table II-17). Contrarily, roots of treated plants with Cr showed no significant decline of flavonoid content which was less influenced by the metal than by Cd and Cu stress (Table II-17). Polyphenol content declined only in roots and leaves of contaminated plants with 100 mg/kg Cd (Table II-17). According to two-way ANOVA, it was influenced by tissues and concentration of Cd and Cr. Thereby, no correlation between the two factors has been shown in young date palm plant exposed to cupric stress.

Table II-17. Monitoring of elements of oxidative and antioxidative state of date palm plantlets subjected to different Cd, Cu and Cr concentrations. Results shown as mean ± SD. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test. where small letters show differences between tissues (in columns) and major letters show differences between concentrations (in rows). Means not showing the same letter are statically different.

		H_2O_2 µmol/g FW		**TBARS nmol/g FW**		**CAT µmole H_2O_2/mg Protein**		**APX**		**SOD U/mg Protein**		**Polyphenol %**		**Flavonoid %**		**Tanins %**	
		Root	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root	Leaves
Cd (mg/kg)	0	156.1aA (4.4)	244.6bA (5.8)	44.9aA (2.9)	56.5bA (4.0)	1.6aA (0.0)	1.7aA (0.2)	8.7aA (0.2)	9.9bA (0.6)	444.1aA (6.2)	550.1bA (28.7)	100aA (0.00)	100aA (0.00)	100aA (0.00)	100aA (0.00)	100aA (0.00)	100aA (0.00)
	10	270.4aB (1.3)	381.9bB (22.0)	62.5aB (4.7)	70.4bAB (0.7)	2.7aB (0.1)	3.5bB (0.0)	12.5aB (0.1)	13.8bB (0.2)	590.3aB (9.3)	612.3bB (8.5)	114.8aB (4.7)	153.2bB (3.8)	201.5aB (3.3)	201.1aB (6.1)	132.4aB (4.19)	113.8bB (3.4)
	50	568.2aC (12.2)	599.6bC (1.7)	94.1aC (4.1)	86.8aC (9.7)	1.5aA (0.0)	1.8bC (0.0)	12.7aB (0.2)	14.7bB (0.1)	540.0aB (13.0)	542.8aA (28.1)	134.6aC (1.4)	145.0aB (10.6)	186.3aB (9.8)	205.2aB (6.5)	109.5aA (5.8)	150.0bC (5.4)
	100	709.5aD (18.7)	598.5bC (6.5)	98.3aC (3.0)	82.2bBC (2.7)	0.9aC (0.0)	0.4bD (0.0)	6.1aB (0.1)	11.4bC (0.7)	310.6aC (10.0)	373.6bC (22.4)	72.0aD (4.3)	83.7bC (2.8)	85.4aC (5.9)	60.6bC (8.0)	55.8aC (4.2)	64.9bD (1.7)
Two way ANOVA main effects																	
Tissues		0.000*		0.000*		0.000*		0.000*		0.000*		0.000*		0.000*		0.000*	
Concentration		0.000*		0.626		0.000*		0.000*		0.000*		0.000*		0.520		0.000*	
Tissues x concentration		0.000*		0.000*		0.002*		0.003*		0.001*		0.000*		0.000*		0.000*	
Cu (mg/kg)	0	156.1aA (4.4)	244.6bA (5.8)	44.9aA (2.9)	56.5bA (4.0)	1.6aA (0.0)	1.7aA (0.2)	8.7aA (0.2)	9.9bA (0.6)	444.1aA (6.2)	550.1bA (28.7)	100aA (0.00)	100aA (0.00)	100aA (0.00)	100aA (0.00)	100aA (0.00)	100aA (0.00)
	50	202.9aB (4.6)	248.4aA (20.9)	44.3aA (3.4)	63.5bAB (3.1)	2.0aB (0.1)	2.0aAB (0.1)	10.9aB (0.1)	11.8bB (0.2)	583.8aB (13.8)	633.5bB (17.7)	116.7aA (7.0)	127.2aB (3.8)	238.1aA (6.6)	247.8aB (1)	148.4aB (7)	151.8aB (6)
	100	319.4aC (17.8)	325.0aB (25.0)	53.5aA (2.31)	69.2bBC (3.17)	2.4aC (0.1)	2.4bBC (0.0)	12.5aBC (0.1)	13.7bC (0.2)	660.8aC (12.3)	717.1bC (9.5)	151.5aB (6.3)	185.2bC (12.5)	271.2aA (18.1)	184.8bC (11.4)	162.6aC (6.5)	179.8bC (2.8)

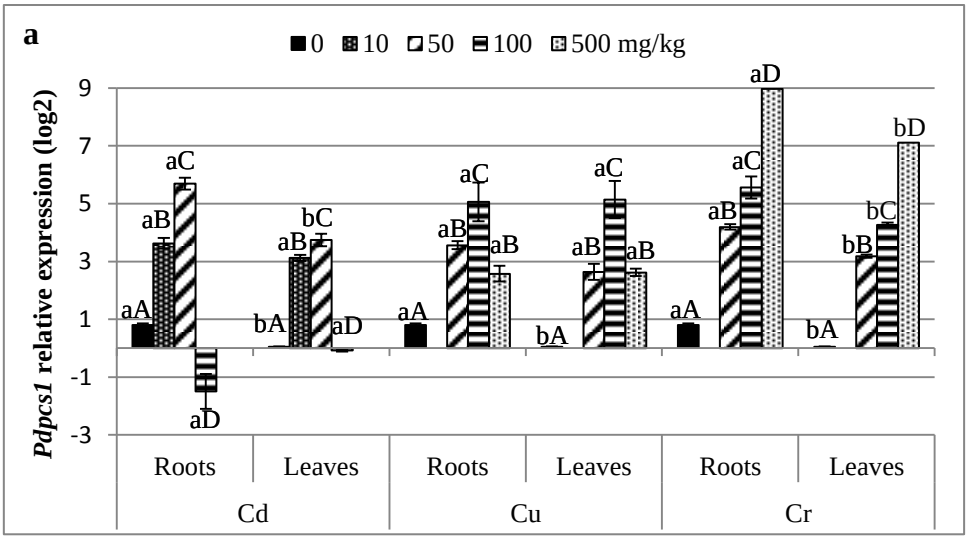
	500	429.1aD (20.2)	464.3aC (15.4)	79.5aB (7.5)	88.1aC (0.8)	2.3aBC (0.2)	2.8bC (0.1)	11.9aC (1.4)	14.1aC (0.1)	829.6aB (5.5)	776.0bC (11.)	100.8aA (12.8)	110.2aAB (7.68)	223.7aA (8.2)	94.5bA (12.9)	87aA (4.3)	119.7bD (6.2)
Two way ANOVA main effects																
Tissues		0.000*		0.000*		0.000*		0.000*		0.000*		0.000*		0.613		0.000*	
Concentration		0.015*		0.000*		0.340*		0.001*		0.000*		0.990		0.539		0.004*	
Tissues x concentration		0.352		0.175		0.780		0.453		0.350		0.363		0.648		0.034*	
Cr (mg/kg)	0	156.1aA (4.4)	244.6bA (5.8)	44.9aA (2.9)	56.5bA (4.0)	1.6aA (0.0)	1.7aA (0.2)	8.7aA (0.2)	9.9bA (0.6)	444.1aA (6.2)	550.1bA (28.7)	100aA (0.00)	100aA (0.00)	100aA (0.00)	100aA (0.00)	100aA (0.00)	100aA (0.00)
	50	183.9aA (18.1)	268.5bA (16.0)	45.1aA (1.9)	59.0bA (1.9)	2.1aAB (0.0)	1.8aAB (0.1)	11.0aB (0.1)	11.4bB (0.1)	503.5aB (7.6)	576.0bA (9.7)	120.2aB (1.2)	152.6bB (2.6)	152.5aB (3.6)	130.7bB (1.3)	169.6aB (3.5)	153.0bB (5.9)
	100	317.7aB (3.2)	347.9aB (32.7)	60.6aB (7.5)	72.6aB (2.4)	2.4aBC (0.3)	2.2aBC (0.1)	12.6aC (0.3)	12.2aB (0.2)	617.1aC (26.8)	684.5bB (11.1)	160.6aC (6.7)	217.8bC (0.4)	272.8aC (8.3)	210.2bC (8.9)	127.0aC (3.5)	181.3bC (6.2)
	500	589.4aC (11.7)	520.9bC (27.6)	83.0aC (2.7)	87.1aC (0.8)	2.7aC (0.2)	2.3aC (0.1)	14.5aD (0.4)	14.5aC (0.3)	730.7aD (11.6)	787bC (16.6)	130.1aD (1.0)	113.6bD (9.4)	199.00aA (4.1)	151.6bA (1.5)	103.5aA (3.3)	112.4aA (6.7)
Two way ANOVA main effects																	
Tissues		0.000*		0.000*		0.000*		0.000*		0.000*		0.000*		0.000*		0.000*	
Concentration		0.229		0.005*		0.065		0.154		0.001*		0.342		0.519		0.177	
Tissues x concentration		0.022*		0.440		0.219		0.149		0.640		0.029*		0.900		0.470	

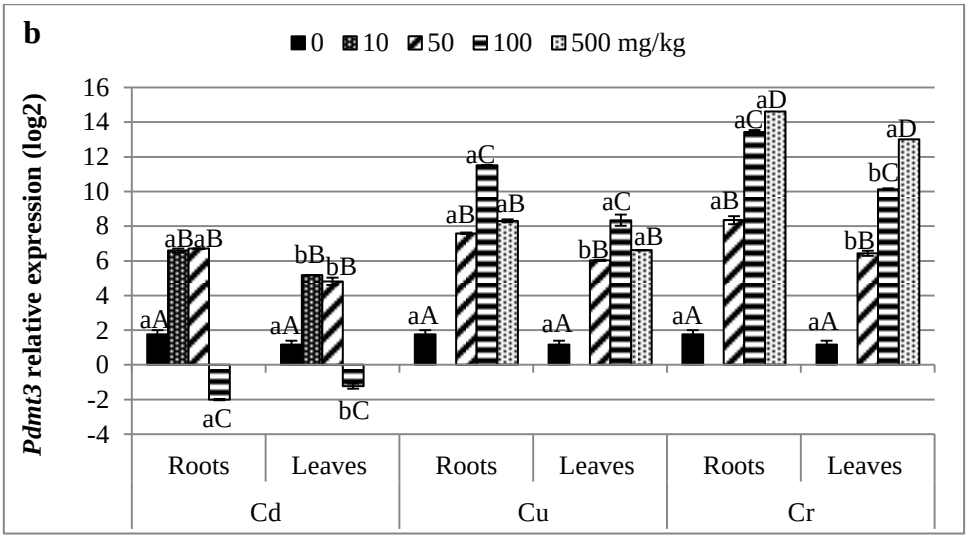
3.4. Profiling of heavy metal related gene transcripts in date palm

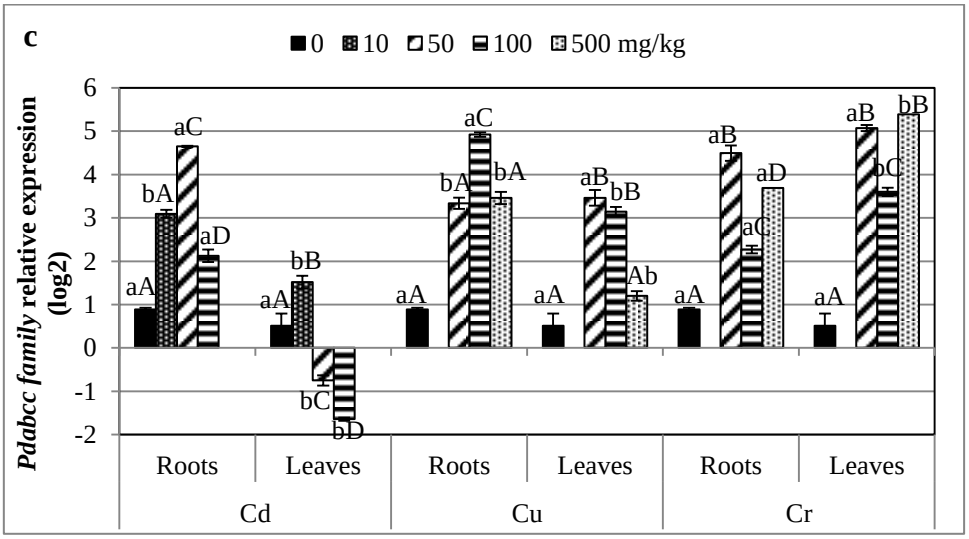
Quantitative real-time PCR (qPCR) was performed on six genes; *Pdpcs1*, *Pdmt3*, *Pdabcc family*, *Pdhma2*, *PdNramp6* and *Pdmate5*; in roots and leaves of date palm young plantlets stressed during two months with Cd, Cu and Cr. The expression level of genes did not significantly differ between tissues of non-treated plants except for *Pdpcs1* and *Pdmate5*, which were predominantly expressed in roots (Fig. II-25). However, both metal concentration and tissue types were in correlation and influenced the expression of all tested genes ($p < 0.05$; Fig. II-25). Among them, the highest expression level (14.61) was observed for *Pdmt3* in leaves exposed to high level of Cr (Fig. II-25b). Whereas, the highest transcription factors of more than 80, 110 and 150 folds (data not shown) were obtained for *Pdpcs1* in leaves of 50 mg/kg Cd, 100 mg/kg Cu and 500 mg/kg Cr exposure respectively. Thus, *Pdpcs1* transcript was found most strongly expressed at low amount of Cd, before it depressed at 100 mg/kg Cd. Gene down-regulation was also observed in *Pdmt3* and *Pdmate5* in roots and leaves of high Cd-exposed plants (Fig. II-25b and f). Only Cd exposure depressed some genes expression. We did not shown gene down-regulation in date palm exposed to Cu and Cr. On the other hand, *Pdhma2* was overexpressed in roots and maintain significantly positive gene expression even at high Cd stress (Fig. II-25d). This latter gene was more induced by Cr^{VI} ions. Its expression increased with increasing Cu-stress in contrast to the other tested genes which decreased at 500 mg/kg Cu (Fig. II-25). As flavonoid transporters, *Pdmate5* gene expression was in correlation with flavonoid accumulation in date palm cells. Gene expression patterns of MATE5 significantly varied between tissues (Fig. II-25f). It was down-regulated at high Cd concentration especially in leaves part of the plant. However, gene expression pattern was induced in Cr-treated leaves by almost 4 folds. The *Nramp* metal transporter gene expression has been monitored (Fig. II-25e). In the present study, the Cd^{2+}, Cu^{2+} and Cr^{6+} ions induced *PdNramp6* expression mainly in plant roots. Chromium was the best *PdNramp6* expression inducer. Based on this finding, we suggest a novel gene expressed in plants in response to metal stress; *PdNramp6.*

4. Discussion

Looking at metal amount in tested samples from Sfax field, especially Cd accumulation in soils and metal content in leaves samples of date palm plants near phosphogypsum piles, we have evidenced tested area is highly contaminated by HMs.







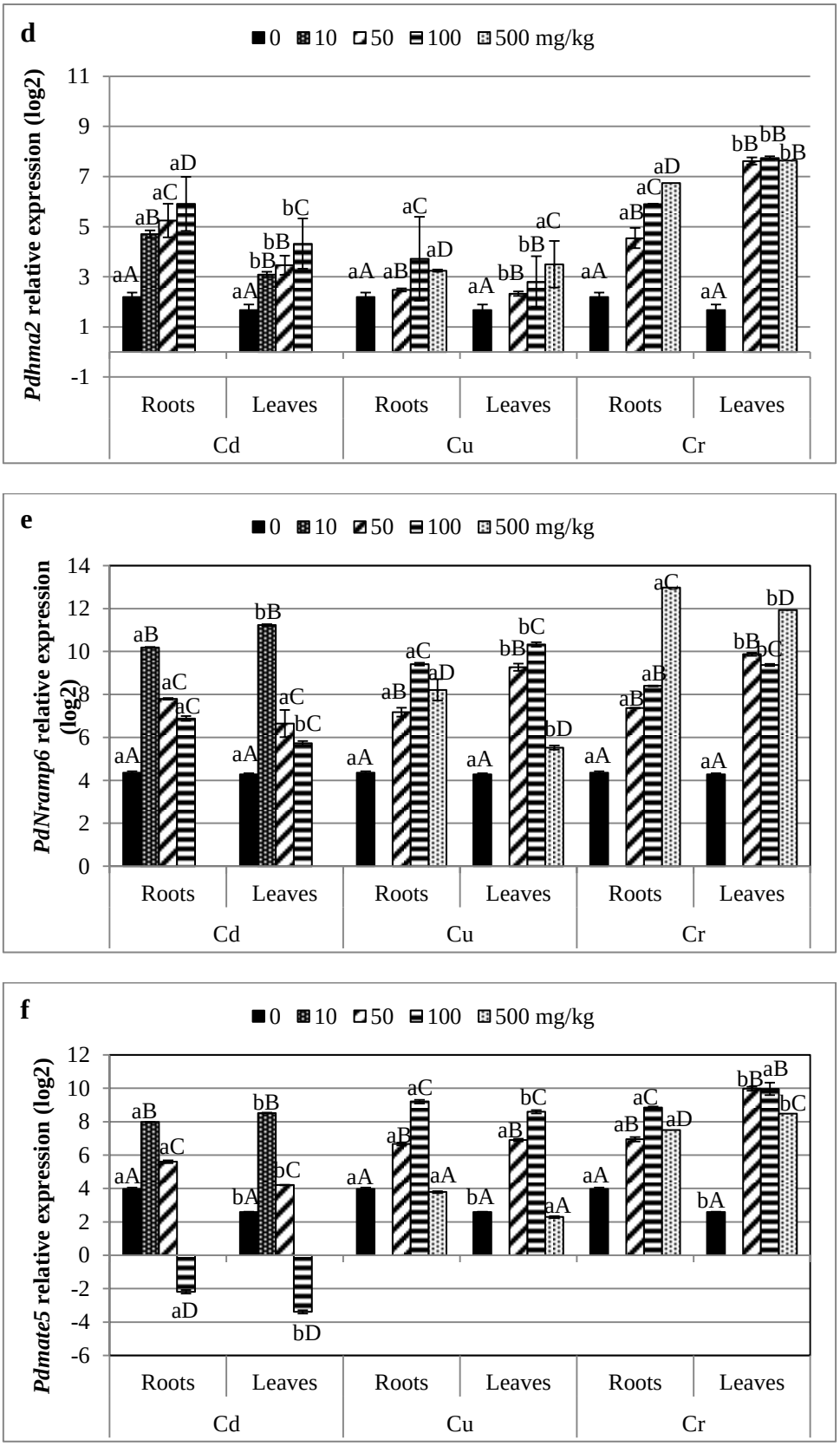


Figure II-25. Transcription factor of *PdPCS1* (a), *PdMT3* (b), *PdABCC* (c), *PdHMA2* (d), *PdNramp6* (e) and *PdMATE5* (f) exposed to Cd, Cu and Cr. Data presented are means ± standard error of three independent experiments. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test; where small letters show differences between tissues and major letters show differences between metal concentrations. Means not showing the same letter are statically different.

Considered as the most industrial city of Tunisia, the Southern edge of the Sfax city suffered from long time ago by particles emission and uncontrolled waste storage like PG piles of SIAPE factory. It was found that the latter released around 4.5 t/day of particulate matter with high amounts of sulphate, phosphorous compounds and HMs largely exceeding the permissible emission standards (Bahloul et al., 2015). Arid climate of Sfax and low annual precipitations caused the concentration of metals mainly in the atmosphere. Metal particle in the atmosphere may be directly retained by plant leaves or absorbed from soils.

Thus, to further investigate plants' potential to uptake and transport metals, *ex-situ* assays of date palm growth in soil doped with increasing concentrations of Cd, Cu and Cr were performed. Results shown in Table II-15 presented the great capacities of the plant to accumulate Cd. Bioaccumulation factor of Cd ranged from 1.07 to 1.72, suggesting important bioaccumulation of Cd from soil especially in soils with relatively low Cd amounts. Plant ability to absorb metal may be related to its great translocation factor linked to Cd (Table II-15). However, the abundance of one metal can disturb the uptake of others as metals compete with each other. Cd can disturb Mn uptake by the plant (Table II-16). Similarly, (Dong et al., 2006) indicated significantly negative correlation between Cd and Mn uptake of tomato plants exposed to Cd-stress. Such antagonism may restraint of leaf photosynthesis and plant growth (Nazar et al., 2012). Chromium induced stress enhanced Mn accumulation in roots of the plant, while it decreased it in the leaves (Table II-16). In fact, previous reports showed that mobilization of micronutrients, especially Mn, into the rhizosphere is due to its acidification and complexation with the organic acids (citrate) in various plant species (Millaleo et al., 2010). Yet, citric acid with its strong affinity to form complexes with heavy metals, reduce the mobility of metals to the shoots. Thus, this correlation between plant ions uptake induce concentration of metal ions in roots. On the other hand, efficient uptake of Cu^{2+} is essential for plant life. The obtained values of BCF of copper in roots of date palm at low metal amount (1.37) as well as those of TF (0.9) suggested that plants needs to accumulate and stabilize metal within its compartment. However, increasing copper stress reduce metal absorption and translocation especially at 500 mg/kg Cu. Date palm regulated intracellular copper levels by regulating copper uptake in harsh copper conditions.

The ability of organisms to adapt metabolically to metal stress in the environment has taken the attention of researchers for years (Sreekanth, 2010) (Tangahu et al., 2011). H_2O_2

accumulation in plant is a key regulator in a numerous physiological processes (Bright et al., 2006). It is characterized by a relatively long half-life (1 ms) and an ability to penetrate the plasma membrane as an uncharged stable molecule (Wang et al., 2006). Because of its photoactive nature, bio-energetic lifestyle and the abundance of polyunsaturated fatty acids (PUFA) in the chloroplast envelope, chloroplasts naturally form ROS during photosynthesis which occur at photosystem I and II (PS I and PS II) (Gill and Tuteja, 2010). The risk of oxidative damage naturally exists in plant cells where oxidant accumulation are more important in green cells (Gill and Tuteja, 2010). Thus, in non-treated leaves, H_2O_2 level was 1.5 time more important than in roots of date palm plantlets (Table II-17).

However, H_2O_2 production by cells is considered as comrade and rival of plant tolerance to stress. With no ability to produce ROS directly, the mechanism of Cd-induced oxidative stress is different from other form of stress (Hasanuzzaman et al., 2017). Cd-exposition of date palm plantlets caused accumulation of oxidant. In fact, H_2O_2 amount in roots of young plants grown in 100 mg/kg of Cd increased more than 4 times (Table II-17) as a response to the high concentration of metal in roots of more than 45 mg/kg DW (Table II-17). This may due to the fact that Cd decrease protective enzymatic defense mechanism, especially SOD activity, which decreased significantly with increasing Cd stress in roots and leaves parts of date palm (Table II-17). The reduction of SOD activity may be due to the inverse relationship between the H_2O_2-accumulation and enzyme production. This may lead to inactivation of SOD enzyme by H_2O_2 molecules. Furthermore, Romero-Puertas et al. (2002) explained the overproduction of H_2O_2 and O_2 under Cd stress by the fact that metal decrease in Calcium (Ca) result in Cu/Zn SOD down-regulation. Cu/Zn SOD enzyme dismutate $O^{-2}\bullet$ to H_2O_2. Furthermore, it has been found that Cd treatment raised the protein carbonylation level in pea plants as a consequence of oxidative damage (Rodriguez-Serrano et al., 2009). It also may displaces iron (Fe) from proteins and increases free Fe that is responsible for ROS generation (Hasanuzzaman et al., 2017).

However, with a positive correlation between tissues and metal concentration ($p < 0.05$), APX activity enhanced parallel to H_2O_2 accumulation in cells (Table II-17). In fact, various environmental stimuli, such as H_2O_2 can modulate the expression of APX encoding genes (Rosa et al., 2010). Similarly, coffee cells increased APX activity at the lower Cd concentration (Gomes-junior et al., 2006). While, in pea plants no significant difference in enzyme activity under Cd stress has been shown (Romero-Puertas et al., 2002). At high metal

amount, APX activity dropped in roots which may be due to glutathione (GSH) depletion and a subsequent reduction in the ascorbate–glutathione cycle (Gomes-junior et al., 2006). In addition, the GSH reduction could be caused by phytochelatin (PCs) synthesis induction caused by Cd^{2+} ions. Indeed, in the present research, *Pdpcs1* gene encoding PCs synthesizer enzyme; phytochelatin synthase PdPCS1, showed the highest induction in roots treated with 50 mg/kg Cd stress (Fig. II-25) were APX activity started to be reduced (Table II-17). At high Cd stress, cells suffered toxicity decreasing ROS scavenging enzymes (CAT, APX and SOD). Furthermore, as an indicator of free-radical formation, date palm Cd-stressed plants produced TBARS, indices of lipid peroxidation. Increasing TBARS accumulation in accordance to increasing Cd accumulation and H_2O_2 in tissues confirmed the oxidative state of Cd-treated cells. Moreover, phenolic content of date palm cells was significantly affected by high Cd concentration (Table II-17). Indeed, phenolic compounds accumulation in cells with their antioxidant properties represents a major key factor of inducing defense mechanisms of plants through the phenylpropanoid pathway (Ramakrishna and Ravishankar, 2011). Their syntheses in *P. dactylifera* were initiated very quickly from the lowest metal amount. Significantly, higher accumulation of non-enzymatic oxidant scavengers was noted in leaves of date palm treated with Cd except for flavonoid content which showed the highest induction in leaves of plants treated with 50 mg/kg Cd (Table II-17). Flavonoids are known to be implicated to provide protection to plants growing in soils containing toxic metals amount (Winkel-shirley, 2001). It is oxidized by peroxidase, and it act especially, in H_2O_2-scavenging (Sharma et al., 2012).

On the other hand, copper is a transitional element and it is known to be more effective causing oxidative damage in cells (Cho et al., 2005). Despite of its great accumulation in plant tissues, it was found that Cu was less active than Cd as a producer of oxidative stressor viz. hydrogen peroxide. The fact that, Cu^{2+} ions are used as cofactors by numerous proteins and it is also required by the ethylene receptor for proper signaling, may explain in part this oxidative state of cells. Yet, SOD activity increased gradually, when metal concentration increased and reached more than 40 % and 20 % of control at 500 mg/kg Cu in roots and leaves respectively (Table II-17). Cu ions are required in the chloroplast as cofactors for SOD producing Cu/Zn SODs under control by microRNAs (Abdel-ghany and Pilon, 2008). The latter are regulated by the transcription factor SPL7 (squamosa promoter binding protein like) showing highly *spl7* expression in the Cd and Cu response in Arabidopsis (Gielen et al.,

2016). Contrarily, a slight enhancement of CAT and APX activity with no significant differences between 100 and 500 mg/kg Cu concentration suggests, that produced protein amounts are not sufficient to decompose the excess of the H_2O_2 radicals. Similarly to the behavior of *Moso bamboo* a Cu-hyperaccumulator plant, under cupric stress (Chen et al., 2015a), the results indicated that *P. dactylifera* can induce the activity of essential component of plant enzymatic antioxidant system, to avoid oxidative damage induced by Cu toxicity. Though, high Cu stress has caused overproduction of ROS caused oxidative burst, decreased enzymes activity and damaged biomolecules like membrane lipids (Table II-17).

Plantlets incubated in 100 mg/kg of Cu secreted polyphenols, flavonoid and tannins as additional mechanisms of self-protection to reduce toxic effect of Cu^{2+} providing stress tolerance (Table II-17). Polyphenols, are known to be involved in ROS scavenging due to their chemical structure which also help to bind with HMs especially Fe and Cu (Yadav et al., 2016). Their enhancement under Cu stress might be due to shikimate dehydrogenase overproduction. The enzyme is responsible for production of Shikimate acid, a key precursor for the synthesis of alkaloid and other secondary metabolism compounds (Yadav et al., 2016). In addition, flavonoids are shown to be produced in oxidative stress conditions, where transition metals ions like Fe and Cu are involved. With their chelating properties and their ability to locate and neutralize ROS from cells, flavonoids have higher reducing capacity for Cu ions and act as ROS scavengers (Mira et al., 2002). Similar increase in total flavonoids content was shown in the medicinal plant *Orthosiphon stamineus* exposed Cu stress (Mamat et al., 2015).

As being a strong oxidizer and highly mobile, Cr in its hexavalent form may cause severe phytotoxic effect. It enhanced H_2O_2 and TBARS level in cells (Table II-17). Cr^{VI} is catalytically more active than Cd and less active than Cu, and is able to generate ROS via Fenton reaction (Ahemad, 2015). However, to date, its participation in such reaction is not clearly understood. As shown by (Ahemad, 2015) Cr^{VI} toxicity related to its easy diffusion through the membrane cells, producing free radicals. The relationship shown between Cr^{VI} accumulation and oxidant generation can be understood as a negative influence of increasing metal accumulation on redox balance. The increase in H_2O_2 may be interpreted as a consequence of the enhancement of SOD activity which is in correlation with the respective Cr-accumulation in plant (Table II-15). In fact, Fe-SOD was found to be the predominant form of Cr-induced SOD in stressed plants (Chatterjee et al., 2015). However, excess Cr has

been shown to interact with essential nutrient like Mn and Zn (Table II-16) and especially Fe (Dube et al., 2003). It decreased the uptake and translocation of iron ions, decreasing in Fe-SOD enzyme induction. While, unlike in Cd and Cu-treated tissues, APX continued to overproduce throughout Cr concentrations and especially at the highest metal level (Table II-17). This Cr plant response specificity defended the potential of APX to gain oxidative damage. Similar founding have been reported in other plant species (Shanker and Pathmanabhan, 2004 ; Chatterjee et al., 2015) suggesting that APX might have provide sufficient antioxidant defense against H_2O_2 generation. However, in rice treated roots by Cr^{VI}, numerous genes encoding peroxidase enzymes family were down-regulated (Dubey et al., 2010a). Cr salt also induced the biosynthesis of secondary metabolites especially polyphenol and flavonoids. The highest induction was accrued in roots for 100 mg/kg Cr (Table II-17). (Dubey et al., 2010a) reported that the most upregulated genes are related to biosynthesis of secondary metabolites especially flavonoid biosynthesis. Similar founding has been shown in *Brassica juncea* exposed to Cr stress (Singh and Sarita, 2005).

These plant biochemical data including integrative biomarkers for HMs stress were further supplemented by more sensitive intra-individual responses. We monitored expression of six different specific genes in response to Cd, Cu and Cr stress in date palm. Specificity of plant HMs chelators and transporters may be achieved by differential regulation at transcriptional and post-transcriptional. Only Cd at high concentration repressed some gene expression (Fig 2). It negatively influenced the expression of *Pdpcs1*, *Pdabcc* family and *Pdmate5*. However, the higher *Pdpcs1* transcription factor of more than 80 occurred in leaves of 50 mg/kg Cd inducing the biosynthesis of phytochelatin synthase enzyme (PCS). (Cobbett and Goldsbrough, 2002b) showed that PCS is constitutively expressed and post-translationally activated, especially by Cd^{2+}. In addition, hypocotyles of date palm exposed to different Cd concentrations showed that increasing metal amount adversely affect *Pdpcs* expression (Chaâbene et al., 2017). Simultaneously, non-significant *Pdpcs* down-regulation was shown after long exposure period on high Cr^{VI} stress (Chaâbene et al., 2017). In different plant tissues, increasing chromium concentration increased gene expression and the highest induction factor of *Pdpcs1* was observed at high amount (500 mg/kg Cr) ,especially in date palm leaves (Fig. II-25a). Under the same concentration, copper reduced gene transcription factor by half (Fig. II-25a). The synthesis of the small metal binding peptides; phytochelatins (PCs) under control of PCS play a role in cellular homeostasis and trafficking of essential

nutriment particularly Cu and Zn. There are also required for detoxification of toxic HMs, as Cd. PCs bind HM ions to form high-molecular-weight (HMW) to be transported into the vacuoles across the tonoplast by the ABCC type transporters. This intracellular transport is important to ensure sufficient levels to the necessary compartments while safely storing metals under times of excess. A correlation between *Pdpcs1* and *Pdabcc-type* expression was deduced in Figure II-25 a and c and calculated by Statistica Pairwise Correlation coefficient (*r*). It was found that at 10 and 50 mg/kg Cd, *r* was 0.954 and 0.990 respectively. Furthermore, our results suggested that Cu and Cr ions were more likely to be uptaken and translocated into vacuoles than Cd ions, which is in accordance to metal concentrations in plant tissues (Table II-15) and may explain in part plant defense mechanisms developed to support excess metal. Furthermore, chromium plant resistance is explained by the overexpression of *MT3* (Fig. II-25b). MT3 phytochelatins are some-what confusingly, described as PCs. Indeed, few researches investigated genes involvement in plant HMs detoxification. In sorghum Cr^{VI} tolerant variety, a higher transcription rates of the MT3 has been obtained (Shanker et al., 2004). Others suggested that ROS and H_2O_2 produced under Cr stress acted as a signal to induce MT mRNA transcription. This is in accordance to our results which showed that H_2O_2 level in date palm treated with Cr and Cu, increased by increasing metal amount (Table II-17) where *Pdmt3* was also more responsive under Cu stress than under Cd stress suggesting that gene could be specifically involved in Cu detoxification similarly to other species (Ramesh et al., 2009). Also, MTs participate in controlling the concentration of ROS that would activate defenses, e.g. via the mitogen-activated protein kinase MAPK cascade (Finatto et al., 2015). The complex HM-MT is then translocated into vacuole by ABCC transporters. However, the P-type ATPases proteins transport a variety of cations across cell membranes. The HM-transporting (HMA) subfamily of P_{1B}-ATPase containe different genes. HMAs involved in HMs uptake are known to be more selective than the other transporter proteins. HMAs gene belongs to the CDF (cation diffusion facilitator) protein family and is involved in Zn and Cd transport. Interest was focused on *hma2*, which respond differently to different metal stress (Fig. II-25d). In natural conditions, the expression of *Pdhma2* was significantly ($p < 0.05$) similar in the roots and shoots accordingly to wheat plants (Tan et al., 2013). It increased significantly with increasing metal stress, even at high metal amount. *Pdhma2* transcription factor reached 2.6 and 2.5 in roots and leaves of 100 mg/kg Cd-treated plants respectively. Studies in Arabidopsis showed the involvement of *Athma2* and *Athma4* in Zn/Cd translocation through xylem loading, as they act as pumps to

efflux Zn/Cd out of cells suggesting a putative role for HMA2 in long-distance transportation. Yet, *Pdhma2* transcription factor was much higher under chromium conditions and it was induced since 50 mg/kg Cr. However, no significant effect of chromium concentrations on gene expression has been shown in plant leaves compared to root tissues (Fig. II-25d). The gene maintained high expression level during metal presence in tissues. This founding suggested high *hma2* sensitivity to Cr in date palm. In literature, no involvement of HMA2 in chromium uptake has been found yet. However, HMA5 encoding gene was upregulated by Cr^{VI} ions and it was indispensably implicated in the regulatory network responsive to Cr stress in radish (Liu et al., 2015). Similarly, Cr induced *Nramp6* expression by almost 3 folds in date palm roots and leaves (Fig 2e). The *Nramp6* was more expressed in regulating iron homeostasis and metal transport. The target gene expression enhancement under chromium stress may be explained by the fact that the forms of Cr^{VI} are reduced by Fe^{2+} uptake (Finatto et al., 2015). It was also induced in Cd stressed-plantlets, especially at low metal amount. Member 6 of Nramp family encoding gene is not much studied before. Yet, in rice, *Nramp1* was found to be highly expressed in roots and shoots of treated plants with Cd contrarily to *Nramp5* (Ishimaru et al., 2012). In addition, transporter gene belongs to the family of citrate transporters (Multi antimicrobial extrusion protein MATE family protein); *Pdmate5* expression responded to metal stress. MATE transporters are localized in both, vacuoles and the plasma membrane, and are involved in a wide range of biological processes in plants, such as transport of secondary metabolites especially flavonoids, alkaloids and anthocyanidin, detoxification of heavy metals, iron translocation and aluminum (Al) detoxification and efflux of plant hormones such as abscisic acid (Liu et al., 2016). In our study, *Pdmate5* transcription enhanced under stress except at high Cd level (Fig. II-25f). There is in correlation to flavonoids comportment under Cd stress. Similarly, MATE was found to be upregulated under chromium stress in rice as a transporter of flavonoids (Dubey et al., 2010a). Because flavonoid composition is different in each plant species, the transport activity of MATE transporters could be different. However, in rice, MATE were proved to be involved in copper resistance by secreting Cu-conjugates as secreting Alcitrate in response to aluminium stress (Ritter et al., 2014b). MATE were also up regulated under copper stress in grapevine (Leng et al., 2015).

5. Conclusions

Face to atmospheric emission of industrial manufactories of Sfax region, plants around

phosphate fertilizer factory suffered from the stress. Heavy metals emitted in the atmosphere constitute a dangerous source of contamination of ecosystem. Focusing on date palm as perennial plant that has been exposed to contamination for a long time, Cd, Cu and Cr levels in plant leaves exceed harmfully the permissible limits of WHO (1996). Nevertheless, plant continues to survive in such environment. Moreover, the investigation for strategies for detoxification of heavy metals in the date palm made it possible to identify integrative biomarkers for each metal tested. Stressed cells have shown accumulation of H_2O_2 and TBARS mainly in roots of Cd-exposed plantlets. To avoid its detrimental effects, CAT and APX activities of Cd and Cr-stressed cells enhanced. However, APX activity was more induced in response to excess copper. More specific HMs plant responses showed that Cd, Cu and Cr stimulate plant differently. Genes' induction regulatory networks play crucial roles in copper homeostasis and Cd and Cr detoxification. Cadmium stress induced *Pdpcs1* more than *Pdmt3* gene. It enhanced the expression of genes encoding PdHMA2 and PdNramp6 to translocate metal to leaves part of the plant. Thus, TF of Cd-plantlets was greater than one. In addition, under Cd stress *mate5* expression was induced in roots. MATE may mobilize secondary metabolites like flavonoid and citrate efflux to stressed cells. Thus, it may reduce Mn absorption and translocation. Transcriptional plant behavior to copper stress showed marked increase in the expression of *Pdpcs1, Pdhma2* and *PdNramp6*. However, chromium stress further induced *Pdmt3* expression. The identification of the mechanisms underlying metal accumulation in date palm can provide the genetic tools for engineering more efficient plants for phytoremediation

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Troisième Partie

DISCUSSION GENERALE

Les palmiers domestiques présentent une large gamme de phénotypes qui différent par la couleur des fruits, les teneurs en sucre, le temps de floraison et autres traits agronomiques. Le Maghreb oriental est le seul producteur de la variété Deglet Nour qui est la plus cultivée en Tunisie, le plus souvent en monoculture monovariétale. L'espèce se trouve dans les écosystèmes oasiens surtout à Nefzaoua, Djerid, Gafsa et Gabes. Des parcelles de palmeraies peuvent se retrouver auprès des industries génératrices de pollution dans le Sud du pays. La zone côtière Sud du Grand Sfax a souffert depuis longtemps des émissions atmosphériques et des rejets de déchets chargés de contaminants métalliques issus principalement des usines polluantes de la SIAPE. Cette usine émet de grandes quantités d'aérosols contenant des métaux lourds à partir de ses terrils de phosphogypse. Les terrils qui représenteraient plus de 15 millions de tonnes de phosphogypse stockés directement à l'air libre sont une source majeure de contamination métallique vers l'environnement (air, sol et eau ; Zairi and Rouis, 1999). Dans ce contexte, nous avons étudié la biodisponibilité de certains ETMs de **la zone industrielle de Sfax** chez le **palmier dattier cv Deglet Nour**.

Dans le cadre de ce travail de thèse, une première étude de caractérisation des teneurs métalliques au niveau des sols et des palmiers de la zone industrielle de Sfax a été réalisée. Les analyses des éléments métalliques dans les sols des horizons superficiels autours de la SIAPE montrent une légère contamination cadmique. De nombreuses études ont été entreprises dans divers pays du Maghreb sur la contamination par les ETMs des sols, des plantes, des eaux et des sédiments résultant de l'activité minière (Greter and Vedy, 2010). Le degré de contamination métallique des sols est souvent évalué à partir de teneurs totales en ETMs déterminée dans l'horizon superficiel (Baize et al., 2002). Selon Li and Thornton, (2001), il s'agit bien d'une approche valide pour étudier le degré et l'ampleur de la contamination dans le système sol-plante. Cependant, très souvent, en bordure de plateau, suite à l'érosion, les sols sont tronqués partiellement et les horizons profonds deviennent les horizons de surface (avec de plus fortes teneurs en matières organiques) et la connaissance des teneurs des horizons profonds est souvent indispensable pour interpréter celles des horizons labourés. Notons que les racines des plantes puisent des éléments dans tous les horizons et pas seulement dans les horizons superficiels, ceci est particulièrement vrai pour le palmier dattier dont les racines s'enfoncent très profondément. **La biodisponibilité des ETMs** vis-à-vis des plantes peut s'apprécier via le dosage des teneurs métalliques dans les tissus végétaux. Les teneurs des métaux testés (Cd, Cu et Cr) dans les feuilles de palmier

dattier du site dépassent les normes règlementaires (WHO, 1996). Ils révèlent une forte **contamination atmosphérique** de la zone. Cette contamination est peut être absorbée directement par les parties aériennes de la plante mais on peut aussi suggérer une contamination des horizons profonds par infiltration des métaux ou même par contamination de la nappe souterraine.

La mise en évidence des **mécanismes métaboliques** mis en œuvre pour répondre au stress abiotique, ainsi que l'identification des **gènes associés aux voies de détoxication des métaux** constituent des outils de choix pour 1-mieux comprendre les mécanismes mis en œuvre par la plante lors d'un épisode de pollution et 2-identifier une batterie de **biomarqueurs d'expression** potentiellement utilisables en biosurveillance des sols contaminés. Ainsi, l'objectif de notre projet était de faire un bilan des performances morphologiques et physiologiques du palmier dattier cv Deglet Nour lors d'une exposition à des ETMs aux regards des réponses infra-individuelles (transcriptomiques et biochimiques).

Malgré l'importance du palmier dattier dans le grand Maghreb, très peu d'informations sont disponibles concernant le **potentiel génomique** de l'espèce. De nombreuses études transcriptomiques sur la réponse au stress ont été effectuées chez des plantes (Matsui et al., 2008; Zeller et al., 2009) mais peu de choses sont connues sur les réponses au stress métalliques des plantes non-modèles, en particulier *Phoenix dactylifera*, qui a montré un grand potentiel d'accumulation du Cd (Chaâbene et al., 2013). Le recours aux nouvelles méthodes de séquençage de type **RNA-seq** d'une banque soustractive d'ADNc de la variété Deglet Nour a été réalisée. Après classification fonctionnelle et annotation des gènes par GO et KEGG en se référant aux données transcriptomiques d'**Arabidopsis** publiées dans les banques de données, l'analyse de la banque ADNc de l'espèce étudiée a révélé **des différences d'expression des voies métaboliques au cours des réponses transcriptionnelles** lors des stress cadmiques et cuivriques. Pour la **détoxication** des ions métalliques, Deglet Nour active davantage sa machinerie métabolique d'autant plus sous l'effet des ions Cd^{2+} (Fig. II-2). Les fréquences des gènes impliqués dans la production des **composants de la cellule végétale** intervenants surtout dans la **construction cellulaire** sont les plus élevées, atteignant plus de 90 % (Fig. II-2) et 79 % en cas de stress par le Cd et le Cu (Tableau III) respectivement. Toutefois, les gènes codants pour la production de **composants intracellulaires** qui ont été classés deuxième en cas de stress cuivrique avec une fréquence qui dépasse 69 %, n'étaient pas observés en cas de stress cadmique. L'absence d'expression

de ces gènes impliqués dans la construction intracellulaire peut être due à l'absence des études sur ces gènes chez Arabidopsis exposé au stress par le Cd. Également, Magali et al. (2009) ont montré que sous l'effet du Cd, les mécanismes de régulation de la transcription chez les cellules de la cyanobactérie *Synechocystis* tendraient à limiter la charge métabolique représentée par l'expression de certains gènes en les réprimant. En plus, l'analyse de la banque transcriptomique de palmier dattier a prédit la surexpression des gènes intervenants dans la construction des organelles nécessaires pour surmonter le stress métallique.

Tableau III. Classification de l'ontologie génique des transcrits assemblés chez le palmier dattier exposé au stress cuivrique.

Composant Cellulaire	
Cellule	79.23 %
Intracellulaire	69,11%
Partie cellulaire	59.41 %
Organelle	59.41 %
Cytoplasme	48.59 %
Processus Biologiques	
Processus cellulaires	58.83 %
Régulation biologique	24.33 %
Processus métaboliques	17.56 %
Réponse au stimulus	12.43 %
Régulation des processus métaboliques et cellulaires	13.91 %
Réponse aux produits chimiques	12.43 %
Fonction Moléculaire	
Liaison	49.91 %
Transducteur de signal	2.64 %
Transducteur moléculaire	1.53 %

Les gènes impliqués dans des **fonctions moléculaires** incluant ceux qui interviennent dans des **fonctions de liaisons protéiques**, d'ADN, d'ATP ou autres, sont les plus exprimés dans les deux cas de stress. Ainsi, les gènes codants pour les protéines chaperonnent et les protéases sont fortement induites conformément à des études ultérieures réalisées sur *Synechocystis* (Magali et al., 2009). Ils sont suivis des gènes intervenants dans des **activités catalytiques** en cas de Cd alors qu'on cas de Cu, les gènes participants dans la **transduction du signal** ont été classés deuxième avec une fréquence de 2.64 % (Tableau III). La troisième catégorie des gènes étudiés appartient à des **processus biologiques**. En effet, les gènes

codants des fonctions dans des **processus cellulaires** ainsi que ceux intervenants dans la réponse au stimulus sont les plus exprimés (Fig. II-2, tableau III). Ils sont d'autant plus exprimés en cas de stress par le Cd. Cependant, sous l'effet des ions Cu^{2+}, les gènes de **régulation biologique** ont été classés deuxième avec une fréquence de 24.33 % (Tableau III). Ensuite, les gènes qui participent à des **processus métaboliques** avec une fréquence de 17.56 % sont d'autant plus élevés en cas de stress par le Cd. Ils ont résulté la surexpression d'enzymes intervenant dans le **métabolisme du glutathion** tels que L-ascorbate peroxydase (APX), la glutathionne S-transférase (GST), la glutathion peroxydase (GP) et la phytochélatine synthase (PCS). La comparaison du transcriptome de *Phoenix dactylifera* et *Arabidopsis* a permis d'identifier des orthologues potentiels responsables de la réponse aux stress métalliques chez le palmier dattier. En particulier, six gènes codants pour des transporteurs et chélateurs de métaux *(Pdpcs1*, *Pdmt3*, *Pdabcc*, *Pdhma2*, *PdNramp6*, *Pdmate5)*.

Afin de suivre les mécanismes de réponse du palmier dattier cv Deglet Nour, des études *ex-situ* en conditions contrôlées ont été réalisées. L'interprétation des résultats de ce type d'expérimentation (**de biosurveillance**) dépend des informations disponibles concernant l'exposition, la toxicocinétique des substances chimiques étudiées, leur toxicité (les effets attendus) et **les relations dose-effet et dose-réponse**. Afin de limiter les interférences, le suivi des réponses du palmier aux stress métalliques a été fait sur des tissus homogènes de l'espèce Deglet Nour issue de **culture *in vitro*** c'est-à-dire des **vitroplants** mais aussi sur des **hypocotyles** issues de germination des graines et des racines et feuilles de plantules de palmier.

L'intensification, la standardisation et la mécanisation d'une production agricole s'accompagne d'une homogénéisation des variétés. Considérée comme « true-to-type », l'homogénéisation permet d'avoir des variétés réduites à un génotype afin d'atteindre des performances supérieures, si l'on recherche les performances maximales adaptées à milieu donné. Le développement de **variétés homogènes** cumulant des gènes d'adaptation aux stress métalliques étaient un de nos objectifs sachant que l'expression phénotypique d'un génotype dépend des conditions environnementales dans lesquelles se sont développés les individus. Cette interaction entre le génotype et l'environnement est très importante car elle signifie que l'expression d'un gène n'est pas indépendante du milieu dans lequel ce gène s'exprime. Le suivi de l'expression de *Pdpcs1*, *Pdmt3*, *Pdabcc*, *Pdhma2*, *PdNramp6*, *Pdmate5* dans des

explants exposés à différentes concentrations de $CdCl_2$, $CuSO_4$ ou K_2CrO_4 a été réalisé par la méthode **PCR quantitative**. La PCR en temps réel est une méthode puissante mais délicate car afin d'établir des contrôles appropriés pour la mesure de l'expression de gènes, il est indispensable de disposer de gènes de références validés. Six gènes de références identifiés chez le palmier dattier cv Deglet Nour (*Pdefα1*, *Pdaldh1*, *Pdact*, *Pdsand*, *Pdtub6* et *Pdtbp1),* ont donc été testés en conditions de stress cadmique, cuivrique ou par le chrome. Ces métaux lourds sont connus par leurs effets néfastes sur l'expression des gènes de références dans de nombreux modèles végétaux (Lee et al., 2013; Morgante et al., 2011; Teng et al., 2012). A chaque fois, les gènes de référence adéquats ont été validés et utilisés pour obtenir une mesure fiable des niveaux **d'expressions des gènes** candidats. L'analyse des **profils d'expression génique** pour chaque condition de stress a montré des différences de comportement entre gènes, type de métal et temps d'exposition. Tous les gènes candidats étaient **sensibles** et ont répondu aux différents types de stress étudiés. *Pdpcs* était le plus exprimé surtout en cas de stress par le Cd ou le Cr. *Pdmt* a montré une sensibilité intéressante aux ions cuivriques qui stimule également l'expression de *Pdhma2*. L'introduction des ions Cr^{6+} dans les cellules juvéniles de palmier dattier ont induit l'expression des *Pdabcc* et *Pdmate5*. Les gènes codants pour des **transporteurs inter et intracellulaires** comme *Pdabcc*, *Pdmate5* et *PdNramp6* répondent le plus au stress cadmique. Toutefois, des baisses d'expression géniques (répressions) ont été observées à fortes concentrations en Cd (1 mM) lors d'expositions longues (> à 40 jours). Ces baisses peuvent être rapprochées de la différence de développement des explants de palmier dattier qui peut être due aussi à l'accumulation de proline et de sucres solubles totaux dans les cellules (Hassine and Bouzid, 2008). Les concentrations de cuivre et de chrome utilisées ont maintenu un niveau d'expression élevé pour les gènes codants des **chélateurs et des transporteurs de métaux lourds**, surtout à faibles doses. Toutefois, malgré le fait que les cultures soient exemptes de pathogènes, les cultures d'explant en milieu riche en Cu (0.2 et 2 mM) ou Cr (2 mM) à long terme ont montré une **contamination endophytique**. Les microorganismes développés présentent des aspects différents. Chaque type de stress a exprimé un endophyte particulier. Les communautés microbiennes endophytes jouent un rôle significatif dans la croissance et le développement des plantes hôtes en milieu optimal ou stressé. Elles jouent un rôle important dans la croissance des plantes et l'absorption de métaux tel que le Cu et le Cr.

L'**homogénéité** d'un peuplement végétal présente cependant certains risques. Le premier

risque est la plus grande fragilité aux changements comparé à un peuplement génétiquement hétérogène comprenant des génotypes adaptés à différentes conditions. L'**hétérogénéité** génétique d'un peuplement peut donc être une solution pour gagner en stabilité, mais qui fait perdre en rendement. Il est donc envisageable d'étudier les effets du stress abiotique sur des hybrides simples de la variété Deglet Nour, en l'occurrence des graines de datte de Deglet Nour. De plus, à cause de plusieurs défis de la biotechnologie du palmier dattier, le temps de génération long, le risque de contamination des cultures *in vitro*, l'incapacité de distinguer les pieds mâle et femelle à un stade précoce, etc., les investigations faites sur les graines nous ont aidées à surmonter ces empêchements.

L'application de stress métallique sur la germination des graines de palmier dattier cv Deglet Nour a montré que les mécanismes oxydants (H_2O_2, TBARS) et antioxydants (CAT, SOD) augmentent linéairement avec l'augmentation de la concentration métallique et du temps d'exposition. **La fluctuation de l'équilibre redox** à des concentrations élevées de Cd et du Cu essentiellement, a perturbé le système de défense causant des **dommages oxydatifs**, surtout après une longue exposition. À ce niveau, des ajustements appropriés de l'expression de gènes codants pour des chélateurs métalliques *Pdpcs1* et *Pdmt3* ont été observés. L'augmentation du niveau d'expression de ces gènes apparaît également comme une ligne de défense supplémentaire contre l'introduction des xénobiotiques en excès et qui est impliquée dans l'homéostasie des métaux et reflète la capacité des graines à germer. Les expressions géniques de *Pdpcs* et *Pdmt* se sont globalement révélées **temps et doses-dépendantes**. Conformément aux travaux antérieurs de Zhang et al. (2005) effectués sur l'ail, *Allium sativum*, ces gènes codants PdPCS et PdMT sont contrôlés au niveau du transcriptome. La baisse des niveaux d'expression à fortes concentrations est à corréler avec les nécroses et surtout un blocage de l'élongation des hypocotyles observés. Toutefois, les conditions d'expérimentations, le stade d'étude (germe/plantule) et la variabilité génétique de la population de palmier dattier cv Deglet Nour a engendré des similarités mais aussi des différences (discordances) de résultats tant biochimiques que moléculaires. Par exemple, l'évolution des teneurs en oxydants (H_2O_2, TBARS) qui augmentent parallèlement à l'augmentation du stress appliqué et du temps d'exposition a été la même entre les hypocotyles et les plantules (issues de germes de palmier dattier cv Deglet Nour) exposés au stress cuivrique. En revanche, les activités enzymatiques de la CAT et de la SOD qui étaient élevées à forte exposition des plantes au Cu, ont diminué dès le premier mois de stress par 2

mM Cu au stade de germination. De plus, l'expression du gène *Pdmt3* qui était fortement **sous-exprimé** à fortes teneur en Cu^{2+}, a présenté une légère diminution mais qui demeure supérieure au contrôle après 4 mois de stress par 500 mg/kg de Cu chez les plantules. Même la chute dramatique de l'expression de *Pdpcs* observée au cours de la germination des graines n'a pas été observée au stade de croissance des plantules. Les différences expérimentales n'ont pas eu de conséquences sur l'expression des gènes codants pour des chélateurs en conditions de stress cadmique. En revanche, l'augmentation des ions Cr^{6+} dans les cellules végétales a augmenté l'expression de *Pdpcs1* à la fois dans les racines et les feuilles de palmier dattier contrairement aux hypocotyles. D'autre part, en culture *in vitro,* les explants de Deglet Nour ont présenté une meilleure induction de certains gènes par les ions Cd^{2+} contrairement à la population hybride en particulier pour le gène *Pdpcs1*. La diminution observée après une forte exposition au Cd de l'expression de *Pdhma2* et *PdNramp6* n'était pas retrouvée chez les plantules.

Le profil d'expression génique et biochimique présenté par le palmier dattier exposé à de faibles concentrations de Cd lui confère un potentiel d'accumulation métallique important et de bonnes capacités de réponse. Dans nos conditions expérimentales, les facteurs de bioconcentration (BCF) et de translocation (TF) racines/parties aériennes étaient >1. Ces résultats ont également mis en évidence une plus grande capacité d'accumulation du Cd comparés au Cu et au Cr dont les facteurs de translocation étaient de 0.9. Les résultats montrent un transfert plus au moins important des métaux du sol vers les racines et moins important vers les feuilles. Ces données confèrent au palmier dattier un caractère «**phytostabilisateur** » pour le Cu et le Cr et un caractère « **phytoéxtracteur** » pour le Cd. Toutefois, plus les concentrations métalliques du sol sont importantes, plus le transfert des métaux vers les racines des plantes diminuent.

Quatrième Partie

CONCLUSIONS ET PERSPECTIVES

1- L'utilisation de nouvelles approches et méthodologies pour la construction d'une banque soustractive d'ADNc de palmier dattier cv Deglet Nour et les investigations exhaustives *in silico* sur les bases moléculaires de la réponse au stress métallique (Cd, Cu, Cr) ont généré des informations utiles et ont fourni une base solide pour l'étude des mécanismes de régulation de l'accumulation et de la tolérance des métaux lourds dans les feuilles et les racines à l'échelle moléculaire.

2- Une batterie de biomarqueurs de stress métalliques montrant une réponse dose- et temps-dépendante a été identifiée. Tous les gènes candidats sélectionnés au cours du présent projet ont montré des différences d'expression sous l'effet du stress et peuvent être considérés comme biomarqueurs. Toutefois, certains gènes ont montré des préférences et une sensibilité meilleure à certains métaux. Les ions cadmiques, en stimulant l'activité APX et l'expression de *Pdpcs1*, *Pdhma2*, *PdNramp6* et *Pdmate5* permettent de les caractériser comme des biomarqueurs potentiels pour la surveillance de la contamination de l'environnement dans les milieux arides terrestres contaminés. Les ions cuivriques stimulent plus les biomarqueurs SOD, *Pdmt3* et *Pdhma2*. En cas d'exposition au Cr^{6+}, les biomarqueurs SOD, *Pdmt3*, *Pdabcc* et *PdNramp* peuvent être considérés comme de bons biomarqueurs de stress.

3- Les profils transcriptionnels et les stratégies de défense ont été étudiés chez des individus constituants une population homogène (vitroplants, plantules) et chez des graines présentant une diversité génétique. Les différences ou discordances observées ne permettent pas de conclure à une différence du potentiel de résistance entre populations hybrides et homogènes. Toutefois, phénotypiquement, on peut suggérer un caractère stable de la diversité génétique capable de renseigner un statut durable de l'écosystème. Par ailleurs, des teneurs élevées de Zn et de Pb ont été retrouvées dans les échantillons du site d'étude. **Il conviendrait d'étudier également la biodisponibilité de ces ETMs dans les tissus de palmier dattier et leurs effets éventuels.**

4- L'apparition de bactéries endophytiques dans des milieux de culture en réponse à de fortes doses de Cu ou de Cr fournit une nouvelle approche de phytoremédiation associée à ces endophytes. **Ainsi, il est nécessaire d'identifier ces endophytes et d'étudier leur potentiel de tolérance et les mécanismes impliqués.**

5- Les profils métabolomiques et transcriptionnels de moindre puissance associés à une résistance moins grande des graines comparées aux plantules suggèrent une sensibilité

plus grande à la pollution au stade graine.

6- Dans un but de phytoremediation, le potentiel de phytoextraction et de phytoaccumulation de Cd et de phytostabilisation de Cu et de Cr par le palmier dattier défend l'utilisation de l'espèce dans un contexte de phytoremediation destiné à la purification d'écosystèmes d'importante contamination métallique. Il peut être exploité dans un dispositif expérimental « bio-station » dont l'objectif est de permettre à l'espèce de pousser dans des biotopes (sols ou eaux) pollués mais pas dans un but de biosurveillance. **Toutefois, afin d'éviter tout risque du passage de la contamination vers les niveaux trophiques supérieurs, surtout humains, il est nécessaire de chercher la teneur métallique au niveau des fruits, des dattes du palmier dattier exposé au stress. De plus, il est envisageable de chercher des espèces à cycle de vie court et pouvant servir comme des bioindicateurs. Nous avons entamé nos investigations sur une espèce génétiquement proche au palmier dattier pour les gènes relatifs au stress métallique ; La massette à larges feuilles, *Typha latifolia*, qui a montré un pouvoir d'accumulation important de Cd, Cu et Pb.**

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ANNEXES

I. Figures et tableaux supplémentaires

Tableau II-S1. Détails des résultats de la cartographie KEGG

KEGG	Transcripts numbers
Metabolisme	
Carbohydrate metabolism	
Pyruvate metabolism	195
Pentose phosphate pathway	153
Amino sugar and nucleotide sugar metabolism	278
Citrate cycle (TCA cycle)	128
C5-Branched dibasic acid metabolism	6
Glycolysis / Gluconeogenesis	283
Starch and sucrose metabolism	514
Inositol phosphate metabolism	145
Butanoate metabolism	107
Propanoate metabolism	82
Ascorbate and aldarate metabolism	75
Galactose metabolism	160
Fructose and mannose metabolism	101
Glyoxylate and dicarboxylate metabolism	192
Energy metabolism	
Methane metabolism	155
Oxidative phosphorylation	95
Nitrogen metabolism	91
Sulfur metabolism	83
Lipid metabolism	
Steroid hormone biosynthesis	14
Fatty acid biosynthesis	54
Fatty acid elongation	36
Steroid biosynthesis	36
Glycerolipid metabolism	210
Fatty acid degradation	126
Synthesis and degradation of ketone bodies	29
Glycerophospholipid metabolism	223
Cutin, suberine and wax biosynthesis	16
Ether lipid metabolism	47
Sphingolipid metabolism	94
Arachidonic acid metabolism	52
Biosynthesis of unsaturated fatty acids	55
Linoleic acid metabolism	16

alpha-Linolenic acid metabolism	90
Nucleotide metabolism	
Purine metabolism	535
Pyrimidine metabolism	179
Amino acid metabolism	
Phenylalanine, tyrosine and tryptophan biosynthesis	77
Cysteine and methionine metabolism	166
Lysine degradation	79
Valine, leucine and isoleucine degradation	91
Tryptophan metabolism	132
Valine, leucine and isoleucine biosynthesis	26
Arginine and proline metabolism	141
Metabolism of other amino acids	
Cyanoamino acid metabolism	107
beta-Alanine metabolism	114
Taurine and hypotaurine metabolism	44
Glutathione metabolism	152
Valine, leucine and isoleucine biosynthesis	26
Arginine and proline metabolism	141
Tryptophan metabolism	132
Valine, leucine and isoleucine degradation	91
D-Alanine metabolism	6
D-Glutamine and D-glutamate metabolism	4
Lysine degradation	79
Cysteine and methionine metabolism	166
Phenylalanine, tyrosine and tryptophan biosynthesis	77
Lysine biosynthesis	36
Phenylalanine metabolism	146
Glycine, serine and threonine metabolism	190
Selenocompound metabolism	47
Tyrosine metabolism	80
Alanine, aspartate and glutamate metabolism	182
Phosphonate and phosphinate metabolism	8
Histidine metabolism	22
Metabolism of cofactors and vitamins	
Ubiquinone and other terpenoid-quinone biosynthesis	36
Biotin metabolism	14
Lipoic acid metabolism	13
Pantothenate and CoA biosynthesis	50
One carbon pool by folate	60
Porphyrin and chlorophyll metabolism	102
Nicotinate and nicotinamide metabolism	49
Vitamin B6 metabolism	14
Riboflavin metabolism	83

Retinol metabolism	46
Thiamine metabolism	15
Folate biosynthesis	26
Metabolism of terpenoids and polyketides	
Biosynthesis of vancomycin group antibiotics	24
Biosynthesis of siderophore group nonribosomal peptides	1
Biosynthesis of ansamycins	2
Tetracycline biosynthesis	8
Terpenoid backbone biosynthesis	63
Monoterpenoid biosynthesis	17
Limonene and pinene degradation	25
Diterpenoid biosynthesis	7
Carotenoid biosynthesis	21
Zeatin biosynthesis	30
Sesquiterpenoid and triterpenoid biosynthesis	10
Geraniol degradation	36
Polyketide sugar unit biosynthesis	27
Insect hormone biosynthesis	1
Biosynthesis of other secondary metabolites	
Aflatoxin biosynthesis	8
Phenylpropanoid biosynthesis	221
Flavonoid biosynthesis	77
Anthocyanin biosynthesis	2
Flavone and flavonol biosynthesis	44
Stilbenoid, diarylheptanoid and gingerol biosynthesis	7
Isoquinoline alkaloid biosynthesis	43
Novobiocin biosynthesis	21
Benzoxazinoid biosynthesis	1
Penicillin and cephalosporin biosynthesis	5
Tropane, piperidine and pyridine alkaloid biosynthesis	43
Betalain biosynthesis	1
Indole alkaloid biosynthesis	8
Glucosinolate biosynthesis	11
Streptomycin biosynthesis	76
Butirosin and neomycin biosynthesis	12
Caffeine metabolism	2
Carbapenem biosynthesis	2
Xenobiotics biodegradation and metabolism	
Steroid degradation	11
Drug metabolism - other enzymes	95
Drug metabolism - cytochrome P450	63
Metabolism of xenobiotics by cytochrome P450	61
Aminobenzoate degradation	99
Naphthalene degradation	24

Chloroalkane and chloroalkene degradation	40
Toluene degradation	22
Xylene degradation	3
Fluorobenzoate degradation	2
Bisphenol degradation	3
Styrene degradation	21
Ethylbenzene degradation	13
Caprolactam degradation	40
Atrazine degradation	3
Glycan biosynthesis and metabolism	
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	4
Glycosaminoglycan degradation	40
Lipopolysaccharide biosynthesis	6
Peptidoglycan biosynthesis	8
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	4
Glycosphingolipid biosynthesis - lacto and neolacto series	6
Glycosphingolipid biosynthesis - ganglio series	34
Glycosphingolipid biosynthesis - globo series	24
N-Glycan biosynthesis	25
Other glycan degradation	80
Various types of N-glycan biosynthesis	22
Other types of O-glycan biosynthesis	7
Environmental Information Processing	
Signal transduction	
mTOR signaling pathway	51
Phosphatidylinositol signaling system	154
Immune system	
T cell receptor signaling pathway	56
Genetic Information Processing	
Translation	
Aminoacyl-tRNA biosynthesis	77

Tableau II-S3. Classification fonctionnelle KOG des transcrits de *P. dactylifera*

KOG class	count	description
A	822	RNA processing and modification
B	189	Chromatin structure and dynamics
C	534	Energy production and conversion
D	354	Cell cycle control, cell division, chromosome partitioning
E	574	Amino acid transport and metabolism
F	146	Nucleotide transport and metabolism
G	812	Carbohydrate transport and metabolism
H	146	Coenzyme transport and metabolism
I	525	Lipid transport and metabolism
J	877	Translation, ribosomal structure and biogenesis
K	947	Transcription
L	336	Replication, recombination and repair
M	229	Cell wall/membrane/envelope biogenesis
N	7	Cell motility
O	1638	Posttranslational modification, protein turnover, chaperones
P	419	Inorganic ion transport and metabolism
Q	304	Secondary metabolites biosynthesis, transport and catabolism
R	1776	General function prediction only
S	902	Function unknown
T	1701	Signal transduction mechanisms
U	997	Intracellular trafficking, secretion, and vesicular transport
V	94	Defense mechanisms
W	48	Extracellular structures

Y	75	Nuclear structure
Z	568	Cytoskeleton

Tableau II-S4. Prédiction de transcriptions codantes pour des métallothionéines dans la banque cDNA de *P. dactylifera*

SeqName	Hit-Desc	GO-ID	Term
M01000000928	metallothionein 2a	GO:0050896	response to stimulus
	metallothionein 2a	GO:0005507	copper ion binding
M01000000001	metallothionein 3	GO:0065008	regulation of biological quality
	metallothionein 3	GO:0005507	copper ion binding
	metallothionein 3	GO:0050789	regulation of biological process
	metallothionein 3	GO:0044763	single-organism cellular process
	metallothionein 3	GO:0044765	single-organism transport
	metallothionein 3	GO:0042221	response to chemical stimulus
	metallothionein 3	GO:0006970	response to osmotic stress
	metallothionein 3	GO:0007275	multicellular organismal development
	metallothionein 3	GO:0016043	cellular component organization
M01000091854	metallothionein 3	GO:0065008	regulation of biological quality
	metallothionein 3	GO:0005507	copper ion binding
	metallothionein 3	GO:0050789	regulation of biological process
	metallothionein 3	GO:0044763	single-organism cellular process
	metallothionein 3	GO:0044765	single-organism transport
	metallothionein 3	GO:0042221	response to chemical stimulus
	metallothionein 3	GO:0006970	response to osmotic stress
	metallothionein 3	GO:0007275	multicellular organismal development
	metallothionein 3	GO:0016043	cellular component organization
M01000068119	metallothionein 3	GO:0065008	regulation of biological quality
	metallothionein 3	GO:0005507	copper ion binding
	metallothionein 3	GO:0050789	regulation of biological process
	metallothionein 3	GO:0044763	single-organism cellular process
	metallothionein 3	GO:0044765	single-organism transport
	metallothionein 3	GO:0042221	response to chemical stimulus
	metallothionein 3	GO:0006970	response to osmotic stress
	metallothionein 3	GO:0007275	multicellular organismal development
	metallothionein 3	GO:0016043	cellular component organization
M01000005797	metallothionein 3	GO:0010363	regulation of plant-type hypersensitive response
	metallothionein 3	GO:0005507	copper ion binding
	metallothionein 3	GO:0009750	response to fructose stimulus
	metallothionein 3	GO:0009651	response to salt stress
	metallothionein 3	GO:0009926	auxin polar transport
	metallothionein 3	GO:0040007	growth
	metallothionein 3	GO:0009963	positive regulation of flavonoid biosynthetic process
	metallothionein 3	GO:0008361	regulation of cell size
	metallothionein 3	GO:0010015	root morphogenesis
	metallothionein 3	GO:0006612	protein targeting to membrane

	metallothionein 3	GO:0006833	water transport
	metallothionein 3	GO:0005576	extracellular region
	metallothionein 3	GO:0046686	response to cadmium ion
	metallothionein 3	GO:0006878	cellular copper ion homeostasis
	metallothionein 3	GO:0007389	pattern specification process
M01000000928	metallothionein 2a	GO:0050896	response to stimulus
	metallothionein 2a	GO:0005507	copper ion binding

Tableau II-S5. Prédiction des enzymes putatives impliquées dans le métabolisme du glutathion à partir du transcriptome de *P. dactylifera*

Pathway	Seqs in Pathway	Enzyme	Ezyme ID	Seqs of Enzyme	Sequences names in P.dactylifera library
Glutathione metabolism	152	glutamyl transpeptidase	ec:2.3.2.2	6	Contig1332, Contig4691, M01000044471, M01000088028, M01000028794, M01000063984
	152	synthase	ec:6.3.2.3	4	Contig5825, M01000017470, M01000029430, M01000039431
	152	ligase	ec:6.3.2.2	23	Contig1389, Contig4492, Contig6020, Contig6747, Contig6748, M01000000939, M01000009387, M01000013016, M01000001837, M01000012147, M01000002826, M01000001834, M01000048550, M01000000155, M01000053641, M01000001952, M01000037010, M01000007183, M01000017193, M01000045549, M01000047783, M01000052393, M01000011282
	152	peroxidase	ec:1.11.1.9	25	Contig1221, Contig2604, Contig2688, Contig3969, Contig3970, Contig4204, Contig5531, M01000026426, M01000070465, M01000067358, M01000035843, M01000012203, M01000068002, M01000010722, M01000012997, M01000073857, M01000003729, M01000056907, M01000000305, M01000005616, M01000068644, M01000045862, M01000012347, M01000011464, M01000002147
	152	thioredoxin peroxidase	ec:1.11.1.15	2	M01000087495, M01000079778
	152	peroxidase	ec:1.11.1.11	19	Contig160, Contig4137, Contig4907, M01000006915, M01000008458, M01000012875, M01000089469, M01000069053, M01000026584, M01000041038, M01000065099, M01000054532, M01000003915, M01000044282, M01000008993, M01000033950, M01000007087, M01000003375, M01000047504
	152	dehydrogenase (ascorbate)	ec:1.8.5.1	3	Contig1453, M01000087052, M01000008424
	152	reductase	ec:1.17.4.1	2	Contig1926, Contig3436
	152	(ATP-hydrolysing)	ec:3.5.2.9	1	M01000005635
	152	dehydrogenase (NADP+)	ec:1.1.1.49	6	Contig206, Contig571, Contig5011, Contig5968, M01000004678, M01000015631
	152	dehydrogenase (NADP+-dependent, decarboxylating)	ec:1.1.1.44	16	Contig448, Contig1177, Contig2937, Contig4433, Contig4687, M01000003566, M01000045047, M01000086927, M01000010747, M01000049247, M01000072042, M01000052781, M01000047094, M01000079607, M01000090081, M01000006466
	152	dehydrogenase (NADP+)	ec:1.1.1.42	12	Contig993, Contig5441, M01000082940, M01000008170, M01000048671, M01000058578, M01000072200, M01000059570, M01000005648, M01000046337, M01000001539, M01000023104
	152	synthase	ec:2.5.1.22	14	Contig1326, Contig6288, Contig6289, Contig6290, Contig6291, M01000010279, M01000019661, M01000067863, M01000067269, M01000000659, M01000049568, M01000045605, M01000021608, M01000018779
	152	transferase	ec:2.5.1.18	29	Contig249, Contig1221, Contig1526, Contig1668, Contig2604, Contig2688, Contig3969, Contig3970, Contig5531, M01000035016, M01000025648, M01000048983, M01000070465, M01000048252, M01000035843, M01000012203, M01000068002, M01000003729, M01000071509, M01000000305, M01000087328, M01000048550, M01000069842, M01000005616, M01000068644, M01000045862, M01000012347, M01000002147, M01000009665
	152	synthase	ec:2.5.1.16	14	Contig1326, Contig6288, Contig6289, Contig6290, Contig6291, M01000010279, M01000019661, M01000067863, M01000067269, M01000000659, M01000049568, M01000045605, M01000021608, M01000018779
	152	reductase	ec:1.8.1.7	14	Contig3749, Contig5649, Contig5957, Contig5958, M01000003566, M01000045047, M01000049247, M01000072042, M01000052781, M01000047992, M01000006466, M01000086902, M01000086624, M01000067993

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
PvPCS1          ATGGCCGTAG CTGGTCTATA CAAGCGTGTC TTGCCTTGCC CACCTGCAGT
AyPCS1          ATGGCTGTAG CTAGTGTCTA CAAGCGTGTG TTGCCTTGCC CGCCTGCCAT
AsPCS1          ATGGCGCTTG CGGGACTTTA TCGTCGAGTT CTCCCGTCAC CCCCGGCCGT
AtPCS           ATGGCTATGG CGAGTTTATA TCGGCGATCT CTTCCTTCTC CTCCGGCCAT
TlPCS           ATGGCGGAGG CAGGTCTTTT CCGGCGAATC CTCCCCTCTC CACCCGCGAT
PdPCS           ATGGCGGTCG CCGGTCTCTA TCGGCGAGCT CTCCCCTCTC CGCCGGCGAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
PvPCS1          GGAACTCGCT TCTCAGGAAG GGAAGGTACT CTTTGCGGAG GCCCTCGCAG
AyPCS1          CGAATTTGCA TCCCAAGAAG GGAAGGTACT CTTTGCTGAA GCTCTCTCCG
AsPCS1          CGAGTTTGCC TCGACTGAGG GAAAGAAACT TTTCGCTGAA GCTCTACAAA
AtPCS           CGACTTTTCT TCCGCCGAAG GCAAGCTAAT CTTCAATGAA GCGCTTCAGA
TlPCS           CGAGTTCGCT TCTCCCGAGG GAAAGTGCCT CTTCTCTGAT GCCCTTCATA
PdPCS           CGAGTTCGCC TCAGTTGAAG GAAAGCGACT CTTCTCTGAA GCCTTTCAAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
PvPCS1          ATGGGTCCAT GAATGGATTC TTTAAGCTAG TCAGTACTTT CCAAACTCAG
AyPCS1          ATGGAACCAT GAATGGATTT TTCAGATTGA TCAGCACTTT TCAAACGCAA
AsPCS1          ATGGTACGAT GGAAGGATTT TTTAAGTTAA TTTCCTGCTT CCAGACTCAG
AtPCS           AAGGAACTAT GGAAGGATTT TTCAGGTTGA TTTCGTATTT TCAGACACAA
TlPCS           ATGGAACGAT GGAAGGGTTT TTCAAATTGA TTTCGTACTT CCAGACACAG
PdPCS           GTGGAACCAT GGAAGGATTT TTCAAATTAA TTTCCTACTT CCAAACACAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
PvPCS1          GCGGAACCAG CTTATTGCGG GCTTACTACT CTTGTAGTTG TGCTGAATGC
AyPCS1          GCTGAACCAG CTTATTGTGG GCTCAGTACC CTCACAGTTG TGCTCAATGC
AsPCS1          TCTGAACCTG CTTATTGTGG TCTGGCTAGT CTATCTATGG TGTTAAATGC
AtPCS           TCCGAACCTG CGTATTGTGG TTTGGCTAGT CTCTCAGTGG TGTTGAATGC
TlPCS           TCGGAGCCTG CTTACTGTGG ACTGGCCACT TTGTCTGTCG TCCTGAATGC
PdPCS           TCAGAGCCTG CTTACTGTGG ATTGGCTAGT TTGTCAGCTG TCCTGAATGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
PvPCS1          CCTCTCTGTA GATCCGGGTC GCAGATGGAA AGGCCCTTGG AGGTGGTTTG
AyPCS1          CCTCTCCATC GATCCCGGTC GCAAGTGGAA AGGTCCCTGG AGGTGGTTCG
AsPCS1          CCTTGCAATT GATCCAGGAA GAAAGTGGAA AGGCCCTTGG AGATGGTTCG
AtPCS           TCTTTCTATC GATCCTGGAC GTAAATGGAA AGGGCCTTGG AGGTGGTTTG
TlPCS           TCTTGCGATA GATCCTGGTA GAAAATGGAA AGGCCCCTGG AGATGGTTTG
PdPCS           TCTTGCTATA GATCCAGGAA GAAAGTGGAA AGGACCTTGG AGATGGTTTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
PvPCS1          ATGAATCAAT GCTTGATTGC TGTGAGCCTC TGGAGAATGT GAAGAAAAAT
AyPCS1          ATGAATCAAT GCTAGATTGC TGCGAGCCTC TCGAGAAGGT GAAAAAGAAT
AsPCS1          ACGAGTCAAT GTTAGATTGT TGCGAACCTT TGGAAAAAGT CAAAGAAGAA
AtPCS           ATGAATCAAT GTTGGATTGC TGCGAACCTC TGGAAGTAGT GAAGGAAAAA
TlPCS           ATGAGTCCAT GCTGGATTGT TGTGAACCTT TGGAGAAAAT CAAAGCTGAA
PdPCS           ATGAGTCCAT GTTGGACTGC TGCGAACCTT TGGATAGAGT CAAAGCTGAA



                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
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PvPCS1      GGAATTACAT TTTCCAAGGT GTCTTGTTTG GCACAATGTG CTGGGGCTTC
AyPCS1      GGCATCACAT TTACAAAAGT GACCTGCTTG GCACAATGTT CAGGGGCCTC
AsPCS1      GGAATCACAT TTGGGAAAGT TGCATGCTTG GCCCATTGTG CCGGTGCTAA
AtPCS       GGCATTTCAT TTGGAAAAGT TGTCTGTTTG GCTCATTGTT CAGGAGCAAA
TlPCS       GGCATCACCT TTGGAAAAGT TGCATGCTTG GCTCATTGTG CTGGARCTAA
PdPCS       GGCATCACAT TTGGCAAACT TGCTTGCTTG GCTTACTGTG CTGGAGCTAA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
PvPCS1      AGTCCAAGCT TTTCGTGCCA ATGAAAGCTC ACTGGATTTG TTTCGATCAT
AyPCS1      GGTCCAAGCT TTTCGTGCCA ATCAAAGCTC GCTGGATTTG TTTCGGTCCT
AsPCS1      TGTTCAAGCT ATTCGTACAA GCCAAGGCAG TCTTGAGGAT TTCCGCCAGC
AtPCS       AGTTGAGGCT TTCCGTACAA GTCAGAGCAC CATTGATGAT TTCCGCAAAT
TlPCS       AGTCGAAGCT TTTCGTGCAA ATCAAAGCAC CATAGATGAC TTCCGTAAAC
PdPCS       AGTTGAAGCT TATCGTACAA GTCACAGCTC CGTTGATGAC TTCCGTAACC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
PvPCS1      TCGTGGAAAC CTGTGCTTCT TCTGAAGACC ACCATCTTGT TGTTTCATAC
AyPCS1      TTGTGGAAAC TTGTGTTTCT TCTGATGACC ACCATCTTAT TGTTTCTTAT
AsPCS1      ATATTATCAG ATGCACTTCT TCTGATGATT GCCATGTGAT CACATCGTAC
AtPCS       TTGTCGTCAA ATGCACGAGT TCTGAGAATT GTCATATGAT CTCAACATAT
TlPCS       ATGTCATACA GTGTACTTCC TCTGAAGATT GTCATTTGGT TGTGTCTTAC
PdPCS       ATGTTATAAA ATGTACTTCC ACTGAGGACT GCCATCTGAT TGCATCATAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
PvPCS1      GATAGGCAGA TACTGAAACA GACAGGCACT GGACATTTCT CACCCGTGGG
AyPCS1      GATAGGCGGC CACTGAAACA GACGGGCACG GGACATTTTT CTCCTTTGGG
AsPCS1      AACCGAAAAG CTTTCGGTCA GACTGGAACT GGACATTTTT CACCAATTGG
AtPCS       CACCGAGGTG TATTTAAGCA GACTGGGACT GGTCACTTTT CACCTATTGG
TlPCS       AGCAGAAAAC TTTTCAAACA GACAGGATCT GGACATTTTT CACCTATTGG
PdPCS       CATAGGAAGC CTCTCAAGCA GACAGGAAGC GGTCATTTTT CACCAATTGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
PvPCS1      TGGGTATCAT CGCCACAAGG ATATGGCTTT GATTTTGGAT GTTGCACGTT
AyPCS1      TGGCTATCAT CGGCAAAAAG ATATGGCATT GATTTTAGAT GTTGCTCGTT
AsPCS1      TGGTTATCAC AAAGGAAGTG ATATGGCACT CATTTTAGAC ACTGCACGTT
AtPCS       TGGCTATAAT GCTGAGAGAG ATATGGCTTT GATTCTTGAT GTTGCTCGTT
TlPCS       TGGTTATCAT TCTGGAAAAG ACATGGTGCT CATTTTGGAT GTCGCCCGTT
PdPCS       TGGCTACCAT GCTGGAAGTG ACATGGTACT CATTTTGGAT GTCGCTCGTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
PvPCS1      TCAAGTATCC CCCTCATTGG GTACCTCTTT CTCTGCTGTG GGAGGCGTTA
AyPCS1      TCAAGTATCC CCCTCATTGG GTACCTCTTT CTCTGTTGTG GGATGCAATG
AsPCS1      TCAAATATCC TCCTCATTGG GTCCCACTTC AACTTCTTTG GGAGGCTATG
AtPCS       TCAAGTATCC CCCTCACTGG GTTCCTCTTA AACTTCTTTG GGAAGCCATG
TlPCS       TTAAGTACCC ACCACATTGG GTTCCACTGG AACTTCTTTG GGAAGCCATG
PdPCS       TTAAATATCC TCCTCACTGG GTCCCCCTCG AACTTCTTTG GGAAGCCATG
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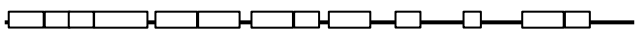
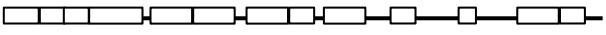
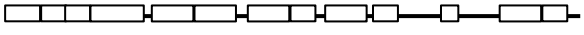
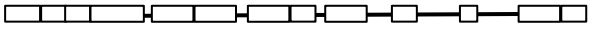
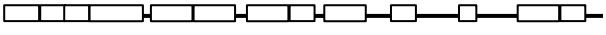
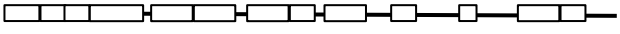
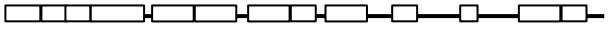
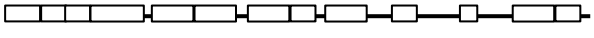
Figure II-S1. Comparaison des séquences nucléotidiques de PCS de fougères; *Pteris vittata* (Pv), *Athyrium yokoscense* (Ay) et de monocotylédones ; *Allium sativum* (As), *Arabidopsis thaliana* (At), *Typha latifolia* (Tl) et *Phoenix datylifera* (Pd) ont été réalisées par l'alignement multiple de la BioEdit.

Sequence ID	Length	Blocks
A	530	
AsPCS1	506	
AtPCS	485	
AyPCS1	488	
B	500	
PdPCS	528	
PvPCS1	512	
TlPCS	507	

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>PCSxxxx__ family
8 sequences are included in 13 blocks

            PCSxxxx__A, width = 31
                   A      3 MAVAGLYRRVLPSPPAIEFASSEGKQLFSEA
                AsPCS1      1 MALAGLYRRVLPSPPAVEFASTEGKKLFAEA
                 AtPCS      1 MAMASLYRRSLPSPPAIDFSSAEGKLIFNEA
                AyPCS1      1 MAVASVYKRVLPCPPAIEFASQEGKVLFAEA
                     B      1 MAVAGLYRRVLPSPPAIEFASSEGKRLFSEA
                 PdPCS      1 MAVAGLYRRALPSPPAIEFASVEGKRLFSEA
                PvPCS1      1 MAVAGLYKRVLPCPPAVELASQEGKVLFAEA
                 TlPCS      1 MAEAGLFRRILPSPPAIEFASPEGKCLFSDA

            PCSxxxx__B, width = 31                * *  *
                   A (0)     34 LQNGTMEGFFKLISYFQTQSEPAYCGLASLS
                AsPCS1 (0)     32 LQNGTMEGFFKLISCFQTQSEPAYCGLASLS
                 AtPCS (0)     32 LQKGTMEGFFRLISYFQTQSEPAYCGLASLS
                AyPCS1 (0)     32 LSDGTMNGFFRLISTFQTQAEPAYCGLSTLT
                     B (0)     32 LQNGTMEGFFKLISYFQTQSEPAYCGLASLS
                 PdPCS (0)     32 FQSGTMEGFFKLISYFQTQSEPAYCGLASLS
                PvPCS1 (0)     32 LADGSMNGFFKLVSTFQTQAEPAYCGLTTLV
                 TlPCS (0)     32 LHNGTMEGFFKLISYFQTQSEPAYCGLATLS

            PCSxxxx__C, width = 27
                   A  (   0)     65 MVLNALAIDPGRKWKGPWRWFDESMLD
                AsPCS1  (   0)     63 MVLNALAIDPGRKWKGPWRWFDESMLD
                 AtPCS  (   0)     63 VVLNALSIDPGRKWKGPWRWFDESMLD
                AyPCS1  (   0)     63 VVLNALSIDPGRKWKGPWRWFDESMLD
                     B  (   0)     63 VVLNALAIDPGRKWKGPWRWFDESMLD
                 PdPCS  (   0)     63 AVLNALAIDPGRKWKGPWRWFDESMLD
                PvPCS1  (   0)     63 VVLNALSVDPGRRWKGPWRWFDESMLD
                 TlPCS  (   0)     63 VVLNALAIDPGRKWKGPWRWFDESMLD

            PCSxxxx__D, width = 51
        A  (   0)     92 CCEPLEKVKAKGITFGKVACLAHCAGAKVEAFRTNQSTIDDFRKHVIKCTS
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AsPCS1  (   0)    90 CCEPLEKVKEEGITFGKVACLAHCAGANVQAIRTSQGSLEDFRQHIIRCTS
 AtPCS  (   0)    90 CCEPLEVVKEKGISFGKVVCLAHCSGAKVEAFRTSQSTIDDFRKFVVKCTS
AyPCS1  (   0)    90 CCEPLEKVKKNGITFTKVTCLAQCSGASVQAFRANQSSLDLFRSFVETCVS
     B  (   0)    90 CCEPLEKVKAEGITFGKVACLAHCAGAKVEAFRTNQSTIDDFRKHVIRCTS
 PdPCS  (   0)    90 CCEPLDRVKAEGITFGKLACLAYCAGAKVEAYRTSHSSVDDFRNHVIKCTS
PvPCS1  (   0)    90 CCEPLENVKKNGITFSKVSCLAQCAGASVQAFRANESSLDLFRSFVETCAS
 TlPCS  (   0)    90 CCEPLEKIKAEGITFGKVACLAHCAGXKVEAFRANQSTIDDFRKHVIQCTS

          PCSxxxx__E, width = 39       *    *
                 A (1)   144 EDCHLIASYHRKVFKQTGTGHFSPIGGYHAGRDMVLILD
               AsPCS1 (1)   142 DDCHVITSYNRKAFGQTGTGHFSPIGGYHKGSDMALILD
                AtPCS (1)   142 ENCHMISTYHRGVFKQTGTGHFSPIGGYNAERDMALILD
               AyPCS1 (1)   142 DDHHLIVSYDRRPLKQTGTGHFSPLGGYHRQKDMALILD
                    B (1)   142 EDCHLIASYHRKPFKQTGTGHFSPIGGYHAGRDMVLILD
                PdPCS (1)   142 EDCHLIASYHRKPLKQTGSGHFSPIGGYHAGSDMVLILD
               PvPCS1 (1)   142 EDHHLVVSYDRQILKQTGTGHFSPVGGYHRHKDMALILD
                TlPCS (1)   142 EDCHLVVSYSRKLFKQTGSGHFSPIGGYHSGKDMVLILD

          PCSxxxx__F, width = 36    ***
                 A  (   0)   183 VARFKYPPHWVPLPLLWEAMNTVDEATGHRRGFMLI
               AsPCS1  (   0)   181 TARFKYPPHWVPLQLLWEAMKYEDPATGYPRGFMLI
                AtPCS  (   0)   181 VARFKYPPHWVPLKLLWEAMDSIDQSTGKRRGFMLI
               AyPCS1  (   0)   181 VARFKYPPHWVPLSLLWDAMNTIDEATGKSRGFMMI
                    B  (   0)   181 VARFKYPPHWVPLPLLWEAMNTVDEATGHPRGFMLI
                PdPCS  (   0)   181 VARFKYPPHWVPLELLWEAMDTIDEATGHHRGFMLL
               PvPCS1  (   0)   181 VARFKYPPHWVPLSLLWEALNSVDEATGKSRGFMMI
                TlPCS  (   0)   181 VARFKYPPHWVPLELLWEAMNTVDKATGCLRGFMLI

          PCSxxxx__G, width = 36
                 A  (   9)   228 LYTLSCRHESWVSMAKYLMDDVPLLLKSEDIKDVQE
               AsPCS1  (   9)   226 LYTLSCRHESWVQTAKYLMDDVPILLKKANLNTVQD
                AtPCS  (   9)   226 LYTLSCKDESWIEIAKYLKEDVPRLVSSQHVDSVEK
               AyPCS1  (   9)   226 LFTLSCKDERWRAMCKHILEEVPELLKTTNLTSTGQ
                    B  (   9)   226 LYTLSCRHESWVSMAKYLMDDVPILLKSEDLNDVQE
                PdPCS  (   9)   226 LYTLSCCHESWVSMARYLIDDVPILLKSEDLSSVPE
               PvPCS1  (   9)   226 LFTLSCKDQRWRAMCKYLLEEVPYLLKSRGFTTVEE
                TlPCS  (   9)   226 LYTLSCRHESWLSMVKYLIDDVPIILKSGSLGDAPS

          PCSxxxx__H, width = 28
                 A  (   0)   264 VLSVVFTSLPANLGDFIKWVAEVRRQED
               AsPCS1  (   0)   262 VLSLIFKSLLSNAGDFIKWVAEVRRPEE
                AtPCS  (   0)   262 IISVVFKSLPSNFNQFIRWVAEIRITED
               AyPCS1  (   0)   262 VIHTVFDSLPADIASFVKWIVEVKLAED
                    B  (   0)   262 VLSLLFKSLPANAGDFIKWVAEVRRQEE
                PdPCS  (   0)   262 VLLLLFKSLPANAGDFIKWIAEVRRQEE
               PvPCS1  (   0)   262 VVYTVFGSLPADVASFVKWIVEVKPAVN
                TlPCS  (   0)   262 VLLLLIKSLPANAGDFIKWFAEVRRQEE

          PCSxxxx__I, width = 33
                 A  (   4)   296 LSEEEKERLAIKEEVLKQVRETELFKHVTEWLS
               AsPCS1  (   3)   293 LSKEEKERLAIKEIVLQQIRETKLYKYVSEWLS
                AtPCS  (   4)   294 LSAEAKSRLKLKQLVLKEVHETELFKHINKFLS
               AyPCS1  (   1)   291 VDRDETERLHYKSKVLQQLRQTTVFALITKWVE
                    B  (   4)   294 LSKEEKERLAIKEEVLQQVRETELFKYVTEWLS
                PdPCS  (   4)   294 LSKEEEERLAAKEEVLQQVRETELYKFVADLVS
               PvPCS1  (   5)   295 VDRDETERLRYKSKVLQQLRQTRALALITKWME
                TlPCS  (   4)   294 ISKEEKERLALKEEVLQQVYETELYKVVKNVLS
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PCSxxxx__J, width = 21
       A  (  14)   343 NDSLSEIAASVCCQGAEILSG
  AsPCS1  (  14)   340 KDSLTDIAASVCCQGALLLAG
   AtPCS  (   4)   331 EDSLTYAAAKACCQGAEILSG
  AyPCS1  (   8)   332 NESLPNVIMQACCQGAAAFTG
       B  (  14)   341 NDSLSEIAASVCCQGAALLSG
   PdPCS  (  14)   341 NDSLSEIAASVCCQGAAILTG
  PvPCS1  (   8)   336 DESLQTAILQACCQGAAAFRG
   TlPCS  (  14)   341 KDFLPEIAATVCCQGAALLTG

PCSxxxx__K, width = 16
       A  (  40)   404 EQGVDMLVPSSPTKSS
  AsPCS1  (  38)   399 EQGVDMLVPTSPSKSH
   AtPCS  (  36)   388 EQKVDLLVPSTQTECE
  AyPCS1  (  39)   392 EQVIDALVPKISTLNT
       B  (  36)   398 GNGVDMLVPTSPTKSS
   PdPCS  (  38)   400 EQGVDMLVPESSAKSK
  PvPCS1  (  40)   397 EQVIDALVPKRPSSGN
   TlPCS  (  38)   400 EQGVDMLVPVSPTKSS

PCSxxxx__L, width = 36
       A  (  18)   438 DVLTVLLLALPPQTWSGIKDEKLLQEINSLVSTENL
  AsPCS1  (  18)   433 DVLTILSLALFTNSWFDISNKKLLDEIRALVSFQNL
   AtPCS  (  11)   415 DVFTALLLALPPQTWSGIKDQALMHEMKQLISMASL
  AyPCS1  (  19)   427 DLLAILLLALPTSMWEDIPNREVHSEICRIVSREEL
       B  (  17)   431 DVLTVLLLALPPNTWLGIKDEKLLAEIQSLVSTENL
   PdPCS  (  18)   434 DALTVLLLALPPCTWQGIKDDRLLAEIHGLVSIENL
  PvPCS1  (  27)   440 DLLAVLLLALPPSTWEQIPDLSIHAEISRIVSRKEL
   TlPCS  (  17)   433 NVLTVLLLALPPRTWLDIEDKSLLAEIQGLVSTENL

PCSxxxx__M, width = 25
       A  (   0)   474 PTLLQEEVLHLRRQLHFLKRCQDNE
  AsPCS1  (   0)   469 PDVLQEEVLHLRRQLMFLKKCKDKE
   AtPCS  (   0)   451 PTLLQEEVLHLRRQLQLLKRCQENK
  AyPCS1  (   0)   463 PFELRLEVEHLWEQANLVSRSCSEE
       B  (   0)   467 PDVLQEEVLHLRRQLHFLKRCQDNE
   PdPCS  (   0)   470 PDVLEQEVLHLQRQLHFLKEMQRAR
  PvPCS1  (   0)   476 PHELQLGVEHLFEQAFFVSRSCTEE
   TlPCS  (   0)   469 PDVLQHEVLHLRRQFDFLKRCKNNE
```

Figure II-S2. Blocs de MOTIF concervés. Les acides aminés en gras se rapportent à la triade catalytique et l'astérisque est utilisé pour les acides aminés constitutifs du second substrat.

Tableau II-S6. Gènes de *PCS* trouvés dans NCBI et PlantGDB

Taxonomy	Gene Name	Species	Protein ACC	Protein length aa
Cyanobacteria	NoPCS	*Nostoc sp.*	242	BAD10973.1
	NpPCS	*Nostoc punctiforme*	243	ACC81212.1
	CsPCS	*Cylindrospermum stagnale*	245	AFZ23969.1
	CmPCS	*Chamaesiphon minutus*	238	AFY91394.1
	CyPCS	*Cyanothece sp.*	244	ACL46092.1
	MvPCS	*Microcoleus vaginatus*	266	EGK86811.1
	SyPCS	*Synechococcus sp.*	257	AFY62739.1
	CgPCS	*Cyanobium gracile*	243	AFY29492.1
	RlPCS	*Rubidibacter lacunae*	250	ERN41197.1
	LaPCS	*Lyngbya aestuarii*	192	ERT05621.1
Proteobacteria	BrPCS	*Bradyrhizobium sp.*	220	EIG63837.1
	MetPCS	*Methylobacterium nodulans*	217	ACL63176.1
	RhPCS	*Rhizobium sp.*	218	EUB96697.1
	TdPCS	*Thiorhodococcus drewsii*	243	EGV28630.1
	TvPCS	*Thiocystis violascens*	250	AFL73590.1
	EmPCS	*Erwinia mallotivora*	259	EXU76727.1
	ShPCS	*Shewanella violacea*	262	YP_003556713
	PnPCS	*Polynucleobacter necessarius*	239	YP_001155331
	BpPCS	*Burkholderia pseudomallei*	354	YP_001059448
amoeba	DfPCS	*Dictyostelium fasciculatum*	789	XP_004351373
	DdPCS	*Dictyostelium discoideum*	626	XP_635353.1
alga	NaPCS	*Nannochloropsis gaditana*	586	EWM29630.1
	TpPCS	*Thalassiosira pseudonana*	444	AET85547.1
	TpPCS2	*Thalassiosira pseudonana*	340	AGE13359.1
	TpPCS3	*Thalassiosira pseudonana*	354	AGE13358.1
Fungi	CoPCS	*Capsaspora owczarzaki*	527	EFW42781.1
	SpPCS	*Schizosaccharomyces pombe*	414	CAA92263.1
Bilateria	HmPCS	*Hymenolepis microstoma*	636	CDJ12891.1
	EgPCS	*Echinococcus granulosus*	661	CDJ21038.1
	LlPCS1	*Loa loa*	227	XP_003139039
	BmPCS	*Brugia malayi*	413	XP_001902100
	CrPCS1	*Caenorhabditis remanei*	426	XP_003117305
	CePCS1	*Caenorhabditis elegans*	371	AAK62991.1
	AcPCS	*Ancylostoma ceylanicum*	425	AGT57959.1
	EfPCS	*Eisenia fetida*	440	ABR13683.1
	LrPCS	*Lumbricus rubellus*	442	AHC94360.1
protozoa	TtPCS1	*Tetrahymena thermophila*	446	AAY68362.2
Ferns	PvPCS1	*Pteris vittata*	476	AAT11885.1
	AyPCS1	*Athyrium yokoscense*	488	BAB64932.1
Mesangiospermae	CdPCS1	*Ceratophyllum demersum*	501	ADR10438.1
	NnPCS	*Nelumbo nucifera*	505	BAN08523.1
	SaPCS	*Sedum alfredii*	555	AHB86971.1
	SsPCS	*Suaeda salsa*	492	AGC82138.1

	AmPCS	*Amaranthus tricolor*	485	AEW23125.1
	FePCS	*Fagopyrum esculentum*	494	BAF75863.1
	KcPCS	*Kandelia candel*	496	ADK61091.1
	MnPCS	*Morus notabilis*	496	EXB53499.1
	BnPCS1	*Boehmeria nivea*	505	AHC98018.1
	PbPCS1	*Pyrus betulifolia*	497	AEY68568.1
	PbPCS2	*Pyrus betulifolia*	488	AHM93477.1
	PcPCS1	*Pyrus calleryana*	497	AGS56990.1
	LeuPCS	*Leucaena leucocephala*	485	ACL00594.3
	SvPCS	*Sophora viciifolia*	500	AFM38979.1
	LjPCS1	*Lotus japonicus*	501	AAT80342.1
	LjPCS2		477	AAT80341.1
	LjPCS3		479	AAY81941.1
	SrPCS5	*Sesbania rostrata*	233	AAY83876.1
	GmPCS	*Glycine max*	498	AAL78384.1
	TjPCS	*Thlaspi japonicum*	485	BAB93119.1
	TcPCS1	*Thlaspi caerulescens*	485	AAT07467.1
	TcPCS2		455	ABY89660.1
	TcPCS		485	BAB93120.1
	BjPCS	*Brassica juncea*	485	CAC37692.1
	AhPCS1	*Arabidopsis halleri*	485	AAS45236.1
	AhPCS2		454	ADZ24787.1
	AlPCS	*Arabidopsis lyrata*	415	XP_002892190.1
	AtPCS	*Arabidopsis thaliana*	485	AAL66747.1
	AtPCS1		485	AAD16046.1
	AtPCS2		452	AAK94671.1
	ThPCS1	*Theobroma cacao*	505	XP_007050223.1
	StPCS	*Solanum tuberosum*	503	CAD68110.1
	NtPCS1	*Nicotiana tabacum*	501	AAO74500.1
	NgPCS1	*Nicotiana glauca*	501	ABX10958.1
	SaPCS1	*Sonchus arvensis*	491	ACU44656.1
	LsPCS1	*Lactuca sativa*	490	AAU93349.1
	TlPCS	*Typha latifolia*	421	AAG22095.3
	PaPCS	*Phragmites australis*	498	AFU06381.1
	CyoPCS1	*Cynodon dactylon*	504	AAO13810.2
	CyoPCS2		508	AAS48642.1
	TaPCS	*Triticum aestivum*	500	AAD50592.1
	OsPCS1	*Oryza sativa*	473	AAO13349.2
	AsPCS1	*Allium sativum*	506	AAO13809.1
	PdPCS	*Phoenix dactylifera*	528	
Human	STX1B	*Homo sapiens*	158	NM_052874.3

Résumé

Les industries de traitement du phosphate produisant des engrais phosphatés, très présentes dans la zone côtière sud du Grand Sfax en Tunisie, sont à l'origine d'émissions atmosphériques et de rejets de phosphogypse chargés de contaminants métalliques (Cd, Cu, Cr, …) source de contamination persistante des sols. Pour mieux comprendre les effets de cette contamination, une espèce modèle, le palmier dattier ; *Phoenix dactylifera* L., a été choisi. Le présent travail avait pour objectifs d'étudier le comportement de différents tissus (vitroplant, hypocotyle, racine et feuille) de la variété Deglet Nour dans différents contextes de contamination métallique aux moyens de techniques de biotechnologie végétale impliquant la recherche *in silico* et la culture *in vitro* et *in vivo* et à l'aide de deux approches. Une approche individuelle intégrative portant sur de nombreuses mesures de biomarqueurs morphologiques et biochimiques de l'espèce étudiée et exposée à divers stress métalliques. Elle a révélé des fluctuations de l'équilibre redox à des concentrations élevées de Cd et du Cu essentiellement qui, en perturbant le système de défense, ont causé des dommages oxydatifs et des signes visuelles de toxicité (nécrose, chlorose, blocage de l'élongation de l'hypocotyle …). Une deuxième approche, plus moléculaire et mécanistique basée sur l'exploitation d'une banque d'ADNc de la variété Deglet Nour, a permis l'identification et l'expression de gènes de chélateurs (*Pdpcs1*, *Pdmt3*) et transporteurs (*Pdabcc*, *Pdhma2*, *Pdmate5*, *PdNramp6*) de métaux suite à une exposition métallique. L'analyse des profils d'expression génique a montré des différences de comportement entre gènes, tissus, type de métal et temps d'exposition. Tous les gènes candidats étaient sensibles et ont répondu aux différents types de stress étudiés. Toutefois, des répressions de gènes ont été observées à fortes concentrations métalliques et après de longues expositions. Le suivi des niveaux d'expression de ces gènes a permis de mieux comprendre les mécanismes de détoxication des métaux mis en œuvre.

Abstract

The phosphate processing industries for the production of phosphate fertilizers, which are present in the southern coastal zone of the Grand Sfax in Tunisia, caused atmospheric emissions and phosphogypsum discharges loaded with metal (Cd, Cu, Cr, …) contaminants causing persistent soil contamination. To better understand the effects of this contamination, a model species, date palm; *Phoenix dactylifera* L., was chosen. The present study aimed to study the behavior of various tissues (vitroplant, hypocotyl, root and leaf) of the Deglet Nour variety in different contexts of metallic contamination using plant biotechnology techniques involving *in silico* research and *in vitro* and *in vivo* culture using two approaches. An integrative individual approach that used numerous morphological and biochemical biomarker in date palm exposed to various metal stresses. Fluctuations in the redox equilibrium at high Cd and Cu concentrations where shown. It caused disruption in the defense system and oxidative damage revealed by visual signs of toxicity (necrosis, chlorosis, blockage of elongation hypocotyl ...). A second approach, more molecular and mechanistic based on the exploitation of a cDNA library of the Deglet Nour variety, allowed the identification and the expression of genes of chelators (Pdpcs1, Pdmt3) and metal transporters (Pdabcc, Pdhma2 , Pdmate5, PdNramp6). Analysis of gene expression profiles showed differences in behavior between genes, tissues, metal type and exposure time. All the candidate genes were sensitive and responded to the different types of studied stress. However, gene repressions were observed at high metal concentrations after long, long exposures. Monitoring of the levels of expression of these genes made it possible to better understand the detoxification mechanisms of metals in the palm date.

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The phytochelatin synthase gene in date palm (*Phoenix dactylifera* L.): Phylogeny, evolution and expression

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ABSTRACT

We studied date palm phytochelatin synthase type I (PdPCS1), which catalyzes the cytosolic synthesis of phytochelatins (PCs), a heavy metal binding protein, in plant cells. The gene encoding PdPCS1 (*Pdpcs*) consists of 8 exons and 7 introns and encodes a protein of 528 amino acids. PCs gene history was studied using Notung phylogeny. During evolution, gene loss from several lineages was predicted including Proteobacteria, Bilateria and Brassicaceae. In addition, eleven gene duplication events appeared toward interior nodes of the reconciled tree and four gene duplication events appeared toward the external nodes. These latter sequences belong to species with a second copy of PCs suggesting that this gene evolved through subfunctionalization. *Pdpcs1* gene expression was measured in seedling hypocotyls exposed to Cd, Cu and Cr using quantitative real-time polymerase chain reaction (qPCR). A *Pdpcs1* overexpression was evidenced in *P. dactylifera* seedlings exposed to metals suggesting that 1-the *Pdpcs1* gene is functional, 2-there is an implication of the enzyme in metal detoxification mechanisms. Additionally, the structure of PdPCS1 was predicted using its homologue from Nostoc (cyanobacterium, NsPCS) as a template in Discovery studio and PyMol software. These analyses allowed us to identify the phytochelatin synthase type I enzyme in date palm (PdPCS1) via recognition of key consensus amino acids involved in the catalytic mechanism, and to propose a hypothetical binding and catalytic site for an additional substrate binding cavity.

1. Introduction

Heavy metals are typically toxic at submicro- to micromolar concentrations. They displace endogenous metal cofactors from their cellular binding sites, participate in aberrant capping reactions with the thiol groups of proteins and promote the formation of reactive oxygen species (Rea et al., 2004).

The interaction of organisms with toxic metal and metalloid elements has been used as a productive model for physiological, ecological, genetic and evolutionary research for over half a century. In eukaryotic cells, toxic ions appear to be removed from the cytosol mainly by chelation and sequestration (Clemens et al., 1999). Plants resort to a series of defense mechanisms that control uptake, accumulation and translocation of toxic elements. One recurrent general tool for heavy metal detoxification is complexion with amino acids, organic acids, metal-binding peptides and/or sequestration into vacuoles (Hall, 2002). A number of metal-binding ligands and their roles have been reviewed by Rauser (1999). Bacteria, yeasts, worms as well as plants synthesize phytochelatins (PCs) which chelate Cd^{2+}, Cu^{2+} and other heavy metals ions. PCs are $(\gamma Glu\text{-}Cys)_n\text{-}Gly$ (n = 2–11) polymers, discovered about 30 years ago as non-protein cysteine-rich oligopeptides entirely different from metallothioneins (MTs) in structure and biosynthetic pathways (Masahiro, 2005). These cysteine (Cys)-rich polypeptides act as high-affinity metal chelators and facilitate their transport (Cobbett, 2000a). The amount of PCs has been proposed as a biomarker to assess metal stress (Keltjens and Beusichem, 1998).

Phytochelatin synthase (PCS; EC 2.3.2.15) is a γ-glutamylcysteine dipeptidyl transpeptidase which catalyzes the synthesis of PCs using glutathione (GSH, γGlu-Cys-Gly) or previously synthesized PC as substrates (Grill, 1989). In 1999, three groups simultaneously and independently cloned and characterized a small family of genes encoding this enzyme in *Arabidopsis thaliana*, *Schizosaccharomyces pombe* and *Triticum aestivum*, designated *Atpcs1*, *Sppcs1* and *Tapcs1*, respectively (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al.,

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1999). However, PCS polypeptides are much more widely distributed than was suspected in 1999. Systematic sequence database searches disclose PCS-like genes in some diatoms, some downy mildews, some ciliates, chordates, echinoderms, annelids and flatworms (Clemens and Persoh, 2009) as well as in prokaryotes (Rea et al., 2004). In contrast, in the limited number of species investigated, it so far appears that the earliest diverged extant land plants, like bryophytes, mosses and hornworts, do not synthesize PCs under metal stress (Leinenweber et al., 2009; Bleuel et al., 2011). Yet, the presence of metal chelating activity, particularly in the lineages originated during the Devonian period succeeding the palaeoenvironments rich in PC-inducing metal and extended with bryophytes and lycophytes, might represent a remnant of that time (Petraglia et al., 2013).

Furthermore, a large number of protein sequences homologous to PCS have been found over the last decade. The eukaryotic PCS proteins are 50–55 kDa polypeptides that display 40–50% sequence similarity, and contain a highly conserved N-terminal domain, with a papain-like catalytic triad (Vivares et al., 2005; Romanyuk et al., 2006; Rea, 2012) and a variable C-terminal domain (Cobbett, 2000b).

A range of metal ions can induce PCS activity, although with different efficiency. The enzyme is activated by copper, cadmium, arsenic, silver, mercury, zinc and lead ions (Oven et al., 2002). However, the dependence of PCS on the provision of heavy metal ions for activity in media containing GSH and other thiol peptides reflects its requirement for GSH-like peptides containing blocked thiol groups. A possibility of synthesizing PCs in plastids by overexpressing a plastid-targeted phytochelatin synthase has been shown (Picault et al., 2006). All of the former experiments aimed at phytoremediation and tried to enhance heavy metal accumulation in plants. Aside from metal detoxification, new functions have been suggested for PCS and PCs alike. With respect to metals, PCs have been implicated in long distance transport of Cd (Gong et al., 2003), predominantly in the phloem (Mendoza-Cozalt et al., 2008). They may also serve additional functions in glutathione metabolism. PCS catalyzes the first step in the degradation of GSH conjugates (Blum et al., 2010). The participation of AtPCS1 in glucosinolate metabolism and consequently in plant innate immunity has been reported (Clay et al., 2009).

To draw attention to diversity and growth of plants that routinely experience multiple major stresses (for example, both aridity and heavy metal contamination of soil), this work addresses one of a very few trees that can tolerate the desert environment; date palm (*Phoenix dactylifera* L.). Around the world, this plant includes more than 1500 cultivars (FAO, 2002). It has a minimal water demand, tolerates harsh weather and high levels of salinity (Diallo, 2005). In fact, it is more salt tolerant than any other fruit crop (FAO, 1982). A recent paper demonstrated a Cd-hyperaccumulation phenotype of the plant (Zayneb et al., 2013). The recent completion of the date palm cv. Deglet Nour genome sequencing project (Rekik et al., 2013), together with its automated annotation process, enabled us for the first time to examine *Pdpcs in silico* and *in vivo*. This enabled application of bioinformatics methods to unravel the molecular mechanisms of PdPCS function under metallic stress.

The aims of the present study were: 1- perform *in silico* characterization of the *Pdpcs*, 2- predict the protein structure and 3- test the hypothesis that the enzyme functions in metal detoxification mechanisms in date palm.

2. Materials and methods

2.1. Plant material and cDNA library construction (mRNA isolation, cDNA synthesis and cloning)

The date palm, *Phoenix dactylifera* cultivar “Deglet nour”, was used as the RNA source. Leaves were collected from a large number of plant samples and ground to a fine powder in liquid nitrogen, and homogenized in total RNA extraction buffer, according to Cathala et al. (1983). Total mRNA was selected by oligo (dT) cellulose chromatography, using the mRNA Purification Kit (QIAGEN) according to manufacturer's specifications. Double stranded cDNA was synthesized using a cDNA synthesis Kit (QIAGEN), following manufacturer's instructions. cDNAs were ligated to *Eco*RI adaptators, cloned into the *Eco*RI restriction site of pUC 18 and used to transform JM 105 cells. Bacteria were plated on LB agarose medium containing 50 mg/ML ampicillin, 0.1 µM IPTG and 0.1% X-Gal. Fifty white colonies were randomly selected out of 1500 and screened for the presence of recombinant plasmids with inserts greater than 400 base pair. Then, a larger scale preparation of DNA plasmids from numerous colonies was performed as described by Lebrun et al. (1988).

2.2. Isolation, cloning and sequencing of PCR products of PCS cDNA

BLASTn searches in NCBI (http://www.ncbi.nlm.nih.gov/) with default parameters allowed retrieval of *PCS* homologous from various organisms. Multiple alignment using BioEdit version 7.0.5.3 of some clones revealed homology with a region of *pcs* cDNA. We searched *pcs* domains (Pfam with E value $< 1e\text{-}5$) against the recovered clone nucleotide sequences and other sequenced plant genes/genomes using HMMER v3.0 and validated the candidate *Pdpcs1* genes using BLASTp against plant PCS protein sequences of UniProt (http://www.uniprot.org/). Degenerate primers were synthesized from the conserved domain found (Table 1). Completion of the other domains and 5’untranslated region (UTR) of putative *pcs* was done using the rapid amplification of 5’cDNA ends (5’ RACE) kit (Invitrogen) according to manufacturer's instructions. The first strand cDNAs were synthesized from mRNAs using gene-specific primers designed to amplify the *Pdpcs1* gene sequence found using PerlPrimer v 1.1.21. The sequences of the primers were 5’ GTCTCTATCGGCGAGCTCTC 3’ and 5’TAGACTCTCAGAATAGG AGTCCA 3’. In brief, first strand cDNA synthesis was done with Superscript TM II RT, RNA template removed by RNAse mix, purified in SNAP columns, followed by homopolymeric tailing of cDNA and then amplification of the target cDNA. The PCR product was cloned in the pGEMTeasy vector.

2.3. Reconstruction of duplication history

A cladogram representing the species tree was constructed sing the information on species relationships available at the Angiosperm Phylogeny Site (www.mobot.org/mobot/research/apweb/). Reconstruction of the gene duplication and loss history of *pcs* were performed, using NOTUNG v2.6 (Yang, 1998), through reconciliation of the species tree with the *pcs* gene trees. In order to obtain the most parsimonious estimation of duplication and loss events, the reconciliation of the species and gene trees was followed by routing and rearranging branches with more than 75% bootstrap support.

2.4. Phylogenetic analysis

The phylogeny of PCS proteins was obtained from 84 sequences, representing all major phylogenetic lineages. *Homo sapiens* syntaxin 1B (STX1B), protein (NM_052874.3) was used as an outgroup. We selected

Table 1
Primer sequenced used in 5’ RACE PCR.

PdPCS3: 5’GTCTCTATCGGCGAGCTCTC 3’
PdPCS4: 5’ACCTCCTGTTCAAGAACATCC 3’
PdPCS5: 5’AAAGTGGAACCATGGAAGGA 3’
PdPCS6: 5’GGTACAAGCATATCAACTCCT 3’
PdPCS7: 5’AGGAAGAAAGTGGAAAGGA 3’
PdPCS8: 5’TGTCTCCTAACTTCTGCAATCC 3’
PdPCS9: 5’ATATTGGCATGTTTCTTCGTGACC 3’
PdPCS10: 5’GAAGGAAACTCAAACCTACCCGA 3’
PdPCS11: 5’CTTAAGCTTGACCCATCTTCCT 3’

the Jones-Taylor-Thornton model to obtain the phylogenies using the Maximum Likelihood (ML) method. We ran the ML analysis with 100 bootstrap replicates using PHYLIP 3.69 (http://evolution.genetics.washington.edu/phylip.html).

2.5. Prediction of ancestral PCS proteins

Ancestral sequence reconstruction (ASR) was used to reconstruct ancestral proteins at specific nodes of the PCS phylogenetic tree. Several different methods of ASR were compared to determine which method resulted in minimal apparent biases for the PCS dataset. The ancestral sequences were predicted using Ancescon (Cai et al., 2004) with a maximum likelihood (ML) tree calculated using PhyML as reference for the reconstruction.

2.6. Construction of 3D PCS model

Amino acid composition and molecular weight of PdPCS1 were determined via the ProtParam tool (http://www.expasy.ch/tools/protparam.html). The server Phyre2 (protein homology/analogy recognition engine), which is an improved version of Phyre, was used. After creating the profile of non-redundant sequence, the secondary structure was predicted. Using the profile and model of secondary structure a full length 3D model was generated. The program threads the protein sequence through a PDB file and searches for the possible alignments.

A PdPCS1 molecular model was generated using the coordinates of the *Nostoc* NsPCS structure as the template in Discovery Studio 4.0 (Accelrys) as described previously (Wang et al., 2009). The resulting docking conformation was visualized using PyMOL.

2.7. RNA isolation and cDNA synthesis for quantitative PCR

Thirty day old Deglet nour date hypocotyls have been used for total RNA extraction. Seeds and seedlings were exposed from day 0 to control or contaminated growth medium (containing 0.02 mM of $CdCl_2$, or 0.02 mM $CuSO_4$ or 0.02 mM of K_2CrO_4). RNAs were isolated with the Plant RNeasy mini kit (Qiagen, Courtaboeuf, France), including the on-column DNase digestion step. Concentration and purity of the RNA samples was determined using a Spectrophotometer (SPECTROstar Nano Microplate Reader). RNA quality was visualized using a method adapted from Masek et al. (2005). Reverse transcription was performed on 1 µg of total RNA from each sample using random hexamer primers and the Thermo Scientific Maxima H Minus First strand cDNA Synthesis Kit according to manufacturer's instructions.

2.8. Reference genes identification and qPCR amplification

From the previously constructed Deglet nour date palm cDNA library we have identified six reference gene candidates using HMMER v3.0. Primer sequences were designed using Primer 3 plus on line software (Rozen and Skaletsky, 2000) and verified using Net Primer and Beacon Designer programs (Table S1). PCR products were cleaned with the EZ-10 spin column DNA gel extraction kit (Biobasic, Markham, Canada) and amplicons were directly sequenced. We identified and examined the expression patterns of elongation factor α1 (*Pdefα1*), aldehyde dehydrogenase type 1 (*Pdaldh1*), actin (*Pdact*), SAND family protein (*Pdsand*), Tubilin β6 (*Pdtub 6*) and TATA-box-binding protein (*Pdtbp*) genes in response to Cd, Cu or Cr stress conditions. Quantitative real-time PCR (qPCR) analysis of gene expression was performed on reverse transcribed RNAs extracted from *Phoenix dactylifera* using MESA Blue qPCR Master Mix reagent kit (Eurogentec, Seraing, Belgium). qPCR reactions were performed with a LightCycler 480 Real Time PCR system (Roche Diagnostics, Mannheim, Germany). Expression levels and amplification efficiencies (E) of each gene, reference (Rg), and target genes (Tg) were determined according to previously described procedures (Brulle et al., 2006). Reference genes were selected using BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004), and geNorm (Vandesompele et al., 2002) algorithms as per Brulle et al. (2014).

2.9. Relative quantification and expression level

Real-time PCR efficiencies (E) were calculated from the given slope of the standard curve according to the equation $E = 10^{(-1/slope)}$. E values ranged from 1.91 to 2 (with 100% = 2) and calculated from standard curve. Moreover, *PdPCS* expression levels were calculated compared to those of the selected reference genes. The average of the 3 most stable reference genes in each stress condition was used to calculate expression of candidate gene levels according to Brulle et al. (2014). Expression ratio (R) was calculated according to the formula $R = (ETg)^{CPTg} / (Eref)^{CPref}$.

3. Results

3.1. Gene identification and genomic structure

The screening of a cDNA library of *P. dactylifera* leaves obtained previously in the lab was performed. A 5764 base pair sequence encoding PdPCS1 (*Pdpcs*) was found, including 8 exons and 7 introns determined with means of Eukaryotic GeneMark.hmm and GSDS 2.0 software (Lomsadze et al., 2005; Fig. 1). The codon sequences were collected and verified using the FGENESH 2.6 software and BLASTx. The PCR amplification conditions were successfully optimized using the degenerate primers indicated in material and methods. Its product was sequenced and a 1587 base pair mRNA sequence encoding *Pdpcs1* was obtained.

BLASTx searches for additional *pcs* cDNA sequences were performed using the date palm phytochelatin synthase mRNA as a query sequence. From the NCBI and PlantGDB databases we selected 84 sequences of the PCS family from different taxa. *pcs* homologues were found in basically all monocots and dicots for which an appreciable amount of sequence data is available. Sequences from 45 taxa in eight families of angiosperms have been collected (Supplemental Table S2). Also, *pcs* genes were found in Bilateria, bacteria, fungi and ferns. We identified two sequences from algal species *Nannochloropsis gaditana* and *Thalassiosira pseudonana* and two more from amoeba species *Dictyostelium fasciculatum* and *Dictyostelium discoideum*. Nucleotide sequence alignment showed homology between *Pdpcs1* and other *pcs* sequences higher than 70%, and largely confined to highly conserved regions in the 5' terminal sequence in which nucleotide identity was as high as 84–

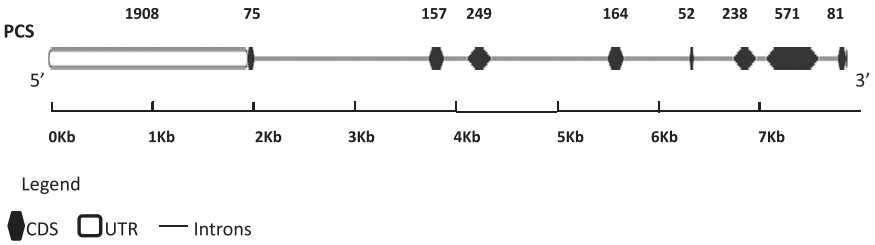

Fig. 1. Genomic organization of the *Pdpcs1* gene. Black boxes: exons, lines: introns, empty boxes: untranslated region (UTR) and numbers: nucleotide position.

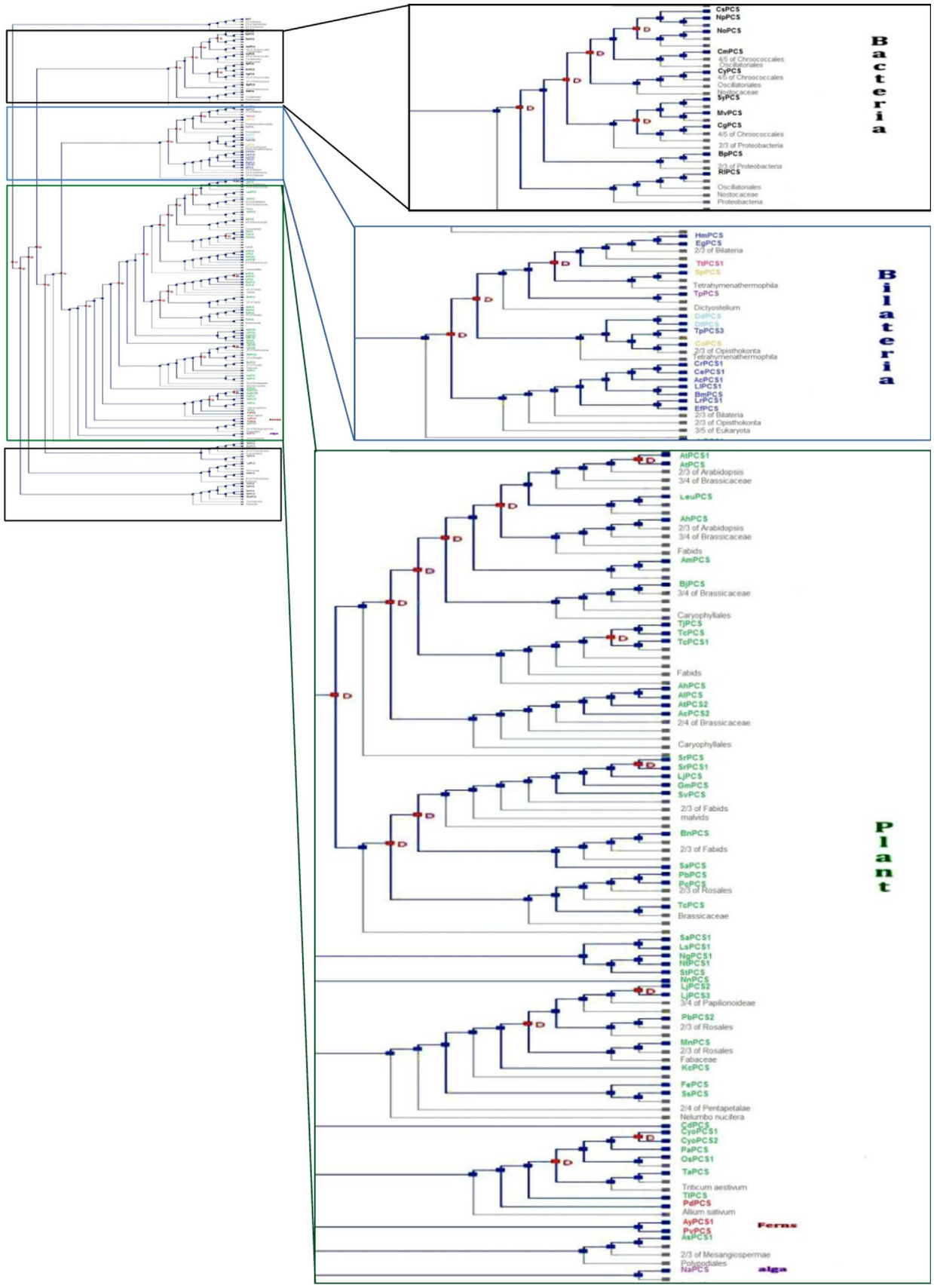

Fig. 2. The phylogeny of *pcs* indicates multiple independent duplication events throughout its evolutionary history. The reconciled *pcs* tree obtained using NOTUNG v2.6, has a duplication/loss score of 27.5. Node" D" for duplication event and grey writing for loss event.

95%. It presented 75% identity to the *pcs* sequence from *Typha latifolia*, and 73% identity to that of *Nelumbo nucifera*.

3.2. pcs *gene duplication events*

Numerous studies have revealed different fates for duplicate genes in plant evolution (Kramer et al., 2004; Causier et al., 2005; Yamaguchi et al., 2006). The reconstruction of *pcs* gene duplication and loss history was performed using NOTUNG v2.6 (Vernot et al., 2008). Based on the reconciled phylogeny (Fig. 2), we have estimated 30 independent gene duplication and 109 gene loss events during its diversification. Sequences were duplicated first into two different groups; Proteobacteria and cyanobacteria. Dissimilar to bacteria groups, the Bilateria contain members of algae, fungi and protozoa *pcs* genes (Fig. 2). Moreover, plants clade contains relatively large numbers of *PCS* homologues compared to the three first clades. Eleven gene duplication events in *pcs* appear toward interior nodes of the reconciled tree and 4 gene duplication events appear toward the terminal nodes.

3.3. *PCS protein phylogeny and ancestral sequence prediction*

In order to assess PCS distribution in nature, a phylogenetic tree based on the full length of 84 protein sequences has been built. The tree was made using PhyML v3.6 software based on a matrix using Maximum Likelihood method with 100 bootstrap replications. The sequences were grouped into 4 main clades that reflect the phylum of origin of the PCS sequences (Fig. 3). About 62% of branches are strongly supported (> 75% bootstrap). In addition, protein-tree analysis showed a remarkably wide and scattered distribution of PCS within the eukaryotic branches. Amoeba, fungi, algae and two bilateria species (HmPCS and EgPCS) were all strongly supported as monophyletic groups and were sisters to all other bilaterian protein sequences with 78% bootstrap support (Fig. 3, Clade II). In addition, the tree showed that PdPCS1 branches (79% bootstrap support) in a common sub-clade together with homologues from other monocots, including *Typha latifolia*, *Triticum aestivum*, *Oryza sativa*, *Phragmite australis* and *Cynodon dactylon*, and separated from the sub-clade that includes fern homologues (79% bootstrap support).

To provide more direct observation of PCS protein evolutionary history, the sequence of ancient protein has been predicted using the ANCESCON program with default parameters. In our study, the common ancestor of all PCS amino acid sequences was presented in node **A** (Fig. 4), where branches join, and the most recent common ancestor (MRCA) to PdPCS1 was represented in node **B** (Fig. 4). Surprisingly, protein sequences of bacteria (Cyanobacteria, Proteobacteria) and Bilateria have diverged significantly, relative to the ancestral protein (node A) and to Mesangiospermae amino acid sequences, suggesting that these sequences have diverged so far that evolutionary relationships are no longer readily apparent.

For truly related proteins, even those which are quite divergent, it is often possible to identify locally conserved regions that are important for structure and/or function. Therefore, our next approach was to search for such motifs using Block Maker (Henikoff et al., 1995). Major blocks of conserved sequences have been identified. Thirteen distinct motifs were found (Supplementary Fig. S2) and were highly conserved especially in the N-terminal region (residues 12–218) compared to the C-terminus. The aligned sequences containing the first 7 motifs which constitute the conserved N-ter domain confirmed previous results and represented 95% of the homology between PCS proteins (Supplementary Fig. S2).

3.4. *PdPCS1 molecular model*

The putative PdPCS1 protein sequence contains 528 amino acids, has a predicted relative molecular weight of 58.39 kDa and theoretical isoelectric point of 6.13. The high instability index (II; 43.91) classifies the protein as unstable.

The tertiary structure PdPCS1 was predicted *in silico* by using the *Nostoc* sp. phytochelatin synthase NsPCS partial structure as a template (Fig. 5). Kinetic studies indicated a substituted enzyme mechanism with the creation of two substrate binding sites in PCS, a high and a low-affinity binding site for GSH to provide the energy for the formation of the new peptide bond in the presence of Cd^{2+}(Vatamaniuk et al., 2002). Five amino acid residues that are highly conserved in plant PCS, namely the cysteine residues at positions 56, 90, 91, 109 and 113, have been found in our PdPCS1 sequence. The first catalytic site includes Cys 56 His 162 and Asp 180 (Fig. 5a). Three pairs of cysteine residues (amino acids 331 – 332, 351 – 352 and 417 – 418) are present in the C-terminal variable domain and are suggested to be form one or more heavy metal binding sites (Vatamaniuk et al., 2002).

Furthermore, Ju-Chen et al. (2013) proposed that a cavity in proximity to the active site of AtPCS might represent the second substrate binding site in which PC molecules were generated from a γGlu-Cys unit and a Cd-GS2 molecule as a second substrate. Our PdPCS1 model showed a similar cavity (Fig. 5b). The second substrate binding cavity is shaped and surrounded by several key amino acids strictly conserved among the PCS sequences, including two basic (Arg 152 and Lys 185), one acidic (Glu 52), three aromatic (Tyr 55, Phe 184 and Tyr 186), and two amide residues (Gln 50 and Gln 157; Fig. 5b).

3.5. *Reference gene validation and* Pdpcs1 *gene expression*

In order to analyze qPCR measurements, internal control namely reference genes that exhibit minimal expression variation in our experimental conditions, are needed. Gene expression measurement on hypocotyls of date palm exposed to 30 days of heavy metal stress revealed many genes whose expression is influenced by this stress, including some reference gene candidates. The three most stable reference genes in each condition determined with BestKeeper, NormFinder and geNorm algorithms were used to normalize target gene expression. Under control conditions, *PdACT*, *PdTBP* and *PdSAND* were found to be the most appropriate reference genes for normalizing transcript level in palm date tissues. Under conditions of Cd exposure, *PdTBP*, *PdACT* and *PdEFα1* were the most stable reference genes; for Cu exposure, *PdEFα1*, *PdALDH1* and *PdTUB6* showed no significant expression variation; under Cr stress, *PdALDH1*, *PdACT* and *PdEFα1* showed constant expression even at higher metal concentrations. To assess the reliability of the selected control genes, we determined the expression profile of *PdPCS1* in date palm normalized against its geometric mean. Fig. 6 depicts gene expression for control and stress conditions (2.27 units) of mRNA of hypocotyl harvested 30 days' post-germination. *PdPCS1* expression responded to all tested heavy metals. Transcript levels were more induced by cadmium than copper, but the largest induction (3.87 fold more than the control) was observed with seedlings treated with K_2CrO_4 (Fig. 6).

4. Discussion

The date palm genome has 36 chromosomes (2 n = 2x = 36), with and estimated size of 550 Mb (Malek, 2010) to ~658 Mb (Al-Dous et al., 2011). Compared to many other crop species, there has been relatively little investment in date palm molecular genetics research, resulting in the serious constraint of an under-developed infrastructure of genetic and genomic tools (Zhao et al., 2013). Interestingly, molecular mechanisms involved in palm resistance to heavy metals remain unexplained. For these reasons, cDNA extraction was performed in order to get an idea of date palm gene diversification looking essentially for biomarkers of metallic stress in *Phoenix dactylifera*.

4.1. PdPCS1 *gene isolation and evolution*

The assembling of nucleotide fragments provided a full length *Pdpcs1* mRNA covering 3495 nucleotides, including a 1587 base pair

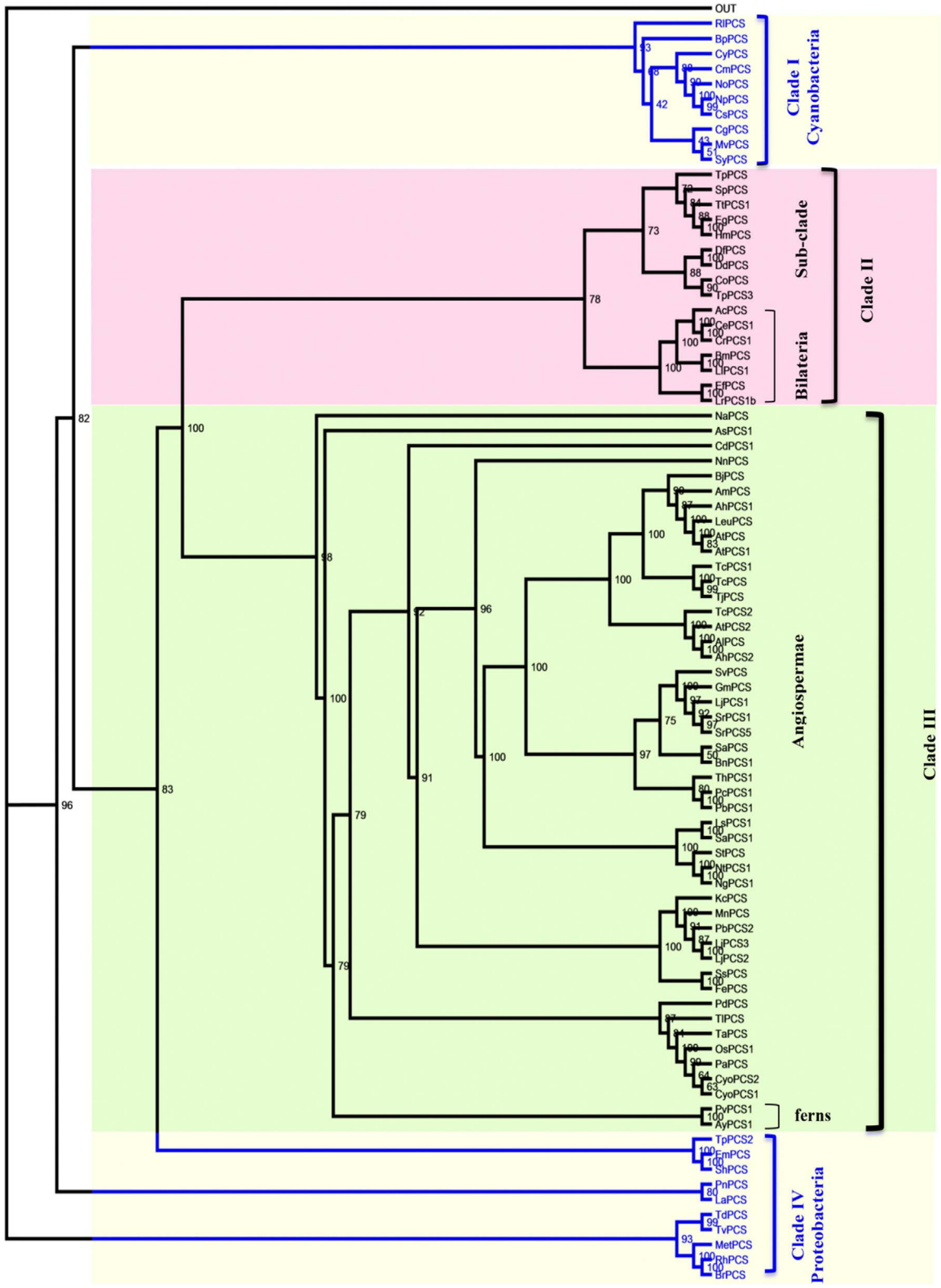

Fig. 3. Phylogenetic relationships of the phytochelatin synthase proteins from NCBI and the PlantGDB database. The tree was constructed from the alignment using ClustalW and the Maximum Likelihood method with PhyML v3.6. The root was placed using the Human syntaxin 1B protein as outgroup. Numbers on branches indicate the percentage of 100 bootstrap replicates that support the adjacent node; low bootstrap support (< 50%) was not reported.

ORF, and 1908 nucleotides of 5′ untranslated region (5′ UTR) containing a TATA box region (Fig. 1). The gene sequence consists of 5764 base pair, 8 exons and 7 introns, is longer than other plant *pcs* genes such as that from pear (*Pyrus betulaefolia*; You et al., 2012), however, it has the same introns and exons number as *Pbpcs1* and *Ljpcs1*. Yet, *Atpcs1* consists of nine exons. Exons 1, 2, 4 and 5 of *Pdpcs1* gene have identical sizes with those in *Atpcs1*, *Ljpcs1* and *Pbpcs1* (75, 157, 164 and 52 base pair (You et al., 2012)).

A BLAST search of the NCBI recovered *pcs* genes from many taxa. Data clearly support the notion that *pcs* genes are present at least in all

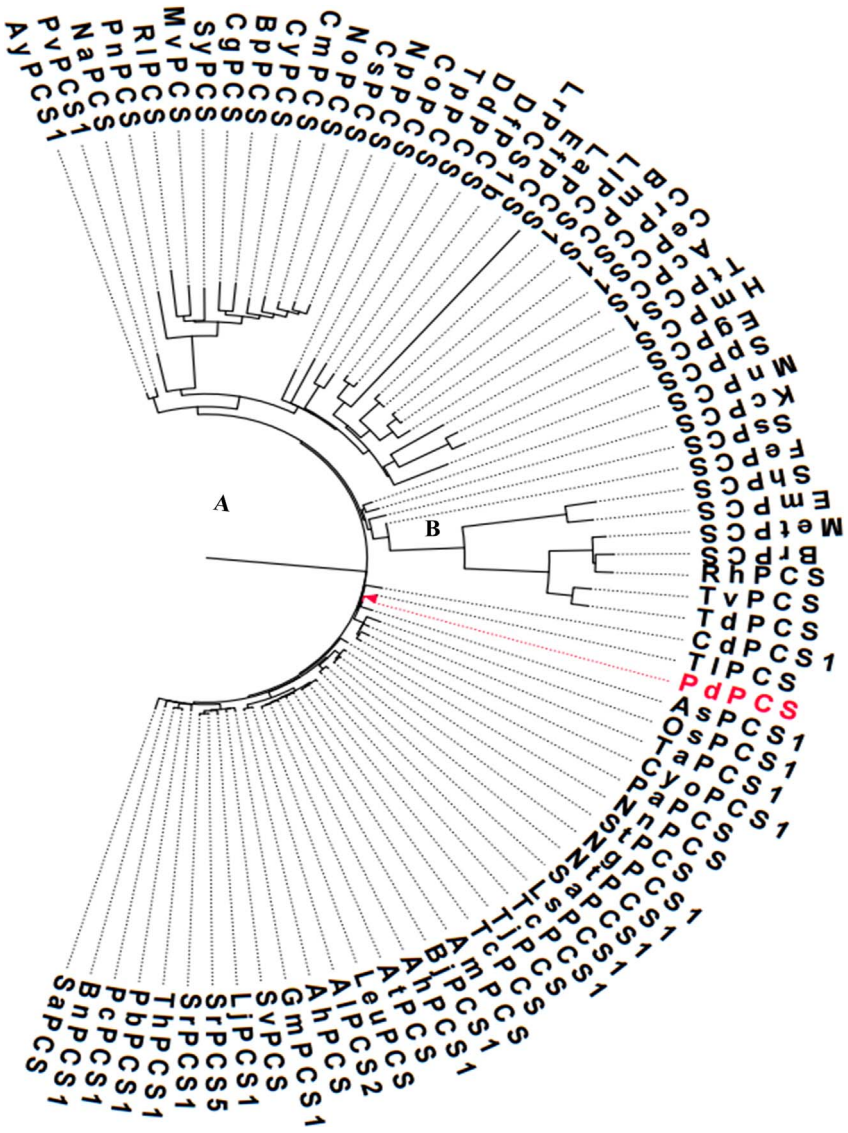

Fig. 4. The tree representing ancestral sequence reconstruction constructed using the ANCESCON program. Evolutionary distances are shown to scale. Nodes A (the ancestor of all PCS amino acid sequences) and B (most recent common ancestor to PdPCS1) are indicated.

higher plants including angiosperms. However, even in the PlantGDB database, no *pcs* sequences from gymnosperms were recovered. Nevertheless, much still remains to be learned about the evolutionary outcome of duplicated genes regarding their impact on genetic pathway and trait evolution (Preston and Hileman, 2013). Gene duplication is common in plants and plays a key role in trait evolution (Airoldi and Davies, 2013). The resulting Notung tree (Fig. 2) showed multiple independent duplication events which occurred throughout the evolutionary history of the *pcs*. During its diversification, *pcs* was subject to 109 gene loss and 30 gene duplication events. Losses were predicted from several lineages during evolution including bacteria, fungi, Bilateria and Angiospermae which may simply represent unidentified genes. However, duplication of *pcs* appears likely in some species such *Arabidopsis*, *Triticum* and *Allium*. The first duplication was retained in Proteobacteria and Cyanobacteria, indicating a likely sub-functionalization. In fact, species development and especially photosynthetic potential acquired by Cyanobacteria may be the cause of the functional evolution of *pcs* gene through the partitioning of ancestral functions. Moreover, the heterogeneity of the Bilateria evident in the notoung tree may be due to animal *pcs* genes which are sparsely found across the tree because they are not found in all animal phyla; and major sub-groups within phyla may be lacking *pcs* homologues. In the other hand, the *pcs* plant clade includes eleven gene duplication events toward interior nodes of the reconciled tree and four gene duplication events toward the terminal nodes indicating that these latter events were more recent in evolutionary time (Fig. 2). All four duplications appear in species that have a second copy of *pcs* suggesting that this gene evolves through subfunctionalization. Moreover, one of the strongest evolutionary pressures for land colonization by plants (and by other phototrophic organisms, such as lichens) arose from their potential access to much greater amounts of nutritive ions from surface rocks, compared with aqueous environments (Ligrone et al., 2012). As a consequence, land colonization may have resulted in a need to carefully regulate trace element homeostasis and to minimize the risk of accumulation of toxic concentrations of certain metal(loid)s, thus leading to the evolution of response mechanisms among which PC synthesis plays a central role (Sanità diToppi and Gabbrielli, 1999). In fact, climate change and the increase of heavy metals widespread in ecosystems may require plants to modify their defense machinery. Furthermore, dimensional increases of the enzyme length were found in angiosperms as demonstrated in Supplemented material S1. Similar findings were presented by Rea (2012) and further explored by Petraglia et al. (2013), who found that improve of enzyme functionality can be mainly ascribed to increases in the length of the C-terminal domain.

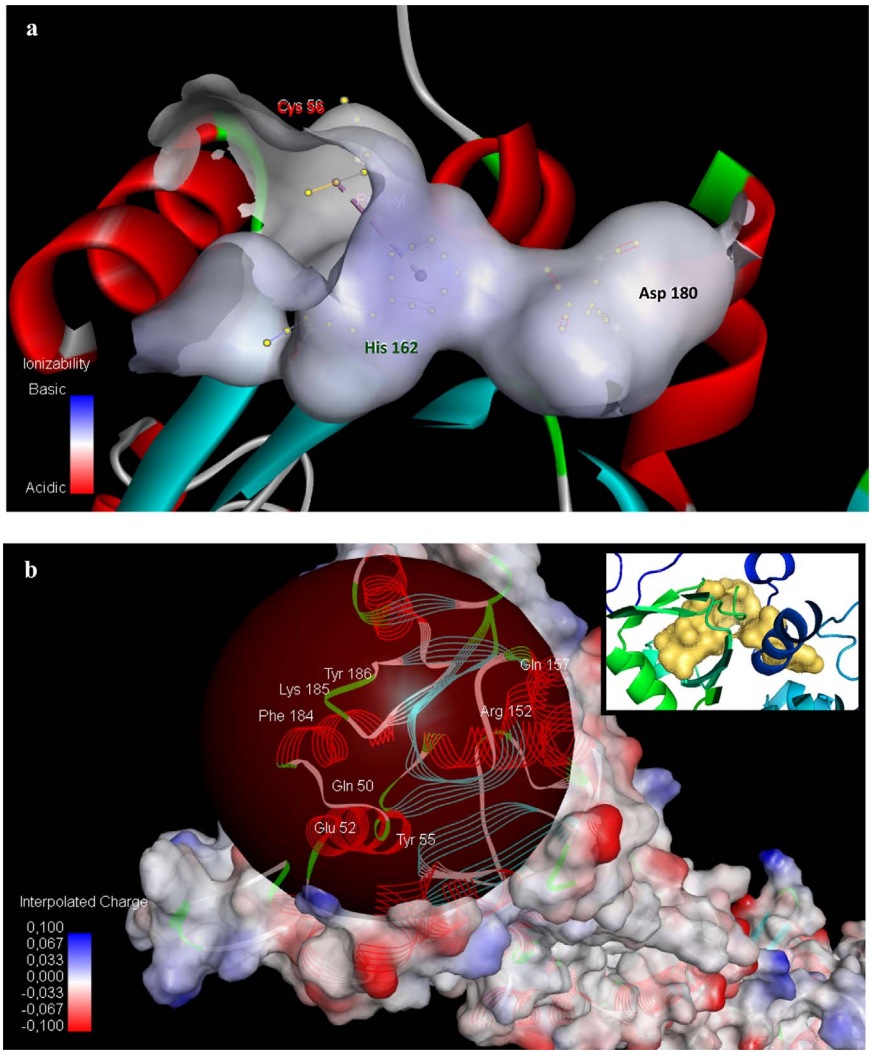

Fig. 5. An overview of the molecular model of PdPCS1, with the putative second substrate binding cavity. (a) The catalytic triad (Cys 56, His 162, and Asp 180) is also shown. (b) The eight conserved residues involved in the formation of the cavity (Gln 50, Arg 152, Gln 157, Phe 184, Lys 185, and Tyr 186, Glu 52 and Tyr 55). The structures were predicted using the coordinates of *Nostoc* sp. phytochelatin synthase NsPCS as a template in Discovery Studio 3.0 (Accelrys). The resulting docking conformation was visualized using PyMOL.

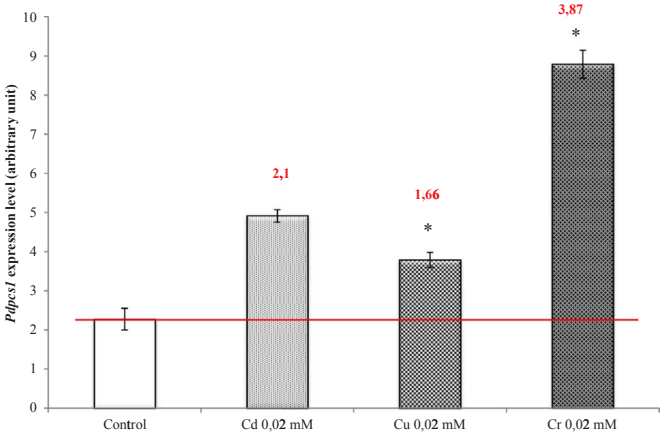

Fig. 6. *Pdpcs1* gene expression levels in *Phoenix dactylifera* seeds hypocotyl of 30 days of germination under Cd, Cu or Cr stress conditions. Data presented are means ± standard error of three independent experiments. * $P < 0.05$ indicates experimental mean is significantly different from the control (Student's *t*-test).

Examination of the phylogenetic tree (Fig. 2) reveals that monophyletic groups are widely scattered, which may suggest, as suggested by Clemens, (2006), that the *pcs* was acquired via horizontal gene transfer (HGT) between eukaryote lineages. However, fern *pcs* genes (*Athyrium yokoscense Aypcs1* and *Pteris vittata Pvpcs1*) as well as *Pdpcs1* showed fewer duplication events and include a highly conserved domain (Supplementary Fig. S1). We hypothesize that fern *PCS* homologues might be an example of an intermediate evolutionary form supporting previous findings that angiosperm homologues are highly derived, while the date palm *PCS* is primitive and classified as one of the world's first cultivated fruit trees representing an ancient group of fruit trees (Zhao et al., 2013).

4.2. PCS protein phylogeny

The PHYLIP generated protein tree showed a distribution of the PCS across many phyla including Bilateria (Fig. 3). These findings contradict previous reports (Clemens, 2006) suggesting that PCs have not been detected in any organisms carrying PCS other than plants, fungi and algae. However, to date, PCS homologues have been identified in only a very small number of animal species (and biochemical characterization of recombinant PCS is so far restricted to proteins from *C. elegans*, *S. mansoni*, and *A. ceylanicum*). In fact, apparent *PCS* homologues are found across the deepest divisions of the Metazoa (Bundy and Kille, 2014). Still, within the phylum Nematoda, Rigouin et al. (2013) looked at the parasitic species *A. ceylanicum*, and showed that the AcPCS also produced PCs when expressed in *S. cerevisiae*. Furthermore, they reported close homologues of PCS in other parasitic nematode species,

including *Brugia malayi*, *Loa loa*, and *Ascaris suum*. Unlike the situation for *C. elegans*, though, exposing the flukes to Cd did not result in any detectable PCs in tissue extracts (Cui et al., 2007). Nonetheless, this distribution argues that it is likely to be found in more animal species in the future.

PCS clade III (Fig. 3) proteins are characterized by their large size including seed plants and ferns which formed a sub-clade. Ferns and angiosperm strongly supported monophyletic groups, but their basal relationships were not fully resolved. They shared a common ancestor suggesting a probability that too many reversals of nucleotide substitutions prohibit a reliable phylogenetic reconstruction (Fig. 4). Yet, ancestral sequence reconstruction (ASR) has proved a useful experimental tool for studying the diverse structure and function of proteins (Harms and Thornton, 2010). To date, ASR has been applied to several enzymes (Perez-jimenez et al., 2011), including photo-reactive proteins (Field and Matz, 2010), viral proteins (Kaiser and Malik, 2007) and a number of peptides (Hult et al., 2008).

The PHYLIP tree (Fig. 3) showed first divergence of the bacterial PCS protein which suggests that evolutionary relationships are obscured due to the shorter PCS length sequences (Supplemental Table S2). In fact, *PCS* genes from prokaryotes, including two *Nostoc* species and five of proteobacterial origin, are approximately one-half the length of their cognates from eukaryotes because they lack the variable C-ter domain (starting from approximately amino acid residue 220; Rea et al., 2012). This domain is generally less well conserved between PCS proteins and is present only in eukaryotic, but not prokaryotic species (Rigouin et al., 2013). The occurrence of the half-size PCS proteins in prokaryotes is extremely scattered. Out of more than 2000 analyzed bacterial genomes only 38 have *pcs* genes (Clemens and Persoh, 2009). Remarkably, even sister strains often differ in the possession of PCS-like genes. Information on metal tolerance of these strains or their ability to synthesize phytochelatins is, to our knowledge, not available.

Therefore, we are interested in major blocks of conserved sequences. We have identified thirteen highly conserved motifs, especially in the N-terminal region (residues 12–218; Supplementary Fig. S2). The N-ter represents the catalytic active domain of PCS (Cys 56, His 162 and Asp 180; Supplementary Fig. S2), whereas, C-ter PCS function is not completely understood. It was suggested to function in metal regulation of protein activity in the *Arabidopsis thaliana* PCS (Romanyuk et al., 2006) and recently, Rigouin et al. (2013) shown that the C-ter domain of *Schistosoma mansoni* PCS is neither involved in metal sensitivity nor in the modulation of protein activity. However, the lack of significant overall sequence identity between the members of these groups could indicate that these proteins arrived at a common function by convergent evolution from different progenitors.

4.3. PdPCS1 structure

In AtPCS1, Cys 56, His 162 and Asp 180 were demonstrated to be able to catalyze a transpeptidation (Rea et al., 2004) and so were confirmed to be the catalytic triad, explaining the mechanism of γ-glutamylcysteine synthesis (Ju-Chen et al., 2013). Indeed, in our model (Fig. 5a), the same residues have been found describing the first site of PdPCS1 acylation. Residue Cys 56 (which is 100% conserved in all known PCS) was subsequently proven to be indispensable for PC synthesis activity and to represent the first site of Cd^{2+} independent acylation (Clemens, 2006). It catalyzes the removal of Gly on the first GSH, resulting in the formation of the γGlu-Cys acyl-enzyme intermediate.

For the second binding cavity (Fig. 5b), the positive charge of Lys 185 might attract the carboxyl group, leaving the free amino group of the second substrate available to attack the acylated γGlu-Cys on the active site and produce PC (Ju-Chen et al., 2013). The carboxyl group on Glu 52 might couple with the cationic Cd through ionic interaction, whereas the aromatic group on Tyr 55 might produce a cation-π complex with Cd (Singh et al., 2009). Arg 152 and Lys 185 are two basic amino acids that might play roles in interaction with the second GSH. However, Lys 185 might directly interact with the second substrate, whereas Arg 152 stabilizes the binding site structure through noncovalent bonds with residues His 162, Asp 180, and Tyr 186 (Ju-Chen et al., 2013). Gln 50 may stabilize the tetrahedral transition state assumed by the first substrate. Modifications of the chain length at this residue completely abolish catalytic activity (Rea, 2012). Our hypothesized interaction between Thr 49 and Arg 183 may act to connect B-loop 1 and B-loop 4. This interaction is triggered by protein phosphorylation and might be critical for the formation of a functional catalytic site (Ju-Chen et al., 2013).

Based on the model of the N-ter structure of AtPCS1, we gained some information on the tentative second substrate binding site on PdPCS1. Differences between bacterial proteins, with lower synthetic activity and lack of the C-ter, and eukaryotic PCS proteins in structure and activity likely reflects the bi-functionality of eukaryotic phytochelatin synthases as peptidases and transpeptidases (Clemens and Persoh, 2009). Recent observations indicate that PCS serves physiological functions aside from metal detoxification. They are hypothesized to be involved in Zn homeostasis, plant defense responses which rely on surveillance systems for nonself recognition and may act as a peptidase in indole glucosinolate metabolism (Clemens and Persoh, 2009).

4.4. PdPCS1 *gene expression pattern*

The hypothetical detoxification mechanism catalyzed by the *Phoenix dactylifera* PCS has yet to be demonstrated. We used an *in silico* approach to identify key features of the protein and molecular expression techniques to investigate whether the gene is active. Based on the present study, we provide evidence that *Pdpcs*1 gene is part of the tolerance mechanism to heavy metal stress in seedling hypocotyls. The expression levels of *Pdpcs1* gene were analyzed by quantitative real-time PCR (Fig. 6). This technique has been adopted in a wide range of scientific areas and research (Pfaffl et al., 2004) because on its advantages in sensitivity, specificity and dynamic range (Bustin, 2000). However, evaluation of qPCR data requires identification of stable reference genes to serve as internal controls. In this study we identified the three most stably expressed housekeeping genes for each tested condition and we used them in normalizing the gene expression levels. Although several studies have used qPCR for analysis of date palm gene expression, none has validated reference genes before. Under control conditions, *PdPCS1* expression was 2.27 arbitrary units 30 days after germination. *PdPCS1* expression was induced by a fairly low Cu^{2+} concentration (0.02 mM), and even more so by Cd^{2+} and especially Cr^{6+}(Fig. 6). Similarly, the transcript levels of phytochelatin synthase type 1 of *Laetuca sativa* (*Lspcs1*) on leaf and root were all much higher under cadmium stress (Zhenyan et al., 2005). Yet, *Avicennia germinans* phytochelatin synthase gene *Avpcs* expression showed a significant increase under copper conditions from the first hours of stress application (Gonzalez-Mendoza et al., 2007). Authors suggested that this rapid increase in *Avpcs* expression confirm that it was the most active gene involved in the regulation of copper in *A. germinans* leaves. Authors demonstrated that the time influence the transcript levels of *Lspcs1* to reach peak level. Leaves required 12 h to exhibit maximum expression level, while root expression peak after 2 h in the Cd treatment (Zhenyan et al., 2005). However, *Phoenix dactylifera* seed germination was completed within 40–45 days after generating a cotyledon leaf developed from the hypocotyls used for RNA extractions. Indeed, previous research reported absence of metal-sequestering phytochelatins under Cr^{6+} stress (Shanker et al., 2004). Manipulation of enzymes involved in the PC biosynthesis pathway (*GS*, *Tapcs1*, *Atpcs1*) leads to higher PC production, and consequently higher heavy metal tolerance and accumulation in plants (Gasic and Korban, 2007) and bacteria (Sauge-Merle et al., 2003).

5. Conclusion

Phytochelatins are essential for toxic element detoxification in plants, some fungi, and animals. The fact that the responsible enzymes, phytochelatin synthases (PCS), are in most species constitutively expressed and so widespread in nature, raises questions. The *pcs* gene trees barely reflected the currently accepted phylogenetic relationships, as the genes of major lineages did not cluster together. Yet, enzyme function was proven for three model systems from three different kingdoms (plant, fungi, animals), as well as prokaryotes. EST data clearly support the notion that *pcs* genes are present in all higher plants. In addition, based on the identification of the consensus amino acids surrounding the active site, we hypothesize a possible binding and catalytic mechanism for the second substrate binding cavity on PdPCS1. Data mining and an *in silico* approach led to the identification and description of a *Pdpcs1* in date palm and to a prediction of its protein structure. The results reported by qPCR showed that *Pdpcs1* gene is not only present but fully operational in *P. dactylifera*. Its level of expression may vary in seedlings exposed to metals suggesting that *Pdpcs1* gene product is involved in tolerance to metals.

Conflict of interest

The authors declare that they have no conflict of interest and there are responsible for the content and writing of the article.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2017.02.020.

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Environmental Toxicology

Copper Toxicity and Date Palm (*Phoenix dactylifera*) Seedling Tolerance: Monitoring of Related Biomarkers

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Abstract: Date palm (*Phoenix dactylifera*) seeds were exposed to different copper (Cu) solutions to examine plant stress responses. Low Cu concentrations (0.02 and 0.2 mM) caused an increase of seed germination, whereas higher Cu amounts (2 mM) significantly inhibited seed germination, delayed hypocotyl elongation, increased seedling mortality, and reduced the germination index by more than 90%. Metal-related toxicity symptoms appeared after 15 d of 2 mM of Cu exposure. Biochemical activities such as amylase activity and redox balance elements were examined to study the relationship between external Cu amount and internal plant response. The present study showed that amylolytic activity was dose- and time-dependent. Likewise, H_2O_2 production increased after exposure to Cu, which was correlated with thiobarbituric acid reactive substance (TBARS) accumulation. Furthermore at low Cu concentrations, superoxide dismutase (SOD) and catalase (CAT) activities increased, suggesting that date palm seed stimulated its metal homeostasis networks. However, the highest cupric ion amounts increased cell oxidant accumulation and reduced enzyme production. Gene expression level measures of *P. dactylifera* phytochelatin synthase (Pd*pcs*) and *P. dactylifera* metallothionein (Pd*mt*) encoding genes have been carried out to investigate the implication of PdPCS and PdMT proteins in Cu homeostasis and/or its sequestration. *Phoenix dactylifera* metallothionein induction reached a peak after 30 d of exposure to 0.2 mM of Cu. However, it was down-regulated in plants exposed to higher Cu concentrations. In the same conditions, *Pdpcs* was overexpressed during 1 mo of exposure before it decreased thereafter. These observations provide a new insight into date palm cell response to Cu, a metal that can be toxic but that is also an essential element. *Environ Toxicol Chem* 2017;9999:1–10. © 2017 SETAC

Keywords: Date palms; Seed germination; Amylase activity; Antioxidative responses; Biomarkers' gene expression

INTRODUCTION

Metals are natural components of terrestrial systems occurring in soil, rock, air, water, and organisms. Several transition metals, including zinc (Zn), manganese (Mn), and copper (Cu), are essential constituents of physiological processes in living organisms including plants. However, their presence in higher amounts makes them not only dangerous poisons for plant life but also toxic pollutants for the environment [1]. The potential toxicity of trace metals in soil depends on their mobility and bioavailability. Naturally, the most radical change in the availability of some transition metal ions was shown when the earth's atmosphere became oxygenic. It dramatically reduced the bioavailability of some transition metals such as iron (Fe) and made others, primarily Cu, more available [2].

Copper is an essential redox-active transition metal involved in many biochemical processes. However, exposure to excess Cu has detrimental eects on plant growth. Among other things, it may reduce biomass, affect root growth and morphology, induce chlorotic symptoms on older leaves, and cause dark-red margin necrosis. Moreover, a decrease of the photochemical activity was shown because of a decrease in the electron transfer rates caused by cupric ions' occupation of several sites of photosystem II particularly susceptible to these ions [3]. Redox properties of Cu^{2+} might increase the production of toxic reactive oxygen species (ROS), particularly the highly reactive hydroxyl radical, via the Haber–Weiss reaction by the biodegradation of membranes and biotransformation of metallic moieties [4,5]. Mechanisms involved in the management of ROS have been extensively studied because they can help to avoid oxidative damages [6,7]. Among these homeostatic mechanisms, plants Q2 promoted the Foyer–Halliwell–Asada pathway, for instance, glutathione reductase and ascorbate peroxidase (APX) responsible for the scavenging of ROS produced in part by superoxide dismutase (SOD). The enzymes, which react with

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superoxide radicals to convert them to O_2 and H_2O_2, represent an early line of defense. Activities of enzymes implied in ROS production and degradation have been extensively studied in numerous plant and animal species to estimate the impact of contaminants, and especially metals, and/or to identify biomarkers of exposure [8].

Exhibiting high affinities to ions, phytochelatins and metallothioneins have been characterized as the 2 most efficient heavy metal binding ligands in plant cells [9]. It has been shown that amounts of phytochelatins increase in response to many heavy metals, including Cu, which constitute a strong activator of enzymes' biosynthesis both in vivo and in vitro [10]. A clear role in Cu detoxification has been shown [11]. During the exposure of plants to metals, phytochelatins are synthesized from glutathione by phytochelatin synthase (PCS) activities [12]. Phytochelatin synthase activities have been well described in plants, which makes the characterization of the genes encoding these enzymes attractive [13]. These dumbbell-type enzymes contain 2 metal-binding and cysteine-rich domains. In addition, metallothioneins are small cysteine-rich proteins produced directly by mRNA translation. Heavy metals such as Cu and arsenic (As) greatly induced the expression of the metallothionein gene (*mt*) in *Festuca rubra* [14] and *Fucus vesiculosus* [15], providing evidence to heavy metal detoxification of metallothionein proteins in these plant species.

Previously cited molecules (ROS, antioxidant enzymes, chelators, etc.) offer a battery of potential biomarkers. Nevertheless, further identification of specific molecular, biochemical, physiological, and morphological changes in plants after cupric stress is necessary. Biomarkers constitute a powerful tool for measuring the extent of exposure and predicting the impacts of metal contaminants in biota [16]. For example, gene expression variations may be "early-warning" signals, enabling a more vigorous environmental monitoring [17] and constitute a suitable and well-established approach for analyzing the effects of heavy metals' exposures [18].

However, very little is known about date palm (*Phoenix dactylifera*) responses to metal-related stress during germination and in the early growing stages. At these stages, seedlings are weak, tender, and equipped with sensing mechanisms that allow them to assure germination when environmental factors are favorable. Hence they can be affected by adverse conditions, making heavy metal tolerance testing at the crop germination stage a key step for the plants' establishment under any prevailing environment [19]. In the present study, Cu toxicity and seedling tolerance were assessed in date palm using individual and infra-infra-individual biomarkers.[Q3]

MATERIALS AND METHODS

Experimental layout, treatment, and seedling growth parameters' measurement

Seeds from the Deglet Nour date palm cultivar were used. Mature seeds were washed extensively in water then surface sterilized with sodium hypochlorite solution. Under sterile conditions, 100% of commercial sodium hypochlorite was added to seeds for 10 min; then 50% of the diluted solution was added for 10 min; and finally 25% more of the diluted sodium hypochlorite was added during the[Q4] same time period. Seeds were then rinsed 3 times for 10 min with distilled water. Earlier, seeds were exposed to direct contact with 0, 0.02, 0.2, or 2 mM of Cu solutions prepared in distilled water using cupric sulfate ($CuSO_4$ $5H_2O$; Sigma-Aldrich) and kept in the dark at 26 °C for 2 mo. Seedlings (10 per replicate; 3 biological replicates per concentration) were excised from seeds at 12, 15, 30, 45, and 60 d after germination.

The inhibition rate of seed germination and seedling growth was calculated using the formula of Ahn et al. [20] (treated samples results/control results × 100).

Germination associate parameters were calculated by using formulas described by Chaâbene et al. [21]. Germination percentage represents the number of germinated seeds/total number of seeds × 100. Speed of germination equals n1/d1 + n2/d2 + n3/d3, and so on, where n is the number of germinated seeds and d represents the number of days. Mean daily germination equals the total number of germinated seeds/total number of days. Mean germination time equals $\Sigma dn/\Sigma n$ where n is the number of seeds germinated on day d and d represents the number of days counted from the beginning of germination. Peak value equals the highest number of seeds germinated/number of days. Seedling mortality equals the number of germinated seeds/total number of seeds × 100. Germination index was measured using the formula: germination index percentage $= \frac{NE \times LE}{NC \times LC} \times 100$ where NE is x, LE is x, NC is x, and LC is x.[Q5]

Estimation of H_2O_2 content and lipid peroxidation

Hydrogen peroxide concentration was determined as previously described by Elleuch et al. [22]. The levels of lipid peroxidation (LPO) products were evaluated as thiobarbituric acid reactive substance (TBARS) aldehydes according to Rustérucci et al. [23]. The concentrations of TBARS were calculated using an extinction coecient of 155 $mM^{-1}cm^{-1}$.

Enzyme analysis

Hypocotyl fresh tissue (0.5 g) was homogenized in 1.5 mL of potassium phosphate buffer 0.1 M, pH 7 containing 4% polyvinylpyrrolidone and centrifuged at 10 000 *g* for 30 min and the supernatant was used as enzyme extract. Total protein content was determined spectrophotometrically according to the method of Bradford [24], using bovine serum albumin as a standard.

Amylase activity, antioxidative enzyme assays, and protein content measurement

Amylase activity was measured as described by Elleuch et al. [22]. The reaction was performed at 60 °C and pH 7 for 10 min. The reaction mixture contained 0.5% (w/v) starch in 25 mM phosphate buffer pH 7 and the enzyme solution in a final

volume of 1 mL. The activity of total amylase enzymes was measured colorimetrically by estimating the concentration of reducing sugar formed (maltose) determined by the 3,5-dinitrosalicylic acid method [25].

Assays of antioxidant enzyme activities in hypocotyl samples were prepared for catalase (CAT) and SOD analyses by homogenization of the frozen tissue with a mortar and pestle in a potassium phosphate buffer 0.1 M, pH 7 (1.5 mL, 0.5 g fresh wt) to extract soluble protein. The homogenate was centrifuged at 13 000 *g* for 15 min. All operations were performed at 4 °C.

Superoxide dismutase activity was assayed using the photochemical nitro blue tetrazolium method and measured according to Beyer and Fridovich [26]. Catalase was determined as described by Aebi [27].

RNA isolation, cDNA synthesis, housekeeping genes validation for Pdpcs *and* Pdmt *expression measurement*

Frozen hypocotyl tissues (~ 100 mg) were ground in liquid nitrogen using a tissue homogenizer. Extraction of RNA and synthesis of cDNA were performed as per Chaâbene et al. [28]. A mixture of frozen seedling tissues of all experimental time points and of all experimental conditions was used for RNA extraction and cDNA synthesis to clone candidate housekeeping genes. Using the previously designed specific primers for housekeeping genes——elongation factor α1 (*Pdefα1*), aldehyde dehydrogenase type 1 (*Pdaldh1*), actine (*Pdact*), SAND family protein (*Pdsand*), Tubiline β6 (*Pdtub6*), and TATA-box-binding protein (*Pdtbp*) and candidate genes *Pdpcs* and *Pdmt* [28]——we performed the quantitative real-time polymerase chain reaction (qPCR) amplification method according to Brulle et al. [29]. The expression levels and amplification efficiencies of reference (housekeeping) and target genes were determined according to previously detailed procedures [29]. To estimate the expression stability of reference genes, we measured the relative quantities of crossing point values of genes obtained using the formula of Hellemans et al. [30]. As shown by Martins et al. [31] different algorithms and statistical analyses were applied to evaluate the expression stability of the reference genes of *P. dactylifera* plants in seedlings under control and Cu toxicity conditions. The procedures of the analyses strictly followed the manuals of BestKeeper [32], NormFinder [33], and geNorm [34] algorithms. The geometric mean of the 3 most stable reference genes in cupric condition was used to calculate expression of target gene levels according to Brulle et al. [35]. Absolute quantification of gene expression levels are in log2. Expression level of control samples versus expression level of exposed samples was normalized to calculate relative fold expression, as discussed by Bernard et al. [8].

Statistical analysis

The data in all experiments are the averages of 3 replicates. All statistical analyses were conducted using STATISTICA Ver 10. Significant differences among parameters were tested using Tukey's honest significant difference test after one-way and 2-way analysis of variances with exposure time and metal concentrations as the 2 factors. A probability value of $p < 0.05$ was considered statistically significant.

RESULTS

Copper effects on date palm seeds' germinability and their relation to physiological perturbations

Date palm hypocotyl always appeared after 12 d in darkness. Germination percentage varied widely (Figure 1A). Low Cu amounts (0.02 and 0.2 mM $CuSO_4$) did not affect long-term seed germination, whereas high Cu concentration (2 mM) significantly reduced seed germination by approximately 20%. The highest germination rate (germination percentage = 100%) was observed after 12 d for seeds exposed to 0.2 mM of Cu and was slightly higher than in control (97%) and in 0.02 mM of Cu (97%) exposed groups. Seed soaking in 2 mM of Cu reached its maximum germination percentage (80.5%) after 20 d of exposure (Figure 1A). The hypocotyl length of treated and untreated date palm seeds was monitored and is shown in Figure 1B. It was found that, except for the highest Cu concentration (2 mM), there were no important differences among treatments. After 60 d of exposure, the best growth (i.e., corresponding to negative inhibitory rate of hypocotyl length) was observed for seeds exposed to 0.02 mM of Cu. Hypocotyl growth was significantly affected by excess Cu. Indeed, a slight inhibitory effect was reported for seeds exposed to 0.2 mM and a strong inhibition of seedling growth (i.e., highest inhibitory rate of hypocotyl growth) was observed when seeds were exposed to

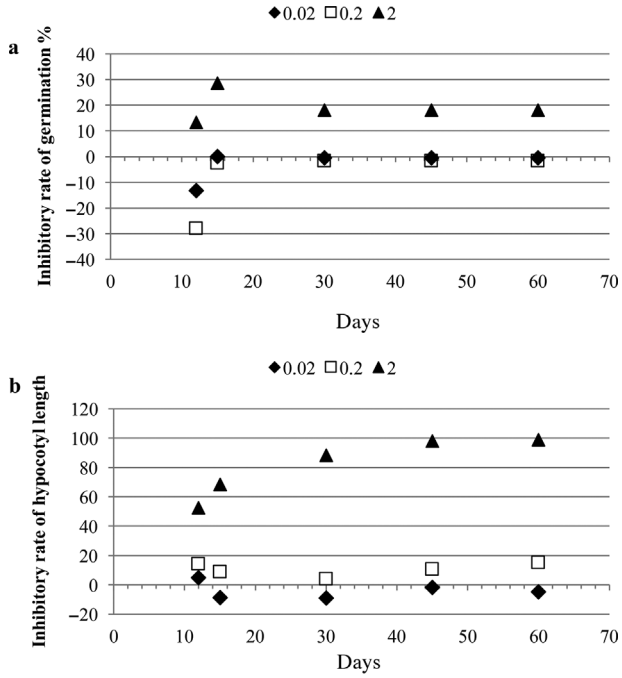

FIGURE 1: Copper effect (**a**) on the inhibitory rate of germination percentage of date palm seeds and (**b**) on the inhibitory rate of hypocotyl length. Data presented are mean ± standard error of 3 independent experiments.

TABLE 1: Copper (Cu) effect on different physiological parameters of date palm (*Phoenix dactylifera*) seed germination

Cu (mM)	SG (s/d)	MDG (s/d)	MGT (d)	PV (s/d)	SLM (%)	GI (%)
0	159.36 ± 5.6	0.163 ± 0.01	287.77 ± 6.4	0.49 ± 0.16	2.78 ± 0.33	100
0.02	167.2 ± 4.3	0.164 ± 0.04	289.28 ± 4.6	0.49 ± 0.03	2.78 ± 0.33	98.04
0.2	172.32 ± 5.3	0.166 ± 0.00	293.93 ± 2.4	0.66 ± 0.00	0 ± 1	84.36
2	138.09 ± 5.9	0.134 ± 0.01	234.11 ± 3.3	0.40 ± 0.02	3.05 ± 2.5	8.37

SG = speed of germination; MDG = mean daily germination; MGT = mean germination time; PV = peak value; SLM = seedling mortality; GI = germination index.

2 mM of Cu. More precisely, after exposure to 2 mM of Cu, hypocotyl elongation stopped after 15 d (Figure 1B). For instance, hypocotyl length was 28.6, 30, 24.3, and 0.3 cm in 0, 0.02, 0.2, and 2 mM of Cu-contaminated media, respectively. Moreover, hypocotyl became considerably or completely necrotic at the end of the treatments with 0.2 and 2 mM of Cu, respectively. Thus it was found that hypocotyl growth was more sensitive to metallic stress than to germination percentage. Additional physiological parameters were measured to investigate what happens during date palm seed germination (Table 1). With a slight enhancement in speed of germination, seeds exposed to 0.02 mM of Cu behaved similarly to untreated seeds and presented almost the same values in mean daily germination, mean germination time, peak value, seedling mortality, and event in the germination index (Table 1). Mean germination time was found to be similar (287, 289, and 294 d) for seeds exposed to the lowest concentrations of Cu (0, 0.02, and 0.2 mM, respectively) but significantly lower (234 d) for seeds exposed to 2 mM of Cu. This indicates that date palm seeds required a long time to accomplish their germination and that addition of Cu may slightly accelerate seed emergence. Moreover, at 0.2 mM of Cu, seedling mortality was nil compared with 2.78 in control conditions, suggesting also that this metal may stimulate seed germination. Maximum speed of germination (172.32 s/d) was recorded in seeds exposed to 0.2 mM of Cu, and it declined by 15% in response to 2 mM of Cu. All the considered parameters improved at 0.2 mM of Cu and declined at higher concentration except for the germination index. The latter parameter showed a harmful decline by more than 90% after treatment with 2 mM of Cu (Table 1). The low value of the germination index obtained confirmed the slow rate of germination obtained at high Cu stress. It also significantly decreased mean daily germination, mean germination time, and peak value.

Copper effects on amylase activity

Date palm (*P. dactylifera*) hypocotyls were used as starting material for monitoring protein amylolytic activities. During the long phase of imbibition or the first 15 d of germination in darkness, amylase levels in seeds were similar for all Cu treatments and comparable with the control (Figure 2). Amylase level presented a steep rise up to 60 d post-germination in seeds exposed to 0, 0.02, and 0.2 mM of Cu and the maximum (38.14 U) was found in hypocotyls exposed to 0.2 mM of Cu. Nevertheless, at higher Cu concentration (2 mM), total amylolytic activity was severely affected (Figure 2); this was observed clearly after 60 d of exposure (amylase activity = 6.18 U compared with 25.09 U under control conditions).

Redox balance perturbation under Cu stress

Under standard conditions, H_2O_2 content in germinating date palm seeds increased gradually over time (Table 2). In the samples treated with 0.02 or 0.2 mM of Cu, the pattern was similar but H_2O_2 contents were always higher than under control conditions. For example, H_2O_2 concentration reached a maximum of 291.08 μmol/g fresh weight after 60 d of exposure to 0.2 mM of Cu. At the same time, H_2O_2 concentration was 182.28 μmol/g fresh weight in control, whereas H_2O_2 content was strongly enhanced in samples treated with 2 mM of Cu. A significant boost of 2.5, 3.6, 2.8, and 3.07 times after 15, 30, 45, and 60 d (respectively) was noted (Table 2). This accumulation of H_2O_2 resulted in deterioration of membrane lipids illustrated by the significant increase in TBARS accumulation observed in cells.

Catalase, the main H_2O_2-scavenging enzyme in plants, exhibited an enhancement of its activity in all cupric conditions compared with control during the first 15 d of exposure. For instance, CAT activity was 2 times higher in seedlings exposed to 0.2 mM of Cu and 3 times higher in seedlings exposed to 2 mM of Cu than in control (Table 2). Catalase activity continued to increase significantly over time in control, 0.02, and 0.2 mM of Cu conditions until the end of the exposure period. In cupric treatment, it was always greater than the corresponding control. The highest enzyme activity level (7.47 ± 0.09 μmol H_2O_2/mg protein) was measured after 60 d of exposure to 0.2 mM of Cu, when the H_2O_2 level in hypocotyl cells was 1.5 times higher than the control. The initial increase in CAT activity was also reported in seedlings exposed to 2 mM of Cu during 12 and 15 d but was followed by a significant drop. Catalase activity remained much lower than in control until the end of the exposure period (Table 2), maintaining heavier redox balance in its oxidative side.

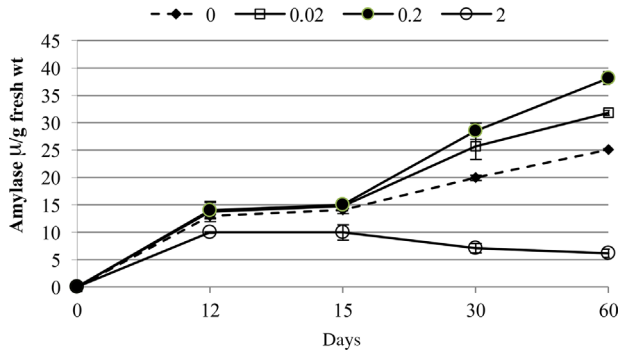

FIGURE 2: Amylase activity in date palm seeds exposed to copper treatment for 60 d. Data presented are mean ± standard error of 3 independent experiments.

TABLE 2: Monitoring of elements of oxidative and antioxidative state of date palm seeds subjected to different copper concentrations Results shown as mean ± standard deviation. Differences between groups are shown as results of one-way analysis of variance post hoc Tukey's test[a]

	Cu (mM)	12 d	15 d	30 d	45 d	60 d
H_2O_2 μmole/g fresh weight						
	0	145.24 ± 4.9a A	158.67 ± 2.3a A	153.56 ± 1.4a AB	195.01 ± 2.9a C	182.28 ± 0.7a BC
	0.02	149.77 ± 5.9a A	149.14 ± 6.8a A	208.78 ± 1.7b BC	227.55 ± 3.6b CD	249.60 ± 0.5a D
	0.2	174.42 ± 2.2a A	190.99 ± 14.1b AB	275.85 ± 7.2c BC	294.18 ± 5.4c C	291.08 ± 4.1a C
	2	279.21 ± 11b A	403.23 ± 9.5c B	552.71 ± 5.2d C	555.68 ± 7.5d C	559.01 ± 7.09b C
CAT activity μmole H_2O_2/mg Protein						
	0	1.66 ± 0.23a A	2.09 ± 0.00a A	3.71 ± 0.3a BC	4.20 ± 0.14a CD	4.88±0.04a D
	0.02	1.97 ± 0.31a A	2.7 ± 0.03b B	5.85 ± 0.19b B	5.6 ± 0.21b B	5.77±0.05b B
	0.2	2.52 ± 0.67a A	4.5 ± 0.7c B	6.36 ± 1.56b C	6.24 ± 0.34b C	7.47±0.09c C
	2	2.89 ± 0.12b A	4.99 ± 0.00d B	2.09 ± 0.28c C	1.95 ± 0.07c C	0.99±0.26d D
TBARS nmole/g fresh weight						
	0	33.67 ± 1.8a A	38.52 ± 2.0a AC	42.43 ± 0.7a BC	52.56 ± 0.6a D	53.08±1.5a D
	0.02	39.75 ± 3.1a A	42.87 ± 1.5a A	66.57 ± 0.8b B	55.56 ± 0.6a C	59.07±1.3a C
	0.2	59.35 ± 0.9b A	56.10 ± 1.2b AB	56.73 ± 3.8c AB	61.10 ± 1.5b AC	71.57±0.8b C
	2	75.06 ± 1.4c A	81.27 ± 2.4c AB	84.2 ± 0.2d BC	90.51 ± 0.7c C	97.09±2.7c C
SOD activity U/mg Protein						
	0	3.00 ± 0.01a A	4.04 ± 0.06a B	9.81 ± 0.16a C	9.95 ± 0.21a C	10.04 ± 0.06a C
	0.02	3.87 ± 0.17b A	4.82 ± 0.1b A	14.35±1.91bc B	15.05±0.21b B	16.65±0.22b B
	0.2	4.07 ± 0.03b A	5.10 ± 0.14cb A	13.23±1.08ac B	16.35±0.63b C	18.17±0.09c C
	2	4.99 ± 0.01c A	3.99 ± 0.0a B	3.04±0.06d C	2.75±0.35c CD	2.14±0.05d D

[a]Lowercase letters show differences between concentrations (in columns) and uppercase letters show differences in time exposure (in rows). Means not showing the same letter are statically different.
Cu = copper; TBARS = thiobarbituric acid reactive substance(s); CAT = catalase; SOD = superoxide dismutase.

Moreover, some necrotic signs in tissue were observed in hypocotyls grown at 2 mM of Cu.

The activity of SOD, an enzyme implied in the elimination of superoxide radicals in cells, presented a pattern almost similar to CAT activity. In controls, SOD activity increased in a linear manner over time. Enzyme activity in hypocotyls exposed to 0.02 and 0.2 mM of Cu was increased significantly by 65 and 80%, respectively (Table 2) at the end of the treatment. Yet higher Cu treatment (2 mM) induced critical reduction in enzyme activity by almost 80% after long-time exposure, inducing TBARS accumulation and growth blockage. These results showed that date palm seeds developed effective protection strategy by expressing CAT enzyme more than SOD activity.

Internal reference gene validation for qPCR analysis

Six housekeeping gene candidates (*Pdefα1*, *Pdact*, *Pdsand*, *Pdaldh1*, *Pdtub6*, and *Pdtbp*) were selected for stability analyses in *P. dactylifera* subjected to cupric stress. The average of cycle threshold (Ct) values and expression levels of each candidate reference gene in 10 different date palm hypocotyl stressed samples were transferred to software, as fully described by Brulle et al. [35]. To ensure the unchanged expression of reference genes under Cu conditions, BestKeeper, geNorm, and NormFinder algorithms were used to select the best stable internal control genes for qPCR normalization. The transcription levels of *Pdefα1* and *Pdaldh1* were higher than the 4 other genes indicated by lower average Ct values of 24.32 and 24.43, respectively (Figure 3), whereas *Pdtbp* displayed the lowest expression crossing point value of 28.68. Ranking expression levels of the 6 genes of the 10 samples were *Pdefα1* > *Pdaldh1* > *Pdact* > *Pdsand* > *Pdtub6* > *Pdtbp* (Figure 3).

The BestKeeper algorithm generated a Pearson's correlation coefficient (*r*). In fact, the most stably expressed gene is the one with the highest BestKeeper correlation coefficient. This algorithm ranked candidate reference genes as: *Pdefα1* > *Pdtub6* > *Pdaldh1* > *Pdact* > *Pdtbp* > *Pdsand* (Table 3). However, geNorm software, with a different approach, provided *M*

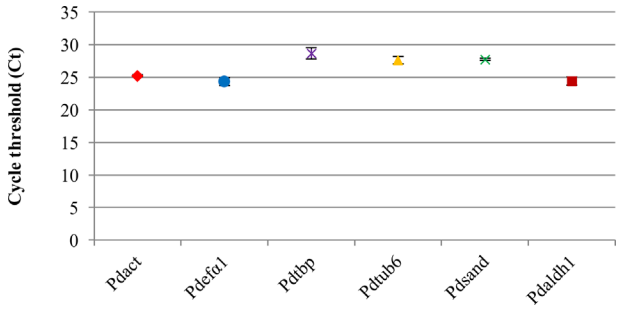

FIGURE 3: Cycle threshold (Ct) of candidate reference genes for each experimental condition. Data presented are mean ± standard error of 3 independent experiments. *Pdact* = *Phoenix dactylifera* actine; *Pdefα1* = *Phoenix dactylifera* elongation factor α1; *Pdtbp* = *Phoenix dactylifera* TATA-box-binding protein; *Pdtub6* = *Phoenix dactylifera* Tubiline β6; *Pdsand* = *Phoenix dactylifera* SAND family protein; *Pdaldh1* = *Phoenix dactylifera* aldehyde dehydrogenase type 1.

value——mean pairwise variation among one gene compared with the others. It considered the gene with the lowest *M* value the most stable gene. Thus, it recommended the use of *Pdtub6*, *Pdtbp*, and *Pdact*. Regarding the variations among algorithm results, we also tested the NormFinder software [36]. According to Expósito-Rodŕguez et al. [37], the top ranked gene is the gene with the lowest standard deviation value. Indeed, NormFinder ranked the 4 most stable genes similar to the geNorm software. Consequently, in the present study, the average Ct values of *Pdtub6*, *Pdtbp*, and *Pdact* were used for qPCR normalization of date palm tissues exposed to cupric stress.

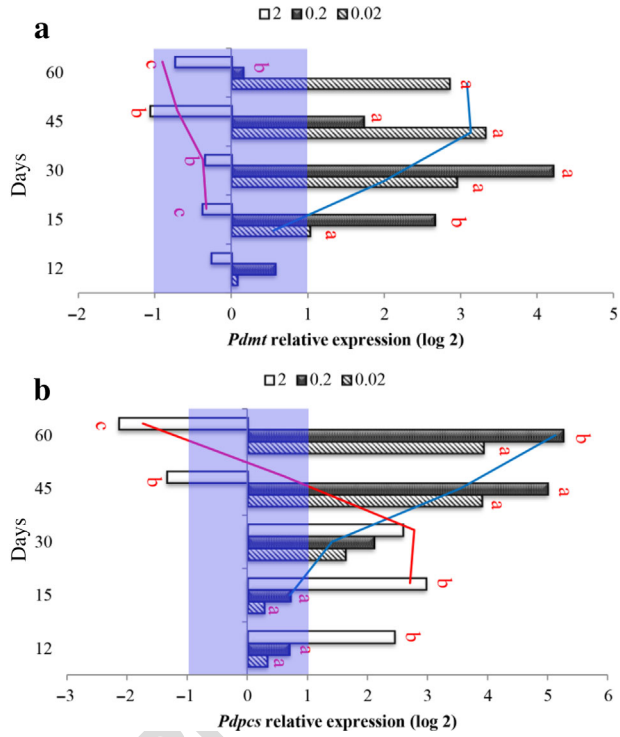

FIGURE 4: Relative expression patterns of (a) *Pdmt* and (b) *Pdpcs* exposed to copper treatment. Blue areas show basal gene variations. Data presented are mean ± standard error of 3 independent experiments. Differences among groups are indicated as results of the Tukey post hoc test. Red lower case letters denote differences among concentrations among time points. Means not displaying the same letter differ statistically. *Pdmt* = *Phoenix dactylifera* metallothionein; *Pdpcs* = *Phoenix dactylifera* phytochelatin synthase.

Pdpcs *and* Pdmt *expression under Cu stress*

To better understand the molecular basis of date palm seeds' tolerance to Cu treatments, the levels of expression of 2 selected genes (*Pdpcs* and *Pdmt*) were measured after scaling with values of untreated samples. Both genes were induced from the first days of germination with 0.02 and 0.2 mM of Cu (Figure 4). The expression of *Pdmt* was detailed by a biphasic curve. The first 45 d of exposure to 0.02 mM of Cu caused linear induction of *Pdmt*, whereas later it decreased up to the level of 1.4-fold higher than in control conditions. These gene inductions observed in the presence of Cu could not be correlated to significant morphological alterations in palm date seed germination after 60 d of exposure. A maximum transcription level of *Pdmt* was observed in hypocotyls exposed to 0.2 mM of Cu (Figure 4A). The *Pdpcs* transcription level increased with augmented exposure time, reaching its maximum at the end of the treatment with 0.02 and 0.2 mM of Cu (Figure 4B). Similar to *Pdmt* relative expression under 0.2 mM of Cu stress, *Pdpcs* was shown with a strong induction (5.25) compared with corresponding control. At a lower copper amount, *Pdpcs* up-regulation reached a plateau at long exposure time. A coordinated expression of *Pdpcs* and *Pdmt* can be suggested to explain at least partially the Cu resistance of *P. dactylifera* seeds at low concentrations. At high Cu concentration (2 mM), destructive effects and gene expression alterations were observed. To illustrate, *Pdmt* and *Pdpcs* were

TABLE 3: BestKeeper Pearson's correlation coefficient (*r*), geNorm *M* values, and NormFinder standard deviation values of 6 date palm (*Phoenix dactylifera*) housekeeping genes

	Pdefα1	*Pdaldh1*	*Pdact*	*Pdsand*	*Pdtub6*	*Pdtbp*
Pearson's correlation coefficient (*r*)	0.886*	0.65*	0.624	0.188	0.781*	0.596
geNorm *M* values	0.330	0.334	0.103*	0.183	0.030*	0.096*
NormFinder SD values	0.471	0.397	0.102*	0.334	0.289*	0.210*

*Denotes the most stably expressed gene values.
M values = mean pairwise; SD = standard deviation; *Pdefα1* = *Phoenix dactylifera* elongation factor α1; *Pdaldh1* = *Phoenix dactylifera* aldehyde dehydrogenase type 1; *Pdact* = *Phoenix dactylifera* actine; *Pdsand* = *Phoenix dactylifera* SAND family protein; *Pdtub6* = *Phoenix dactylifera* Tubiline β6; *Pdtbp* = *Phoenix dactylifera* TATA-box-binding protein.

down-regulated from the beginning of the treatment and continued to depress over time.

DISCUSSION

In general, a seed is known to be stress sensitive, limiting the establishment of a plant [38]. Depending on the manner of seed responses to heavy metals' exposure, germination ability——the first interface for material exchange between plant and environment——is a useful parameter for the decision of tolerance [39]. Germination is the result of complex cellular and molecular mechanisms.

In Tunisia, several sources of metal pollution exist, especially mining activity in the northern and central parts of the country (phosphate fertilizer industry). This type of activity may be a source of environmental damages caused by migration of trace metals from phosphogypsum stock piles to the surrounding environment (soil and water) [40]. These areas are characterized by significant concentrations of metals, especially Zn, lead (Pb), and Cu in soils [41]. A sequential extraction method and then soils' chemical properties analyses revealed higher concentrations of trace metals in topsoil samples (0–20 cm) than in subsoil samples (20–40 cm and 40–60 cm) [Q9][42]. Furthermore, the mobility and bioavailability were important for Cu, creating a problem of agricultural and environmental significance [42], especially for date palm trees growing in contaminated environments in the east-central part of Tunisia.

The response of seeds to Cu stress varied. Dose–response relationships of metal inhibitory eects on seed germination and growth are mainly comparable with those in *Arabidopsis* [38] and cucumber [43]. Date palm seed germination as well as *Arabidopsis* and cucumber seed germination declined with increasing Cu concentration. Also, the fact that at high Cu level the germination rate was better suggests that the date palm seed coat covering the embryo plays a role in selective penetration of metal into cell sap, as in *Arabidopsis* seeds exposed to Cu^{2+}, Zn^{2+}, and Pb^{2+} [38]. Furthermore, failure to germinate or to grow when seeds were exposed to metals may be a result of the failure of subcellular organelles to adjust metal content in cells [44]. Also, it may be caused by metal interference with cell division [45]. The authors go further and explain the seedling growth affection to chromosomal aberrations and abnormal mitosis caused by metal high toxicity [46,47].

To gain more information about the dynamics of the *P. dactylifera* seed germination process, physiological related indices were measured. These indices may be useful to estimate the degree of success of a species in a changing environment based on the capacity of their collected seed to spread and to germinate. However, date palm seed started to germinate after 12 d. It required an extended period of time to avoid mechanical restriction exerted by the seed coat. Certainly, physiological parameters measured in samples treated with 0.02 mM of Cu did not significantly dier from that of the control (Table 1). Despite this, 0.2 mM of Cu clearly improved speed of germination, mean germination time, mean daily germination, seedling mortality, and peak value and the germination index declined by more than 15%. This may be attributed to the cumulative effect of Cu caused by the increasing time of exposure, suggesting better tolerance of the plant in Cu-induced toxicity when exposed to the lower concentration. Furthermore, measured indices under high metal concentration showed an evident concentration-dependent inhibition of germinability. The germination index declined significantly by 91.63%, showing a large decrease in speed of germination. In fact, the initiation of radical emergence necessitates high seed water content so that the metabolic activity necessary for germination can occur. Still, the response of seeds to this hydration varies; depending on its capacity to control interior water level, the alteration of seed germination can be explained by a modification in water absorption. Actually, cupric ions have a toxic effect by creating an external osmotic potential preventing water uptake [48], similar to Na^+ and Cl^- ions [49]. Lack of water absorption may be added to reduction of meristematic cells by accelerating break down of stored food materials in seeds exposed to Fe and cadmium (Cd) [50]. Such an important phenomenon is correlated to amylase activity in seeds that decreased in seedlings exposed to 2 mM of Cu. Amylase activity was increased in seeds exposed at lower concentration (Figure 2). Amylase, a member of the hydrolyzing enzymes group, becomes extensively activated during germination to hydrolyze starch reserve stored in the endosperm. This step is necessary to release glucose and fructose that will be used by the embryo for respiration and other anabolic reactions. This parameter is a key molecular marker for the monitoring of early events in plant development [21]. In the present study, and in line with the findings of Robledo et al. [51] and Fendri et al. [52], we showed that the amylolytic activity was dose- and time-dependent. Undeniably, enzyme activity increased linearly up to 60 d post germination in 0.02 and 0.2 mM of Cu. Nevertheless, an excess supply of Cu affected the mobilization of reserve materials affecting seedling size [50]. Similarly, suppressed amylase activity was observed in pea seeds exposed to Cu [53], garlic seeds exposed to arsenic (As) [47], and sunflower seeds exposed to Cd [54]. Interestingly, amylase activity is an indicator of the gibberellic acid level in germinating seeds [55]; gibberellic acid is a classical plant hormone that mediates cell elongation and seed germination [56].

During cupric stress exposure, a seed activates its metabolic machinery depending on the redox properties of the metal and catalyzes the formation of free radicals through Fenton-type reactions [57]. Because this metabolic response is highly dependent on the plant species, ROS content constitutes a common physiological biomarker of multiple abiotic stressors. Although the half-lives of ROS are usually extremely short [58], H_2O_2 is relatively stable and can penetrate the plasma membrane as an uncharged molecule. In the present study, a marked increase in H_2O_2 was noted in treated date palm seeds (Table 2). Under physiological steady-state conditions, it increased slightly over time in accordance with hypocotyl elongation and development causing surface and water limitation, which may stimulate ROS production. In addition, excessive cupric ions (2 mM) significantly multiplied the H_2O_2 level by 2.5-fold within only 15 d of exposure (Table 2), which can directly inhibit CO_2 fixation [59]. Regarding the oxidative level in cells,

close correlation with a moderate degree of production of TBARS——a biomarker of LPO used as an indicator of membrane damage [60,61] to H_2O_2 content——was shown. In fact, the small increase of TBARS contents at low Cu treatment suggests that the date palm was well protected against oxidative damage under 0.02 mM of Cu. However, 0.2 mM of Cu affected the cell membrane only after extension of Cu exposure time, enhancing TBARS accumulation by more than 30% after 60 d. This situation promoted necrosis, which may be partially caused by Cu-mediated Fe^{2+} deficiency. Decrease of free Fe ions released by a hydrolyzing enzyme from its organic component may also decrease seed germination and radical growth. In accordance with the previously-mentioned physiological parameters, the amount of TBARS production was not directly correlated with H_2O_2 concentrations. It described a steady-state level more pronounced in samples treated with 2 mM of Cu than with 0.2 mM of Cu (Table 2). At the same time, 0.02 mM of Cu did not exhibit a significant reduction compared with untreated samples. A more destructive situation was presented by samples exposed to the highest metal concentration. Reactive oxygen species and TBARS accumulation from the first days of germination under 2 mM of Cu suggested plant sensitivity to a high Cu amount that ultimately resulted in hypocotyl elongation inhibition and finally in cell death because such a metal amount was found to be critical for plant defensive biomolecules. The latter include a scavenging enzyme that often presented to limit free radicals' availability. Reactive oxygen species, in the form of superoxide anion radicals (O_2^-), was dismutated by SOD activity to prevent the oxidative damage. Superoxide dismutase constitutes the first line of defense in plants, exhibited by plant metal homeostasis networks. Seeds exposed to low metal concentrations stimulated SOD activity in 0.2 mM of Cu. The promoted enzyme amount was necessary to control free radicals' availability and equilibrate redox balance. However, SOD activity should not be considered alone in metal homeostasis systems. Additional implication of CAT was shown. With a low affinity compared with APX, CAT catalyzes the destruction of H_2O_2 molecules only in peroxisomes [62]. It increased at a low Cu level and decreased significantly after 30 d of exposure to 2 mM of Cu. Inhibited CAT activities could decrease membranes' stability and CO_2 fixation by blocking several enzymes of the Calvin cycle in chloroplasts [63]. Conversely, increase in both enzymes in stressed plants with lesser concentrations of the metal indicated a constant detoxification of ROS and enhanced oxidative stress tolerance.

NormFinder and geNorm software algorithms showed that *Pdtub6*, *Pdtbp*, and *Pdact* were overall the most stable reference genes under our experimental conditions. Displaying insensibility to Cu, Cd, or chromium (Cr) stress, *Pdtbp* was ranked among the best 3 stable reference genes [28]. In the present study, we confirmed the potential regulation of such traditionally used reference genes in various physiological states [60,61]. For example, the[Q10] *EFα1* gene, which was reported to be stably expressed in different plants such as the date palm [28] and the potato [64] during abiotic stress, showed sensibility to cupric ions.

Two successive normalizations——against the geometric means of the top 3 best identified housekeeping genes and against target gene expression level in control conditions——allowed description of the expression profiles of 2 heavy metals specific biomarkers (PCS and metallothionein) catalyzing the uptake of essential elements above the threshold level at which they become toxic in date palm seeds. Increased resistance to heavy metals stress by overexpressing PCS and metallothionein genes was extensively reported [8,28] but not in *P. dactylifera* seeds subjected to Cu treatment. Candidate genes showed discriminative expression profiles among $CuSO_4$ concentrations (Figure 4). Quantitative real-time polymerase chain reaction results indicated that *Pdpcs* and *Pdmt* responded strongly to low Cu concentrations. Gene expression levels were significantly induced, suggesting their prominent involvement in Cu tolerance either by increasing production of Cu-sequestering protein or by maintaining the local redox balance as a preventive action to the deleterious Fenton chemistry reactions [65]. Our results are in agreement with the hypothesis of Zhang et al. [66], who reported that PCS is controlled at the transcriptional level. Also, the *Avicennia germinans* phytochelatin synthase gene *Avpcs* expression showed a significant increase under Cu conditions from the first hours of stress application [67]. The authors suggest that this rapid increase in *Avpcs* expression confirms that it was the most active gene involved in the regulation of Cu in *A. germinans* leaves.

Likewise, Bernard et al. [8] and Huguet et al. [68] showed metallothionein gene stimulation in response to oxidative stress induction in other species. Nevertheless, the *Pdmt* mRNA level was lower compared with the *Pdpcs* mRNA level at the end of treatment with 0.02 and 0.2 mM of Cu. Our findings disagreed with others [69–71] who suggested that the metallothionein gene is organ-specifically expressed only in roots. Evident symptoms of Cu toxicity were shown at the germination stage of date palm seeds.

CONCLUSIONS

Regarding the biological significance of Cu response in germinating date palm seeds under increasing metal stress, it was shown that at low Cu concentration (0.02 mM) only changes in *Pdpcs* and *Pdmt* gene expression were significant. Under an increasing Cu amount (0.2 mM), both oxidative (H_2O_2, TBARS) and antioxidative (CAT, SOD) mechanisms became informative. The induction of genes involved in the synthesis of metal-chelating peptides is not only involved in metal homeostasis but also constitutes a defense against excess of Cu. However, synchronous fluctuation in the redox balance at high Cu concentration (2 mM) deregulates seed antioxidative defense systems causing oxidative damage, especially after a long exposure period, leading to necrosis and blockage in hypocotyl elongation. Molecular mechanisms underlying Cu response in date palm seeds deserve further attention to elucidate those by which Cu is distinguished from other ions.

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Data Availability—Data are available from the corresponding author (franck.vandenbulcke@univ-lille1.fr).

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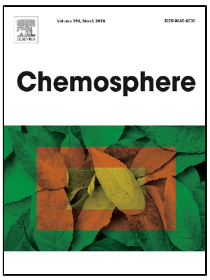

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Insight into the expression variation of metal-responsive genes in the seedling of date palm (*Phoenix dactylifera*)

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ABSTRACT

Phytochelatin synthase and metallothionein gene expressions were monitored via qPCR in order to investigate the molecular mechanisms involved in Cd and Cr detoxification in date palm (*Phoenix dactylifera*). A specific reference gene validation procedure using BestKeeper, NormFinder and geNorm programs allowed selection of the three most stable reference genes in a context of Cd or Cr contamination among six reference gene candidates, namely elongation factor α1, actin, aldehyde dehydrogenase, SAND family, tubulin 6 and TaTa box binding protein. Phytochelatin synthase (*pcs*) and metallothionein (*mt*) encoding gene expression were induced from the first days of exposure. At low Cd stress (0.02 mM), genes were still up-regulated until 60th day of exposure. At the highest metal concentrations, however, *pcs* and *mt* gene expressions decreased. *pcs* encoding gene was significantly up-regulated under Cr exposure, and was more responsive to increasing Cr concentration than *mt* encoding gene. Moreover, exposure to Cd or Cr influenced clearly seed germination and hypocotyls elongation. Thus, the results have proved that both analyzed genes participate in metal detoxification and their expression is regulated at transcriptional level in date palm subjected to Cr and Cd stress. Consequently, variations of expression of *mt* and *pcs* genes may serve as early-warning biomarkers of metal stress in this species.

Keywords Biomarkers - Date palm hypocotyls - Gene normalization - Reference gene validation - Gene expression – Detoxification mechanisms

1. Introduction

Although informative, physicochemical analyses reach their limits as tools in environmental management assessment since speciation and/or bioavailability may strongly influence the impacts on organisms (Amiard et al., 1998). Therefore, it is useful to associate them with measurements of biological parameters in organisms in order to assess sub-lethal and sometimes discrete effects. Plants frequently respond in some measurable and somewhat predictable ways through mechanisms at various levels of biological organization (Bickham, 2000) starting from the community to individual and infra-individual levels. Particular factors involved in those mechanisms (bioindicators, biomarkers) can serve as tools for documenting the biological response to environmental menace and the extent of organism's exposure to chemical contaminants so that preventive measures can be taken. Biomarkers may indicate exposure to specific classes of pollutants such as metals (Marigomez et al., 2002).

Of all non-essential metals, cadmium (Cd) is probably the most common toxicant to plants, animals and humans (Demidenko et al., 2011). Environmental effects of this element have been extensively reviewed (Benavides et al., 2005; McBride, 2003). Briefly, in plants, Cd has major detrimental impacts on physiology and biochemistry inducing growth reduction, on chloroplast ultrastructure causing alteration of photochemical reactions, on mineral nutrition, on carbohydrate metabolism and on oxidative stress enhancement (Expósito-rodríguez et al., 2008; Gill et al., 2011; Shanker and Venkateswarlu, 2009). Because of its great mobility in the plant–soil system, Cd has the potential to accumulate in some crops with a significant potential to contaminate the diets of animals and humans (Gabbrielli, 1999).

Likewise, with its high affinity for living organisms (Campbell, 2005), chromium, especially its hexavalent chemical form (Cr^{6+}), is known to be a very toxic contaminant steadily increasing in the environment due to the development of industry, especially metal processing, chromium plating and leather tanning (Huguet et al., 2004; Wyszkowska, 2002). Coal and Area (2013) reported that Cr^{6+} accumulation was important in the shoots of exposed rice plants, causing chlorosis and compromised photosynthesis. Thus, Cr may interfere with several metabolic processes and poses a threat to the health of organisms. As a result of their accumulation in crop plants, Cr can contaminate the food web. The United States Environment Protection Agency has established a standard limit of 0.01 ppm Cr in foods (Agency, 2009). However, both the Food and Agriculture Organization (FAO) and World Health Organization (WHO) allow 0.05 ppm Cd in food products (Solidum, 2011).

Controlling metal pollution sources is important but insufficient for protecting soil health. Metal specific biomarkers could act as early warning signals allowing a better exposure assessment. The enzymes that synthesize molecules capable of chelating metals are potentially good biomarkers. The main species of chelators in plants are phytochelatins (PCs). PCs are synthesized by phytochelatin synthase (PCS), an enzyme requiring glutathione (GSH) as a precursor (Oa et al., 2008). PCS is encoded by a gene (*pcs*), the expression of which might be influenced by metals (Hall, 2002). The enzyme activation in cells depends on the presence of heavy metal ions such as Cd^{2+}, one of the strongest inducers of *pcs* expression (Vatamaniuk et al., 2000). Over the last two decades, many PCS coding genes have been isolated from a wide range of organisms such as plants (Clemens et al., 1999; Heiss et al., 2003; Oa et al., 2008) and animals (Brulle et al., 2010) and have been documented to contribute in HMs detoxification processes. However, *pcs* is not found in any vertebrate species (Pal and Rai, 2010). Essential metal homeostasis and detoxification also relies on other metal-binding ligands, such as metallothioneins (MTs) (Huguet et al., 2004; Zimeri et al., 2005). Most cells can induce the synthesis of MT in response to HM challenge. Thus, MT content and/or *mt* gene expression are specific biomarker candidates for HMs risk assessment. Although MT and PCS coding genes have been both described in many plant species, few authors studied the control of their expression (Gonzalez-mendoza et al., 2007).

Molecular biology techniques applied to ecotoxicology help to better understand the detoxification and defense mechanisms of organisms. Quantitative real-time PCR (qPCR) is a standard method to study gene expression variation under different experimental conditions. It has been adopted in a wide range of scientific areas and research work (Pfaffl, 2001) because of its advantages in sensitivity, specificity and its extensive range of detection (Bustin et al., 2009). To further improve the reproducibility and reliability of qPCR experiments, Bustin et al. (2009) described approaches to normalize mRNA. It requires the use of at least one endogenous reference gene transcription as internal control for relative quantification (Czechowski et al., 2005). These reference genes are typically constitutive genes with housekeeping (HKG) functions required for the maintenance of basic cellular function. The expression levels of these genes should be stable among cells of different tissues across the developmental stages under normal and adverse conditions (Bustin, 2000; Crismani et al., 2006; Villaseñor et al., 2011). Recently, a number of commonly accepted reference genes presented expression variability in response to some experimental conditions (Chandna et al., 2012; Lilly et al., 2011; Morgante et al., 2011). Consequently, validation of appropriate reference genes is required prior to any quantification (Guénin et al., 2009; Gutierrez et al., 2008; Martins et al., 2016a). Several

statistical algorithms such as geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) have been developed and are now commonly used in HKG validation. Such a validation procedure has not been performed in date palm in a Cd or Cr contamination.

Thus, in this paper, we evaluated, for the first time, the expression stability of six traditionally used reference genes in date palm in order to measure the gene encoding phytochelatin synthase (*Pdpcs*) and metallothionein (*Pdmt*) in date palm, *Phoenix dactylifera* L., subjected to various and environmentally relevant Cd or Cr concentrations.

2. Methods

2.1. Seeds germination and treatments

Mature seeds of the date palm cultivar Deglet Nour were washed with distilled water extensively. Then, under sterile conditions, 100% of commercial sodium hypochlorite was added to seeds for 10 min followed by 50% of diluted solution for another 10 min and completed with more diluted sodium hypochlorite (25%) during the same time. Seeds were then rinsed 3 times with distilled water for 10 min. They were germinated in direct contact with 0, 0.02, 0.2, 1 and 2 mM cadmium solutions prepared in distilled water using cadmium chloride ($CdCl_2$) or with 0, 0.02, 0.2 and 2 mM of chromium solution prepared in distilled water using potassium chromate (K_2CrO_4) and kept in the dark at 26 °C in order to induce germination. Hypocotyls (10 per replicate; three biological replicates per sample) were excised from seeds at 15, 30, 45 and 60 days after germination and used for total RNA extraction.

2.2. Seedling growth parameters measurement

Germination Percentage (GP), Speed of Germination (SG), Mean Daily Germination (MDG), Mean Germination Time (MGT), Peak Value (PV), Seedling Mortality (SLM) and Germination Index (GI) were measured according to previous studies of Chaâbene et al. (2015).

2.3. Gene sequences identification in date palm

From previously constructed Deglet nour date palm cDNA library (Dhieb et al., 2012), we isolated *mt* and reference encoding genes. Homologous sequences of genes of metallothionin (*mt*), elongation factor alpha 1 (*Efα1*), aldehyde dehydrogenase (*aldh1*), actine (*act*), SAND family protein (*sand*), Tubulin beta-6 (*tub6*) and TATA-box-binding protein (*tbp*) from various organisms recognized in NCBI (http://www.ncbi. nlm.nih.gov/) using BLASTn were aligned

against nucleotide sequences from our date palm cDNA bank using BioEdit version 7.0.5.3. Some clones revealed partial homology with selected genes. Following methods described by Chaâbene et al. (2017), we have identified *Pdmt* amplified using the primer pair (5' C GTGATCACCGAGACTGAAA 3' and 5' TTGGTTGTGGAGGAGTGTCA 3') and the six candidate Housekeeping genes (HKGs) using Pfam (with E value <1e-5), HMMER v3.0 and UniProt (http://www.uniprot.org/) programs.

2.4. Total RNA isolation and cDNA synthesis

Deglet Nour date seedling which experienced 0.5, 1, 1.5 or 2 months growth in stressful conditions with $CdCl_2$ or K_2CrO_4 was used for total RNA extraction. RNAs were isolated with the Plant RNeasy mini kit (Qiagen, Courtaboeuf, France), including the on-column DNase digestion step. Concentration and purity of the RNA samples was determined using a Spectrophotometer (SPECTROstar Nano Microplate Reader). RNA integrity was checked using a method adapted from (Masek et al., 2005). Reverse transcription was performed on 1 µg of total RNA from each sample using the random hexamer primers and the Maxima H Minus First strand (Thermo Scientific, USA) cDNA Synthesis Kit according to manufacturer's instructions.

2.5. Gene amplification and sequencing

Primer sequences of candidate genes were designed from the conserved domain found using Primer3Plus (Rozen and Skaletsky, 2000); http://frodo.wi.mit.edu/) and verified using Net Primer and Beacon Designer. Table 1 lists the primer pairs used.

PCR products were cleaned with the EZ-10 spin column DNA gel extraction kit (Biobasic, Markham, Canada). Amplification products were sequenced in both directions by Genoscreen Company (Pasteur Institute, Lille, France). Using CodonCode Aligner and Sequencher, a contig was assembled and used to design qPCR primers (Table 2).

2.6. Real time qPCR amplification

Quantitative real-time PCR (qPCR) analysis of gene expression was performed on reverse transcribed RNAs extracted from *Phoenix dactylifera* using MESA Blue qPCR Master Mix reagent kit (Eurogentec, Seraing, Belgium). Gene-specific qPCR primers were designed with Primer3Plus with special settings selection. Primers verified with Net Primer and Beacon Designer, are listed in Table 2. qPCR reactions were performed with a LightCycler 480 Real

Time PCR system (Roche Diagnostics, Mannheim, Germany) according to previously described procedures (Brulle et al., 2014). Real-time PCR efficiencies (E) were calculated from the given slope of the standard curve according to the equation $E = 10^{(-1/slope)}$. E values ranged from 1.91 to 2 (with 100% = 2) and calculated from a standard curve. *Pdpcs* and *Pdmt* relative expression levels were normalized to those of the reference genes selected after gene expression validation according to previously described procedures (Pfaffl, 2001). Absolute quantification of genes expression levels are in log2. Relative expression level of control samples vs relative expression level of exposed samples was normalized to calculate relative fold expression according to Bernard et al. (2016).

2.7. Expression stability analysis

Performance of the six genes as potential reference genes for date palm was assessed in samples divided into three experimental sets of two treatments; including samples not subjected to stress conditions. To estimate the expression stability of reference genes, the relative quantities of Cq values of genes obtained using the formula of (Hellemans et al., 2007) were used. As per Martins et al. (2016b) different algorithms and statistical analysis were applied to evaluate the expression stability of the reference genes of *P. datylifera* plants in seedling under stress conditions. The analysis procedures strictly followed the manuals of used algorithms. Reference genes were validated using BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004) and geNorm (Vandesompele et al., 2002) following (Brulle et al., 2014) procedure.

2.8. Statistical analysis

The relative expression ratios between control and treated plants were transformed and subjected to a one-way and two-way ANOVA.

3. Results

3.1. Target gene identification and HKGs selection

In addition to previously identified full length *Pdpcs* in date palm (Chaâbene et al., 2017), a second heavy metal biomarker candidate has been identified: a metallothionein mRNA sequence of 387pb (*Pdmt*) was identified with via Eukaryotic GeneMark.hmm, FGENESH 2.6 and BLASTx. Following the same procedure, six candidate HKGs were selected. They were named *Pdefα1*, *Pdaldh1*, *Pdact*, *Pdsand*, *Pdtub6* and *Pdtbp*, following the nomenclature of *P. dactylifera* (Table 1). The latter genes exhibit highly conserved domains which allowed NCBI

database exploration using Blastn and design of primers for PCR amplification. Generated sequences for candidates and HKGs from date palm var. Deglet Nour after PCR product sequencing and re-amplification were used to evaluate gene expression in qPCR.

3.2. Reference gene amplification efficiency and expression levels

qPCR analysis of gene expression was performed on reverse transcribed mRNA extracted from hypocotyl samples of *Phoenix dactylifera* seedlings exposed in various metallic conditions. Transcript abundance was evaluated by qPCR. All products showed a single peak in the melting curve analysis using the LC480. The calculated amplification efficiency for primers ranged from 97.5% to 100%, $r^2 \geq 0.95$ (Table 2) and were considered appropriate for qPCR (Zhao, 2005).

Mean values showed that they were moderately abundant and they differed between stress conditions (Fig. 1).

The gene showing the highest expression was *Pdefα1*. *Pdtub6* exhibited the lowest level of expression and was the most stable reference gene showing no expression variability between samples. Indeed, the expression variation of all HKGs, represented by different cycle threshold (Ct) values, in control and chromium conditions was almost the same (Fig. 1). A Ct dispersal was observed under Cd stress for *Pdefα1* having mean Ct value of 24.7 cycles and revealing sensitivity to heavy metal treatment. *Actin*, frequently used as reference gene, showed expression induction in Cd treated simples. As a consequence of this considerable variability among candidate HKGs, a simple comparison of the raw Ct values was inadequate, requiring further analysis in order to identify a suitable combination of reference genes for normalizing expression under specific experimental conditions.

3.3. Reference gene expression stability across different experimental conditions

Expression stability has been initially evaluated by BestKeeper based on the pairwise correlation coefficient and the BestKeeper index (geometric mean of Ct value for each candidate gene (Pfaffl, 2001) Genes with high BestKeeper coefficient of correlation indicates that their expression correlates well with the expression patterns of other reference genes and so were considered as the most stably expressed. The algorithm calculated the stability of the six candidates and present different expression behavior between experimental conditions (Fig. 2). Hypocotyls of non-treated date palm seeds expressed HKGs ranking in order of most stable to least stable: *Pdact*, *Pdefα1*, *Pdsand*, *Pdtbp*, *Pdaldh1* and *Pdtub6* (Fig. 2a). However under

HMs stress, *Pdsand* showed an unstable expression pattern, indicated by a low Pearson's Coefficient value under Cr exposure (Fig. 2c) and a negative value obtained with Cd treatment (Fig. 2b) suggesting gene sensitivity to abiotic stress caused by these toxic elements. Although gene expression variations occur, the same four genes exhibited the highest stability in different samples across different exposure treatments (Fig. 2).

Expression stability was also evaluated using another approach relying on the geNorm algorithm. The stability measure calculated with the geNorm applet (*M* value) is the mean pairwise variation between a gene and the other candidate HKGs. $M < 0.5$ indicates good stability and HKGs with lowest *M* values are the most stably expressed. *Actin* was one of the most stable genes in samples under standard conditions (Table 3). Surprisingly, *Pdtbp* was the least stable HKG in the chromium treatment according to BestKeeper, while geNorm ranked it as the most stable. We cannot explain this contradiction. Also, *Pdtub6*, the third best stable gene in control hypocotyls with *M* value equal to 0.16 has been established as the lowest stable expressed gene by Bestkeeper. This latter gene remained unstable after Cd exposure and the best three stable genes according to geNorm were *Pdtbp*, *Pdact* and *Pdefα1*).

Lastly, NormFinder software was used to re-analyze and confirm results since it is less sensitive to co-regulation (Expósito-rodríguez et al., 2008). This algorithm uses a model-based approach ranking the best candidate reference genes by comparing inter- and intra-group expression variation (Demidenko et al., 2011). For NormFinder, top ranked genes will present the lowest standard deviation values (Demidenko et al., 2011). A gene ranked as stable by geNorm should receive atop four ranking in NormFinder (Simon et al., 2013). As shown in table 3, geNorm and NormFinder produced very similar results suggesting the reliability of analysis. NormFinder confirmed that *Pdtbp* could be used as reference gene across abiotic stress treatments.

To conclude, the described selection procedure allowed identification of suitable reference genes for the present experimental conditions. Yet, the obtained results indicated that *Pdact*, *Pdtbp* and *Pdsand* could be the most appropriate reference genes for normalizing transcription level in non-stressed palm date tissues. Otherwise, the most suitable normalized HKGs for Cd and Cr stressed plant were *Pdtbp*, *Pdefα1*, *Pdact* and *Pdaldh1, Pdefα1*, *Pdtbp*, respectively.

3.4. Determination of the optimal number of reference genes

Two or more HKGs were required for the planned qPCR experiments. The geNorm algorithm was used further to determine the optimal number of HKGs required for reliable

normalization. Different combinations of reference genes were used. According to Vandesompele et al. (2002), Pairwise Variation (V*n*/V*n*+1) between normalization factors (NF) allowed estimation of the best number of reference genes for normalization. The NFs were calculated initially for control genes with the lowest *M* value (most stable gene) and then, in a stepwise manner, other reference genes with lower *M* values were added (Fig. 3).

These analyses indicated that two or three genes with the most stable expression would be optimal for reliable normalization. For samples subjected to chromium stress, *Pdaldh1*, *Pdefα1* and *Pdtbp* would be sufficient for qPCR analysis (V2/3 value = 0.100 < 0.15, the recommended cutoff calculated by Vandesompele et al. (2002) (Fig. 3). However, for the control and Cd treated samples, the pairwise variation V2/3 value (0.19 and 0.204 respectively) was higher than 0.15, indicating that two reference genes were not sufficient for normalization, and a third gene is recommended. Indeed, V3/4 values of 0.102 and of 0.111 were below the threshold, suggesting that the optimal number of reference genes for normalization in different conditions was three (Fig. 3).

This crucial step was necessary to identify the reference genes allowing quantification of *pcs* and *mt* gene expression levels in *P. dactylifera*.

3.5. Pdpcs and Pdmt expression variation in response to heavy metal stress

The expression profiles of the two selected target genes have been normalized using the geometric means of the three best HKGs for each treatment concentration at various time points during a 60 days development period (Fig. 4).

Obtained results proved that candidate genes are not only present but fully operational in *P. dactylifera*. The effects of stressors (Cd or Cr) were tested with a two-way ANOVA, with metal concentration as factor one and time exposure as factor two. In control conditions, both gene expressions enhanced slightly over time (Table 4). Results presented in table 4 indicated that Cd and Cr concentrations and stress exposure time had a significant overall effect on *Pdpcs* and *Pdmt* expression (p =0.000 < 0.05)

Major differences were observed at high Cd and Cr concentration for the longest exposure time periods. Thus, after 15 days of exposure, the expression levels of *Pdpcs* and *Pdmt* increased in all tested conditions (Table 4) except a slight and not significant decrease of *Pdmt* at 2 mM of Cd (Table 4). Interestingly, low Cd^{2+} and Cr^{6+} concentrations (0.02, and 0.2 mM) strongly induced expression of both target genes at various time points. At high metal

concentrations, expression was suppressed. This was more pronounced under Cd stress and can be linked to the severity of toxicity symptoms reported in *P. dactylifera* seedlings, including inhibition of germination. Data can be presented as log2 relative expression after a second normalization and scaling with values of non-treated samples. It varied significantly ($p<0.05$) between treatments (Fig. 4). Both genes were down regulated or up-regulated in both metal treatments indifferent time points. Target genes induction began at the lowest Cd concentration showing remarkable sensitivity (Fig. 4a-b). Increasing metal amount adversely affected gene expressions, especially *Pdmt* (Fig. 4b). After 15 days of exposure, *Pdpcs* expression reached a maximum at 0.2 mM Cd and decreased to a stable, low level after that time (Fig. 4a). In addition, at this concentration *Pdmt* relative expression was up-regulated (3-folds) after 30 days of treatment (Fig. 4b). Yet, at high Cd levels (1 and 2 mM), a significant ($p<0.05$) down-regulation starting from the first days of exposure for *Pdmt* (Fig. 4b) and after 45 days for *Pdpcs* (Fig. 4a) was observed. Suppression of *Pdpcs* and *Pdmt* expressions in date palm seedlings under these conditions resulted in a severe Cd toxicity phenotype (Fig. 5a). The influence of chromium exposure on gene expression suggests the existence of a regulation of *pcs* and *mt* at transcriptomic level. After 60 days of treatment with 2 mM Cr^{6+} a significant down-regulation in *Pdpcs* expression was observed (Fig. 4c). *Pdmt* gene expression in response to Cr^{6+} ions increased linearly with exposure time but not in a dose-dependent manner (Fig. 4d). A mild decrease in *Pdmt* gene expression was observed after two months of exposure to 2 mM Cr (relative to 0.02 mM; Fig. 4d). The highest *Pdmt* level of expression was seen at 2 mM Cr^{6+}. Thus, during chromium exposure, coordination in genes expression was more pronounced. Moreover, significant morphological alterations in date palm seedlings grown for 60 days at the highest concentration of Cr was not reported.

3.6. Metal effects on date palm seeds germinability and its related physiological perturbations

Because the ability of a plant to resist or tolerate toxicity to heavy metals depends on its ability to maintain germination in a polluted environment (Peralta et al., 2001), *P. dactylifera* seeds germination was monitored during 60 days of continual exposure to cadmium or chromium solutions. The seeds, which start to grow after 10 days of darkness, showed significant resistance to xenobiotics. Indeed, 92% and 84% of seed germination under 1 mM Cd and 2 mM Cr respectively was registered against 77.33% in control suggesting that metal induced germination process (Fig. 5). This promoting effect on seed germination continued to improve along treatment, whereas, high cadmium concentration (2 mM) significantly inhibited seeds germination by more than 13%. The highest promoting effect; GP = 99.5% (Fig. 5a) and 100%

(Fig. 5a), was achieved by 0.02 mM Cd and 0.2 mM Cr respectively came up to 15 days. Hypocotyl length was more sensitive to metallic stress than germination percentage. It was found that, except at high Cd or Cr concentrations, there was no significant difference between treatments (Fig. 5). Hypocotyl growth was dramatically inhibited when exposed to Cd (Fig. 5b). The lowest hypocotyl length with the highest inhibitory rate (98.97) was obtained after 60 days of exposure to 2 mM Cd.

Hypocotyl elongation was stopped at 0.3 cm after 15 days of Cd treatment (Fig. 5b) and hypocotyl also becomes considerably or completely necrotic at the end of the treatment with 0.2, 1 and 2 mM Cd respectively (Fig. 6a). Some necrotic marking are also shown after a long treatment with 2 mM Cr (Fig. 6b).

4. Discussion

The date palm was chosen because of its economic and social importance. For these reasons, countries such as Tunisia ranked it as a research priority for further development of crop production (Hammadi et al. 2015). Furthermore, this species has an additional ecological importance due to its use for biomonitoring of pollutants in many countries (Thomas 2002; Al-khlaifat and Al-khashman 2007). The emergence of heavy metal (HM) contamination in arid countries associated especially with re-use of wastewater (Lazarova and Bahri, 2004) or the presence of phosphate mining activity (Magni et al., 2005) is creating new environmental challenges for the plant. Moreover, date palm showed a great potential for Cr uptake (Enas et al., 2010), removing of HMs from wastewater and has been classified by (Zayneb et al., 2013) as a Cd hyperaccumulator. More recently, identification and involvement of a *pcs* type I gene in HM chelation has been reported in date palm (Chaâbene et al., 2017).

Monitoring the existence of contamination in terrestrial environments typically involves frequent chemical analyses of environmental samples (soil, air, water). Biological data can be an effective supplement for these techniques, giving rise to the term "biomonitoring" (Bone et al., 2010). Biomonitoring consists not only of measuring contaminants in organisms, but also measuring physiological, biochemical or molecular parameters which can provide information on the performance of the organisms (Weeks, 1995). In fact, biomarker analysis can constitute an effective monitoring network, sensitive to a wide range of xenobiotics, including heavy metals (Monteiro et al., 2009). It provides an early and specific response which will be a critical element for any restoration or protection project. Physiological responses to environmental stressors are accompanied by changes in specific gene expression profiles that can be used as

biomarkers (Brulle et al., 2010). The regulation of gene expression represents the first level of integration between environmental stress and genome. The isolation of the genes encoding PCS and MT from the HM tolerant (Layla and Ahmed, 2011) and Cd hyperaccumulator *P. dactylifera* (Zayneb et al., 2013), enable us to explore their involvement in metal detoxification. It is not easy to draw general rules since the expression of target genes may vary within plant species, tissue and even cultivars. In *Arabidopsis* different isoforms for each *mt* type has been identified showing differential gene expression patterns suggesting that they have specialized functions in different tissues (Garcı et al., 1998). *Pdmt* is similar to *mt3*-like mRNAs detected in developing embryos in *Arabidopsis* (Dong, and Dunstan, 1996) and to other *mt3* from other plant species.

Due to its sensitivity, accuracy and practical simplicity, qPCR technology is widely used in gene expression analysis (VanGuilder et al. 2008). Measuring target mRNA abundance with this method requires selection of suitable internal controls that have relatively stable expression irrespective of treatment to control for non-specific variation between testers and obtain biologically meaningful data (Czechowski et al. 2005; Crismani et al. 2006). Because HKG expression can vary considerably (Bustin 2001; Warrington et al. 2000), universal reference genes qPCR normalization which are stable under all experimental conditions do not exist (Gutierrez et al. 2008; Artico et al. 2010).

Therefore, validation of reference genes is required to avoid inaccurate data interpretation and false conclusions (Silveira et al., 2009). Despite the potential of date palm to take up and accumulate HMs (Layla and Ahmed, 2011), there was no information on the expression stability of reference genes in the plant during exposure to HMs stress. Previous study on date palm (Saidi et al., 2009) relied on classical reference genes without any prior validation. To our knowledge, the present investigation is the first to assess selection of potential reference genes suitable for gene expression quantification in *P. dactylifera* exposed to Cd or Cr. The candidate housekeeping genes that were evaluated in this study were identified with bioinformatics software detecting similarity to various genes expressed in plants. Stability of six candidate reference genes was evaluated in date palm subjected to abiotic stress caused by Cd and Cr, which are known to affect HKGs expression in numerous plant models (Morgante et al. 2011; Teng et al. 2012; Lee et al. 2013). Expression patterns have been analyzed with various mathematical methods relying on different algorithms (Bestkeeper, geNorm and NomFinder) in order to identify the most stable reference genes (Rapacz, 2013). Robledo et al. (2014) suggested that none of these programs is currently considered the best one. However, previous studies preferred geNorm which select similarly expressed reference genes by a pairwise

approach (Andersen et al. 2004; Wood et al. 2008). Results presented in Table 3 showed good stability of all investigated candidate genes with *M* value inferior to 0.5 (Hellemans et al., 2007). Although the algorithms of these software-programs are different, very similar results have been obtained with NormFinder software. Indeed, the most stable HKGs found by these two programs were the same in all samples but they ranked differently. This difference was expected and has been previously observed in other plants (Almeida et al. 2010; Demidenko et al. 2011). Under controlled environments, the classical reference gene actin, was ranked first by geNorm and NormFinder (Table 3) and further confirmed by BestKeeper algorithm (Fig. 2a). Its validity as an internal standard has been previously questioned and changed by *PDA* in Northern blot analysis (Teste et al., 2009). Under the approach taken in this work, the three algorithms recommended the use of *Pdtbp*, *Pdact* and *Pdefα1* for Cd stress samples. These genes were insensitive to metal stress and stayed stable during the entire exposure period. Different outcomes have been produced in samples with chromium treatments. The ranking of reference gene stability identified by BestKeeper was different to that produced by geNorm and NormFinder. The Tata Box Protein encoding gene (*Pdtbp*) showed stable expression, according to both geNorm and NormFinder (Table 3). While, with low correlation ($r = 0.065$), BestKeeper ranked it as the least stable gene (Fig. 2c). The fact that expression of gene generated contradictory results among software programs discourages its use as a reference for qPCR normalization. Furthermore, a previous study proved that a conventional normalization based on a single HKG may lead to erroneous normalization (Vandesompele et al., 2002). Our approach determined a normalization factor indicating the number of HKGs required for reliable normalization. Figure 3 clearly shows that three stable control genes allowed accurate normalization of samples.

In order to alleviate the stress, re-establish internal homeostasis and antioxidant capacity and minimize the detrimental effects of nonessential metals, plants have developed highly effective mechanisms to avoid or to regulate the introduction of HM ions, which employ metal transporters, chaperones and chelators such as PC(s) and MT(s). Identification of protein markers could serve as a good starting point for revealing new aspects of HM stress in plants and may complement the set of biological indicators traditionally used to evaluate the impact of metal contamination in natural ecosystems. Because it offers great potential for deployment in bioassays for evaluating environmental risk as well as in monitoring the effectiveness of remediation processes in contaminated environments with respect to biological site disturbances indications, toxicologists frequently study the effects heavy metals by monitoring the variation of the expression genes know to act as toxicity biomarkers (Benhamed et al.,

2016). qPCR results normalization with a proper selection and evaluation of potential reference genes for each experimental condition and after scaling with values of non-treated samples revealed significant differential *Pdpcs* and *Pdmt* transcription levels in response to treatment with metals. The present study provides important evidence that *Pdpcs* and *Pdmt* genes are part of the tolerance and detoxification mechanisms for HMs in palm date hypocotyl tissues.

In fact, organism's deficient in PCS become unable to tolerate HMs-induced toxicity or display hypersensitivity to excess metals (Pal and Rai, 2010). Both time and metal concentration influenced target gene expression. In addition, according to two-way ANOVA (Table 4), a significant metal stress and time exposure interaction for both genes was detected (p range from 0.000 and 0.019 < 0.05). Data showed that genes transcript levels increased significantly in hypocotyls of seeds treated with low Cd^{2+} or Cr^{6+} concentrations compared to controls. This is in accordance to previous research suggesting that PCS and MT were induced and regulated by several stimuli notably HMs at the transcriptional level (Clemens et al., 1999). Indeed, *Pdpcs* was rapidly induced in seedlings 15 days after germination (Fig. 4a). Number of transcripts continued to increase significantly in tissues exposed to low Cr concentrations (Fig. 4c) and was still up-regulated after exposure to 0.02 mM Cd (Fig. 4a). This is in agreement with the hypothesis that *pcs* is controlled at the transcriptional level as proposed for garlic (Zhang et al., 2005). Increased tolerance to HM stress by *pcs* overexpression in *Tuberculous granuloma* has been reported (Zhu et al., 2003). Furthermore, the rapid reaction in the gene expression may play a pivotal role in immediate HM detoxification and may act as a part of the protection mechanism against oxidative stress caused by this xenobiotic in garlic as reported by (Zhang et al., 2005). However, gene expression was strongly dependent on metals ions concentration. At high Cd concentrations, both genes displayed decreased mRNA concentration compared to controls (Fig. 4a, b). At these highest concentrations seed germination and hypocotyls' growth were inhibited (Fig. 5a, b, c). This observation is similar to previous findings in other plant species (Kohler et al. 2002; Gonzalez-mendoza et al. 2007). A similar observation can in part be done where palm date seedlings exhibited toxicity symptoms only after 60 days of exposure to 2 mM Cr^{6+} (Fig. 5d). The fact that *Pdpcs* was found to be more highly expressed under chromium stress contrasts previous results indicating that PCs were not involved in Cr^{6+} detoxification (Dubey et al. 2010; Shanker and Venkateswarlu 2009).

The coordinate expression of *Pdpcs* and *Pdmt* genes can give a part of the explanation of the high resistance of *P. dactylifera* seedlings to HMs. The transcription of *Pdpcs* in response to Cd reached a maximum after 15 days of exposure to 0.2 mM. It was significantly inhibited after 45 days of 2 mM Cd treatment and after 60 days of 1 mM Cd treatment which were highly

inhibitory to hypocotyl elongation, most probably because the toxicity level has been reached. *Pdmt* expression level was less strongly induced (compared to *Pdpcs*) in seedlings exposed to Cd. In fact, it has been found in *Arabidopsis* that PCs were involved in HM detoxification only under MTs deficiency (Zhou and Goldsbrough, 1994). Under chromium treatment, mRNA accumulation was more induced and *Pdpcs* was less sensitive to high Cr concentration than *Pdmt*. Under experimental conditions of 2 mM Cr, hypocotyls suffered necrosis and slight growth inhibition (Fig 5 and 6). The observed *mt* gene overexpression may play a pivotal role in Cr resistance. In addition to its high affinity for HMs, MT is commonly involved in a protective mechanism which appears to be the dominant metal detoxification pathway (Zhou and Goldsbrough 1994; Yu et al. 1994). It may maintain local redox balance, either by sequestering HMs or by otherwise preventing potentially deleterious Fenton chemistry reactions (Zhang et al., 1999). However, it was reported to be more specifically a response to metals, rather than oxidative stress (Ramesh et al., 2009). Similar findings have been described by Shanker and Venkateswarlu (Shanker and Venkateswarlu, 2009) who showed an increase of MT amounts as compared to the absence of metals sequestering PCs under Cr stress.

5. Conclusions

The present research gave an insight into the molecular basis of date palm heavy metals resistance. To the best of our knowledge, this is the first work investigating acclimation mechanisms involved in seed germination and in seedlings exposed to Cd or to Cr. Molecular mechanisms were studied through the quantification of the expression levels of two genes encoding proteins involved in metal detoxification, namely PCS and MT. Results displayed that *pcs* and *mt* genes were regulated at the transcriptional level in date palm subjected to heavy metal stress. Based on this study, the overexpression of genes responsible for Cd and Cr uptake, translocation, and sequestration can explain the exceptional metal tolerance of date palm, which can be observed in highly contaminated areas and which could be successfully exploited in phytoremediation.

Conflict of interest

Authors disclose that there are no relationships or interests that could have direct or potential influence or impart bias on the work. The authors declare that they have no conflict of interest and there are responsible for the content and writing of the article.

Authors' contributions

Amine Elleuch designed the research; Zayneb Chaâbene, Agnieszka Rorat and Imen Rekik Hakim performed research and analyzed the data; Zayneb Chaâbene wrote the paper, C. Douglass Grubb proofread the paper and improved the grammar and the English. Pr. Mejdoub Hafedh and Pr. Franck Vandenbulcke followed the work and participate in article writing. All authors read and approved the final manuscript.

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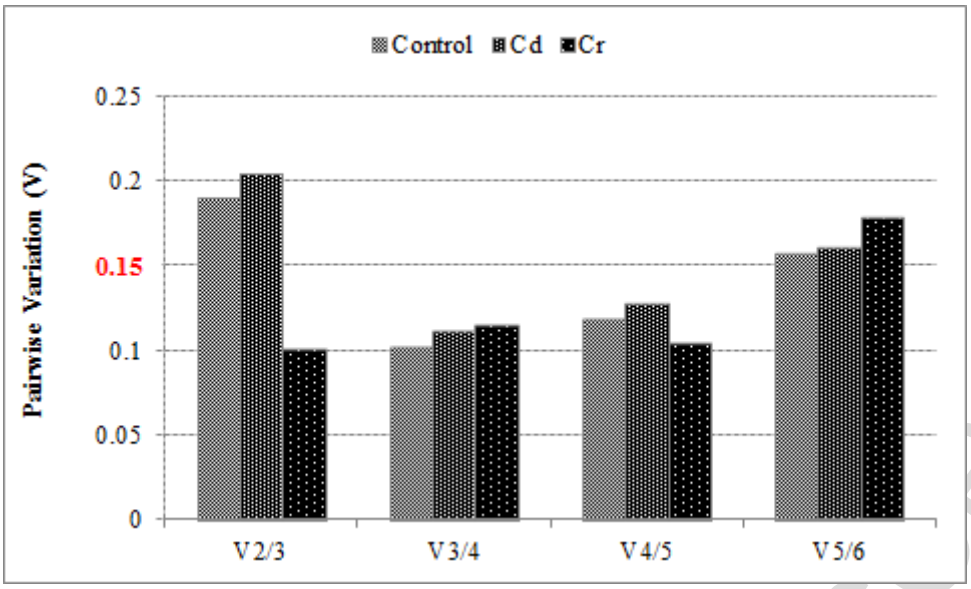


Fig. 3. Predicted geNorm pairwise variation of the six candidate reference genes to determine the optimal number of HKGs required for qPCR data normalization. 0.15 represent the recommended cutoff calculated by Vandesompele et al. (2002).

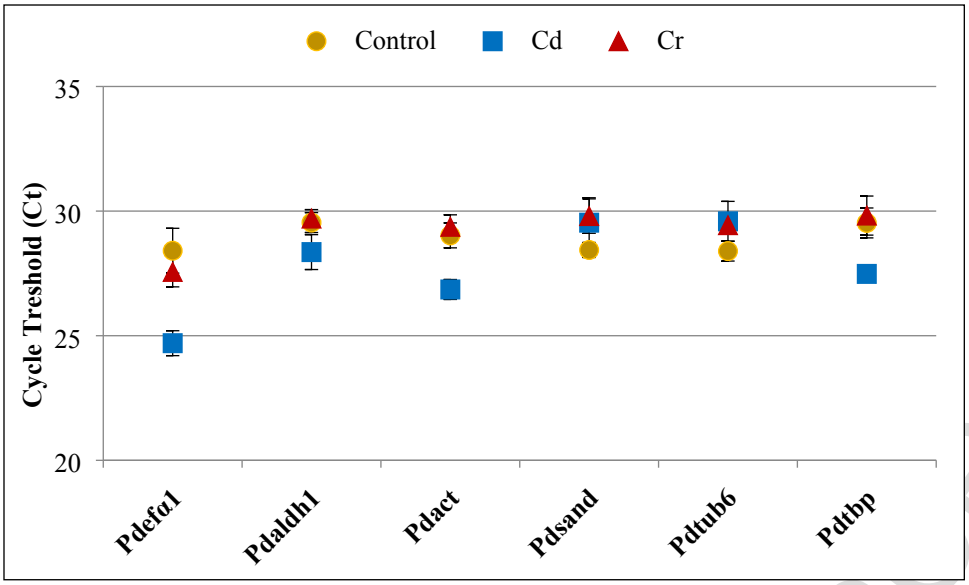
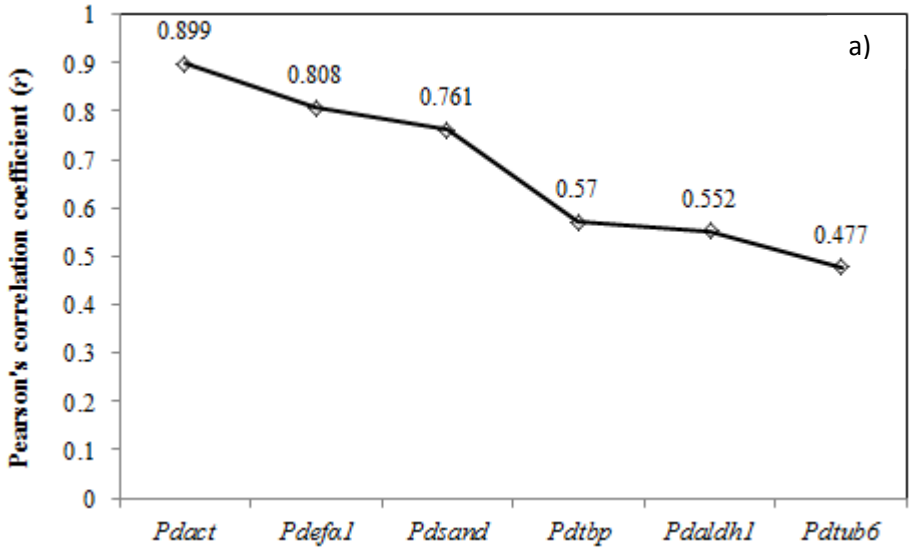
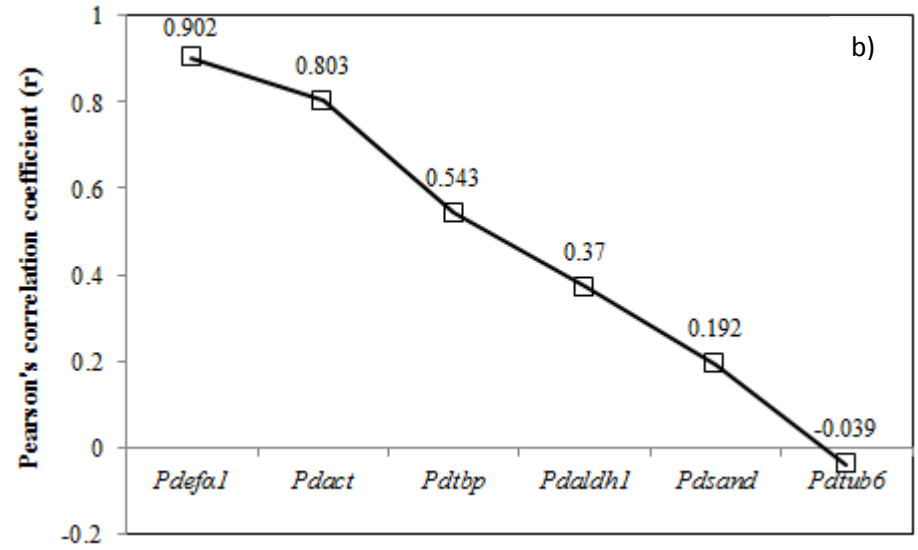
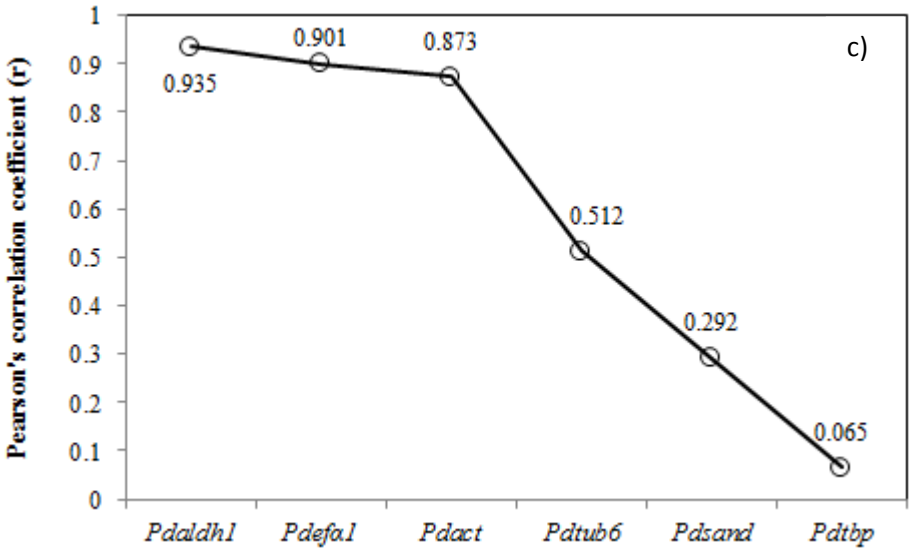


Fig. 1. Cycle treshold (Ct) of candidate reference genes for each experimental condition. Data presented are means ± standard error of three independent experiments.







Most stable ← → Least stable

Fig. 2. Ranking and expression stability of candidate reference genes measured by BestKeeper for samples exposed to control (a), cadmium (b) and chromium (c) treatment.

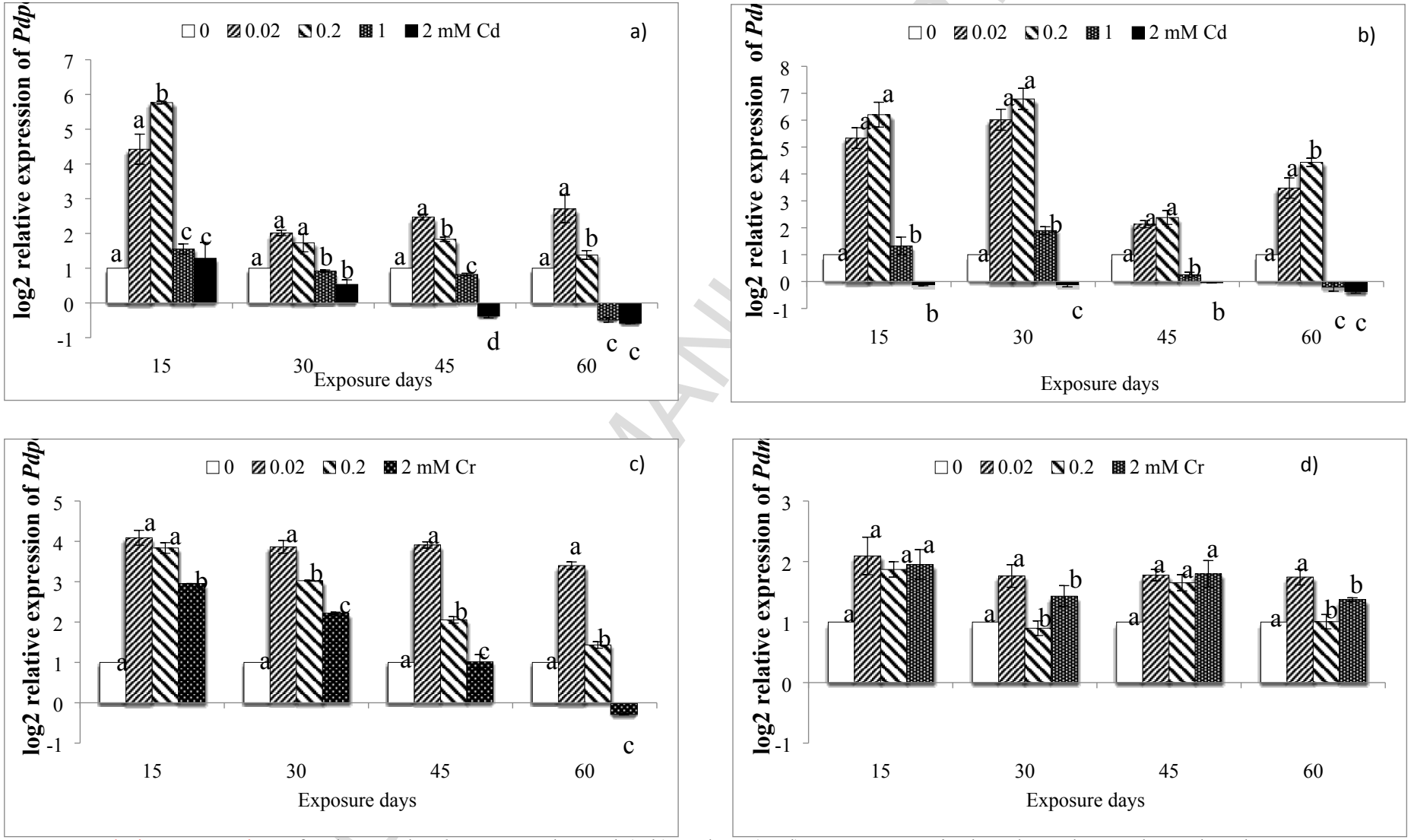


Fig. 4. Log2 relative expression of *Pdpcs* and *Pdmt* exposed to Cd (a,b) and Cr (c, d) stress respectively. The red area shows basal genes variations. Data presented are means ± standard error of three independent experiments. Differences between groups are shown as results of one-

way ANOVA post-hoc Tukey's test; where small letters show differences between concentrations. Means not showing the same letter are statically different.

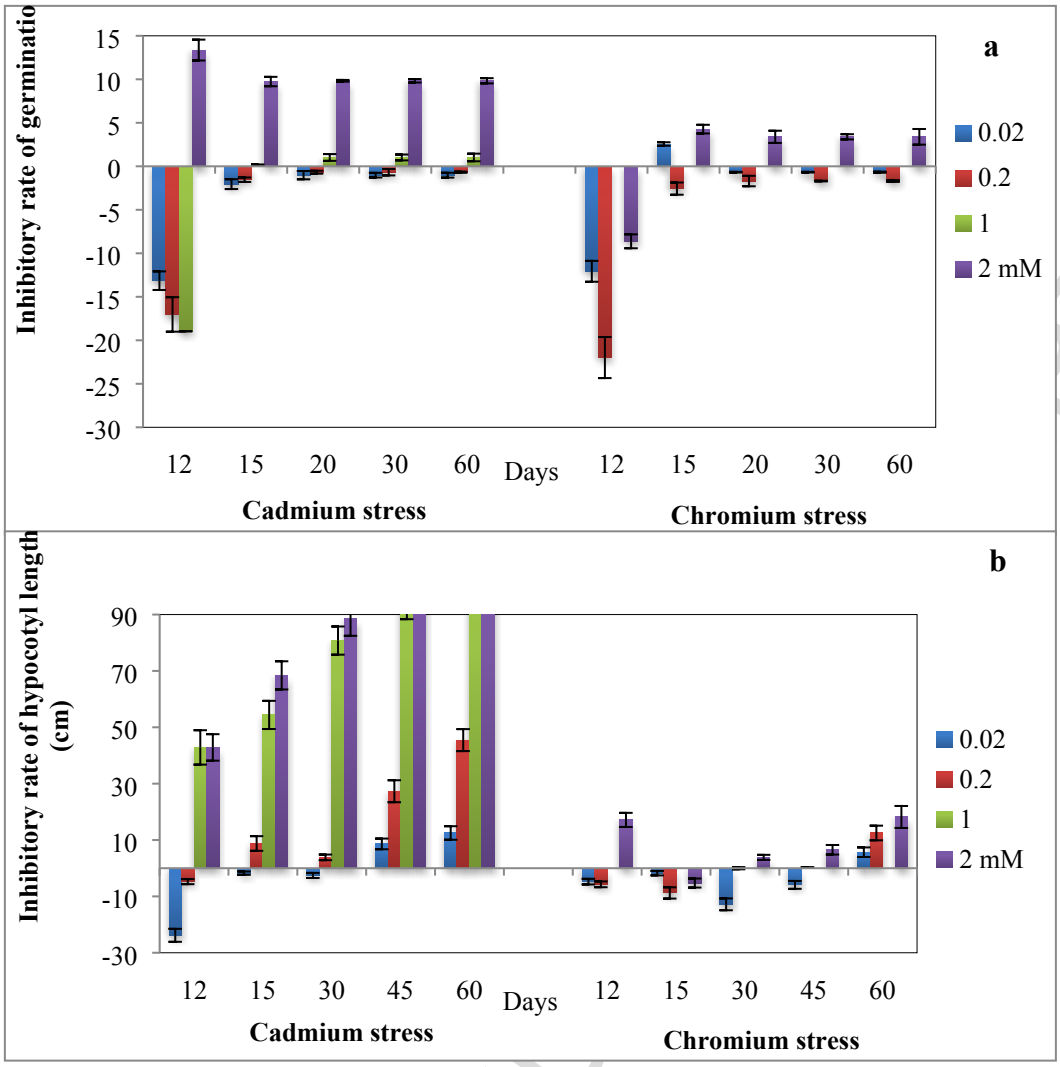


Fig. 5. Effects of different Cd or Cr concentrations on germination percentage (a) and hypocotyl length inhibition (b) have been shown. Data presented are means ± standard error of three independent experiments.

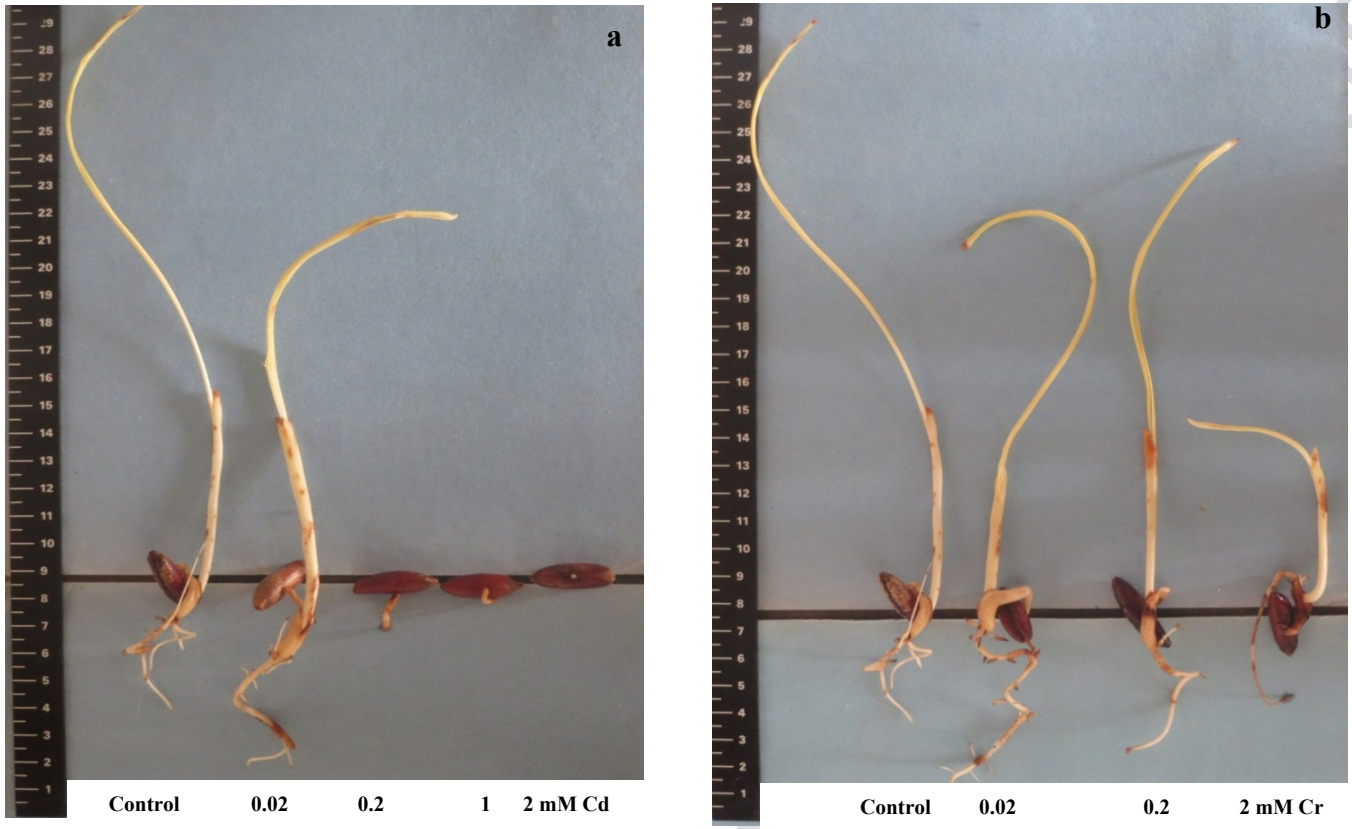


Fig. 6. Photograph of 2 months seedling of date palm exposed to Cd (a) and Cr stress (b).

HIGHLIGHTS

- Reference genes were selected in date palm hypocotyls exposed to Cd and Cr
- Phytochelatin synthase gene was upregulated under Cd and Cr stress
- Metallothionein less responsive to heavy metal stress than phytochelatin synthase
- Identification of potential biomarkers of HMs contamination

Table 1. Primer sequences used for cloning of candidate and reference genes.

Name	Functional description	Amplification length (bp)	qPCR specific Primer sequence (5'→3')	Length (bp)	Tm	GC %	Contig Length (bp)
Pdefα1	Elongation factor 1-alpha	235	F : CTTGACTGCCACACCTCTCA	20	60.02	55.0	237
			R : TGATGACACCAACAGCCACT	20	60.16	50.0	
Pdaldh1	Aldehyde dehydrogenase	239	F : CAACCGTGGATGTGTCTGTC	20	60.00	55.0	241
			R : GTCGTACCAAGCCACCAACT	20	60.04	55.0	
Pdact2	Actine 2	265	F : AGGTGCCCAGAGGTTCTTTT	20	60.11	50.0	165
			R : AGCAATACCAGGGAACATGG	20	59.81	50.0	
Pdsand	SAND family protein	218	F : TGTGAGGCATTGAGGGAACAA	20	57.7	47.6	129
			R : TTCCATAAGCAAGAGGAAGGCA	20	57.6	45.5	
Pdtub6	Tubulin beta-6	236	F : GCCTCAGTGAATTCCATCTCAT	20	60.5	45.5	138
			R : TGCAGAACAAGAACTCCTCCTAC	20	59.9	47.8	
Pdtbp	TATA-box-binding protein	251	F : TGATCCGAGCGTACTTCCTT	20	59.84	50.0	152
			R : CCCTTCTGGCATTGTTCCTA	20	60.1	50.0	
*Pdmt**	Metallothionin	107	F : GTGCGTGAAGAAGGGAAATG	20	60.4	50.0	107
			R : ACTTGCAGTCAGGCTCCGTA	20	60	55	
*Pdpcs**	Phytochelatin synthase	171	F : GCTGCGAACCTTTGGATAGA	20	60.4	50.0	113
			R : TCAGATGGCAGTCCTCAGTG	20	60	55.0	

* Primer sequences from Chaâbene et al. (2017)

Table 2. qPCR Primer sequences of the candidate and reference genes used for relative gene expression analysis.

Name	Amplification length (bp)	qPCR specific Primer sequence (5'→ 3')	Length (bp)	Tm	GC %	PCR Efficiency ±SD
Pdefα 1	153	F : TGACTGCCACACCTCTCACATTGC	24	59.4	54.2	2±0.01
		R : ACAACCATGGGCTTGGTGGGAA	22	59.1	54.5	
Pdaldh 1	149	F : ACCGTGGATGTGTCTGTCGTGGAT	24	59.9	54.2	2±0.01
		R : AAGTCGGTGGACACCAAGTCCTCT	24	59.3	54.2	
Pdact 2	129	F : TTTCCAGCCGTCCCTCATTGGAA	23	58.2	52.2	1.95±0.03
		R : GTTGATCCTCCACTGAGCACAACG	24	58.0	54.2	
Pdsand	120	F : CTGCTTATCTTGACAAAGTCAGTTC	25	58.7	40.0	1.98±0.02
		R : CCAGCTGAAGGAATGAATCAG	21	59.8	47.6	
Pdtub 6	183	F : ATCCATTCCCTCCCCTGTAT	20	59.5	50.0	2.02±0.04
		R : GGATCCCCAACAATGTGAAG	20	60.2	50.0	
Pdtbp	152	F : TTGCTCCTGTACAAACCATTTT	22	58.7	36.4	1.99±0.01
		R : GGACTGCAAGTTGGACCTTA	20	57.8	50.0	
*Pdmt**	107	F : GTGCGTGAAGAAGGGAAATG	20	60.4	50.0	1.99±0.02
		R : ACTTGCAGTCAGGCTCCGTA	20	60	55	
*Pdpcs**	113	F : GGCAGTCCTCAGTGGAAGTA	20	57.9	55	2±0.00
		R : TGCTTGCTTGGCTTACTGTG	20	60.2	50.0	

* Primer sequences from Chaâbene et al., (2017)

Table 3 geNorm *M values* and NormFinder SD values of six date palm HKGs. The most stably expressed genes values are presented in fold.

		Pdefα1	*Pdaldh1*	*Pdact*	*Pdsand*	*Pdtub6*	*Pdtbp*
GeNorm *M* values	Control	0.20	0.30	**0.01**	0.19	**0.16**	**0.12**
	Cd	**0.15**	0.20	**0.19**	0.19	0.21	**0.02**
	Cr	**0.10**	**0.09**	0.12	0.18	0.40	**0.07**
NormFinder SD values	Control	0.450	0.608	**0.021**	**0.395**	0.432	**0.247**
	Cd	**0.367**	0.502	**0.41**	0.473	0.523	**0.071**
	Cr	**0.127**	**0.113**	0.155	0.247	0.502	**0.198**

Table 4

Log2 relative expression pattern of *Pdpcs* and *Pdmt* exposed to Cd and Cr stress. Results shown as mean ± SD. Two-way ANOVA main effects shown as *p* values. Differences between groups are shown as results of one-way ANOVA post-hoc Tukey's test. where small letters show differences between concentrations (in columns) and major letters show differences in time exposure (in rows). Means not showing the same letter are statically different.

		Pdpcs				***Pdmt***			
		15	**30**	**45**	**60**	**15**	**30**	**45**	**60**
Cd	0	1.03aA (0.05)	2.03aA (0.05)	2.13aA (0.01)	2.04aA (1.14)	0.45aA (0.36)	0.46aA (0.48)	1.57aB (0.16)	0.92aA (0.10)
	0.02	4.58bA (0.44)	4.11bA (0.15)	5.27bA (0.18)	5.54bA (0.81)	2.45bA (0.63)	2.77bA (0.18)	3.35bA (0.20)	3.21bA (0.34)
	0.2	5.97cA (0.03)	3.52bB (0.52)	3.90cB (0.13)	2.82aB (0.25)	2.85bA (0.21)	3.12bA (0.18)	3.74bA (0.40)	4.09cB (0.14)
	1	1.60dA (0.15)	1.87aA (0.06)	1.75aA (0.07)	-1.00cB (0.12)	0.60aA (0.15)	0.87cA (0.06)	0.40cA (0.14)	-0.19dB (0.14)
	2 mM	1.33dA (0.47)	1.08aA (0.26)	-0.80dB (0.11)	-1.19cB (0.02)	-0.05aA (0.01)	-0.05cA (0.03)	-0.07cA (0.00)	-0.34dB (0.05)
Two way ANOVA main effects									
Concentration				0.000*				0.000*	
Time				0.000*				0.000*	
Concentration x Time				0.000*				0.000*	
Cr	0	2.03aA (0.05)	2.27aA (0.27)	2.37aA (0.32)	2.91aA (0.07)	1.68aA (0.68)	2.460aA (0.48)	2.57aA (0.16)	3.25aA (0.07)
	0.02	8.32bA (0.37)	8.78bA (0.35)	9.26bA (0.18)	9.91bB (0.26)	3.53aA (0.52)	4.33bcA (0.46)	4.57bA (0.24)	5.66bB (0.42)
	0.2	7.81bA (0.26)	6.88cB (0.02)	4.86cC (0.18)	4.17cC (0.23)	3.15aA (0.21)	2.21aAB (0.29)	4.24bAC (0.34)	3.27aA (0.38)
	2 Mm	6.00cA (0.01)	5.04dB (0.05)	2.4aC (0.42)	-0.82dD (0.05)	3.29aA (0.41)	3.51acA (0.42)	4.62bA (0.56)	4.46cA (0.09)
Two way ANOVA main effects									

Concentration	0.000*	0.000*
Time	0.000*	0.000*
Concentration x Time	0.000*	0.019*