Université de Lille 1 – Sciences et Technologies

Diplôme d'Habilitation à Diriger des Recherches

Evolution de l'adaptation locale en environnement anthropisé : le cas des pseudométallophytes

Hélène Frérot-Pauwels

Présenté le 10 octobre 2016 devant la commission d'examen composée de :

Laurence Després, professeure, Université Grenoble Alpes	**Rapporteure**
Christine Dillmann, professeure, Université Paris Sud	**Rapporteure**
Marc Hanikenne, Chercheur Qualifié du FNRS, Université de Liège	**Examinateur**
Henk Schat, professeur, Université Libre d'Amsterdam	**Rapporteur**
Sébastien Thomine, directeur de recherche CNRS, I2BC	**Examinateur**
Pascal Touzet, professeur, Université de Lille – Sciences et Technologies	**Examinateur**
Denis Vile, chargé de recherche, INRA Montpellier	**Examinateur**

TABLE DES MATIERES

CURRICULUM VITAE

Hélène FREROT

Maître de Conférences de l'IUT-A de Lille (section 67)

Née le 25 novembre 1977 à Agen (47)

Mariée, deux enfants

1. CURSUS

- Depuis 2013 : titulaire de la Prime d'Excellence Scientifique
- Depuis 2006: **Maître de Conférences** rattachée à l'IUT-A de l'Université de Lille, recherche effectuée dans l'unité UMR CNRS 8198 Evolution-Ecologie-Paléontologie
- 2005-2006: **Attaché Temporaire à l'Enseignement et à la Recherche** au laboratoire Génétique et Evolution des Populations Végétales (devenu unité EEP)
- 2001-2004: **Thèse de doctorat en Biologie de l'Evolution et Ecologie** de l'Ecole Nationale Supérieure d'Agronomie de Montpellier

  Titre: "Aspects génétiques et écologiques de la tolerance aux métaux lourds et de l'hyperaccumulation chez *Thlaspi caerulescens* (Brassicaceae). Perspectives en phytoremédiation."

  Directeurs de thèse: José Escarré (CNRS) & Christophe Petit (Université Montpellier II)

  Laboratoire: Centre d'Ecologie Fonctionnelle et Evolutive - CNRS Montpellier

  Financement: Bourse des Ingénieurs, CNRS & Région Languedoc-Roussillon
- 2000-2001: **Master en Biologie de l'Evolution et Ecologie** de l'Université Montpellier II
- 1998-2001: **Diplôme d'Ingénieur en Agronomie** de l'Ecole Nationale Supérieure d'Agronomie de Montpellier

2. PUBLICATIONS ET PRODUCTIONS SCIENTIFIQUES DEPUIS LA THESE

Les noms en bleu correspondent à des étudiants de thèse ou de M2 encadrés.

a. ARTICLES DANS DES REVUES INTERNATIONALES OU NATIONALES AVEC COMITE DE LECTURE

1. Meyer CL, Pauwels M, Briset J, Godé C, Salis P, Bourceaux A, Souleman D, **Frérot H***, Verbruggen N*. Potential preadaptation to anthropogenic pollution: evidence from a common QTL for zinc and cadmium tolerance in metallicolous and non-metallicolous accessions of *Arabidopsis halleri*. *New Phytologist*. (in press). ***contributions équivalentes**
2. Bapst-Kostecka AA, Waldmann P, **Frérot H**, Vollenweider P. (2016). Plant adaptation to metal polluted environments—Physiological, morphological, and evolutionary insights from *Biscutella laevigata*. *Environmental and Experimental Botany*. 127:1-13.
3. Bonin A, Paris M, **Frérot H**, Bianco E, Tetreau G, Després L. (2015). The genetic architecture of a complex trait: resistance to multiple toxins produced by *Bacillus thuringiensis israelensis* in the dengue and yellow fever vector, the mosquito *Aedes aegypti*. *Infection, Genetics and Evolution*. 35: 204-213.

4. Gonneau C, Genevois N, **Frérot H**, Sirguey C, Sterckeman T. (2014). Variation of trace metal accumulation, major nutrient uptake and growth parameters and their correlations in 22 populations of *Noccaea caerulescens*. *Plant and Soil*. 384(1-2): 271-287.
5. Pauwels M, **Frérot H**, Souleman D & Vandenbulcke F. (2013). Using biomarkers in an evolutionary context: Lessons from the analysis of biological responses of oligochaete annelids to metal exposure. *Environmental Pollution*, *179*: 343-350.
6. Escarré J, Lefèbvre C, **Frérot H**, Mahieu S, Noret N. (2013). Metal concentration and metal mass of metallicolous, non metallicolous and serpentine *Noccaea caerulescens* populations cultivated in different growth media. *Plant and Soil*. 370(1-2): 197-221.
7. Pauwels M, Vekemans, Godé C, **Frérot H**, Castric V, Saumitou-Laprade P. (2012). Nuclear and chloroplast DNA phylogeography reveals vicariance among European populations of the model species for the study of metal tolerance, *Arabidopsis halleri* (Brassicaceae). *New Phytologist*. 193(4): 916-928.
8. Mahieu S, **Frérot H**, Vidal C, Galiana A, Heulin K, Maure L, Brunel B, Lefèbvre C, Escarré J, Cleyet-Marel JC. (2011). *Anthyllis vulneraria/Mesorhizobium metallidurans*, an efficient symbiotic nitrogen fixing association able to grow in mine tailings highly contaminated by Zn, Pb and Cd. *Plant and Soil*. 342(1-2): 405-417
9. **Frérot H**. (2011). A challenge for hyperaccumulating plant models: 'cycling' as fast as *Arabidopsis thaliana*. *New Phytologist* 189(2): 357-359.
10. Escarré J, Lefèbvre C, Raboyeau S, Dossantos A, Gruber W, Cleyet-Marel JC, **Frérot H**, Noret N, Mahieu S, Collin C, van Oort F. (2011). Heavy metal concentration survey in soils and plants of the Les Malines mining district (Southern France): implications for soil restoration. *Water, Air and Soil Pollution*. 216:485–504.
11. **Frérot H**, Faucon MP, Willems G, Godé C, Courseaux, Darracq A, Verbruggen N, Saumitou-Laprade P. (2010). Genetic architecture of zinc hyperaccumulation in *Arabidopsis halleri*: the essential role of QTL x environment interactions. *New Phytologist* 187(2): 355-367.
12. Willems G, **Frérot H**, Gennen J, Salis P, Saumitou-Laprade P, Verbruggen N. (2010). Quantitative trait loci analysis of mineral element concentrations in an *Arabidopsis halleri x Arabidopsis lyrata petraea* F-2 progeny grown on cadmium-contaminated soil. *New Phytologist* 187 (2): 368-379.
13. Shahzad Z, Gosti F, **Frérot H**, Lacombe E, Roosens N, Saumitou-Laprade P, Berthomieu P. (2010). The Five AhMTP1 Zinc Transporters Undergo Different Evolutionary Fates towards Adaptive Evolution to Zinc Tolerance in *Arabidopsis halleri*. *Plos Genetics* 6(4): e1000911.
14. Meyer CL, Kostecka AA, Saumitou-Laprade P, Créach A, Castric V, Pauwels M, **Frérot H**. (2010). Variability of zinc tolerance among and within populations of the pseudometallophyte species *Arabidopsis halleri* and possible role of directional selection. *New Phytologist* 185(1): 130-142.
15. Sarret G, Willems G, Isaure MP, Marcus MA, Fakra SC, **Frérot H**, Pairis S, Geoffroy N, Manceau A, Saumitou-Laprade P. (2009). Zinc distribution and speciation in Arabidopsis halleri x Arabidopsis lyrata progenies presenting various zinc accumulation capacities. *New Phytologist* 184(3): 581-595.
16. Pauwels M, Willems G, Roosens N, **Frérot H**, Saumitou-Laprade P. (2008). Merging methods in molecular and ecological genetics to study the adaptation of plants to anthropogenic metal-polluted sites: implications for phytoremediation, *Molecular Ecology*. 17 (1): 108–119.
17. Pauwels M, Roosens N, **Frérot H**, Saumitou-Laprade P. (2008). When population genetics serves genomics: putting adaptation back in a spatial and historical context. *Current Opinion in Plant Biology*, 11 (2): 129-134.
18. Pauwels M, **Frérot H**, Bonnin I, Saumitou-Laprade P. (2006). A broad-scale analysis of population differentiation for Zn tolerance in an emerging model species for tolerance study: *Arabidopsis halleri* (Brassicaceae). *Journal of Evolutionary Biology*. 19(6): 1838-1850.

b. CONFERENCES SUR INVITATION

1. **Frérot H**, Pauwels M. (2015). Mécanismes d'adaptation des végétaux à la pollution métallique: les modèles pseudométallophytes. Colloque « Les êtres vivants face aux pollutions », BRGM Orléans.
2. Decombeix I, Saumitou-Laprade P, **Frérot H**. (2011). QTL de tolérance au zinc chez *Arabidopsis halleri*. Ecole Chercheurs Génomique et Diversité des Caractères à Déterminisme Complexe, La Colle-sur-Loup.
3. **Frérot H**, Vidal C, Mahieu S, Escarré J, Maure L, Brunel B, Lefèbvre C, Heulin K, Galiana A, Cleyet-Marel JC. (2009). Phytostabilisation de déchets miniers utilisant les interactions spécifiques entre des plantes méditerranéennes métallicoles et la symbiose Rhizobium / Fabaceae. *Réseau Francophone d'Echanges et de Valorisation en Ecologie de la restauration*. Avignon.

c. COMMUNICATIONS AVEC ACTES DANS CONGRES NATIONAL

Non oratrice :

1. Roosens N, Shahzad Z, Lacombe E, **Frérot H**, Créach A, Berthomieu P, and P Saumitou-Laprade (2008). Identification des gènes candidats de la tolérance au zinc chez la pseudométallophyte *Arabidopsis halleri* par l'intégration des données de génomique comparative et de transcriptomique disponibles chez Arabidopsis. 7e Colloque national BRG : Les ressources génétiques à l'heure des génomes, FRB (ed). Strasbourg, pp. 373-389

d. COMMUNICATIONS ORALES SANS ACTES DANS UN CONGRES INTERNATIONAL OU NATIONAL

En tant qu'oratrice :

1. **Frérot H**, Pauwels M. (2014). Evolution de l'adaptation locale en environnement anthropisé. Réunion annuelle du GDR CNRS « Génétique Quantitative en Populations Naturelles », Toulouse
2. **Frérot H**, Decombeix I, Bourceaux A, Maxime P. (2014). Phenotype-dependent QTLs of zinc tolerance in *Arabidopsis halleri*. 3rd meeting of LOCOMET international network (GDRI CNRS), Lille
3. **Frérot H**, Pauwels M. (2012). Molecular ecology in *Arabidopsis halleri* and *Noccaea caerulescens*. 1st meeting of LOCOMET international network (GDRI CNRS), Lille.
4. **Frérot H**, Pauwels M, Faucon MP, Kostecka A, Meyer CL, Decombeix I, Créach A, Saumitou-Laprade P. (2009). Genetic bases of adaptation to calamine soils in *Arabidopsis halleri* (Brassicaceae): implications for phytoremediation. 14th International Symposium on Toxicity Assessment, Metz.

Non oratrice :

1. Karam MJ, Souleman D, Gallina S, Hanikenne M, Schvartzman S, Pauwels M, **Frérot H**. (2015). From NGS to SNPs for intra-species QTL mapping in *Arabidopsis halleri*. 4th meeting of LOCOMET international network (GDRI CNRS), Lille.
2. Souleman D, Meyer CL, Pauwels M, Vandenbulcke F, **Frérot H**. (2014). Local adaptation to heavy metals in two model species: *Lumbricus terrestris* and *Arabidopsis halleri*. 3rd meeting of LOCOMET international network (GDRI CNRS), Lille.

3. Meyer CL, Briset L, Osterieth L, Souleman D, Godé C, **Frérot H**, Verbruggen N. (2014). Genetic architecture of metal tolerance in *Arabidopsis halleri*. Colloque Ecology BES-SFE, Lille.
4. Decombeix I, Pauwels M, Godé C, Bourceaux A, Glorieux C, Boucherie P, Courseaux A, Créach A, Saumitou-Laprade P, **Frérot H**. (2011). Disentangling demographic processes and local adaptation in the evolution of adaptative traits in the model species *Arabidopsis halleri*. International Botanical Congress, Melbourne.
5. Decombeix I, Loingeville F, Godé C, Pauwels M, **Frérot H**, Bourceaux A, Glorieux C, Boucherie P, Créach A, Saumitou-Laprade P. (2011). Disentangling demographic processes and local adaptation in the evolution zinc hyperaccumulation in the model species *Arabidopsis halleri*. Ecological Genetics Group, London.
6. Pauwels M, Meyer CL, Kostecka A, Decombeix I, **Frérot H**, Créach A, de Laguérie P, Castric V, Saumitou-Laprade P. (2010). Conséquences génétiques de la colonisation des sites calaminaires chez *Arabidopsis halleri* (Brassicaceae), Colloque Ecologie 2010, Montpellier.
7. Decombeix I, Godé C, Bourceaux A, Tailliez A, Capet G, Glorieux C, Créach A, Pauwels M, Saumitou-Laprade P, **Frérot H**. (2010). Vers une meilleure compréhension écologique et évolutive de la tolérance au zinc chez *Arabidopsis halleri*. Colloque Ecologie 2010, Montpellier.

e. COMMUNICATIONS PAR AFFICHE DANS UN CONGRES INTERNATIONAL OU NATIONAL

1. Karam MJ, Souleman D, Gallina S, Hanikenne M, Schvartzman S, Pauwels M, **Frérot H**. (2015). Local adaptation in metal polluted environments: how and how fast does it occur? Seeking answers in the NGS era. SMBEBA, Montpellier.
2. Souleman D, Meyer CL, Briset L, Verbruggen N, **Frérot H**. (2014). Nouvelles avancées sur l'origine évolutive de de la tolérance aux métaux chez l'espèce pseudométallophyte *Arabidopsis halleri* : que nous apprennent les populations non métallicoles ? 36^{ième} Réunion du Petit Pois Déridé, INRA Versailles.
3. Decombeix I, Chevin LM, Bourceaux A, Godé C, Pauwels M, **Frérot H**. (2012). Epistasis versus pleiotropy in the determinism of genetic correlations: a QTL analysis for zinc tolerance traits in *Arabidopsis halleri* (Brassicaceae). 4th International Conference of Quantitative Genetics, Edinburgh.
4. Kostecka AA, **Frérot H**, Saumitou-Laprade P, Grodzińska K. (2010). Is there evidence of local adaptation in metallicolous and non-metallicolous populations of *Arabidopsis halleri*?" *Society of Environmental Toxicology and Chemistry,* Seville.
5. Decombeix I, **Frérot H**, Godé C, Bourceaux A, Boucherie P, Loingeville F, Glorieux C, Courseaux A, Créach A, Saumitou-Laprade P, Pauwels M. (2010). Patron de polymorphisme de l'hyperaccumulation de zinc chez *Arabidopsis halleri*: un cas d'adaptation locale? Colloque Ecologie 2010, Montpellier.
6. Tailliez A, Decombeix I, Saumitou-Laprade P, **Frérot H**, Créach A, Godé C, Bourceaux A. (2009). La détection de QTLs liés à la tolérance au zinc dépend-elle du milieu de culture et du stade phénologique ? 31^{ième} Réunion du Petit Pois Déridé, Grenoble.

f. OUVRAGES SCIENTIFIQUES / CHAPITRES D'OUVRAGES SCIENTIFIQUES

1. **Frérot H**, de Laguérie P, Créach A, Meyer CL, Pauwels M, Saumitou-Laprade P. (2011). Adaptation to metals in higher plants: the case of *Arabidopsis halleri* (Brassicaceae). In: Tolerance to environmental contaminants. Claude Amiard-Triquet, Philip S. Rainbow, Michèle Roméo (eds). CRC Press Inc.
2. Pauwels M, **Frérot H**, Saumitou-Laprade P. (2010). Pollution et évolution contemporaine: la tolérance aux métaux lourds des végétaux supérieurs. In: Frédéric Thomas, Thierry Lefevre, Michel Raymond (eds), Biologie Evolutive. De Boeck Editions.
3. **Frérot H**, Lefèbvre C, Petit C, Collin C, Dos Santos A & Escarré J. (2009). Adaptation des végétaux aux milieux pollués par les métaux lourds et phytoremédiation. Cas de la Région Languedoc-Roussillon. In: Philippe Cambier, Christian Schvartz, Folkert van Oort (eds), Contaminations métalliques des agrosystèmes et écosystèmes péri-industriels**.** Quae Editions.

3. ACTIVITES D'ENCADREMENT

a. CO-ENCADREMENTS DE THESE

1. Co-encadrement de la thèse de Julien Nowak (50%) : « Evolution expérimentale de la tolérance et de l'hyperaccumulation du zinc chez *Noccaea caerulescens* ». En cours (2015-2018).
2. Co-encadrement de la thèse de Dima Souleman (50%) : « Etude de l'adaptation locale aux milieux pollués par les métaux ». En cours (2012-2016).
3. Co-encadrement de la thèse de Cédric Gonneau (10%) : « Distribution, écologie et évolution de l'hyperaccumulation des éléments en traces par *Noccaea caerulescens* ». Soutenue le 26 mars 2014.
4. Co-encadrement de la thèse d'Isabelle Decombeix (50%): « Apport de l'écologie dans l'étude de l'adaptation d'*Arabidopsis halleri* aux sites polluées par les métaux lourds ». Soutenue le 5 décembre 2011.
5. Co-encadrement de la thèse d'Alicja Kostecka (50%): "Adaptation of *Arabidopsis halleri* to habitat rich in heavy metals in Southern Poland". Soutenue le 25 février 2009.
6. Co-encadrement de la thèse de Claire-Lise Meyer (30%) : "Evolution des populations métallicoles d'*Arabidopsis halleri* (Brassicaceae), étude sur les traits et sur le génome en populations naturelles". Soutenue le 19 juin 2009.

b. AUTRES ACTIVITES D'ENCADREMENT

Encadrement de post-doctorants :

Marie-Joe Karam 2015-2017

Encadrements d'étudiants en M2 :

Michel-Pierre Faucon 2005, Seydou Traore 2007, Bérénice Dambrine 2008, Guillaume Capet 2010, Julien Nowak 2015

Encadrements d'étudiants en M1 :

Bérénice Dambrine et Pauline Goubet 2007, Doriane Canesse 2008, Gabriel Fourel 2009

Autres encadrements :

Florence Loingeville 2011 (Polytech, Lille), Thomas Vandewalle 2012 (SupBiotech, Paris), Mégane Lorgouilloux et Jérémy Armetta 2014 (IUT-A, Lille)

4. ACTIVITES DE RAYONNEMENT ET ATTRACTIVITE ACADEMIQUE

a. PARTICIPATION A DES PROJETS DE RECHERCHE COLLABORATIFS

1. *2015-2018* : ANR ARSENIC « Adaptation et résilience des réseaux d'interactions écologiques face aux changements induits par l'Homme ». Coordination François Massol et Nicolas Loeuille.
2. *2013-2017* : **coordinatrice** de l'ANR Jeunes Chercheurs-Jeunes Chercheuses ELOCANTH « Evolution de l'adaptation locale en environnement anthropisé ».
3. *2012-2014* : **coordinatrice** en France du programme d'échange franco-belge PHC TOURNESOL (2012-2014): « Bases génétiques et épigénétiques de l'adaptation locale chez les plantes hyperaccumulatrices de métaux » (coordination en Belgique : M. Hanikenne).
4. *2011-2014* : Arcir BioImpact « Impacts des changements globaux sur la distribution de la biodiversité en région Nord Pas de Calais », volet « Anthropisation des sols et installation d'espèces nouvelles : le cas d'*Arabidopsis halleri*, une espèce spécifique des sols pollués en région Nord Pas de Calais » dans le cadre de l'opération de recherche soutenue par le GIS « Biodiversité en Nord-Pas de Calais ». Coordination Yves Piquot.
5. *2011-2014* : projet FRB Biodiversité ORDYNORD « Origine et Dynamique de la biodiversité de la flore sur les milieux calaminaires du Nord-Pas de Calais ». Coordination Pierre Saumitou-Laprade.
6. *2011-2013* : Arcir PlanTeq8 « Génétique de l'homéostasie des métaux chez les plantes : le cas du zinc et du cadmium chez Arabidopsis ». Coordination Pierre Saumitou-Laprade.
7. *2009-2010* : **coordinatrice** du projet PEPS MOBILMET, « Exploration des capacités de mobilisation des métaux lourds en phase solide contenus dans les sols pollués calaminaires par la pseudométallophyte modèle *Arabidopsis halleri* ».

b. COLLABORATIONS SUIVIES AVEC D'AUTRES LABORATOIRES

1. Laboratoire de Physiologie et de Génétique moléculaire des Plantes, Université Libre de Bruxelles. Contact : Pr Nathalie Verbruggen.
   - Trois manuscrits co-signés
   - Une participation de part et d'autre dans des jurys de thèse
2. Laboratoire de Génomique Fonctionnelle et Imagerie Moléculaire Végétale, Université de Liège. Contact : Marc Hanikenne, chercheur FNRS.
   - Echanges réguliers dans le cadre de l'ANR JCJC Elocanth
   - Programme d'échange franco-belge PHC TOURNESOL (2012-2014): « Bases génétiques et épigénétiques de l'adaptation locale chez les plantes hyperaccumulatrices de métaux »
3. Laboratoire Sols et Environnement, Université de Lorraine.
   Contact : Thibault Sterckeman, IR INRA et Catherine Sirguey, MCF.
   - Deux manuscrits co-signés (un publié et un soumis)
   - Une participation dans un jury de thèse

c. PARTICIPATION A DES RESEAUX, SOCIETES SAVANTES

1. *Depuis 2010* : membre de la Société Française d'Ecologie
2. *2016-2020* : co-coordinatrice du réseau international GDRI CNRS intitulé LOCOMET (Transport, localization and complexation of metals in hyperaccumulating plants).
3. *2011-2015* : **coordinatrice** du réseau international GDRI CNRS intitulé LOCOMET (Transport, localization and complexation of metals in hyperaccumulating plants).

4. *2011-2015* : participation à l'axe thématique « Adaptation en milieu hétérogène » dans le GDR 3448 GQPN « Génétique Quantitative dans les Populations Naturelles ». Coordination Benoît Pujol et Anne Charmantier.

d. PARTICIPATION A DES INSTANCES D'EXPERTISES SCIENTIFIQUES

1. *2010* : expertise de projet ANR pour appel d'offre cd2i

e. REVISIONS DE MANUSCRITS

15 révisions au total pour *Molecular Ecology, New Phytologist*, *Plant and Soil*, *Planta, Journal of Environmental Quality, Journal of Agronomy and Crop Science, Central European Journal of Biology, Australian Journal of Soil Research, Environmental Engineering and Management Journal*

f. PARTICIPATION A DES JURYS

1. Thèse de doctorat de Cecilia Baliardini (Université Libre de Bruxelles) : « Genetic analysis of cadmium tolerance in *Arabidopsis halleri* : contribution of *CAX1* ». Soutenue le 29 septembre 2015.
2. Thèse de doctorat de Mazhar Iqbal (Vrije Universiteit Amsterdam): « Molecular mechanisms of heavy metal tolerance and accumulation in hyperaccumulating and non-hyperaccumulating metallophytes ». Soutenue le 8 janvier 2013.
3. Thèse de doctorat de Lucie Lovy (Université de Lorraine) : « Hyperaccumulation du Cd par *Noccaea caerulescens* : cinétique, répartition et prédiction ». Soutenue le 29 octobre 2012.
4. Thèse de doctorat d'Isabelle Decombeix (Université Lille 1) : « Apport de l'écologie dans l'étude de l'adaptation d'*Arabidopsis halleri* aux sites polluées par les métaux lourds ». Soutenue le 5 décembre 2011.
5. Thèse de doctorat de Coraline Caullet (Université de Bourgogne) : « Dispersion et adaptation des capselles : *Capsella rubella* et *Capsella bursa-pastoris* dans un agroécosystème ». Soutenue le 13 décembre 2011.
6. Thèse de doctorant de Stéphane Grenier (Université de Poitiers) : « Impact de la plasticité phénotypique et de la sélection sur l'évolution morphologique. Le cas d'une graminée pérenne, *Lolium perenne* L., sous défoliation». Soutenue le 12 décembre 2011.
7. Diplôme Supérieur de Recherche d'Adeline Vazquez (Université Lille 1) : « Identification des bases génétiques de la variation naturelle de la "tolérance" au zinc chez *Arabidopsis thaliana* ». Soutenu le 26 juin 2009.

5. ACTIVITES D'ENSEIGNEMENT ET RESPONSABILITES PEDAGOGIQUES

Enseignements à l'IUT-A de Lille, Département Génie Biologique, première année :

Cours : biologie cellulaire (9h), physiologie végétale (6h), biologie moléculaire (8h)

TP : histologie animale (72h), histologie et physiologie végétale (50h)

TD : génétique mendélienne (7,5h), biologie moléculaire (22,5h), projets personnels encadrés (20h)

Responsabilités pédagogiques :

Co-responsabilité (50%) des plannings de TP/TD de première année et de la gestion des notes de TP/TD de première année

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Toutes les personnes que je vais citer sur cette page ont contribué de près ou de loin à mes travaux de recherche depuis ma thèse. Merci à vous tous de m'avoir suivie sur ce chemin, jalonné, je l'avoue, de nombreux doutes.

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Mes parents, la famille Frérot et la famille Pauwels. Aussi loin que vous puissiez être, de moi ou de mes activités professionnelles, vous êtes mes indispensables repères.

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Julien Nowak (Mr Photoshop), Joseph Denton (Mr so-British), Marie-Joe Karam (Mijou du Liban), Dima Souleman (Doudou de Syrie), Lucas Prost (Mr 19/20), Anne-Laura Patris (Mme Zincon)… Vous avez marqué, vous marquez, ou vous marquerez encore, votre passage dans notre groupe. Cédric Gonneau et Claire-Lise Meyer sont également passés par là. Merci à tous de nous avoir témoigné votre confiance et votre amitié.

Fabrice Roux, jadis mon compagnon de bureau. Quand tu es parti j'ai perdu un ami et un collègue chercheur aguerri, qui me faisait progresser dans mon travail. Merci de m'avoir fait profiter de tes lumières.

Pascal Touzet et Xavier Vekemans, vous avez su m'encourager et me donner l'opportunité d'aller au bout de cette « aventure HDR » dans de bonnes conditions. Pascal, merci pour le coup de pouce avec la thèse de Julien.

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AVANT-PROPOS DU DOCUMENT DE SYNTHESE

Voilà un peu plus de quinze ans que je travaille sur des modèles pseudométallophytes, sur la tolérance au zinc et l'hyperaccumulation du zinc. Il est instructif de remarquer ce qui a changé depuis lors, et ce qui reste inchangé. Ce qui a changé, c'est le contexte de mes recherches. A l'époque de ma thèse notamment, il était de bon ton de justifier les recherches sur les espèces tolérantes et hyperaccumulatrices par les applications en phytoremédiation. Les recherches sur les espèces hyperaccumulatrices devaient conduire à la création d'un cultivar utile en phytoextraction, tandis que les espèces seulement tolérantes, à défaut de servir en phytoextraction, pouvaient servir en phytostabilisation. Plus tard, tandis que mes propres recherches prenaient une tournure plus fondamentale, apparaissait la notion de « Changement Global ». Aujourd'hui, je justifie l'intérêt de mes travaux en invoquant l'anthropisation croissante de l'environnement, et la nécessité de comprendre les capacités d'adaptation des espèces face à l'un des changements environnementaux parmi les plus rapides et les plus drastiques. En revanche, ce qui n'a pas changé, ce sont les questions de fond, et les difficultés expérimentales pour y répondre. Il est toujours question d'élucider les bases génétiques de la tolérance ou de l'hyperaccumulation, de connaître la relation entre ces deux traits, et de découvrir, un jour peut-être, la fonction adaptative de l'hyperaccumulation. Pour tester des hypothèses, il existe toujours de multiples discussions quant aux conditions expérimentales : sol ou hydroponie ? Avec quelle concentration en zinc ? Sulfate ou oxyde de zinc ? Sol artificiellement ou naturellement contaminé ? En conditions contrôlées ou semi-contrôlées ? Mêmes discussions pour les traits à mesurer : trait végétatif ou reproducteur ? Toutes les plantes à un moment donné ou toutes en décalé à un stade de croissance donné ? Les feuilles ou les racines ? Trait quantitatif ou qualitatif ? Trait morphologique ou physiologique ? Trait intégrateur ou l'une des composantes d'un trait complexe ? Feuille du haut ou feuille du bas ? La réponse à ces questions n'est jamais définitive, elle dépend bien souvent de la question posée, et impose de faire des choix, parfois des expériences exploratoires, et éventuellement d'accepter le renversement des hypothèses de départ. Ce document d'HDR présente mon cheminement à travers ces questions.

NB : les publications auxquelles j'ai contribué apparaissent en bleu dans le document.

CHAPITRE 1 : INTRODUCTION GENERALE

Dans ce chapitre, je traiterai du contexte scientifique, au sens large, de mes recherches. J'introduirai progressivement des notions fondamentales que j'appliquerai à mes modèles d'étude dans les chapitres suivants. Néanmoins, afin de ne pas trop disperser mon propos, je vais centrer celui-ci sur (1) le règne végétal, notamment à travers les exemples pris dans la littérature et (2) l'étude des aspects génétiques ou épigénétiques, en négligeant un peu le développement des aspects purement écologiques.

1. DU CHANGEMENT GLOBAL A L'ADAPTATION LOCALE

« Changement Global et adaptation locale ». Cette formule a l'apparence d'une contradiction. Cependant, le Changement Global n'est pas seulement la conséquence d'un vaste désordre climatique imposant un réchauffement moyen de l'ensemble de la planète. Le Changement Global est aussi la conséquence de nombreux facteurs agissant à une échelle locale (de Bello *et al*, 2013). Ces facteurs locaux sont multiples : surexploitation des terres, apport d'espèces envahissantes, fragmentation des habitats, pollution de l'atmosphère, des eaux et des sols (Millennium Ecosystem Assessment, 2005). Se placer à une échelle spatiale suffisamment petite permet l'étude de l'adaptation locale. Une échelle spatiale « suffisamment petite » signifie que, pour une espèce donnée, les populations sont connectées, au moins potentiellement, par des flux de gènes (Kawecki and Ebert, 2004). Au sens strict, l'adaptation locale correspond au cas où chaque population présente les meilleures performances dans son site d'origine par comparaison avec les performances des populations provenant des sites voisins (Kawecki and Ebert, 2004) (Figure 1). De plus, les effets locaux du Changement Global ont nécessairement lieu sur une échelle de temps réduite car ils sont contemporains des activités humaines. Ceci est dû à la nature même du Changement Global, causé directement ou indirectement par ces activités. C'est pourquoi, l'adaptation locale dans un milieu anthropisé ne peut s'étudier que dans le cadre conceptuel de l'adaptation dite « contemporaine » ou « rapide » (Reznick and Ghalambor, 2001; Stockwell *et al*, 2003).

Ainsi, l'étude du Changement Global conduit à l'étude des processus résultant d'une évolution locale et rapide, qui peuvent être adaptatifs, notamment s'ils sont génétiquement déterminés. Une autre issue possible est la plasticité phénotypique (Ghalambor *et al*, 2007; Pigliucci, 2005; van Kleunen and Fischer, 2005). D'importants flux de gènes peuvent favoriser l'évolution de phénotypes plastiques et généralistes, capables de s'ajuster à différentes conditions environnementales, au détriment de phénotypes spécialistes résultant d'une adaptation locale (Figure 2). La plasticité phénotypique pourra également être favorisée si (1) les pressions de sélection en jeu ne sont pas assez fortes pour s'y opposer, (2) les variations temporelles de l'environnement sont fréquentes et le rendent trop fluctuant pour que des spécialistes puissent évoluer, (3) celle-ci n'implique pas d'ajustements physiologiques, morphologiques ou comportementaux trop coûteux pour l'organisme (Kawecki, 2000; Kawecki and Ebert, 2004).

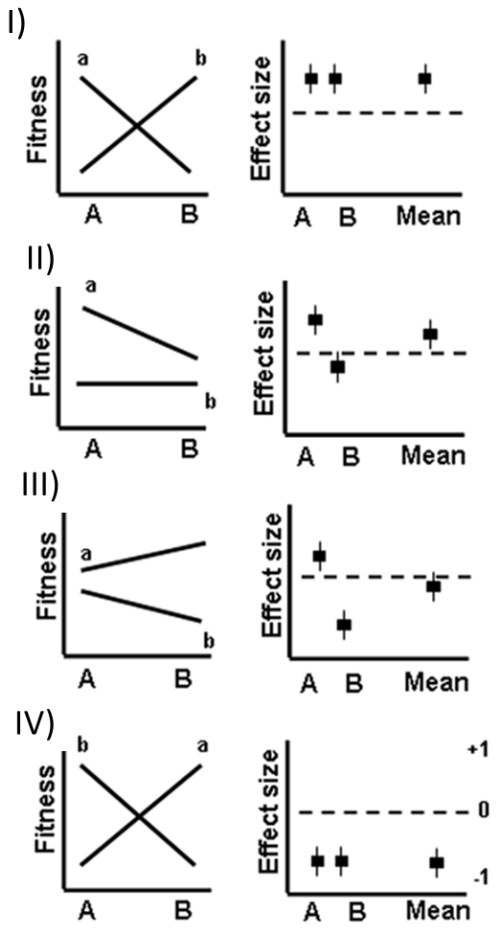

Figure 1: Combinaisons possibles de normes de réaction pour la valeur sélective (« fitness ») et les tailles d'effet correspondantes (« effect size »). Les tailles d'effet mesurent la différence de valeur sélective entre les plantes « étrangères » et les plantes locales (a ou b) pour chacun des sites A ou B. Un effet positif indique que la plante locale a une meilleure performance que la plante étrangère dans son site d'origine. Plusieurs cas sont possibles : (I) les plantes locales sont toujours meilleures que les plantes étrangères dans leur site d'origine, les normes de réaction se croisent, (II et III) les plantes issues de A sont meilleures que les plantes étrangères dans le site A, mais aussi dans le site B, et (IV) les plantes étrangères sont toujours meilleures que les plantes locales, révélant de la maladaptation dans les deux sites. (D'après la méta-analyse réalisée par Leimu et Fischer, 2008).

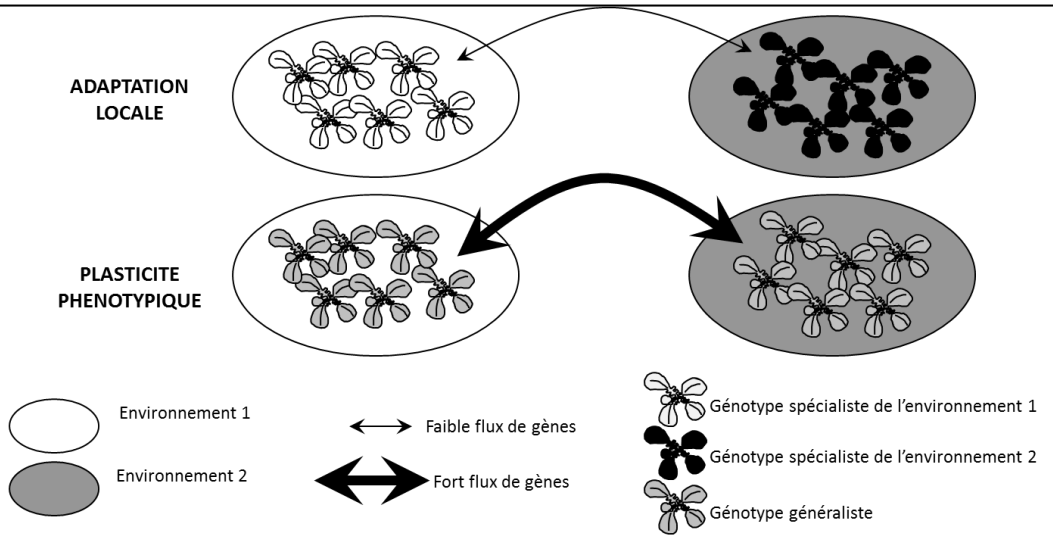

Figure 2: Relation entre flux de gènes et évolution de l'adaptation locale. En cas de faibles flux de gènes entre deux environnements contrastés, des génotypes spécialistes de chaque environnement peuvent évoluer. En cas de forts flux de gènes, l'adaptation locale peut être désavantagée, et ce sont des génotypes généralistes qui se développent dans les deux environnements.

Parmi les facteurs anthropiques responsables du Changement Global, la pollution, qu'elle soit organique ou inorganique, mérite une attention particulière car son impact peut être brutal et mettre immédiatement la survie des populations en danger. Les populations peuvent alors être instantanément soumises à, au moins, une pression de sélection très intense. Dans ce cas, l'adaptation est locale, rapide, et s'effectue en milieu dit « extrême » : les environnements radioactifs, les eaux douces polluées par les pluies acides, les eaux et les sols pollués par des pesticides, des déchets organiques, ou des résidus miniers. Après un tel changement environnemental, la survie des populations ne sera possible que dans certaines conditions démographiques, génétiques et écologiques. Parmi les facteurs génétiques influençant une possible reprise de la croissance démographique, il faut souligner l'importance de la variation génétique initiale (ou « standing variation »), de l'architecture génétique du ou des traits sous sélection, de la corrélation génétique entre ces traits, et des taux de mutation et de migration (Carlson *et al*, 2014).

2. BASES GENETIQUES DE L'ADAPTATION LOCALE EN MILIEU ANTHROPISE

La littérature théorique et empirique sur l'architecture génétique de l'adaptation locale, ou de traits sous sélection, est particulièrement abondante. Les exemples historiques les plus fréquemment cités sont le mélanisme industriel chez *Biston betularia* (Bishop, 1972), la résistance aux insecticides chez *Culex pipiens* (Lenormand *et al*, 1998), et la tolérance au cuivre chez *Mimulus guttatus* (Macnair, 1983). Il suffit à chaque fois d'un ou deux gènes majeurs pour conférer le trait adaptatif indispensable, même si l'adaptation complète requiert secondairement plusieurs changements dans l'organisme et donc le concours de très nombreux gènes à effet mineur (Reznick and Ghalambor, 2001). Il est aujourd'hui largement admis que l'adaptation locale s'appuie sur des caractères quantitatifs polygéniques (Kawecki and Ebert, 2004), théoriquement contrôlés par quelques gènes à

effet majeur, lorsqu'il existe des flux de gènes entre les populations, et de nombreux gènes à effet mineur (Yeaman and Whitlock, 2011). Dès lors, il est possible d'appliquer les méthodes de génétique quantitative à l'étude de l'architecture génétique de l'adaptation locale : Quantitative Trait Loci (QTL) Mapping en population expérimentale (descendances de croisement), et Linkage Disequilibrium (LD) Mapping en population naturelle (et les dérivés du LD Mapping, comme le Genome-Wide Association (GWA) Mapping). Ces deux approches sont basées sur l'association statistique entre phénotype et génotype, et impliquent la mesure d'un ou plusieurs traits. Les méthodes de génétique et génomique des populations prennent également en compte la multiplicité des gènes contrôlant potentiellement l'adaptation locale. Dans ces approches, il s'agit de détecter la « trace » ou la « signature » que la sélection devrait avoir laissé sur le génome, par comparaison avec le résultat attendu sous l'hypothèse d'une évolution neutre : comparaison F_{st}-Q_{st}, scan génomique des valeurs de F_{st}, association entre fréquence allélique et variable environnementale, mise en évidence d'un « balayage sélectif » par l'étude de la diversité nucléotidique ou du niveau de divergence génétique entre espèces proches. Parmi ces méthodes, seule la comparaison F_{st}-Q_{st} nécessite encore la prise en compte du phénotype. Mon propos n'est pas d'entrer dans le détail de chacune de ces méthodes, dont les avantages et inconvénients ont été largement décrits (par exemple : Ehrenreich and Purugganan, 2006; Savolainen *et al*, 2013; Stinchcombe and Hoekstra, 2007). Je souhaite davantage souligner que les approches de génomique des populations – en particulier depuis la généralisation du séquençage haut débit - ont favorisé la recherche des bases génétiques de l'adaptation locale sans prendre en compte de variables phénotypiques (par exemple: Turner *et al*, 2010). Malgré les corrections appliquées et la référence à des modèles d'évolution neutre, ces approches génèrent potentiellement un grand nombre de faux-positifs. Par exemple, une valeur exceptionnellement élevée de F_{st} peut résulter de l'adaptation locale, mais aussi d'effets démographiques (Bierne *et al*, 2013).

Si les techniques modernes de séquençage autorisent les démarches presque exclusivement moléculaires, il faut profiter de cette opportunité, tout en envisageant des combinaisons d'approches. Ainsi, plusieurs types de combinaisons ont été proposées (Ehrenreich and Purugganan, 2006; Purugganan and Gibson, 2003; Stinchcombe and Hoekstra, 2007). Stinchcombe et Hoekstra (2007) proposent d'associer la génomique des populations, le QTL Mapping et le LD Mapping, qui sont des approches exploratoires utilisant des marqueurs génétiques anonymes, et les informations disponibles sur les gènes candidats. En effet, les études fonctionnelles effectuées par ailleurs peuvent mettre en évidence, par exemple, des gènes dont la fonction correspond à un attendu sur les mécanismes sous-tendant l'adaptation étudiée, et/ou dont l'expression est modifiée dans une condition liée à la pression de sélection supposée. Ces gènes sont des candidats potentiels pour devenir le gène causal de l'adaptation. Ehrenreich et Purugganan (2006) n'abordent pas la génomique des populations, mais positionnent les études d'association (QTL et LD Mapping) sur une échelle génomique allant du génome complet à la mutation nucléotidique, et placent les études fonctionnelles aux plus petites échelles génomiques (Figure 3).

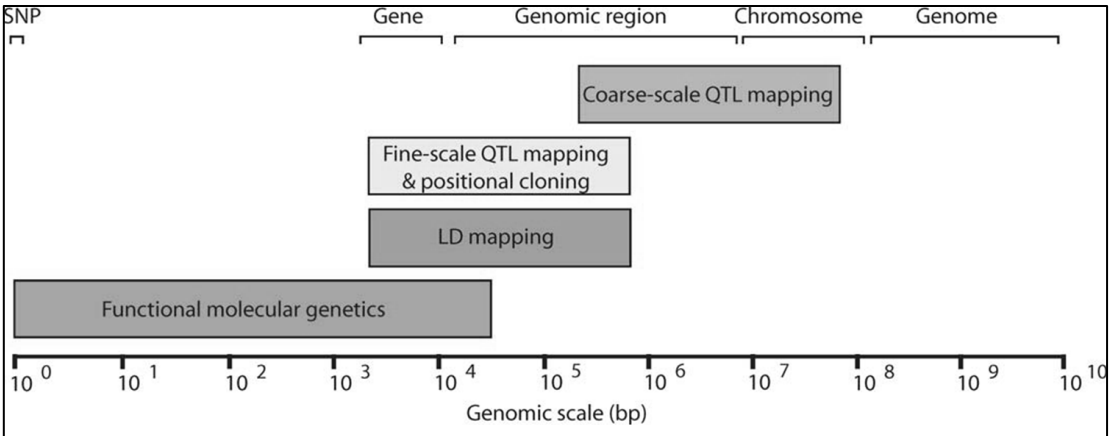

Figure 3: Echelle génomique des différentes méthodes pour isoler les gènes de traits adaptatifs. Entre le QTL Mapping mettant en évidence de larges régions génomiques et le LD Mapping plus précis, il est possible de raffiner les résultats du QTL Mapping par Fine Mapping. Ensuite, le clonage positionnel permet d'établir une liste de gènes candidats présents dans la région QTL dès lors qu'il existe une espèce modèle au génome séquencé et annoté suffisamment proche de l'espèce étudiée. La génétique fonctionnelle consiste à explorer la fonction d'un gène, notamment par des méthodes de constructions génétiques suivies de transformations génétiques. (D'après Ehrenreich and Purugganan, 2006).

Cette démarche a l'avantage de porter plus d'attention aux aspects fonctionnels (notamment, complémentation fonctionnelle dans les levures, constructions génétiques et transformations génétiques) qui contribuent à valider les gènes candidats. Purugganan et Gibson (2003) soulignent l'importance des approches de type « omique », et les placent au centre des autres approches. Les études transcriptomiques et protéomiques peuvent venir en effet compléter les études fonctionnelles dans la démarche de validation des gènes candidats. Néanmoins, même si toutes ces approches, conduites les plus souvent en laboratoire, aboutissent à un seul gène candidat, il reste la question de la pertinence de ce gène en conditions naturelles (Ungerer *et al*, 2007).

3. PHENOTYPE, GENOTYPE ET ENVIRONNEMENT

Une situation très favorable serait donc de pouvoir, dans la même étude, mesurer des phénotypes, tout en prenant formellement en compte des variables environnementales illustrant les pressions de sélection subies, pour finalement détecter les variants génétiques à l'origine de l'adaptation. Cela a été possible pour l'adaptation au climat chez *Arabidopsis thaliana*, grâce à l'utilisation de 948 accessions mondiales, la mesure de 107 phénotypes écologiquement pertinents, la prise en compte de 13 variables climatiques, et l'accès à environ 215000 marqueurs moléculaires de type Single Nucleotide Polymorphism (SNP) (Hancock *et al*, 2011). Chez les espèces pour lesquelles les ressources (moléculaires et autres) sont moins étendues, c'est l'approche de QTL Mapping qui a souvent apporté les résultats les plus significatifs. En utilisant des croisements intra-spécifiques entre accessions localement adaptées, l'architecture génétique de l'adaptation locale a pu être mise en évidence chez des espèces telles que *Mimulus guttatus*, *Avena barbata* ou *Silene vulgaris* (Bratteler *et al*, 2006; Gardner and Latta, 2007; Hall *et al*, 2006; Hall *et al*, 2010; Latta and Gardner, 2009; Latta *et al*, 2010). Ces études de QTL Mapping ont le mérite d'aller au-delà de la

simple question du nombre de gènes et de leur effet. Plusieurs questions sur la complexité potentielle des bases génétiques de l'adaptation locale sont également abordées, comme : (1) l'existence de gènes pléiotropes contrôlant plusieurs traits corrélés entre eux et à la valeur sélective ou (2) la variation de l'effet individuel d'un allèle lié à l'adaptation locale en fonction de l'environnement. Dans le cas de traits corrélés, il est possible de trouver des régions QTL associés à plusieurs traits, mais un doute subsiste souvent entre l'hypothèse d'un gène pléiotrope et l'existence de plusieurs gènes en déséquilibre de liaison (Gardner and Latta, 2007). Quant aux effets alléliques, ils sont le plus souvent évalués par le biais des interactions QTL x environnement (Anderson *et al*, 2011; Colautti *et al*, 2012). Deux situations sont alors décrites : un allèle donné peut être avantageux dans un environnement et désavantageux dans l'autre (hypothèse de pléiotropie antagoniste), ou bien avantageux dans un environnement et évoluer de façon neutre dans l'autre (hypothèse de neutralité conditionnelle) (Figure 4).

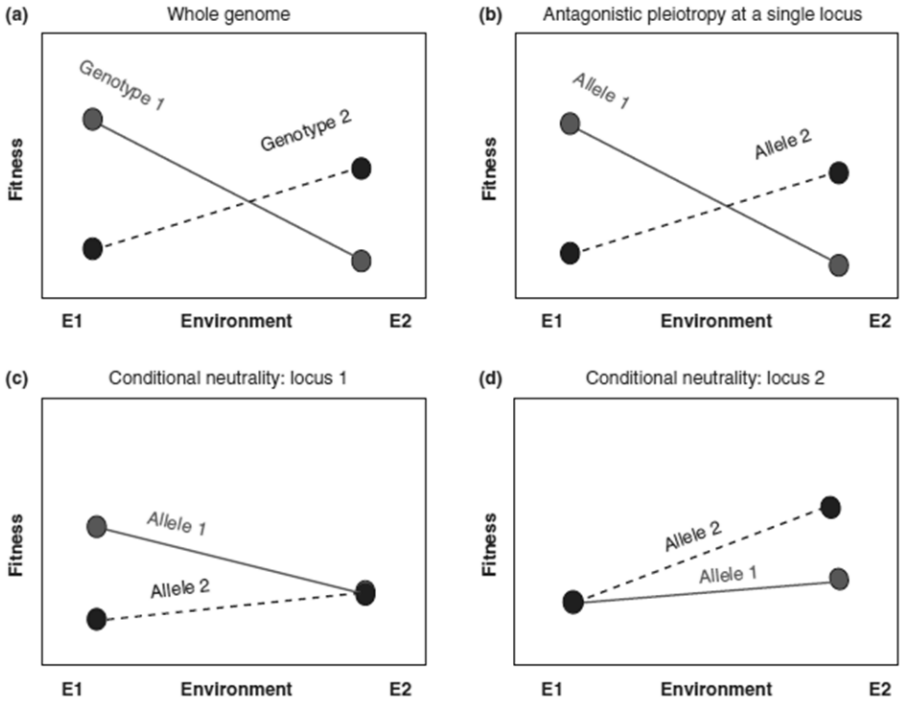

Figure 4: Adaptation locale représentée par (a) une valeur sélective plus élevée pour chaque génotype dans son environnement d'origine, et (b, c, d) différents cas d'effets alléliques individuels. L'adaptation locale peut résulter de (b) pléiotropie antagoniste, où l'allèle natif confère un avantage dans son environnement par rapport à l'allèle « étranger » mais présente un coût dans l'autre environnement, ou (c et d) neutralité conditionnelle, où l'allèle natif confère un avantage dans son environnement sans présenter de coût dans l'autre environnement. (D'après Anderson *et al*., 2011).

Les interactions QTL x environnement peuvent aussi concerner l'environnement dans lequel l'architecture génétique d'un trait est étudiée, c'est-à-dire les conditions expérimentales. Par exemple, dans une étude portant sur les déterminants de la période de floraison chez *A. thaliana*, les auteurs montrent un recouvrement très réduit entre les QTLs détectés en serre et ceux détectés en conditions naturelles (Brachi *et al*, 2010). Ceci illustre bien la nécessité (1) de ne pas considérer comme entièrement généralisables les régions QTL ou les déterminants génétiques découverts en laboratoire, et (2) de valider ces régions QTL ou ces déterminants génétiques dans le contexte naturel des populations.

4. BASES EPIGENETIQUES DE L'ADAPTATION LOCALE

Comme indiqué précédemment, l'adaptation locale, et la différenciation génétique associée, est traditionnellement opposée à la plasticité phénotypique (Figure 2). Plusieurs auteurs proposent de ne plus systématiquement dissocier ces deux processus, notamment lorsqu'il existe une différenciation génétique portant sur les niveaux de plasticité phénotypique (voir : Baythavong and Stanton, 2010). Récemment, une autre forme de différenciation pouvant générer des niveaux variables de plasticité phénotypique a été mise en évidence : les modifications épigénétiques (Johnson and Tricker, 2010). En effet, les changements épigénétiques peuvent activer, réduire ou complètement abolir l'activité de certains gènes, et ce grâce à trois processus non exclusifs : (1) la méthylation des cytosines de l'ADN, (2) la modulation de la structure chromatinienne par acétylation ou méthylation des histones, et (3) l'intervention de petits ARN nucléaires. Le phénomène de méthylation des cytosines est celui qui a été le plus largement étudié. Chez les plantes, il implique l'activité de l'enzyme méthyltransférase, qui réplique les profils de méthylation durant les mitoses et les méioses cellulaires (Takeda and Paszkowski, 2006). Ceci réconcilie la génétique et l'épigénétique, ou la génétique et la plasticité phénotypique, dans la mesure où les profils épigénétiques peuvent être héritables, au sens large « transmissibles aux descendants » (Figure 5). De plus, les changements épigénétiques peuvent être directement induits par des stress environnementaux (Bossdorf *et al*, 2008; Boyko and Kovalchuk, 2011; Feil and Fraga, 2012; Mirouze and Paszkowski, 2011), ce qui souligne leur intérêt pour l'étude de l'adaptation locale aux milieux extrêmes. Des méthodes expérimentales sont dès lors proposées pour parvenir à distinguer les effets génétiques des effets épigénétiques (Bossdorf *et al*, 2008), en partant du principe qu'une « épimutation » répandue dans toute une population peut constituer une réponse adaptative (Johnson and Tricker, 2010).

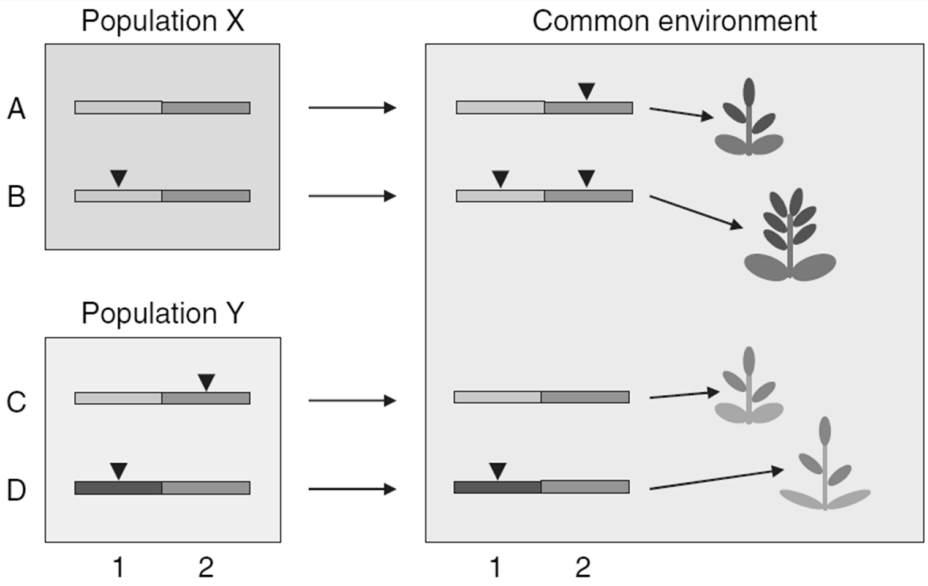

Figure 5: Relations hypothétiques entre génétique, épigénétique et variation phénotypique. Deux gènes (barres horizontales) par individu (A, B, C, D) sont représentés dans deux populations (X, Y). Les triangles représentent les modifications épigénétiques. La variation épigénétique peut avoir lieu au sein des populations (A1 vs B1) ou entre les populations (A2/B2 vs C2/D2). La variation épigénétique peut être indépendante de la variation génétique (A1 vs B1), ou confondue avec la variation génétique (C1 vs D1). Une partie des variations épigénétiques peut être non héritable, et ne pas persister en jardin commun (C2 vs D2). Une autre partie peut être héritable et persister en jardin commun (A1/B1). Si cette variation épigénétique héritable se traduit par une variation phénotypique et une différence de valeur sélective (comme dans le cas présent), elle a une signification écologique et évolutive. (D'après Bossdorf *et al*, 2008).

5. CONTEXTE ET OBJECTIFS DE CE DOCUMENT D'HDR

La catégorie de milieu extrême d'origine anthropique dans laquelle je travaille est celle des milieux métallifères, c'est-à-dire polluées par les métaux. Les métaux[1] représentent une source de contamination particulière, car (1) ils sont persistants – ou non-biodégradables - dans l'environnement (Peralta-Videa *et al*, 2009), et (2) une partie d'entre eux sont des oligo-éléments métalliques essentiels aux processus biologiques (fer, cuivre, zinc, manganèse, magnésium, cobalt, molybdène, nickel…), mais qui deviennent toxiques pour les êtres vivants s'ils sont présents en excès dans leurs cellules (Borovik, 1990). Les oligo-éléments métalliques non essentiels sont toxiques à de très faibles concentrations cellulaires (cadmium, plomb, mercure, thallium…). La contamination par les métaux est qualifiée de primaire si elle résulte de phénomènes naturels, comme les éruptions volcaniques ou les affleurements de roche-mère, dont l'altération entraîne la concentration des éléments métalliques dans le sol (Friedland, 1990). Par exemple, dans les sols serpentiniques,

[1] Il y a encore quelques années de cela, les sites contaminés étaient connus pour être particulièrement enrichis en « métaux lourds ». Cela concernait les éléments de la classification périodique dont la densité dépassait 5 $g.cm^{-3}$, bien que cette valeur ne soit pas unanimement fixée (Duffus, 2002). En réalité, la définition de « métaux lourds » n'a jamais été claire. Le terme a surtout été utilisé par les biologistes, en particulier pour exprimer la toxicité de ces éléments. Pour réintroduire un peu de rigueur dans le vocabulaire, le terme « métaux » a donc été recommandé par les géochimistes (Hodson, 2004). Finalement, le terme « métaux » est aujourd'hui largement employé, au moins autant que « éléments traces métalliques ». Ce dernier exprime le fait que les éléments concernés sont surtout très peu abondants dans la croûte terrestre (moins de 0,1% de la composition totale en éléments).

dérivant de roches-mères ultramafiques, le nickel, le chrome et le cobalt atteignent des concentrations inhabituellement élevées. Les autres sources de contamination sont issues des activités humaines : exploitations minières (de cuivre ou de zinc), industries métallurgiques, épandage de boues d'épuration, utilisation agricole de pesticides, ou réseaux routiers (Bradl, 2005). Par exemple, les déchets miniers, les terrils et les retombées atmosphériques industrielles sont une source particulièrement importante de pollution par le zinc (Zn), le plomb (Pb) et le cadmium (Cd). Les sols pollués par ces trois métaux sont dits calaminaires.

Les espèces végétales capables de faire face aux milieux métallifères sont appelées métallophytes. Certaines espèces sont endémiques des sites métallifères, et sont alors des métallophytes absolues. D'autres espèces, dites pseudométallophytes, sont capables de se développer à la fois sur les sols pollués et non pollués, donnant lieu à des populations métallicoles (M) et non métallicoles (NM), pouvant être géographiquement proches (Antonovics *et al*, 1971). Pour étudier l'adaptation locale aux milieux calaminaires, les espèces pseudométallophytes représentent donc un matériel biologique de choix. Deux pseudométallophytes modèles de la famille des Brassicacées ont émergé ces dernières années (Assunção *et al*, 2003b; Roosens *et al*, 2008b) : *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz et *Noccaea caerulescens* (J. Presl and C. Presl) F.K. Mey (anciennement *Thlaspi caerulescens*). Ces choix ne sont pas étrangers au fait qu'*Arabidopsis thaliana* est aussi une Brassicacée, et que son génome est entièrement séquencé et annoté (The Arabidopsis Genome Initiative, 2000). En particulier, *N. caerulescens* présente 88% d'homologie avec le génome d'*A. thaliana* dans les régions codantes (Rigola *et al*, 2006), tandis qu'*A. halleri* présente 94% d'homologie (Becher *et al*, 2004). Il faut cependant noter que, contrairement à *A. halleri*, *N. caerulescens* présente des populations serpentiniques. Parmi les autres points distinguant ces deux espèces, le régime de reproduction de *N. caerulescens* est mixte (autofécondation et allofécondation possibles, car l'espèce est auto-compatible) tandis que celui d'*A. halleri* est strictement allogame (espèce auto-incompatible) (Mousset *et al*, 2016; Van Rossum *et al*, 2004). Cet aspect est essentiel car il permet de poser l'hypothèse que l'adaptation locale pourrait être plus prononcée chez *N. caerulescens* que chez *A. halleri*, du fait des flux de gènes réduits entre populations M et NM.

C'est dans ce contexte que se situe la suite de ce document. De manière générale, il sera organisé selon une démarche d'abord thématique (un thème par chapitre), puis, au sein de chaque chapitre, de manière chronologique. Je profiterai de ce document pour porter un regard critique sur l'ensemble de mes travaux de recherche. Plus particulièrement, l'objectif du Chapitre 2 est de montrer à quel point il a été possible jusqu'à présent de disséquer les mécanismes génétiques de l'adaptation locale aux milieux calaminaires chez *A. halleri*, une espèce pseudométallophyte modèle. Pour cela, et comme souligné dans le Chapitre 1, je m'appuierai sur une combinaison d'approches. Certaines approches seront directement issues de mon travail de recherche, et d'autres seront des sources d'information sur lesquelles reposent mes propres recherches. J'aborderai également cette question au travers de projets en cours sur *N. caerulescens*. Dans le Chapitre 3, j'aborderai deux aspects sur lesquels j'ai insisté dans le chapitre 1 et qui me paraissent fondamentaux dans l'exploration des bases génétiques de l'adaptation locale : quels phénotypes décrivent le mieux le phénomène d'adaptation locale ? Et quels paramètres de l'environnement constituent le vrai moteur de l'adaptation locale ? Je m'appuierai sur des travaux de recherche déjà mené chez *A. halleri*, ou en cours chez *N. caerulescens*.

CHAPITRE 2 : BASES GENETIQUES ET EPIGENETIQUES DE LA TOLERANCE AU ZINC ET DE L'HYPERACCUMULATION DU ZINC

1. *TOLERANCE ET* HYPERACCUMULATION : DEUX TRAITS QUANTITATIFS, ADAPTATIFS ET POLYMORPHES CHEZ *A. HALLERI* ET *N. CAERULESCENS*

a. DEFINITIONS SUCCINCTES DE «TOLERANCE» ET «HYPERACCUMULATION»

La tolérance aux métaux est définie comme la capacité à survivre et à se reproduire en milieu pollué (Antonovics *et al*, 1971; Macnair and Baker, 1994). La tolérance aux métaux peut donc être comprise comme un trait fondamentalement adaptatif. Avec les premiers tests en laboratoire, permettant de séparer l'effet des métaux de l'effet des autres pressions de sélection agissant dans les milieux métallifères, la tolérance aux métaux est devenue une capacité de l'individu à gérer d'importantes quantités de métaux dans son organisme (Craig, 1977; Nicholls and McNeilly, 1979; Wilkins, 1978). Ces tests se basaient sur la capacité de croissance des racines dans des solutions nutritives comportant des concentrations élevées en métal par rapport à la capacité de croissance en solution contrôle (Baker, 1987; Macnair, 1990; Schat and Ten Bookum, 1992; Wilkins, 1978). Le test séquentiel de tolérance avec observation de l'arrêt de croissance racinaire est celui qui est aujourd'hui le plus communément admis (Figure 6). Ce test présente des avantages pratiques de par l'utilisation d'un seul lot d'individus (par rapport aux tests en concentrations multiples), et repose sur le principe des tests de type dose-réponse (Schat and Ten Bookum, 1992). En effet, la dose à laquelle intervient l'arrêt de croissance racinaire est considérée comme une dose létale, ce qui permet à la fois d'obtenir une valeur de tolérance par individu (appelée « EC100 » pour « effective concentration for 100% growth inhibition ») et de construire des courbes de type dose-réponse pour étudier le comportement moyen d'une population grâce à un paramètre équivalent à un « EC50 ». Ceci implique que la tolérance de chaque individu est estimée par une concentration en métal dans le milieu au-delà de laquelle la survie n'est plus possible.

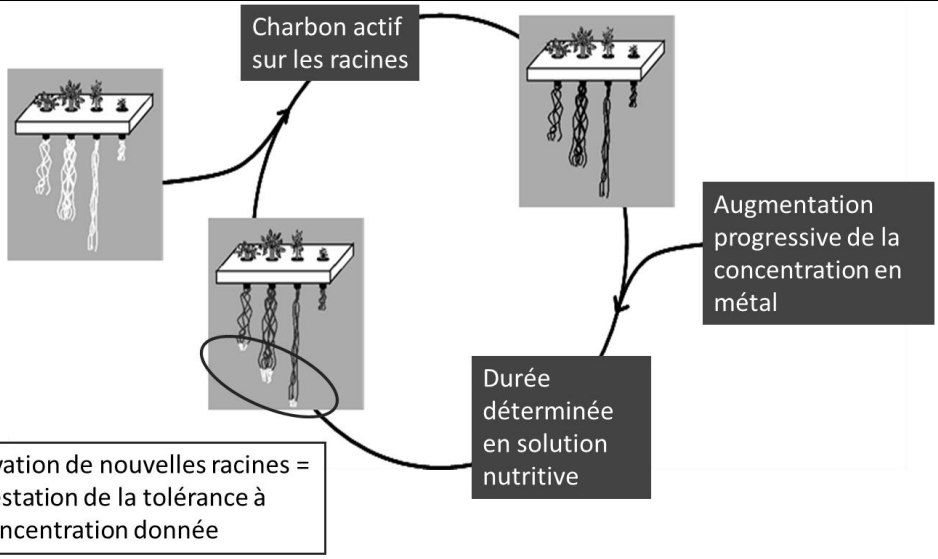

Figure 6: Fonctionnement du test séquentiel de tolérance au métal. Les individus sont placés sur une plaque trouée qui repose sur un pot contenant de la solution nutritive. La solution est au niveau "contrôle" pour tous les individus au départ de l'expérience, et les racines sont teintées en noir par du charbon actif. Après une courte durée en solution, les racines sont observées: des extrêmités blanches indiquent une croissance racinaire. La concentration en métal est alors augmentée et les racines sont reteintées en noir. Après une courte durée en solution, les racines sont de nouveau observée. Le cycle se poursuit jusqu'à l'arrêt de croissance racinaire de tous les individus.

La tolérance aux métaux pose la question de la stratégie de gestion physiologique des métaux dans les tissus[2]. Lorsque le métal entre dans les parties racinaires, soit il pénètre peu ou pas dans les feuilles (stratégie d'exclusion des parties aériennes), soit il pénètre en quantité plus ou moins importante dans les feuilles (stratégie d'accumulation dans les parties aériennes) (Baker, 1981). Certaines espèces accumulent à un niveau censé être presque équivalent au niveau racinaire (espèces indicatrices), d'autres transfèrent activement le métal dans les feuilles, et parfois en quantité très supérieure à celle des racines ou du sol (espèces hyperaccumulatrices). La question de la concentration foliaire à partir de laquelle il est possible de définir une espèce comme hyperaccumulatrice est débattue (van der Ent *et al*, 2013). De même, la question du rôle adaptatif de l'hyperaccumulation est débattue depuis longtemps (Boyd and Martens, 1992), bien que la nature adaptative de ce trait n'ait jamais réellement été mise en doute.

b. Distribution en populations chez *A. halleri* et *N. caerulescens*

A. halleri et *N. caerulescens* sont connues pour être tolérantes au Zn et au Cd, de telle façon que les populations M sont en moyenne plus tolérantes que les populations NM. Les populations NM présentent cependant des niveaux de tolérance substantiels, définissant la tolérance au Zn et au Cd comme constitutive (au sens de : répandue dans toute l'espèce) chez ces deux espèces (Escarré *et al*, 2000; Meerts and Van Isacker, 1997; Meyer *et al*, 2015; Pauwels *et al*, 2006). Chez *A. halleri* en particulier, et grâce à la mise en œuvre du test séquentiel de tolérance, nous avons démontré l'existence (1) d'un saut qualitatif entre tous les individus de l'espèce *A. halleri* et des espèces-sœurs

[2] La stratégie d'évitement total du métal à l'intérieur de l'organisme a été considérée comme inexistante car jamais détectée (Baker, 1981) et reste finalement très peu étudiée.

non-métallophytes, (2) de la variation quantitative de la tolérance au Zn, et (3) de la relative structuration phénotypique de cette variation entre populations M et NM (Figure 7).

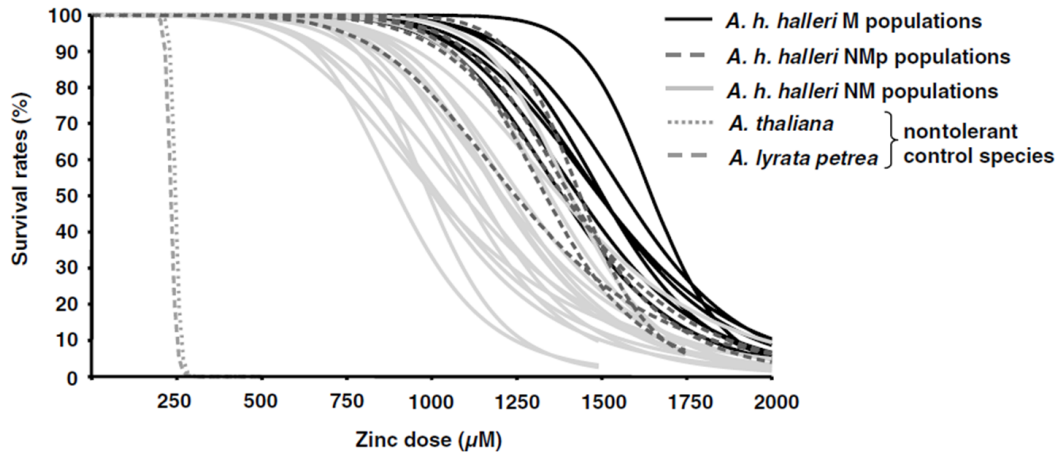

Figure 7 : Courbes de survie des populations M et NM d'*A. halleri* et de représentants d'espèces-soeurs non-métallophytes après ajustement à un modèle sigmoïdal de type dose-réponse. Tous les individus utilisés dans cette expérience ont été soumis à un test séquentiel de tolérance au zinc. Les populations "NMp" représentent des populations NM à proximité de sites pollués. L'étalement des courbes de survie montre que la tolérance au zinc est un caractère quantitatif. (D'après Pauwels *et al*, 2006).

A. halleri est connue pour être hyperaccumulatrice de Zn et de Cd (Bert *et al*, 2002; Meyer *et al*, 2015), alors que *N. caerulescens* est connue pour être hyperaccumulatrice de Zn, Cd et nickel (Assunção *et al*, 2003a; Escarré *et al*, 2013; Escarré *et al*, 2000; Gonneau *et al*, 2014; Meerts and Van Isacker, 1997; Roosens *et al*, 2003). Chez *N. caerulescens*, les études publiées convergent sur le fait que l'espèce est constitutivement hyperaccumulatrice de Zn, en particulier les populations NM lorsqu'elles sont placées en conditions expérimentales. En revanche, chez *A. halleri*, les études publiées jusqu'à présent n'ont pris en compte que peu de populations, ce qui ne permet pas de conclure d'après la bibliographie. J'ai pour ma part eu l'occasion d'analyser les niveaux d'hyperaccumulation du Zn chez plusieurs populations d'*A. halleri* provenant de Pologne ou d'Italie (Decombeix, 2011; Kostecka, 2009). Il semblerait que l'hyperaccumulation du Zn soit un trait largement constitutif et quantitatif chez *A. halleri*, sans distinction très nette entre populations M et NM (Figure 8). Comme pour la tolérance au Zn, seules quelques populations M présentent vraiment des niveaux d'hyperaccumulation vraiment plus faibles (par exemple PL15 et PL17, figure 8A), et quelques populations NM présentent des niveaux d'hyperaccumulation vraiment plus forts (par exemple I29 et I32, figure 8B). La tendance globale ressemble donc à celle mise en évidence chez *N. caerulescens* (Escarré *et al*, 2013; Escarré *et al*, 2000; Gonneau *et al*, 2014; Meerts and Van Isacker, 1997), bien qu'elle paraisse moins claire.

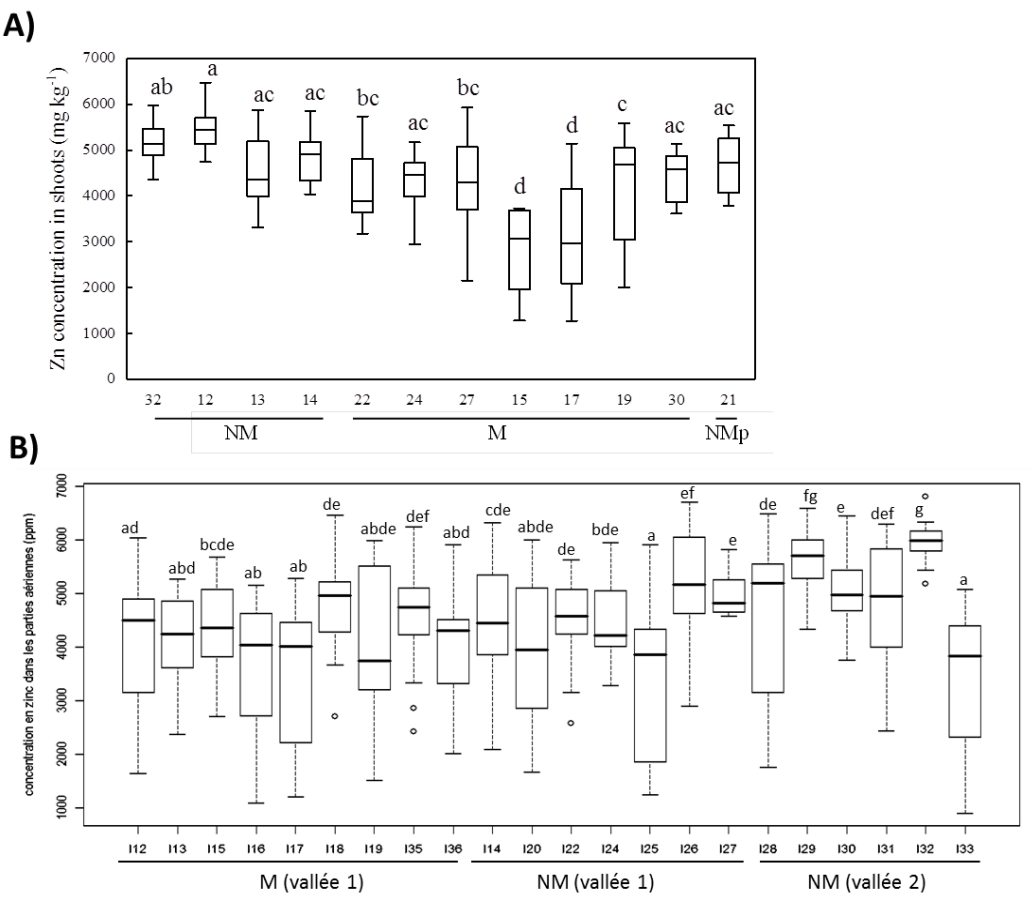

Figure 8 : Box-plots représentant les niveaux d'hyperaccumulation du zinc dans les populations d'*A. halleri* de Pologne (A) et d'Italie (B). Les plantes ont été cultivées en conditions contrôlées, dans du terreau additionné de 1500 $mg.kg^{-1}$ de zinc sous forme de $ZnSO_4$, 7 H_2O (Frérot *et al*, 2010). M : populations métallicoles; NM : populations non-métallicoles; NMp : populations non-métallicoles à proximité de populations métallicoles; vallée 1 : vallée des Alpes italiennes comprenant des populations métallicoles et non-métallicoles proches géographiquement; vallée 2 : vallée des Alpes italiennes comprenant uniquement des populations non-métallicoles, distantes d'environ 40 km de la vallée 1. (D'après les thèses d'Alicja Kostecka, 2009 et Isabelle Decombeix, 2011).

2. ETUDE DES BASES GENETIQUES DE LA TOLERANCE AU ZINC ET DE L'HYPERACCUMULATION DU ZINC CHEZ *A. HALLERI* PAR CROISEMENTS INTER-SPECIFIQUES

a. QTLS DE TOLERANCE ET D'HYPERACCUMULATION DANS LES DESCENDANCES DU CROISEMENT *A. HALLERI X A. LYRATA PETRAEA*

Une stratégie pour parvenir à étudier les bases génétiques de la tolérance et de l'hyperaccumulation est de mettre en ségrégation ces caractères dans des descendances appropriées. Les descendances les plus appropriées pour comprendre les mécanismes génétiques de l'adaptation locale aux sites calaminaires sont issues de croisements entre individus M et NM. Cependant, dans un premier temps, ces descendances n'ont pas pu être produites. En effet, avant l'exploration plus complète de l'aire de répartition d'*A. halleri* (Pauwels *et al*, 2005), il était établi que la variation nécessaire à une mise en ségrégation, notamment de l'hyperaccumulation, n'existait pas chez cette espèce (Macnair *et al*, 1999). Par conséquent, les premiers croisements impliquant *A. halleri* se sont effectués en prenant l'espèce non-métallophyte *Arabidopsis lyrata petraea* comme mère (l'utilisation d'*A. halleri* comme mère n'a jamais été concluante). Il s'agissait de rechercher les déterminants génétiques à l'origine des propriétés métallicoles d'*A. halleri*, c'est pourquoi les premiers croisements était des descendances impliquant seulement un individu métallicole de la population d'Auby (nord de la France). Ainsi, à mon arrivée dans le laboratoire GEPV (aujourd'hui EEP), j'ai produit des descendances impliquant d'autres génotypes d'*A. halleri*, notamment des génotypes non métallicoles. Je reviendrai plus tard sur cet aspect.

La première descendance étudiée à des fins de QTL Mapping était un BC_1, c'est-à-dire un génotype d'Auby croisé successivement sur deux génotypes différents d'*A. lyrata petraea* (provenant d'une population de République Tchèque) afin d'éviter les effets d'une éventuelle dépression de consanguinité. Cette descendance avait conservé les bonnes propriétés de clonage de l'espèce *A. halleri*, ce qui a permis son exploitation dans de nombreuses expériences de phénotypage (Courbot *et al*, 2007; Dräger *et al*, 2004; Sarret *et al*, 2009; Willems *et al*, 2007). Les analyses QTL sur la tolérance au zinc, estimée par un test séquentiel en hydroponie (voir Figure 6), ont révélé trois régions génomiques significativement impliquées dans ce trait (appelées Zntol-1 à 3), expliquant ensemble 29% de la variance phénotypique. Grâce à du fine-mapping et à la technique de clonage positionnel utilisant la carte physique d'*A. thaliana* et la synténie entre les génomes d'*A. thaliana* et *A. halleri* (Roosens *et al*, 2008a; Roosens *et al*, 2008b), des gènes candidats ont pu être mis en évidence. Il s'agit des gènes *AhHMA4*, *AhMTP1*-A et *AhMTP1-B*, dont l'expression avait été préalablement étudiée soit chez *N. caerulescens*, soit directement chez *A. halleri* (Bernard et al, 2004; Dräger et al, 2004; Papoyan and Kochian, 2004; Talke et al, 2006). Ce qui a attiré particulièrement l'attention sur ces gènes, ce sont aussi leurs fonctions et notamment leurs rôles dans le transport des métaux. *HMA4* (Heavy Metal ATPase 4) code pour un transporteur de Zn et de Cd responsable de l'export des métaux hors des cellules racinaires, mais aussi du chargement du xylème conduisant à une translocation vers les parties aériennes (Hussain *et al*, 2004; Mills *et al*, 2003). MTP1 (Metal Transporter Protein 1) code pour un transporteur de Zn responsable en particulier de la détoxication du métal par stockage dans la vacuole des cellules du mésophylle (Dräger *et al*, 2004; Küpper *et al*, 2000). Ces deux gènes font en réalité partie de tout un réseau de gènes de l'homéostasie des métaux, dont l'expression est constitutivement plus élevée chez *A. halleri* par rapport à *A. thaliana*,

ou encore chez *N. caerulescens* par comparaison avec l'espèce non-métallophyte *Thlaspi arvense* (Verbruggen *et al*, 2009).

L'analyse de l'hyperaccumulation du Zn dans le BC_1 n'a pas conduit à l'identification de QTLs significatifs (Pierre Saumitou-Laprade, *pers. com*.), c'est pourquoi une descendance F_2 a été produite. Mon premier travail de recherche en tant que nouvelle arrivante au laboratoire GEPV fût donc de mener à bien la construction de la nouvelle carte génétique et l'analyse QTL de l'hyperaccumulation du Zn, mais aussi d'autres éléments accumulés en collaboration avec le laboratoire de Physiologie et de Génétique Moléculaire des Plantes de l'Université Libre de Bruxelles (*coll.* Nathalie Verbruggen) (Frérot *et al*, 2010; Willems *et al*, 2010). L'ensemble des études sur le BC_1 et la F_2 ont montré qu'une région génomique sur le LG3 fait largement consensus entre les différentes études (Figure 9), bien que les parts de variance phénotypique expliquées soient variables : 42,9% (Cdtol-1) > 21,4% (Cdconc-1) > 14,85% (ZnAcHP-1) > 12,6% (Feconc-3) > 12,2% (Zntol-1) > 7,9% (Znconc-2) > 4,25% (ZnAcLP-1).

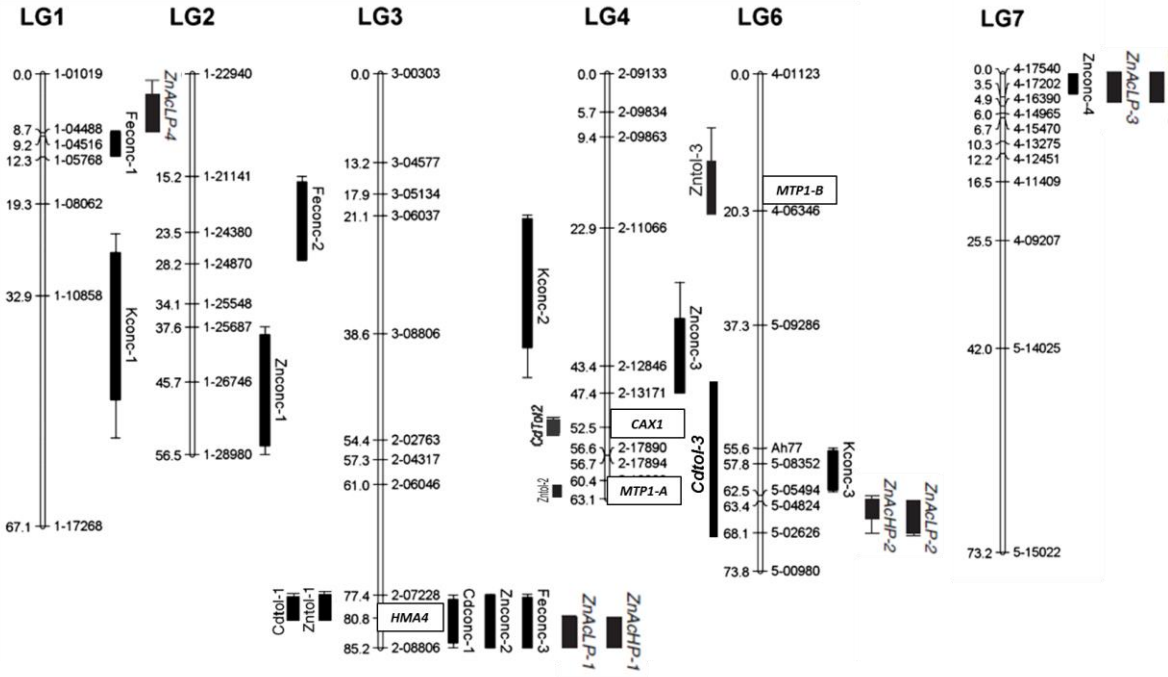

Figure 9 : Bilan des QTLs de tolérance et d'hyperaccumulation détectés dans les descendances de croisements entre *A. halleri* Auby et *A. lyrata petraea*. Zntol-1 à 3 sont les QTLs de tolérance au zinc identifiés dans Willems *et al.* (2007) et Roosens *et al*. (2008a). Cdtol-1 à 3 sont les QTLs de tolérance au cadmium identifiés dans Courbot *et al*. (2007) et Baliardini *et al.* (2015). Les bornes et les intervalles de confiance de Cdtol-3 ne sont pas fournis dans la littérature (Courbot *et al*., 2007). ZnAcLP-1 à 4 et ZnAcHP-1 à 3 sont les QTLs d'hyperaccumulation du zinc identifiés dans Frérot *et al*. (2010), à faible concentration en zinc dans le sol (LP) ou à forte concentration en zinc dans le sol (HP). Znconc-1 à 4, Cdconc-1, Feconc-1 à 3, et Kconc-1 à 3 sont les QTLs d'accumulation du zinc, cadmium, fer et potassium identifiés dans Willems *et al*. (2010), à forte concentration en cadmium dans la solution nutritive. Zntol-1 à 3 et Cdtol-1 à 3 sont représentés à gauche des groupes de liaison, tous les autres sont représentés à droite des groupes de liaison. Le groupe de liaison LG8 n'est pas représenté car il ne porte pas de QTL significatif. Dans les cadres sont indiqués les noms des gènes candidats ayant fait l'objet d'investigations quant à leur rôle dans la tolérance ou l'hyperaccumulation chez *A. halleri*.

Cette région correspond à la position du gène candidat *AhHMA4* (ou plutôt des gènes *AhHMA4* : voir plus loin). De nombreuses données démontrent aujourd'hui que ce gène joue un rôle dans la tolérance au Zn et au Cd et dans l'hyperaccumulation du Zn et du Cd chez *A. halleri via* une surexpression constitutive par rapport à *A. thaliana*. Cette surexpression est due à la combinaison d'une triplication en tandem du gène et d'une modification des séquences promotrices (Hanikenne *et al*, 2008; Krämer, 2010; Talke *et al*, 2006). Les pourcentages de variance phénotypique expliqués par les QTLs suggèrent que le gène *AhHMA4* serait plus impliqué dans la tolérance au Cd que dans la tolérance au Zn (Cdtol-1 par rapport à Zntol-1), alors que les ordres de grandeurs sont similaires pour l'hyperaccumulation du Cd et du Zn (Znconc-1 et ZnAcHP-1). Dans Hanikenne *et al*. (2008), les lignées RNAi d'*A. halleri*, dans lesquelles l'expression d'*AhHMA4* est fortement diminuée, montrent en effet une diminution significative de la tolérance au Cd par rapport aux plantes sauvages d'*A. halleri*, alors qu'elles montrent des niveaux de tolérance au Zn plus variables et parfois aussi élevés que ceux des plantes sauvages d'*A. halleri* (Figure 10). Ce résultat peut paraître anecdotique, mais pour ma part il m'a laissée perplexe quant au rôle d'*AhHMA4* dans la tolérance au Zn, surtout après avoir obtenu une série de résultats négatifs...

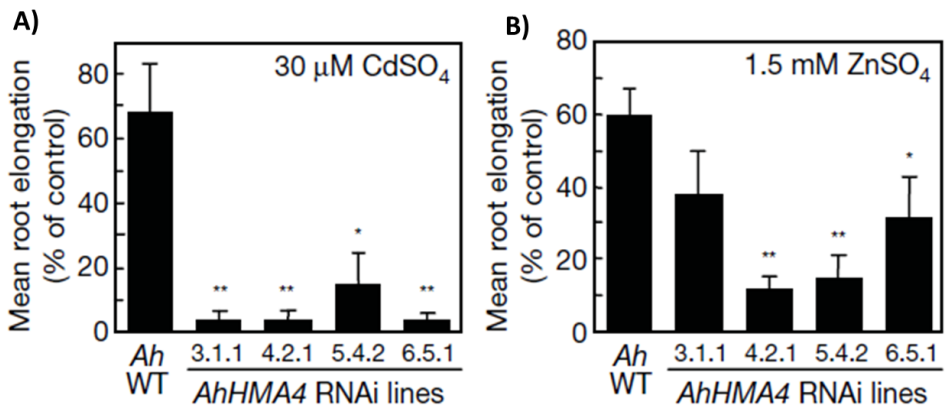

Figure 10 : Caractérisation des lignées RNAi d'*A. halleri,* dans lesquelles l'expression du gène *AhHMA4* est fortement diminuée, pour la tolérance au Cd (A) et au Zn (B). La moyenne et les erreurs standards sont indiquées. N=4 à 6 pour les lignées RNAi, et n=12 à 18 pour les plantes sauvages. *P<0,05; **P<0,01; ***P<0,001. (D'après Hanikenne *et al*., 2008).

b. QUAND *HMA4* NE REPOND PLUS...

La tolérance m'apparaissant de plus en plus comme un trait complexe (voir Chapitre 3), et après avoir démontré que d'autres traits que l'allongement racinaire pouvaient rendre compte du niveau de tolérance d'un individu (Meyer *et al*, 2010), j'ai entrepris de tester à nouveau la tolérance au Zn du BC_1 (Decombeix, 2011). Pour cette expérience, le croisement avait été refait à partir d'un clone de la F_1 et d'un clone du deuxième parent de l'espèce *A. lyrata petraea*, donc *a priori* à l'identique du premier croisement (Pierre Saumitou-Laprade, *pers. com*.). L'expérience consistait à placer des individus du « nouveau » BC_1 en solution nutritive, à raison de 3 clones de chaque génotype dans chaque concentration, aux doses fixes de 1µM (concentration contrôle) et 100µM (traitement contaminé) pendant 4 semaines d'exposition. Les traits mesurés étaient : la longueur racinaire (au début de l'expérience et à la fin de l'expérience), la largeur des feuilles, la longueur des

feuilles, la biomasse sèche racinaire finale, la biomasse sèche aérienne finale et le rendement photosynthétique. Pour tous ces traits, des indices de tolérance ont été calculés, basés sur le rapport entre la valeur d'un individu (*i.e*. un clone) en traitement pollué sur la valeur moyenne des 3 clones placés en condition contrôle (Meyer *et al*, 2010). Le nombre d'individus correctement phénotypés variait entre 106 et 112. La carte génétique a été reconstruite sans grande difficulté à partir de 153 individus génotypés et 54 marqueurs génétiques, de type microsatellite ou SNP. Les résultats ont été pour le moins surprenants. J'ai choisi de ne montrer que ceux concernant les indices de tolérance calculés pour l'accroissement racinaire [(longueur de départ – longueur de fin) / longueur de départ], le rendement photosynthétique et la largeur des feuilles, car les autres traits n'ont pas donné de QTL très robustes (ils étaient par exemple sensibles à la présence de valeurs extrêmes) (Figure 11).

Tout d'abord, nous n'avons obtenu aucun QTL significatif commun avec les précédentes études sur la tolérance au zinc ou l'hyperaccumulation du zinc (Frérot *et al*, 2010; Willems *et al*, 2007; Willems *et al*, 2010). En revanche, les parts de variance phénotypique expliquées étaient du même ordre de grandeur que précédemment, c'est-à-dire moins de 10%, ou exceptionnellement à peine plus de 10% (Tableau 1). Sur le LG7, nous avons identifié une région génomique intéressante car commune à plusieurs traits[3] (Figure 11). Pour expliquer l'origine de ces QTLs chevauchants, il est à ce stade impossible de trancher entre un seul gène pléiotrope contrôlant plusieurs traits ou plusieurs gènes en déséquilibre de liaison. Ensuite, je dirais presque « et par-dessus tout », compte tenu de la réaction de certains de mes pairs, nous n'avons pas obtenu de QTL dans la région des gènes *HMA4* sur le LG3. C'est pourquoi, nous avons tenté de réaliser de nouveau le test de tolérance séquentiel, pour se rapprocher des conditions de l'expérience menée dans Willems *et al*. (2007). Cette expérience, réalisée deux fois indépendamment (par moi-même et Pierre Saumitou-Laprade), n'a jamais permis de retrouver les premiers QTLs identifiés, et pour ma part, je n'ai obtenu qu'une ségrégation très uniforme pour ce trait et aucun QTL significatif. Ensuite, beaucoup plus tard après le test de tolérance, nous avons aussi réalisé en collaboration avec le Laboratoire de Physiologie et de Génétique Moléculaire des Plantes à l'Université Libre de Bruxelles (*coll.* Nathalie Verbruggen), le dosage du zinc dans les feuilles de 60 plantes[4] ayant poussé dans la solution à 100 µM. Le seul QTL significatif identifié se trouve dans la région commune à plusieurs QTLs de tolérance au zinc sur le LG7 (Figure 11).

[3] J'avais, avec Isabelle Decombeix, ma doctorante de l'époque, entrepris un travail très prospectif sur cette région génomique qui aurait fait suite à la publication des QTLs présentés ici. Il était question de valider cette région comme « QTL de corrélation », c'est-à-dire un QTL responsable de la modification des corrélations entre les traits. Pour tester cela, nous avions simplement (probablement un peu trop simplement) réalisé des tests de Mantel entre matrices de corrélation pour les individus *A. halleri/A. lyrata* vs *A. lyrata/A.lyrata* du croisement, et ce, marqueur par marqueur. De fait, le marqueur 4-09130 se distinguait des autres marqueurs, mais nous n'avons jamais réussi à dire si ce comportement était significativement différent du comportement des autres marqueurs.

[4] Nous n'avons pu analyser que 60 plantes pour cause de biomasses trop faibles ou de génotypes manquants. Avec un tel effectif, un manque de puissance statistique n'est pas exclu.

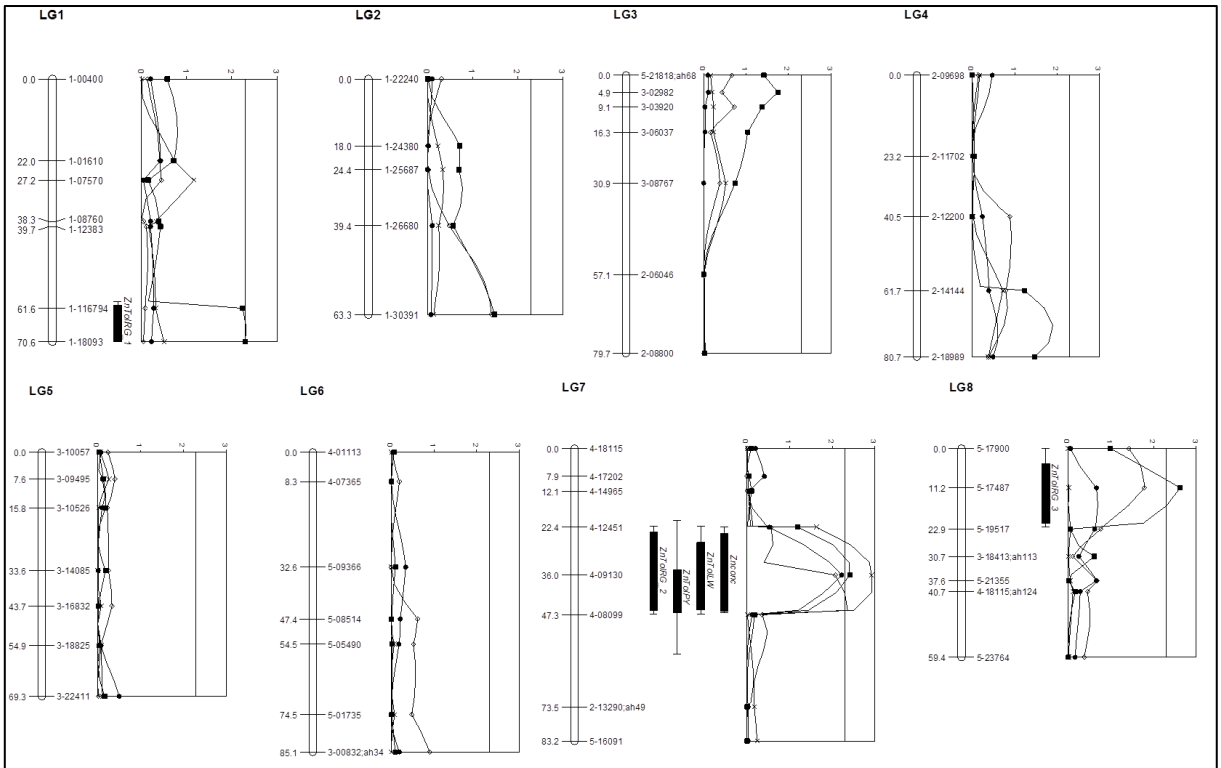

Figure 11 : Résultats du QTL Mapping sur le test de tolérance à doses fixes effectué sur la descendance BC_1 nouvellement produite. Le symbole « carré noir » représente l'accroissement racinaire (RG), le symbole « rond noir » représente la largeur des feuilles (LW), le symbole « losange blanc » représente le rendement photosynthétique (PY) et le symbole « croix » représente la concentration en zinc (Znconc) dans les feuilles des plantes cultivées à 100µM. Le trait noir vertical représente le seuil de LOD score calculé par une méthode de permutation au-délà duquel le QTL est significatif au seuil 5% (2,3 pour tous les traits). Les régions QTL sont indiquées par leur intervalles de confiance à 95%.

Tableau 1 : Synthèse des QTLs significatifs identifiés dans le test de tolérance à doses fixes effectué sur la descendance BC_1 nouvellement produite.

Nom du QTL	Groupe de liaison/marqueur le plus proche	LOD max	Part de variance phénotypique (%)	Additivité
ZnTol_RG1	LG1/1-18093	2,32	6,8	-0,267
ZnTol_RG2	LG7/4-09130	2,41	7,7	-0,290
ZnTol_RG3	LG8/5-17487	2,62	7,5	-0,305
ZnTol_PY	LG7/4-08099	2,63	14,8	-0,0687
ZnTol_LW	LG7/4-09130	2,22	8,9	-0,280
Znconc	LG7/4-09130	2,72	18,3	-435

Ces résultats se sont révélés impossibles à valoriser jusqu'à présent, dans la mesure où je n'ai pas trouvé d'explication claire pour justifier l'absence de QTLs communs avec la première expérience de tolérance (et même d'hyperaccumulation), en particulier l'absence du QTL dans la région d'*HMA4*. Si le test séquentiel de tolérance avait donné des résultats cohérents avec ceux publiés dans Willems *et al.* (2007), j'aurais simplement proposé que l'exposition prolongée à 100 µM mobilise d'autres mécanismes que l'exposition à des doses croissantes en zinc, en tout cas pour ce qui est de la réponse des racines. La réponse des feuilles (largeur et rendement photosynthétique) pouvant faire intervenir aussi de nouveaux mécanismes. De plus, les tests que j'ai réalisés plus tard sur une autre descendance de croisement *A. halleri* x *A. lyrata petraea* impliquant une autre accession que celle d'Auby (voir paragraphe suivant) me montreront que la région *HMA4* peut tout à fait être détectée dans nos conditions expérimentales si elle doit l'être. J'ai donc pensé que ces résultats inattendus pouvaient provenir du matériel biologique. Celui-ci était peut-être de mauvaise qualité, notamment à cause d'une infection virale (*Arabis Mosaïc Virus*) sur les *A. halleri* cultivés en serre, survenue à l'époque de ces expériences, en particulier lorsque les tests séquentiels de tolérance ont été répétés. L'alternative serait d'admettre que les gènes candidats précédemment identifiés n'interviennent pas toujours, bien qu'il s'agisse d'une descendance produite à partir des mêmes parents, soit pour des raisons génétiques, soit pour des raisons épigénétiques. Parmi les raisons génétiques (et après avoir écarté l'hypothèse peu parcimonieuse des mutations), je pense qu'il est possible que d'autres gènes prennent (parfois, mais quand ?) le relai des gènes *HMA4* pour conférer la tolérance au zinc et peut-être aussi l'hyperaccumulation du zinc. Cette idée vient du fait que (1) une très bonne tolérance au Zn est possible même si l'expression d'*HMA4* est fortement diminuée (voir Figure 10), et (2) l'expression d'*HMA4* semble dépendre de la concentration en Zn appliquée, d'après l'interaction QTL x environnement révélée dans Frérot *et al*. (2010). Quant aux raisons épigénétiques, je ne les écarte pas dans la mesure où les parents du croisement ont été bouturés plusieurs années avant d'être croisés à nouveau, ce qui peut avoir donné lieu à des modifications épigénétiques.

Au final, pour les tests séquentiels de tolérance je privilégierais l'hypothèse d'altération du matériel biologique à cause d'une infection virale, et pour le test à dose fixe je privilégierais l'hypothèse d'une moindre intervention d'*HMA4* par rapport à d'autres gènes à la dose de 100 µM. Pour compléter cette discussion, je n'ai malheureusement pas pu trouver d'individus vivants qui me permettraient de vérifier les niveaux d'expression des gènes candidats HMA4 ou *MTP1.* En revanche je projette de vérifier le nombre de copies du gène *HMA4* chez quelques individus de la descendance grâce à une collaboration avec le Centre d'Ingénierie des Protéines de l'Université de Liège (*coll*. Marc Hanikenne).

c. Bases genetiques de la tolerance constitutive chez *A. halleri*

Les travaux présentés jusqu'à présent ne permettent pas de discuter des bases génétiques de l'adaptation locale puisqu'ils engagent des croisements inter-spécifiques. Cependant, il est possible d'aborder la question de l'adaptation locale tout en conservant une approche par croisements inter-spécifiques en multipliant les croisements et, idéalement, en prenant des individus d'*A. halleri* provenant de populations M et NM proches géographiquement. J'ai tenté de mettre en œuvre cette approche, mais je n'ai finalement eu l'occasion d'analyser qu'un seul de tous ces croisements, à partir d'une population NM issue des montagnes des Tatras en Slovaquie (SK2). Cette population est connue comme étant parmi les moins tolérantes au Zn et au Cd, et parmi les plus isolées génétiquement de toute population M, ce qui en fait une bonne représentante des populations NM (Meyer *et al*, 2015; Meyer *et al*, 2010; Pauwels *et al*, 2006; Pauwels *et al*, 2012). Pour une discussion

sur les déterminants génétiques de l'adaptation locale, le rapprochement entre les deux croisements, que j'appellerais BC_1 AU (AU pour « Auby ») et BC_1 SK2, est possible mais pas idéal compte tenu de la distance qui sépare Auby de SK2. En revanche, l'analyse de BC_1 SK2 a donné pour la première fois accès à des informations génétiques sur la tolérance constitutive au Zn et au Cd chez *A. halleri*, c'est-à-dire la tolérance présente à l'échelle de l'espèce. Afin de traiter la tolérance au Zn et au Cd en même temps, ce travail a été réalisé en collaboration avec le Laboratoire de Physiologie et Génétique Moléculaire des Plantes à l'Université Libre de Bruxelles (*coll*. Nathalie Verbruggen) (Meyer *et al*, sous presse).

Les tolérances au Zn et au Cd ont été examinées selon les protocoles décrits dans Willems *et al*. (2007) et Courbot *et al*. (2007), respectivement. Il s'agit encore une fois de tests séquentiels de tolérance, basés sur l'arrêt de l'élongation racinaire. La carte génétique a été construite à partir de 335 individus génotypés pour 60 marqueurs génétiques de type microsatellite ou SNP, et parmi eux, 129 ont été phénotypés pour la tolérance au Zn et 70 pour la tolérance au Cd. Les résultats ont révélé un QTL significatif pour chaque type de tolérance, chacun d'eux recouvrant la région des gènes *HMA4* sur le LG3 (Figure 12). Le QTL de tolérance au Zn expliquait 22,6% de la variance phénotypique, tandis que le QTL de tolérance au Cd expliquait 31,2% (Tableau 2). Certains QTLs non significatifs sont également apparus sur les groupes de liaison LG1, LG5, LG6 et LG8, correspondant dans certains cas à des QTLs précédemment identifiés (Tableau 2). Pour appuyer ces résultats, des mesures complémentaires montrent que (1) le gène *HMA4* serait présent en 3 copies chez les individus du BC_1 SK2 portant l'allèle *A. halleri* et (2) aucune différence d'expression n'a été mise en évidence pour l'autre gène candidat *MTP1-A* entre les individus portant l'allèle *A. halleri* ou non (Meyer *et al*, sous presse).

Tableau 2 : QTLs de tolérance au Zn et au Cd identifiés d'après l'analyse du BC_1 SK2, et correspondance avec les QTLs de tolérance précédemment identifiés. Les QTLs marqués en gras sont significatifs. (D'après Meyer *et al*, sous presse).

Nom du QTL	Groupe de liaison/marqueur le plus proche	LOD max	Part de variance phénotypique (%)	Additivité	Correspondance avec d'autres QTLs de tolérance	Référence
SK2_ZnTol1	**LG3/Chr2-08800;Chr2-06046**	**6,85**	**22,6**	**-178**	Zntol-1	Willems *et al.* (2007)
SK2_ZnTol2	LG6/Chr5-08514	1,65	4,6	-79	Cdtol-3	Courbot *et al.* (2007)
SK2_ZnTol3	LG8/Chr5-17487	1,29	3,9	-75	ZnTol_RG3	Ce rapport
SK2_CdTol1	**LG3/Chr2-08800;Chr2-06046**	**4,33**	**31,2**	**-48**	Cdtol-1	Courbot *et al*. (2007)
SK2_CdTol2	LG1/Chr5-01735	1,55	10,5	-27	ZnTol_RG1	Ce rapport
SK2_CdTol3	LG5/Chr3-14085	1,39	7,3	-23		

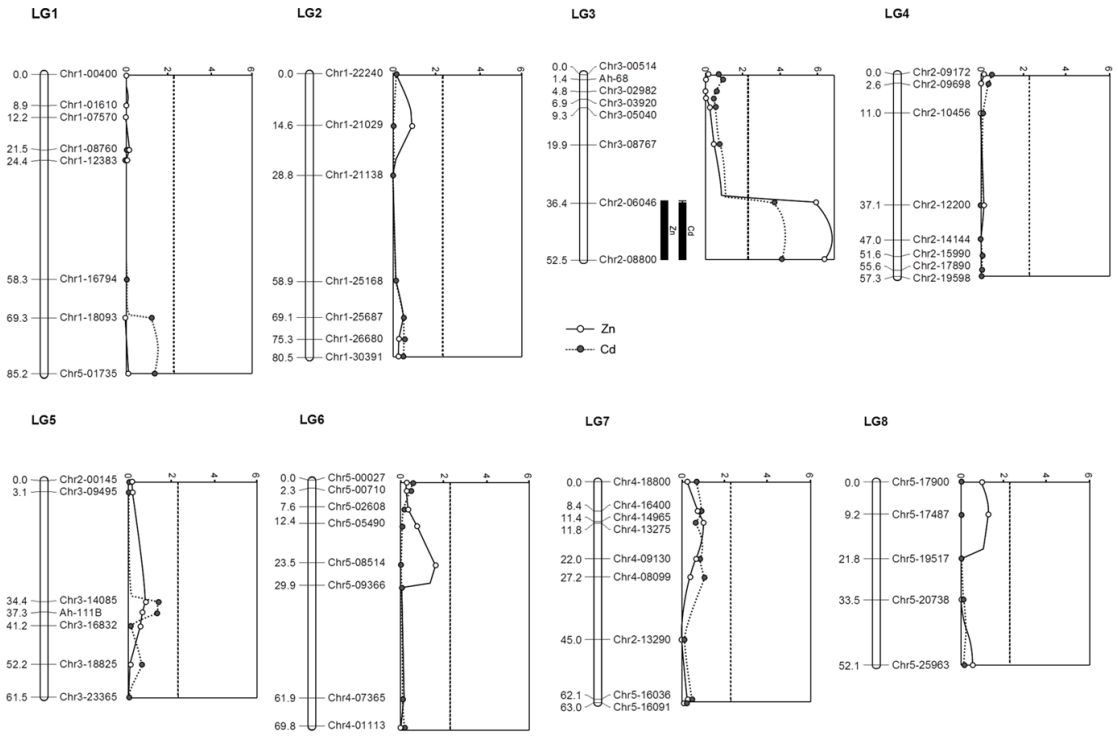

Figure 12 : QTL Mapping de la tolérance au Zn et au Cd dans la descendance BC_1 SK2. Les valeurs de LOD score pour la tolérance au Zn sont représentées par un trait plein tandis que les valeurs de LOD score pour la tolérance au Cd sont représentées par un trait en pointillé. Le trait vertical en pointillé représente le seuil de LOD score au-delà duquel un QTL est considéré comme significatif au seuil 5% (2,3 pour les deux traits). Les QTLs significatifs sont représentés par leur intervalle de confiance à 95%. (D'après Meyer *et al*, sous presse).

Même s'il est toujours possible que les gènes *HMA4* ne soient pas les gènes causaux de la région QTL du LG3, l'ensemble des preuves qui sont aujourd'hui assemblées montrent qu'ils constituent d'excellents candidats pour la tolérance au Zn et au Cd. Leur rôle dans la tolérance constitutive *a priori* apparue lors de la spéciation d'*A. halleri*, ou du moins, avant la colonisation des milieux pollués par les activités humaines, semble se préciser, que ce soit dans notre étude, ou d'après d'autres études d'évolution moléculaire (Hanikenne *et al*, 2013; Roux *et al*, 2011). Inversement, les gènes *MTP1-A* et *MTP1-B*, révélés par l'étude de la tolérance au Zn dans le BC_1 AU, pourrait avoir subi la sélection au moment de la colonisation des sites pollués, probablement afin d'activer la détoxication des métaux par séquestration vacuolaire. Ce dernier scénario est peut-être valable pour de nombreux autres gènes, participant à la « standing variation » présente dans les populations NM de l'espèce *A. halleri* (Meyer *et al*, 2009) à partir desquelles les populations M ont probablement été fondées (Pauwels *et al*, 2005).

Ceci pose évidemment la question du rôle évolutif initial des gènes *HMA4*, et de l'environnement initial d'évolution (pollué *vs* non pollué). Sachant que les gènes *HMA4* interviennent également dans l'hyperaccumulation du Zn et du Cd, et que de nombreuses hypothèses d'évolution de l'hyperaccumulation en milieu non pollué ont été proposées (Boyd and Martens, 1992), leur rôle dans la tolérance pourrait être vu comme une « exaptation » (Gould and Vbra, 1982). Ceci signifie que la tolérance représenterait une nouvelle fonction adaptative pour un seul et même trait qui est

celui de la translocation active du Zn dans les parties aériennes. La translocation du Cd en milieu pollué ne serait alors qu'une conséquence de la similarité chimique entre le Zn et le Cd. Il n'est cependant pas encore exclu que des affleurements comportant des minerais de zinc en quantité importante existaient à l'époque de la spéciation d'*A. halleri*.

3. Etude des bases genetiques et epigenetiques de la tolerance au zinc et de l'hyperaccumulation du zinc chez *A. halleri* et *N. caerulescens* par croisements intra-specifiques

Ces projets s'intègrent dans un projet plus large nommé « ELOCANTH » pour « Evolution of Local Adaptation in Anthropogenic Environments », financé par l'ANR de 2013 à 2017 (appel d'offre Jeunes Chercheurs-Jeunes Chercheuses) et dont je suis la coordinatrice. Ils s'intègrent aussi dans la thèse de Dima Souleman, qui étudie l'adaptation locale aux milieux calaminaires à la fois chez *A. halleri* et chez *Lumbricus terrestris*. Ils s'intègrent également dans le post-doctorat de Marie-Joe Karam, qui est en charge des analyses bioinformatiques, et qui plus globalement étudie les bases génétiques et épigénétiques de l'adaptation locale chez *A. halleri* et *N. caerulescens*.

a. Bases genetiques de la tolerance au zinc et de l'hyperaccumulation du zinc

Chez *A. halleri*, l'étude des populations polonaises et slovaques (Kostecka, 2009; Meyer *et al*, 2010; Meyer *et al*, 2009) et des populations italiennes (Decombeix, 2011) a montré qu'il existait de la variation intra-spécifique, entre populations, pour la tolérance au Zn et pour l'hyperaccumulation du Zn à une échelle régionale (voir aussi Figure 8). Néanmoins, si la différenciation phénotypique entre M et NM n'est pas toujours claire, les populations M restent en moyenne plus tolérantes que les NM, la tendance étant inverse pour l'hyperaccumulation. Quant à la variabilité intra-population, elle est souvent très importante. Dans ces régions, des populations M et NM se développent à proximité les unes des autres avec souvent une faible structuration génétique neutre entre populations (Decombeix, 2011; Kostecka, 2009; Meyer *et al*, 2009). Dans un tel contexte, l'adaptation locale reste possible, peut-être à condition de rechercher une situation suffisamment favorable. Par exemple dans les Alpes italiennes, les populations se répartissent en deux vallées distantes de 40 km environ à vol d'oiseau, dont l'une est entièrement constituée de populations NM (Figure 13).

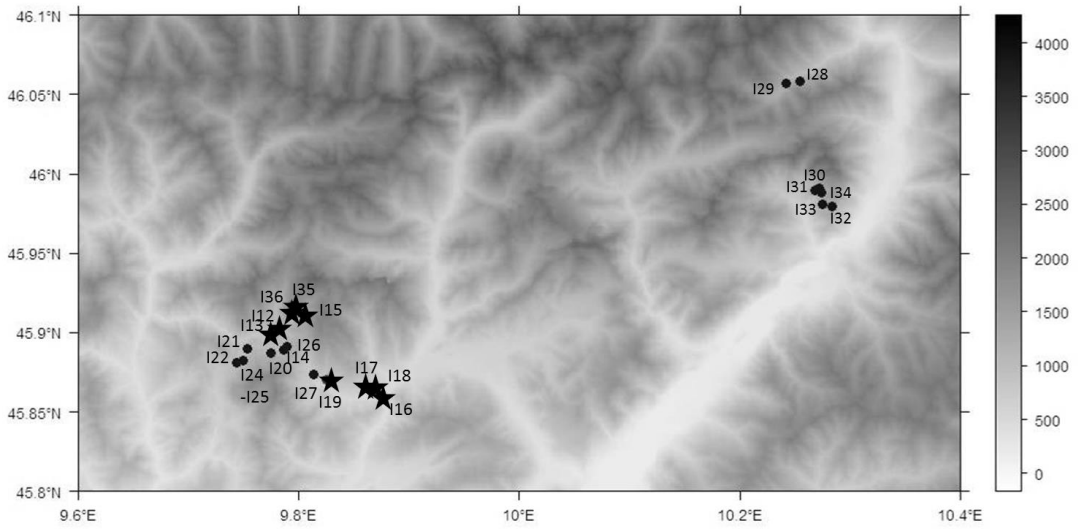

Figure 13 : Localisation des populations métallicoles et non métallicoles dans deux vallées des Alpes italiennes. Les populations métallicoles sont représentées par le symbole "étoile", tandis que les populations non métallicoles sont représentées par le symbole "rond".

En fonction du matériel biologique disponible, et pour maximiser mes chances de détecter un signal d'adaptation locale, j'ai choisi de mettre en culture la descendance F_2 d'un croisement entre une population M de l'une des vallées (I35) et une population NM de l'autre vallée (I30). *A. halleri* étant une espèce auto-incompatible, ce croisement a nécessité l'utilisation de 2 individus I35 et de 2 individus I30, afin de créer 2 descendances (I35 x I30). La descendance F_2 se nomme donc [I30.16xI35.12 (7) x I30.13xI35.6 (9)]. Les teneurs moyennes en Zn et en Cd biodisponibles[5] dans les sols sont des paramètres qui distinguent fortement les deux populations : 11417 $mg.kg^{-1}$ de Zn pour I35 et 23 $mg.kg^{-1}$ pour I30, 38 $mg.kg^{-1}$ de Cd pour I35 et 0,33 $mg.kg^{-1}$ pour I30.

En accord avec l'étude de Meyer *et al.* (2010), nous avons choisi de tester la tolérance au Zn via un test à doses fixes en solution nutritive (10µM pour la concentration contrôle et 2000µM pour le traitement pollué), et de mesurer différents traits comme la largeur des feuilles, la longueur des racines, le rendement photosynthétique, la biomasse sèche finale des feuilles et des racines. La carte génétique a été construite par séquençage haut débit des 4 parents et des deux individus de la F_1 (effectué sur la plateforme GeT-PlaGe de l'INRA de Toulouse) suivi d'un alignement sur le génome provisoire d'*A. halleri* (*coll.* Marc Hanikenne du Centre d'Ingénierie des Protéines, Université de Liège). Les SNPs utilisables pour la cartographie ont été sélectionnés par Marie-Joe Karam, tels que leur score de qualité soit bon (bonne représentativité au sein des reads de séquençage par exemple) et que leur polymorphisme soit compatible avec le suivi de la transmission des allèles « métallicoles » et « non métallicoles » (Tableau 3). Au final, 700 SNPs ont été retenu, parmi lesquels 384 ont été aléatoirement choisis pour la réalisation d'une puce SNP (travail effectué sur la plateforme GENTYANE de l'INRA de Clermont-Ferrand). Près de 170 individus ont été ainsi génotypés, et la carte génétique a pu être achevée avec succès (même démarche que dans : Bonin *et al*, 2015). Les analyses en cours révèlent l'existence d'un seul QTL significatif pour le rendement

[5] Cette mesure nécessite une extraction du métal contenu dans le sol dans un solvant organique. Le solvant utilisé ici est l'acétate d'ammonium-EDTA, et représente un solvant parmi d'autres pour extraire les métaux dits « biodisponibles », c'est-à-dire capables de passer dans la phase liquide du sol et donc accessibles aux plantes.

photosynthétique, expliquant 30% de la variance phénotypique. Ce QTL est présent sur le LG4, dans une région génomique encore jamais mise en évidence jusqu'à présent (Figure 14). Nous poursuivons actuellement ce travail par du fine-mapping.

Tableau 3 : Deux exemples de transmission des allèles « non métallicoles » (0) et « métallicoles » (1), tels que l'origine de chaque allèle reste identifiable dans la F_1. Ces deux cas sont pris en compte dans la sélection des SNPs utilisables pour la cartographie génétique. Ah1-6 : parents et F_1 *A. halleri*.

NM		**M**		**F_1**		**NM**		**M**		**F_1**
Ah1	*	Ah3	=	Ah5		Ah2	*	Ah4	=	Ah6
0/0	&	1/1	→	0/1	**&**	0/0	&	1/1	→	0/1
0/0	&	0/1	→	0/1	**&**	0/0	&	1/1	→	0/1

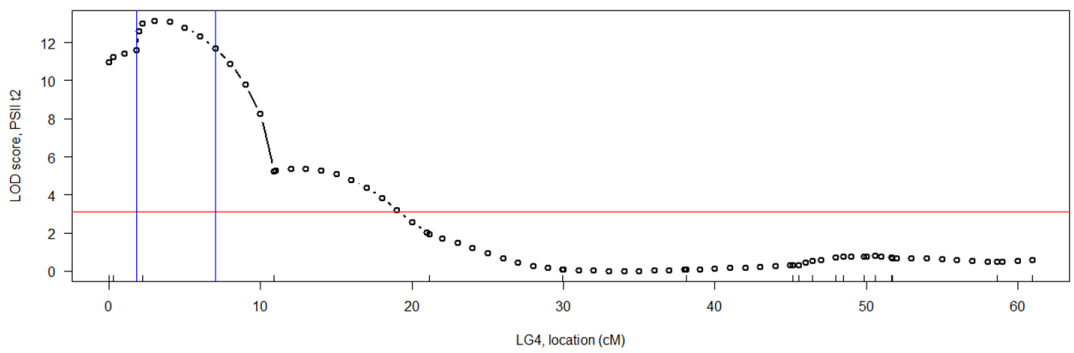

Figure 14 : Résultat des analyses de QTL Mapping pour la tolérance au Zn dans la descendance du croisement intra-spécifique chez *A. halleri*. La tolérance au Zn est mesurée par le rendement photosynthétique du photosystème II. Seul le groupe de liaison 4 (LG4), représentant le chromosome 4 d'*A. halleri*, est figuré ici. Le trait horizontal rouge représente le seuil de LOD score calculé par une méthode de permutation au-délà duquel le QTL est significatif au seuil 5%. Les deux traits verticaux bleus définissent l'intervalle de confiance à 99% du QTL. (Figure fournie par Marie-Joe Karam).

Quant à l'hyperaccumulation du Zn, nous l'avons testée de la même manière que dans Frérot *et al*. (2010), en conditions contrôlées et dans du terreau additionné de 1500 $mg.kg^{-1}$ de zinc sous forme $ZnSO_4$, 7 H_2O. Nous n'avons pas obtenu de QTL pour ce caractère. Nous explorons actuellement les causes de ce résultat négatif, qui proviennent *a priori* d'une distribution trop uniforme entre les différentes classes phénotypiques, due soit (1) à une architecture génétique complexe composée de nombreux gènes à effets mineurs, soit (2) des conditions expérimentales (notamment la concentration en Zn dans le substrat) ne permettant pas l'expression des gènes majeurs.

En parallèle, nous avons également réalisé le croisement d'individus M et NM de l'espèce *N. caerulescens*. Un individu NM issu de la population « Wilwerwiltz » (Luxembourg) a été croisée avec un individu M issu de la population « La Calamine » (Belgique). Ces deux populations sont connues et citées dans la bibliographie (Assunção *et al*, 2003a; Dechamps *et al*, 2008). De plus, contrairement à *A. halleri*, les croisements intra-spécifiques chez *N. caerulescens* se pratiquent depuis longtemps (Assunção *et al*, 2006; Assunção *et al*, 2003c; Deniau *et al*, 2006; Frérot *et al*, 2005; Frérot *et al*, 2003). *N. caerulescens* étant une espèce auto-compatible, il suffit pour obtenir une descendance F_2 de réaliser manuellement une F_1 la première année, et de mettre en place une autofécondation de quelques individus de la F_1 la deuxième année. Une première descendance F_2 est actuellement en cours de phénotypage pour la tolérance au Zn, dans des pots placés en conditions contrôlés et comportant 750 $mg.kg^{-1}$ de Zn ajouté sous forme de sulfate de zinc. Cette concentration en Zn provient des résultats d'une autre expérience que j'aborde au Chapitre 3. Une nouvelle descendance est actuellement en cours de production avec des accessions M et NM du Sud de la France, et incluant le génotype « Ganges » dont le génome est séquencé et annoté (*coll*. Mark Aarts du Laboratoire de Génétique de l'Université de Wageningen). Les cartes génétiques de ces descendances de *N. caerulescens* seront produites grâce à la méthode de Génotypage par Séquençage (*coll.* Sylvain Santoni, INRA de Montpellier).Nous avons également entrepris un travail de plus long terme avec la création de lignées, dont des lignées recombinantes.

Pour conclure sur la présentation de ces travaux en cours, j'ajouterais que l'analyse de *N. caerulescens* se déroule d'une manière très différente de celle d'*A. halleri* dans la mesure où l'espèce n'est pas clonale. Ainsi, il n'est pas possible de multiplier les expériences avec les mêmes génotypes, et les réplicats sont représentés non pas par des clones, mais par des graines issues de la même plante (des « frères », idéalement des « pleins-frères »). Ensuite, la partie végétative des individus de *N. caerulescens* est en une seule rosette (alors qu'*A. halleri* se présente souvent en plusieurs rosettes reliées par des stolons), ce qui autorise les mesures végétatives simples et intégratives comme le diamètre de la rosette. Enfin, de nombreuses démarches expérimentales sur *N. caerulescens* ont considéré les mesures de traits d'histoire de vie incluant les traits reproducteurs (Dechamps *et al*, 2011; Dechamps *et al*, 2007; Dechamps *et al*, 2008; Jimenez-Ambriz *et al*, 2007). Nous nous inscrivons tout à fait dans cet historique en conduisant des expériences dans lesquelles les plantes ont la possibilité de fleurir et de produire des graines. De cette manière, nous espérons accéder à la valeur sélective des plantes, en tant que révélateur du niveau de tolérance au Zn (voir Chapitre 3).

b. Bases epigenetiques de l'adaptation locale

Ce projet est issu d'une collaboration avec Marc Hanikenne, de l'Université Liège, dont l'un des projets-phare est le séquençage du génome d'*A. halleri* et l'exploration chez cette espèce des modifications épigénétiques le long de ce génome en rapport avec l'adaptation aux milieux calaminaires. Ce projet comprend également l'analyse du transcriptome et du lien potentiel entre modifications épigénétiques et modifications d'expression génétique. Nous avons accompagné Marc Hanikenne dans ce projet en mettant en œuvre la culture en pots de deux couples de populations M et NM d'*A. halleri* (un couple en Pologne/Slovaquie et un couple en Italie) en conditions polluées par le Zn (1500 $mg.kg^{-1}$) et non polluées. Après cinq semaines d'exposition, les individus ont été pesés, conditionnés pour les analyses génomiques d'une part et pour les analyses de teneur en métaux d'autre part. Toutes les données ont été acquises à l'Université de Liège grâce à du séquençage haut débit. Les sites méthylés ont été repérés grâce à un traitement de l'ADN au bisulfite qui a pour effet de transformer les cytosines non méthylées en thymines (« Methyl-Seq »). Tous les traitements

bioinformatiques sont effectués par Sol Schvartzman, actuellement en post-doctorat chez Marc Hanikenne.

Nous sommes pour notre part en charge des analyses équivalentes chez l'espèce *N. caerulescens*, et ce projet s'intègre dans le post-doctorat de Marie-Joe Karam. Le plan expérimental est nécessairement différent du précédent puisque *N. caerulescens* ne peut pas être clonée. Il n'a pas été possible non plus de rassembler suffisamment de lignées M et NM pour concevoir un plan expérimental basé sur des génotypes identiques. Nous avons donc mis en culture des graines issues des mêmes plantes dans trois conditions de pollution en Zn (0, 750 et 1500 $mg.kg^{-1}$), pour cinq semaines d'exposition, et ce pour deux couples de populations M et NM (Belgique/Luxembourg et Sud de la France). Nous avons réalisé des mesures de rendement photosynthétique qui montrent que la condition 1500 $mg.kg^{-1}$ provoquent une baisse de rendement très forte chez les populations NM. Nous tentons actuellement de mener à bien l'extraction et le séquençage des génomes choisis, toujours en collaboration avec l'Université de Liège et Marc Hanikenne.

Cette investigation des bases épigénétiques est assez prospective. Bien qu'ayant conçu les protocoles expérimentaux avec le plus grand soin afin d'accéder à la part héritable des modifications épigénétiques due à la méthylation des cytosines, nous ne savons pas à quoi nous attendre en terme de résultats.

CHAPITRE 3 : DES TRAITS COMPLEXES DANS UN ENVIRONNEMENT COMPLEXE

Par comparaison avec le chapitre précédent, ce chapitre comporte davantage de résultats non publiés ou en cours d'acquisition, ainsi que des perspectives de travail.

1. LA TOLERANCE : UN TRAIT COMPLEXE

a. DES TRAITS « MOLECULAIRES » AUX TRAITS D'HISTOIRE DE VIE CHEZ *A. HALLERI*

Les approches QTL présentées dans le chapitre précédent ont été menées parallèlement à un large développement des approches transcriptomiques (Becher *et al*, 2004; Halimaa *et al*, 2014; Hammond *et al*, 2006; Rigola *et al*, 2006; Talke *et al*, 2006; van de Mortel *et al*, 2008; van de Mortel *et al*, 2006; Weber *et al*, 2004; Weber *et al*, 2006) et des approches de physiologie moléculaire (par exemple : Dräger *et al*, 2004; Hanikenne *et al*, 2008; Shahzad *et al*, 2010). L'attention a alors été essentiellement portée sur les gènes de l'homéostasie des métaux et leur rôle dans la tolérance ou l'hyperaccumulation (Clemens, 2001; Verbruggen *et al*, 2009). Chez *A. halleri* particulièrement, du fait de la relative proximité entre les génomes d'*A. halleri* et *A. thaliana* (Roosens *et al*, 2008a), la tolérance aux métaux est ainsi de mieux en mieux comprise au niveau physiologique : la détoxication des cellules racinaires par transfert dans le xylème (rôle des gènes *AhHMA4*), la séquestration dans les vacuoles des cellules foliaire (rôle des gènes *AhMTP1*), la chélation des métaux dans le cytoplasme (rôle des nicotianamines, phytochélatines...). Mon propos n'est pas d'être exhaustive quant aux nombreux transporteurs et chélateurs impliqués dans la tolérance aux métaux, mais de pointer du doigt que l'organisme n'apparaît presque plus dans ces considérations moléculaires. Comment tous ces mécanismes viennent finalement se combiner pour former un individu plus ou moins tolérant aux métaux ?

Lorsque des tests de tolérance sont pratiqués, les études moléculaires se contentent souvent de tests de croissance racinaire (par exemple : Hanikenne *et al*, 2008; van de Mortel *et al*, 2008). D'autres études, plus écologiques que purement physiologiques, ont évalué la tolérance par la production de biomasse ou l'apparition de chlorose foliaire (Assunção *et al*, 2003a; Assunção *et al*, 2003c; Escarré *et al*, 2000; Frérot *et al*, 2003; Meerts and Van Isacker, 1997). Ces études concernent l'espèce *N. caerulescens*, et faisaient partie de ma culture lorsque je suis arrivée au laboratoire GEPV (aujourd'hui EEP). C'est pourquoi, et contrairement aux pratiques courantes pour l'espèce *A. halleri*, j'ai rapidement souhaité mettre en place une caractérisation de la tolérance au Zn chez *A. halleri* par la mesure de traits autres que la croissance racinaire (Meyer *et al*, 2010). Ceci a nécessité de nombreux tests préalables car elle impliquait de travailler à des concentrations en Zn fixes. En choisissant 10 µM et 2000 µM comme concentrations (respectivement « contrôle » et « traitement pollué »), l'estimation du rendement photosynthétique du photosystème II s'est notamment avéré être un trait suffisamment résolutif pour (1) quantifier des variations intra-spécifiques et (2) révéler un signal d'adaptation locale entre populations M et NM. La structuration en population de la variation de ce trait est en effet apparue supérieure à un attendu neutre (Figure 15).

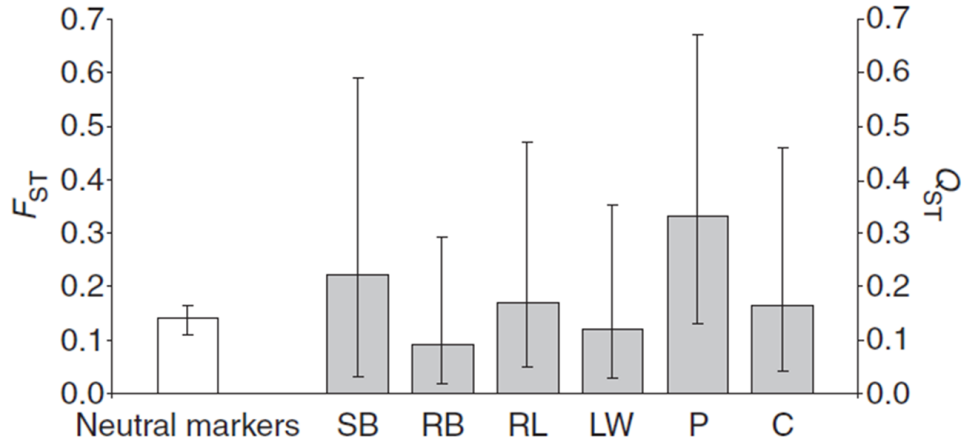

Figure 15 : Comparaison des niveaux de différenciation génétique pour les marqueurs moléculaires neutres (F_{ST}) et les traits quantitatifs (Q_{ST}). Les traits quantitatifs indiqués sont la biomasse aérienne (SB), la biomasse racinaire (RB), la longueur des racines (RL), la largeur des feuilles (LW), le rendement photosynthétique (P), et la quantité de chlorophylle (C). Les lignes verticales représentent les intervalles de confiance à 95%. Les populations impliquées dans cette étude sont toutes issues de la région Pologne/Slovaquie. (D'après Meyer *et al*., 2010).

Ainsi, si la croissance racinaire peut permettre la détection de variations intra-spécifiques (la différence M-NM était significative pour ce trait dans Meyer *et al*, 2010), elle ne peut ne pas fournir à elle seule toutes les informations sur le niveau de tolérance. Des mesures intégratives, telles que la biomasse aérienne ou racinaire, ne semblaient cependant pas mieux refléter les niveaux de tolérance que des traits relativement simples (dans le sens : peu intégrateurs) comme la largeur des feuilles ou le rendement photosynthétique. Décomposer la tolérance, en tant que trait complexe, en traits simples et héritables, comme dans le cas des espèces agronomiques soumises à des stress abiotiques (Tardieu and Tuberosa, 2010), semble donc approprié. Pour l'espèce *A. halleri*, nous avions donc finalement établi des conditions de mesures de la tolérance au Zn plutôt convaincantes (voir aussi : Kostecka, 2009). Cependant, les traits reproducteurs, bien qu'apparaissant explicitement dans la définition historique de la tolérance (voir partie 1.a du Chapitre 2), restaient complètement négligés. Sur ce point, les avancées les plus significatives venaient des publications sur l'espèce *N. caerulescens* (Dechamps *et al*, 2011; Dechamps *et al*, 2007; Dechamps *et al*, 2008; Jimenez-Ambriz *et al*, 2007). Ces études s'attachent à mesurer les traits d'histoire de vie, comme le nombre et la hauteur des hampes florales, le nombre de fruits fertiles, le nombre de graines par fruit, ou le nombre de graines. Inspirés par ces études, nous avons tenté de les appliquer aux populations naturelles d'*A. halleri* (Decombeix, 2011).

Après quelques tests préliminaires, nous nous sommes placés dans les conditions de Dechamps *et al*. (2007), à savoir en pots et en extérieur, avec un traitement non pollué, et deux traitements pollués (1000 et 8000 $mg.kg^{-1}$ de Zn sous la forme ZnO) pour mesurer différents traits végétatifs (notamment : longueur et largeur des feuilles sur une moyenne de 3 feuilles par plante, nombre de rosettes) et reproducteurs (notamment : nombre de hampes florales, hauteur de la hampe la plus grande, nombre de graines). L'expérience complète a échoué car nous avons subi une réduction drastique d'effectif (entre 30% et 70% de mortalité en fonction des populations) dans le

traitement non pollué à cause d'une infestation de *Phyllotetra nemorum* (altise du chou)[6] au 75ième jour de l'expérience. Néanmoins, les résultats obtenus à ce stade étaient intéressants (Figure 16). Les traits reproducteurs (représentés ici par le nombre de hampes florales, Figure 16B) ne montraient aucune différence significative entre les populations, car la variabilité entre les génotypes au sein des populations était très élevée. Pourtant, une tendance à la hiérarchisation des réponses selon les groupes de populations était visible dans les conditions polluées car les populations M (I16, I35) portaient en moyenne plus de hampes florales que les populations NM de la vallée 1 (I14, I22, I27), qui elles-mêmes en portaient plus que la population NM de la vallée 2 (I31). Pour les traits végétatifs (représentés ici par la largeur des feuilles, Figure 16A), ou le rendement photosynthétique (Figure 16C), aucune différence entre écotypes (M *vs* NM) n'est apparue significative. Ceci est probablement dû à la proximité phénotypique (et géographique) entre les populations M et NM de la vallée 1.

Finalement, chez *A. halleri* l'utilisation des traits reproducteurs pour estimer la tolérance au Zn ne semble pas se justifier. Ceci pourrait provenir du fait que, contrairement à *N. caerulescens*, l'espèce *A. halleri* est clonale. Dans ce cas, l'adaptation aux sites pollués pourrait impliquer plutôt les traits végétatifs (dont ceux participant à la reproduction asexuée) par rapport aux traits reproducteurs. Une telle stratégie d'adaptation poserait néanmoins la question de l'estimation de la valeur sélective des individus en termes de nombre de descendants issus de la reproduction sexuée.

[6] Nous n'avons pas eu l'opportunité de doser la quantité de métal dans les feuilles, mais il semblerait que le Zn potentiellement accumulé dans les feuilles des plantes cultivées dans les traitements pollués ait limité l'infestation dans ces traitements.

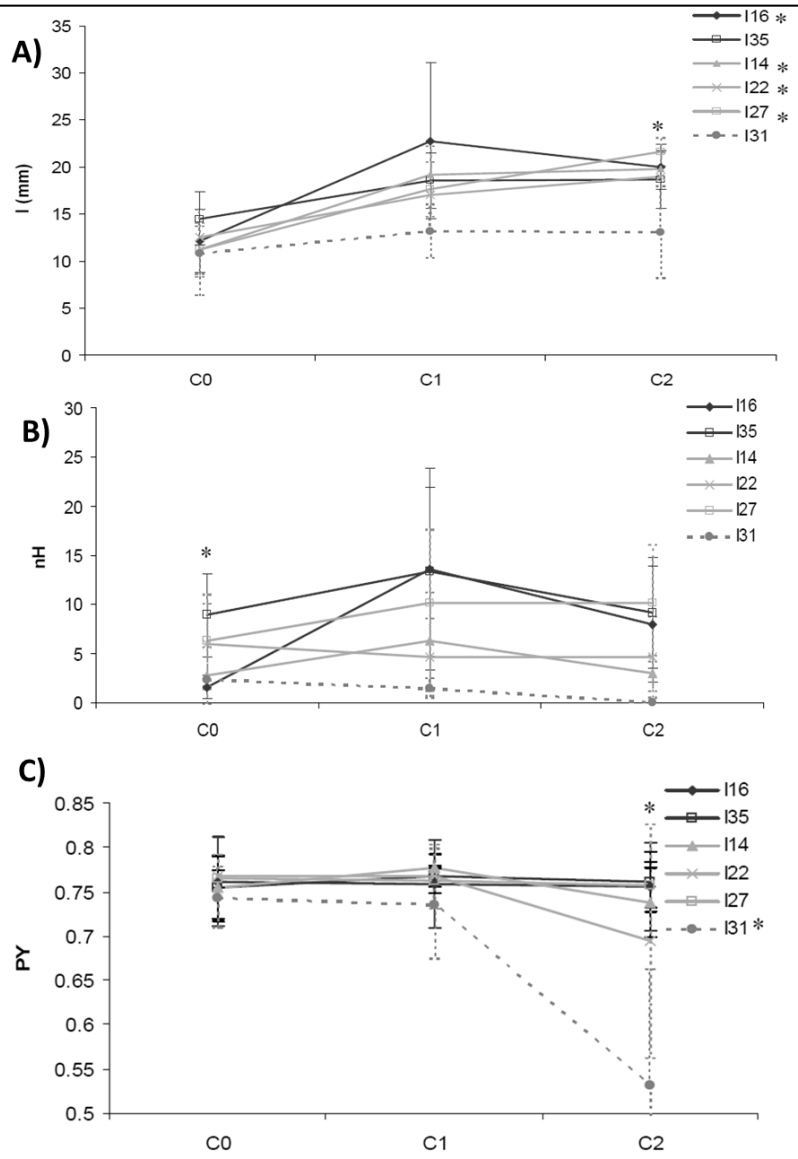

Figure 16 : Résultats du test de tolérance en pots et en extérieur réalisé sur des populations des Alpes italiennes de l'espèce *A. halleri*. Les traits indiqués sont (A) la largeur des feuilles en mm, (B) le nombre de hampes et (C) le rendement photosynthétique. Les données fournies sont enregistrées au 72^{ième} jour de l'expérience, avant l'attaque par l'altise du chou (vers le 75^{ième} jour). Les populations M sont I16 et I35, les populations NM de la vallée 1 sont I14 et, I22 et I27. La population NM de la vallée 2 (sans populations M) est I31. C0 : condition non polluée; C1 : condition polluée à 1000 mg.kg^{-1} de Zn; C2 : condition polluée à 8000 mg.kg^{-1} de Zn. * p<0,01 pour le test de comparaison entre conditions pour une population donnée, ou le test de comparaison entre populations pour une condition donnée. (D'après la thèse d'I. Decombeix, 2011).

b. DES TRAITS D'HISTOIRE DE VIE A LA VALEUR SELECTIVE CHEZ *N. CAERULESCENS*

La définition de la tolérance fait explicitement référence à la survie et la reproduction (voir partie 1.a du Chapitre 2), ce qui suggère que le trait peut être appréhendé par des mesures de valeur sélective. Se pose alors la question plus générale de la manière de mesurer la valeur sélective. Chez *N. caerulescens*, elle a été récemment mesurée *via* le nombre de graines produites par un individu (Dechamps *et al*, 2007; Jimenez-Ambriz *et al*, 2007). Comme la récolte du nombre total de graines produites par un individu est en réalité rarement possible (culture en extérieur et exposition aux intempéries, ou dispositifs de récolte contraignant pour la croissance de la plante…), ce nombre est estimé par la multiplication suivante : nombre d'inflorescences x nombre de fruits par inflorescence x nombre de graines par fruits. Cependant, il manque dans cette estimation quantitative de la valeur sélective (nombre de descendants) la notion qualitative de viabilité des descendants. Pour accéder à la viabilité des descendants, le plus simple est de récupérer le plus de graines matures possible, et d'évaluer les taux de germination, ou encore la survie des plantules.

C'est pourquoi, nous avons mis en place une expérience spécifique pour établir une relation allométrique entre le nombre total de graines produites et différents traits végétatifs et reproducteurs. Nous avons mesuré le nombre de feuilles, le plus grand diamètre de la rosette et son perpendiculaire, la longueur et la largeur des trois plus grandes feuilles, le nombre de hampes florales, la hauteur des hampes florales et celle de la plus grande hampe florale, le nombre total de fruits (en distinguant fruits pleins et fruits avortés), la taille de cinq siliques par hampe florale. La taille des siliques est un trait qui nous a été inspiré par la méthode de mesure de la valeur sélective pratiquée chez *A. thaliana* (Brachi *et al*, 2012). Le nombre total de graines produites par un individu semble être essentiellement corrélé aux traits reproducteurs, en particulier par le nombre de hampes florales, la taille des hampes, le nombre de fruits fertiles et la taille des siliques (Figure 17). Dans nos expériences mettant en jeu l'estimation de la valeur sélective (voir partie 3.b de ce Chapitre), nous avons aussi complété cette « batterie de mesures » par l'évaluation des taux de germination par plante-mère, censés refléter la qualité des graines et la survie des descendants potentiels.

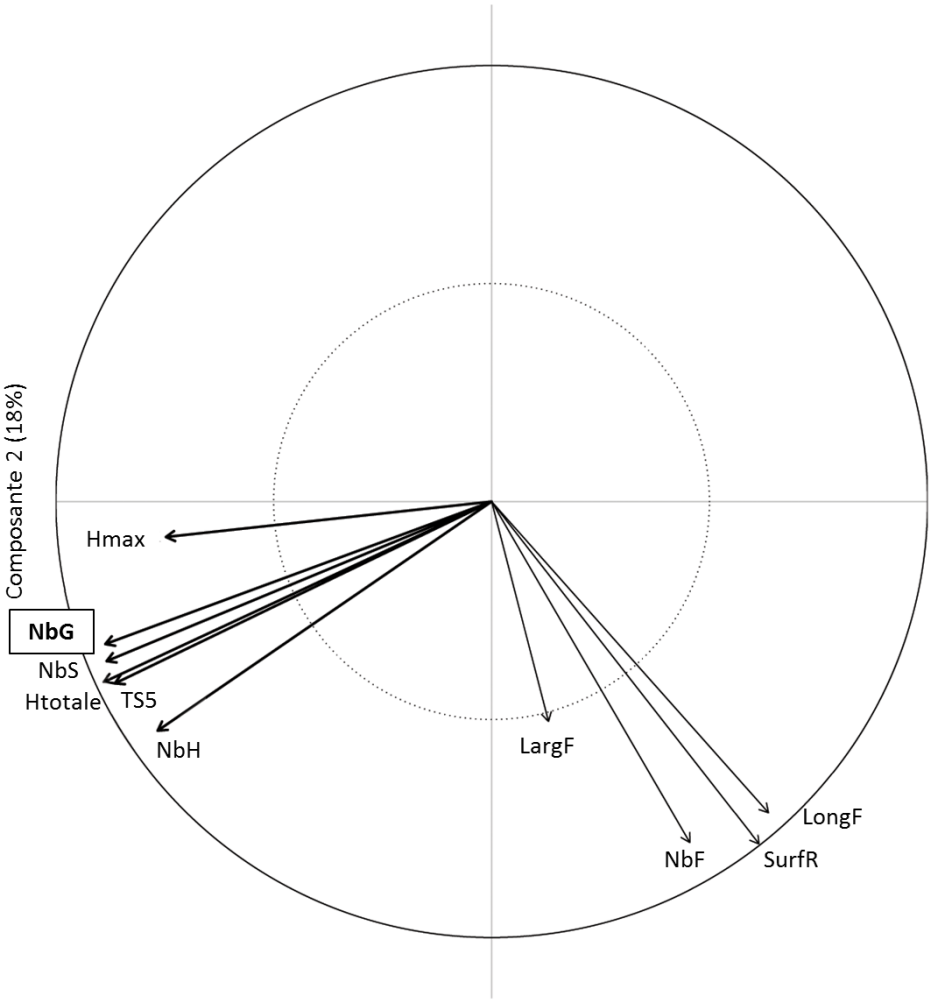

Figure 17 : Résultat de l'analyse en composantes principales sur un ensemble de traits végétatifs et reproducteurs susceptibles d'être corrélés au nombre de graines produites. Les traits végétatifs sont : longueur des 3 plus grandes feuilles (LongF), largeur des 3 plus grandes feuilles (LargF), nombre de feuilles (NbF), surface de la rosette (SurfR). La surface de la rosette est obtenue par calcul de l'aire d'une ellipse ayant pour axe principal le plus grand diamètre de la rosette et pour axe secondaire le diamètre perpendiculaire au premier. Les traits reproducteurs sont : nombre de hampes (NbH), taille de 5 siliques par hampe (TS5), hauteur totale de toutes les hampes (Htotale), hauteur de la plus grande hampe (Hmax), nombre de siliques fertiles (NbS). L'expérience a été réalisé en pots et sans zinc ajouté, avec 72 individus répartis dans 5 populations de Belgique et Luxembourg, dont 2 populations métallicoles et 3 non métallicoles (13 à 15 individus par population). (Figure fournie par Julien Nowak).

Cette démarche m'inspire trois réflexions importantes. Tout d'abord, elle implique une hypothèse sous-jacente, à savoir que les corrélations entre les traits sont stables, et ne dépendent par exemple ni du temps, ni des populations étudiées, ni de l'environnement dans lequel les individus se développent (différents niveaux de pollution, conditions extérieures ou contrôlées…). Nous avons commencé à vérifier certaines de ces conditions, car nous avons mis en place deux expériences d'allométrie en conditions contrôlées, sur plusieurs populations à chaque fois, la deuxième expérience comprenant un sol pollué et un sol non pollué. Nous analysons actuellement les résultats de ces expériences. Nous ne pouvons pas exclure que ces relations soient différentes en conditions extérieures, mais nous ne l'avons pas encore testé. La deuxième réflexion concerne l'estimation de la valeur sélective uniquement *via* des traits reproducteurs, en omettant par exemple la survie. L'estimation de la valeur sélective peut être complétée en utilisant une méthode d'analyse spécifique, appelée « ASTER », qui permet de combiner dans un seul modèle tous les paramètres de la valeur sélective dépendants entre eux et obéissant à des lois statistiques différentes (Geyer *et al*, 2007; Shaw *et al*, 2008). Nous n'avons pas encore appliqué cette méthode à nos données, mais nous serons peut-être amenés à l'utiliser dans le cadre de la thèse de Julien Nowak (voir partie 3.b de ce Chapitre). La troisième réflexion est que l'évolution peut potentiellement porter non sur les traits pris séparément, mais sur la matrice de variance-covariance entre ces traits, appelées communément « matrice G » (Lande and Arnold, 1983). Il s'agit là encore de tout un corpus théorique que je dois acquérir.

2. L'HYPERACCUMULATION : UN TRAIT PAS SI SIMPLE

L'hyperaccumulation est définie par une concentration très élevée en métal dans les feuilles. D'un point de vue pratique, l'hyperaccumulation est donc un trait qui paraît très accessible puisqu'il suffit de récolter la biomasse aérienne, de la laver, de la minéraliser, et de doser la quantité de métal contenue dans une biomasse donnée. Néanmoins, la concentration foliaire en métal à partir de laquelle une plante peut être qualifiée d'hyperaccumulatrice fait l'objet d'un débat compliqué (van der Ent *et al*, 2013). Les raisons de ces complications sont multiples, mais la raison principale est que l'hyperaccumulation est un trait quantitatif soumis aux interactions génotype x environnement (Macnair, 2002; Pollard and Baker, 1996). Par conséquent, la concentration foliaire mesurée dépend des conditions dans lesquelles la plante est cultivée : milieu naturel ou en serre, solution nutritive, sol additionné d'un sel métallique ou encore sol prélevé sur le terrain (Escarré *et al*, 2013; Escarré *et al*, 2011; van der Ent *et al*, 2013). Cela suggère qu'avant de déclarer une espèce comme hyperaccumulatrice, il est nécessaire d'avoir réalisé plusieurs tests dans différents environnements. Il est également nécessaire de prouver que l'hyperaccumulation n'entraîne pas de symptôme d'intoxication, sans quoi la concentration obtenue est forcée par les conditions de l'expérience, au lieu d'être une caractéristique stable apparue au cours de l'évolution. Ces étapes sont maintenant largement dépassées pour *A. halleri* et *N. caerulescens* dont le statut d'hyperaccumulatrice est bien établi. Mon propos n'est donc pas de rediscuter de cela, mais de savoir si la simple concentration foliaire en métal donne vraiment une bonne image de l'hyperaccumulation en particulier chez *A. halleri* et *N. caerulescens*.

Aujourd'hui, une bonne image de l'hyperaccumulation pourrait être représentée par le niveau d'expression de certains gènes connus pour être impliqués dans l'hyperaccumulation. Si par exemple l'hyperaccumulation pouvait être réduite à la translocation active des métaux vers les parties

aériennes, alors le niveau d'expression du gène *HMA4* serait un bon indicateur des capacités d'hyperaccumulation. Cependant, l'étude de Hanikenne *et al*. (2008) met bien en évidence le fait que la transformation d'*A. thaliana* avec *AhHMA4* ne permet pas d'atteindre les niveaux d'hyperaccumulation naturellement présents chez *A. halleri*. De plus, les analyses QTL montrent que d'autres gènes contribuent probablement à l'hyperaccumulation du Zn (Frérot *et al*, 2010). Il reste à illustrer le fait que d'autres mécanismes physiologiques que la translocation du Zn pourraient entrer en jeu dans le phénotype d'hyperaccumulation du Zn. Justement, la descendance F_2 entre *A. halleri* accession Auby et *A. lyrata petraea* a été soumise à des analyses spectroscopiques et microscopiques (*coll.* G. Sarret de l'Université Joseph Fournier, Grenoble) qui ont révélé une corrélation entre le niveau d'accumulation du Zn et (1) le taux de Zn complexé par des acides organiques et (2) le patron de localisation du Zn dans les feuilles (Sarret *et al*, 2009). Chez les individus les plus accumulateurs, le Zn était davantage complexé à des acides organiques, et préférentiellement localisé dans les tissus foliaires par rapport aux vaisseaux, suggérant un meilleur déchargement du xylème et une meilleure séquestration dans les vacuoles, où se trouvent la majorité des acides organiques (Tableau 4). Chez *N. caerulescens*, l'inhibition de la séquestration vacuolaire du Zn dans les racines semble aussi être un mécanisme favorisant l'hyperaccumulation (Kozhevnikova *et al*, 2014).

Tableau 4 : Distribution du Zn dans les feuilles des parents et des descendants du croisement entre *A. halleri* accession Auby et *A. lyrata petraea*. La distribution est estimée par micro-fluorescence à rayons X, sur des feuilles matures et lyophilisées. (D'après Sarret *et al*., 2009).

	Zn content (μmol g^{-1} DW)	Vein : tissue Zn counts[a]	Leaf Zn in each compartment[b] (%)		
			Leaf tissue	Veins	Trichomes
Arabidopsis halleri	97.9	0.8 ± 0.3	76 ± 5	14 ± 5	10 ± 5
Arabidopsis lyrata	6.6	1.9 ± 0.3	54 ± 5	26 ± 5	20 ± 5
$F_{1\text{-}1}$	17.7	2.3	57	34	9
$F_{2\text{-}3}$	12.2	1.0	64	16	20
$F_{2\text{-}4}$	30.8	1.8	54	25	21
$F_{2\text{-}6}$	59.2	1.6	58	24	18
$F_{2\text{-}8}$	170.8	0.6	73	11	16

[a]Ratio of the average Zn counts measured on the veins and on the leaf tissue. [b]Calculated by multiplying Zn counts by the estimated percentage of leaf surface occupied by each compartment (78% for the leaf tissue, 20% for the veins and 2% for the trichomes). This calculation assumes that μXRF at 10 keV probes the whole thickness of the leaf.

En 2010, j'ai été contactée par Anna Kozhevnikova et Ilya Seregin (Timiryazev Institute of Plant Physiology, Moscou) pour établir une collaboration. La publication de Sarret *et al*. (2009) venait de paraître, et j'avais donc en tête l'idée que l'hyperaccumulation pouvait être décomposée en plusieurs traits dépendants du patron de localisation ou du type de complexation du Zn dans la plante. Je pensais qu'à concentration égale dans les feuilles, il était possible de distinguer différentes stratégies d'hyperaccumulation, et que ce serait exploitable à l'échelle d'une descendance et être suivi d'analyses QTL. Nos collègues russes possédaient justement des compétences en marquage histochimique pour localiser précisément le métal dans les tissus. Néanmoins, je savais aussi qu'en France, cela était réalisable par plusieurs autres collègues, dont G. Sarret (Université Joseph Fournier, Grenoble) et M-P. Isaure (Université de Pau et des Pays de l'Adour) grâce à des techniques de

fluorescence des rayons X (Sarret *et al*, 2002; Sarret *et al*, 2009). Voici la série d'événements qui ont conduit à la soumission du projet de réseau international au CNRS (GDRI) sous le nom de LOCOMET (Localisation et Complexation des Métaux chez les plantes hyperaccumulatrices). Ce réseau a démarré en 2012, et a été renouvelé jusqu'en 2020. Outre le fait que cela a globalement dynamisé toutes nos activités de recherche, j'espère toujours que nous aurons finalement un nouvel éclairage sur la définition de l'hyperaccumulation, allant au-delà de la simple concentration en métal. A terme, j'espère que des traits de localisation ou de complexation du Zn montreront de la variation intra-spécifique, ségrégeront dans des descendances de croisements entre individus M et NM, et qu'il sera possible d'en déduire une architecture génétique des mécanismes d'hyperaccumulation correspondant (même si pour le moment, il existe un obstacle purement technique d'application des procédés physiques et histochimiques aux grands effectifs).

Lorsque je suggère une telle approche, je pars du principe que l'hyperaccumulation du Zn est un trait adaptatif, structuré entre populations M et NM. C'est une hypothèse souvent sous-jacente, mais encore jamais rigoureusement démontrée. Le caractère physiologiquement coûteux de l'hyperaccumulation (transport, stockage, détoxication des métaux) a laissé penser qu'il fallait un (ou des) bénéfice(s), même en milieu non métallifère (Pollard *et al*, 2014). De là, plusieurs hypothèses ont été formulées (Boyd and Martens, 1992). L'hyperaccumulation serait soit (1) un mécanisme d'allélopathie consistant à concentrer les métaux autour d'un individu – lors de la décomposition des feuilles sénescentes par exemple - afin d'intoxiquer d'éventuels compétiteurs, (2) un mécanisme de tolérance à la sécheresse en modifiant les équilibres osmotiques dans la plante, (3) la conséquence fortuite de mécanismes d'acquisition d'autres nutriments que les métaux, (4) un mécanisme de défense contre les herbivores et les pathogènes s'intoxiquant au contact des feuilles. Cette dernière hypothèse remporte beaucoup de suffrages (Boyd, 2007). L'hyperaccumulation a été par ailleurs explicitement décrite comme une stratégie de tolérance (Baker, 1981; Lin and Aarts, 2012). Mon propos n'est pas de refaire le point sur ces différentes hypothèses, mais de souligner que la communauté scientifique est tout à fait disposée à attribuer une fonction adaptative à ce trait, qui est potentiellement une réponse à plusieurs pressions de sélection, biotiques et abiotiques. La dernière partie de ce rapport aborde précisément la question des pressions de sélection, notamment abiotiques, qui agissent potentiellement sur l'hyperaccumulation du Zn mais aussi la tolérance au Zn.

3. Et le zinc dans tout ça ?

a. Approche indirecte : le zinc, une pression abiotique parmi d'autres

Dans toutes les expériences que je pratique depuis mes travaux de thèse, j'ai toujours dû créer un ou plusieurs environnements pollués, la plupart du temps en ajoutant du Zn sous forme de sulfate ou, plus rarement, d'oxyde de Zn. Cela dépend de la durée de l'expérience et du besoin en solubilité de ces sels, cette dernière étant très variable (Meerts *et al*, 2003). Je suis toujours partie du principe que ce métal était très abondant dans les milieux calaminaires, et qu'il constituait donc la pression de sélection évidente responsable des différences entre populations M et NM observées. En conditions contrôlées, il est vrai que les réponses, que ce soit d'*A. halleri* ou de *N. caerulescens*, sont le plus souvent cohérentes et répétables, en tout cas en termes de tolérance au Zn. En revanche, l'hyperaccumulation du Zn ne donne pas toujours des patrons très clairs. Chez *N. caerulescens*, les populations M calaminaires montrent des niveaux d'hyperaccumulation du Zn toujours plus faibles en moyenne, et assez peu variables par rapport aux populations NM (ou même serpentiniques)

(Gonneau *et al*, 2014). Chez *A. halleri*, ce patron général reste vrai en moyenne (populations M qui accumulent moins que les populations NM), mais il existe une importante variance inter- et intra-population dans les deux types de populations (voir Figure 8). Malgré cette faible différenciation en conditions contrôlées, je garde à l'esprit que les niveaux d'hyperaccumulation observés dépendent de l'environnement dans lequel ils sont mesurés. Ainsi, avant d'avoir mesuré l'hyperaccumulation du Zn dans plusieurs concentrations en Zn différentes et d'avoir constaté à chaque fois une faible différenciation M-NM, je ne conclurais pas qu'il n'existe aucune adaptation locale relative à l'hyperaccumulation du Zn, et que ce trait n'a pas évolué sous l'effet de la concentration en Zn du sol.

Parmi les approches possibles pour détecter un effet sélectif sur un trait donné, l'approche indirecte consiste à rechercher en conditions naturelles l'existence d'une corrélation entre une variable phénotypique (le trait censé être sous sélection) et des variables environnementales (les potentielles pressions de sélection) (Linhart and Grant, 1996; Merilä *et al*, 2001). Bien que cette approche, basée sur de simples corrélations, soit loin d'être une démonstration définitive, elle présente le mérite d'explorer plusieurs variables environnementales en même temps dont l'influence est évaluée sur plusieurs générations de sélection au travers des individus mesurés en population naturelle. C'est dans cet objectif que nous avons choisi de conduire une étude, non en conditions contrôlées dans un substrat additionné de Zn, mais directement sur le terrain, dans les Alpes italiennes (Decombeix *et al*, en préparation). L'objectif était de rechercher des paramètres abiotiques[7] susceptibles de prédire les niveaux d'hyperaccumulation du Zn et du Cd chez *A. halleri*, en tant qu'hyperaccumulatrice de ces deux métaux, ces niveaux d'hyperaccumulation étant mesurés directement sur des plantes du terrain.

Plus exactement, nous avons échantillonné 21 populations, dont 9 populations M de la vallée 1 (M), 6 populations NM de la vallée 1 (NM1), et 6 populations NM de la vallée 2 (NM2). Nous nous sommes intéressés à un ensemble de paramètres abiotiques : des paramètres pédologiques (granulométrie, pH, concentrations biodisponibles en Zn, Cd, Pb, Ca, Cu, Fe, K, Mg, Na, P), topographiques (pente, altitude, latitude, orientation) et climatiques (humidité absolue). Certains paramètres topographiques (latitude, pente, orientation) n'ont pas été étudiés en tant que tels, mais ont été intégrés dans un calcul d'ensoleillement maximal ($kWh.m^{-2}$). Les paramètres pédochimiques (pH, concentrations en éléments) ont été évalués dans cinq points de prélèvements par population, au niveau desquels un individu d'*A. halleri* a également été récolté et analysé pour la concentration foliaire en Zn et en Cd. En plus des concentrations foliaires, nous avons calculé les facteurs de bioconcentration, qui correspondent à la concentration en un métal dans les feuilles d'un individu, divisée par la concentration en métal dans le sol rhizosphérique correspondant (Lovy *et al*, 2013). Avant tout, le facteur de bioconcentration est une mesure qui reflète la réelle capacité d'absorption et de translocation des métaux d'un individu, et se rapproche de la capacité obtenue en conditions contrôlées par rapport à la simple concentration foliaire en métal.

Les résultats montrent tout d'abord que malgré des concentrations foliaires en Zn et Cd plus élevées, les individus des populations M présentent des facteurs de bioconcentration plus faibles que dans les populations NM (Figure 18A, 18B, 18C, 18D). Ceci confirme par des données de terrain la plus grande capacité des individus issus des populations NM à absorber le Zn et le Cd biodisponibles et à stocker ces métaux dans les feuilles. Pour les Alpes italiennes, cette tendance n'avait été observée qu'en conditions contrôlées, pour le Cd, et seulement pour I28 et I16 (Meyer *et al*, 2015).

[7] Bien entendu, la variation naturelle peut tout à fait dépendre de paramètres biotiques (compétition intra et inter-spécifique, présence d'herbivores…), mais nous avons choisi de commencer par l'étude des paramètres abiotiques.

Ensuite, les concentrations en macro- et oligo-éléments du sol sont variables et diffèrent parfois entre les écotypes M ou NM (Figure 19B, 19D, 19E, 19F et 19H), parfois même entre les groupes de populations NM1 et NM2 (Figure 19A, 19C, 19E et 19G). Les autres paramètres environnementaux sont beaucoup moins variables (d'après les valeurs des coefficients de variation) et parfois ne diffèrent pas significativement entre les groupes de populations (Figure 20B, 20C, 20D, 20E, 20G). Seules les valeurs de pH et d'humidité absolue présentent des différences significatives entre groupes de populations en particulier entre NM1 et NM2 (Figure 20A et 20F). En utilisant ces paramètres environnementaux comme variables explicatives dans des régressions de type « Partial Least Square » (PLS), il est possible d'estimer lesquels seront de bons prédicteurs des concentrations foliaires en Zn ou en Cd et des facteurs de bioconcentration du Zn et du Cd.

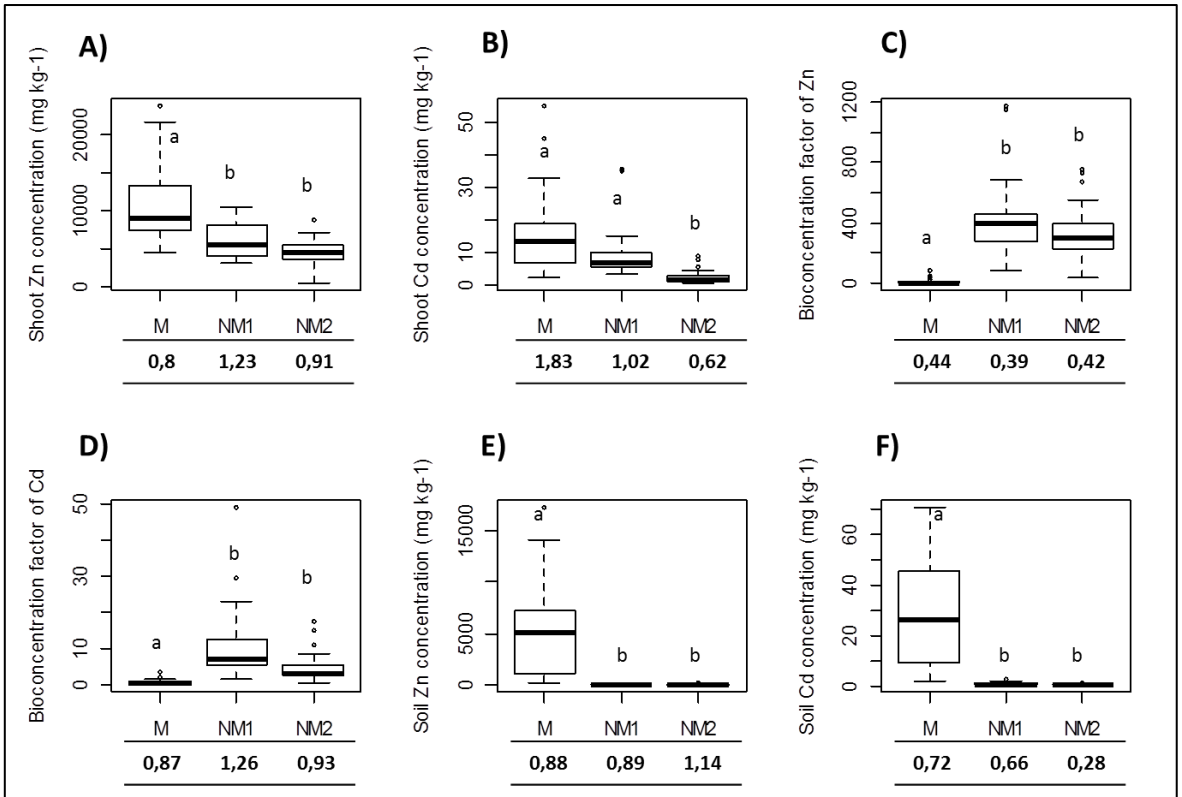

Figure 18 : Concentrations en Zn et en Cd dans les feuilles des plantes prélevées sur le terrain (A et B), facteurs de bioconcentrations dans les feuilles de ces mêmes plantes (C et D), ainsi que les concentrations biodisponibles dans les sols (E et F) auxquelles se rapportent les facteurs de bioconcentration. M=populations métallicoles; NM1=populations non-métallicoles de la vallée 1; NM2=populations non-métallicoles de la vallée 2. Les lettres représentent les différences significatives au seuil 5% obtenues d'après des tests non-paramétriques de Nemenyi. Les tableaux insérés sous les graphes présentent les coefficients de variation par groupe de populations.

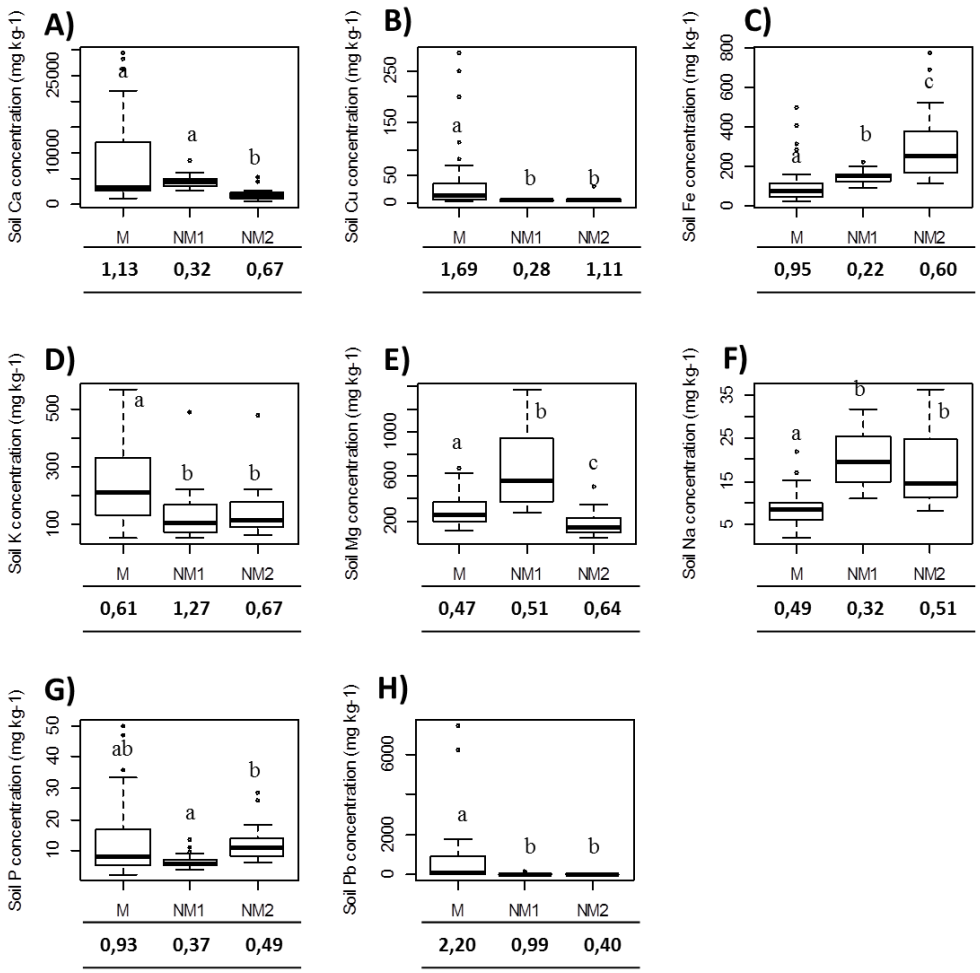

Figure 19 : Concentrations en macro-éléments (A, D, E, F, G) et oligo-éléments (B, C, H) autres que Zn et Cd dans les échantillons de sol rhizosphérique. M=populations métallicoles; NM1=populations non-métallicoles de la vallée 1; NM2=populations non-métallicoles de la vallée 2. Les lettres représentent les différences significatives au seuil 5% obtenues d'après des tests non-paramétriques de Nemenyi. Les tableaux insérés sous les graphes présentent les coefficients de variation par groupe de populations.

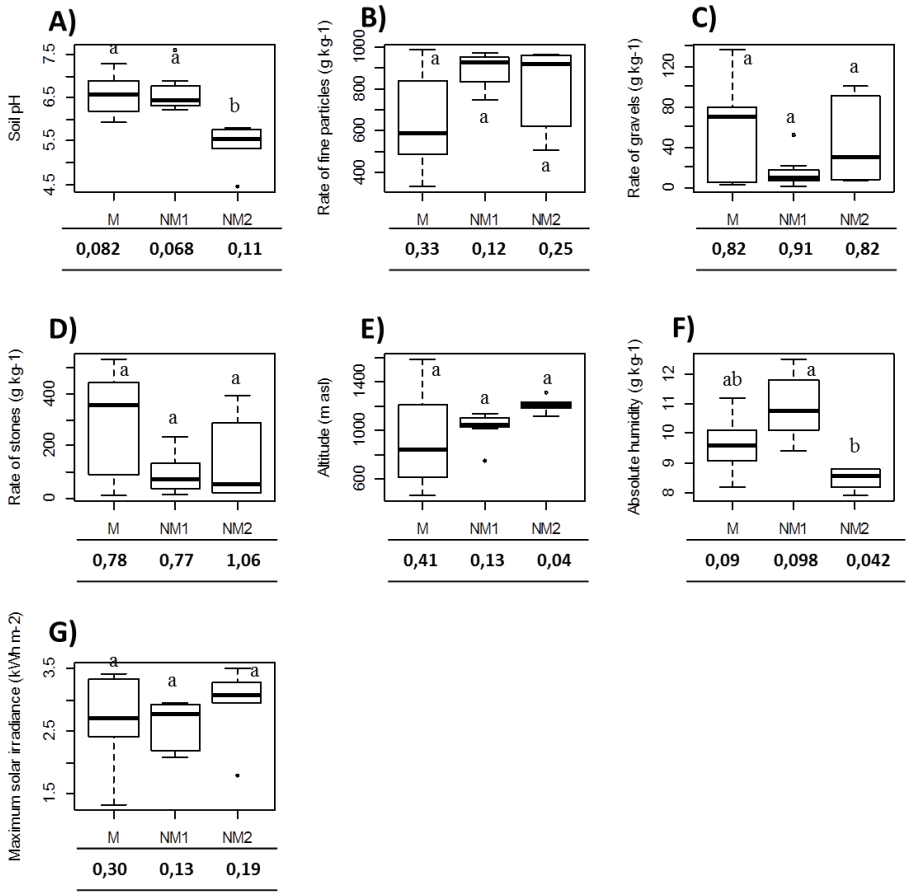

Figure 20 : Paramètres pédologiques (A, B, C, D), topographiques (E) et climatiques (F, G) dans les populations italiennes échantillonnées. M=populations métallicoles; NM1=populations non-métallicoles de la vallée 1; NM2=populations non-métallicoles de la vallée 2. Les lettres représentent les différences significatives au seuil 5% obtenues d'après des tests non-paramétriques de Nemenyi. Les tableaux insérés sous les graphes présentent les coefficients de variation (variance rapportée à la moyenne) par groupe de populations.

Les résultats de la régression PLS montrent que les coefficients de régression dépendent du métal (Zn ou Cd) et du trait (concentration foliaire ou facteur de bioconcentration) (Tableau 5). Notamment, la capacité de prédiction par la régression PLS est probablement très mauvaise pour les traits liés au Cd dans les populations NM puisque le nombre de composantes retenues égal à 0. Ceci suggère qu'en environnement non métallifère, l'accumulation du Cd est corrélée à d'autres variables environnementales que celles considérées ici, ou bien évolue de manière totalement neutre. Ensuite, tous les paramètres environnementaux interviennent tous au moins une fois comme variables explicatives significatives, mais très rarement pour les trois groupes de populations en même temps (Tableau 5, cases jaunes), un peu plus fréquemment pour distinguer les populations M des populations NM (Tableau 5, cases oranges) ou les deux vallées (Tableau 5, cases vertes). Même quand les coefficients de régression sont élevés pour deux ou trois groupes de population en même temps, il peut se produire des inversions de signe témoignant de processus assez complexes.

Tous les résultats montrant une distinction entre les deux vallées (M-NM1 *vs* NM2), soit par inversion de signe, soit par le passage d'un coefficient significatif à un coefficient non significatif (les cases vertes du Tableau 5) pourraient être attribués en partie à l'intensité des flux de gènes. En effet, entre les populations M et NM1 de la vallée 1, les flux de gènes sont importants, tandis qu'ils sont réduits entre les populations de la vallée 1 et celles de la vallée 2 (Decombeix, 2011). L'influence de la structure génétique neutre sur des résultats d'analyses PLS entre variables phénologiques et variables écologiques a été démontrée chez *A. thaliana* (Brachi *et al*, 2013). Néanmoins, même si les échanges de gènes peuvent masquer les effets de la sélection entre les populations M et NM1, et éventuellement effacer les traces d'adaptation locale dans la vallée 1, il reste que les populations NM2 peuvent témoigner des processus qui opèrent spécifiquement en environnement non pollué.

Tableau 5 : Résultats de la régression PLS sur les données centrées réduites. Les 4 variables à expliquer sont [Zn] (concentration foliaire en zinc), FB_Zn (facteur de bioconcentrationd u zinc), [Cd] (concentration foliaire en cadmium),et FB_Cd (facteur de bioconcentration en Cd), en fonction du groupe de populations (M, NM1, NM2). Les R^2 des modèles (en %) pour chaque variable à expliquer (Y) et pour l'ensemble des variables explicatives (X) sont cumulés sur l'ensemble des composantes retenues. Le nombre de composantes retenues (Nb Comp.) a été calculé par validation croisée par la méthode "leave-one-out". En rouge : les coefficients de régression correspondant aux variables explicatives les plus fortement impliquées à la fois dans la régression et dans la projection (Variable Importance in Projection > 0.8). En souligné : les coefficients de régression correspondant aux autres variables explicatives fortement impliquées dans la régression. Les cases en jaune signalent les cas où la variable explicative affecte les 3 groupes de populations. Les cases en orange signalent les cas où les variables explicatives affectent différemment les populations M et NM (NM1+NM2). Les cases en vert signalent les cas où les variables explicatives affectent différemment la vallée 1 (M+NM1) et la vallée 2 (NM2).

	[Zn]			FB_Zn			[Cd]			FB_Cd		
	M	**NM1**	**NM2**	**M**	**NM1**	**NM2**	**M**	**NM1**	**NM2**	**M**	**NM1**	**NM2**
Nb Comp.	**5**	**1**	**4**	**1**	**3**	**13**	**1**	**0**	**0**	**3**	**0**	**0**
R^2Ycum	**78,4**	**59,8**	**91,4**	**37,6**	**88,4**	**99,1**	**42,9**			**74,9**		
R^2Xcum	**76,2**	**27,2**	**69**	**32**	**77,4**	**99,9**	**31,9**			**58,4**		
Intercept	0	0	0	0	0	0	0			0		
Ca	-0.104	-0.042	0.153	-0.054	-0.041	-0.352	0.027			-0.042		
Cd	0.329	0.136	-0.484	-0.075	-0.135	-0.987	0.098			-0.225		
Cu	-0.195	0.094	-0.029	-0.050	-0.255	0.598	0.097			0.107		
Fe	-0.044	0.003	-0.008	0.079	-0.141	0.595	-0.015			0.023		
K	-0.156	-0.092	0.089	0.079	-0.096	-0.393	-0.113			0.017		
Mg	-0.222	-0.078	-0.064	-0.042	-0.080	-0.197	-0.029			-0.171		
Na	0.214	0.101	0.250	0.009	-0.041	0.261	0.027			-0.002		
P	-0.122	-0.042	-0.028	0.000	-0.199	-0.039	0.036			0.084		
Pb	0.271	-0.068	0.133	-0.033	0.161	-0.363	0.078			0.492		
Zn	0.388	0.100	0.484	-0.114	-0.191	0.117	0.080			-0.096		
pH	-0.587	-0.061	-0.089	-0.099	0.006	0.350	0.059			-0.162		
terres fines	-0.135	0.026	-0.267	0.100	0.022	1.361	-0.113			-0.109		
graviers	0.202	0.000	0.211	-0.060	-0.029	0.999	0.085			0.054		
cailloux	0.112	-0.032	0.280	-0.106	-0.019	-1.926	0.115			0.117		
humidité	0.102	-0.171	-0.148	0.005	0.152	1.583	0.016			-0.117		
altitude	0.092	-0.153	-0.152	-0.027	0.080	-1.430	-0.032			-0.260		
ensoleillement	0.398	-0.068	0.312	-0.021	0.066	1.319	-0.040			0.185		

Compte tenu de ces observations générales, et de la complexité des processus en jeu, je soulignerais essentiellement trois points :

1. La concentration en Zn du sol semble être un paramètre influençant positivement et uniformément la concentration foliaire en Zn (ce qui est assez logique), mais ce paramètre influence positivement le facteur de bioconcentration en Zn dans les populations de la vallée 1 (M et NM1) et négativement dans celles de la vallée 2 (NM2). Il est intéressant de noter que ceci reste cohérent avec les hypothèses qui prétendent que l'hyperaccumulation du Zn aurait d'abord été sélectionnée en milieu non pollué, puis nécessairement plus ou moins contre-sélectionnée en milieu calaminaire afin d'éviter l'intoxication.
2. Un autre résultat remarquable est l'influence négative de la concentration en Cd du sol sur le facteur de bioconcentration en Zn, et ce de manière significative uniquement en milieu non pollué (NM1 et NM2). La compétition entre ces deux métaux, et la baisse associée des capacités d'hyperaccumulation du Zn en présence de Cd a déjà été observée plusieurs fois dans la littérature (Assunção *et al*, 2003a; Escarré *et al*, 2013; Roosens *et al*, 2003), et se confirme ici en particulier lorsque les quantités en métaux sont limitées.
3. Enfin, je soulignerais que les concentrations du sol en Cu, Fe et Pb sont significativement corrélées aux facteurs de bioconcentration en Zn uniquement dans les populations NM, mais avec une inversion de signe entre NM1 et NM2, ce qui ne permet pas de proposer une interprétation générale pour les populations NM. Seule la variable « humidité absolue » semble distinguer l'ensemble des populations M des populations NM, bien que les populations NM1 et NM2 soient significativement différentes de ce point de vue (Figure 20F). Evidemment, l'influence de l'humidité absolue peut provenir de sa corrélation à une variable causale que nous n'aurions pas prise en compte. Par exemple, le niveau d'humidité pourrait influencer les effectifs d'herbivores présentes en milieu non pollué, alors qu'en milieu pollué, ces effectifs sont de toute façon réduits (Dechamps *et al*, 2008).

Je garde tout à fait à l'esprit qu'il est délicat d'interpréter l'effet de ces variables environnementales sur les concentrations foliaires ou les facteurs de bioconcentration dans la mesure où (1) les variables portant sur les éléments du sol sont mieux représentées que les autres variables, car mesurées en 5 points différents par population au lieu d'un seul, (2) ces analyses ne mettent en évidence qu'un rôle potentiellement très indirect de ces variables, et (3) nous n'avons pas évalué toutes les variables abiotiques permettant de différencier les groupes de populations, et nous n'avons pas considéré de variables biotiques qui sont probablement aussi très influentes. Néanmoins, d'après cette étude, le rôle de la concentration en Zn dans le sol comme possible pression de sélection ne peut pas être exclu.

b. Approche directe : evolution experimentale sous l'effet de la pression zinc

Une autre manière de démontrer le rôle potentiel du Zn comme pression de sélection est de faire évoluer artificiellement une population « naïve » vis-à-vis de la pollution par les métaux en présence de Zn, et d'observer les variations des niveaux de tolérance au Zn et d'hyperaccumulation du Zn de génération en génération. Cette expérience, en cours actuellement, est un élément majeur de notre projet ANR ELOCANTH, et constitue le sujet de thèse de Julien Nowak. Le dispositif est basé sur quatre populations expérimentales de *Noccaea caerulescens* (Figure 21). Les populations choisies proviennent de la Belgique (populations M de La Calamine et de Prayon) et du Luxembourg (populations NM de Lellingen, Winseler et Wilwerwiltz), et sont largement citées dans la littérature (par exemple : Assunção *et al*, 2003a; Assunção *et al*, 2006; Assunção *et al*, 2003c; Dechamps *et al*,

2011; Dechamps *et al*, 2007; Dechamps *et al*, 2008). Nous avons pris soin de mélanger des individus de plusieurs provenances M ou NM selon les cas afin d'assurer un bon niveau initial de diversité génétique. Le dispositif est conçu pour limiter les flux de gènes entre les populations expérimentales par le biais de « cages » déposées tour à tour sur l'ensemble des populations expérimentales sauf une pendant toute la période de pollinisation. Il est également conçu pour estimer les effets de la dérive génétique par l'installation d'une population expérimentale « témoin », et sachant que l'effectif par population expérimentale est fini, de 98 individus répartis en deux mésocosmes de 49 individus (Figure 21). Grâce à l'estimation de la valeur sélective relative de chaque individu (voir partie 1.b de ce Chapitre), nous en déduisons sa contribution, sous forme d'un nombre de descendants (plus exactement : une proportion d'individus par rapport à une taille de population finie), à la génération suivante. La thèse de Julien Nowak a pour objectif d'étudier la réponse des individus des générations G0, G1 et G2 en conditions contrôlées, et d'appliquer les méthodes de génétique quantitative permettant l'estimation formelle de l'intensité et de la direction de la sélection (Lande and Arnold, 1983).

Pour la conception de cette expérience, nous avons longuement réfléchi à la forme et l'intensité de la pression de sélection à appliquer. Concernant la forme de pollution, pour un meilleur mimétisme avec les conditions naturelles nous aurions pu prélever du sol contaminé sur le terrain et réaliser un mélange avec du terreau pour atteindre le niveau de pollution voulu (voir : Escarré *et al*, 2000; Frérot *et al*, 2005). L'inconvénient de cette méthode est qu'elle ne permet pas de produire un véritable sol témoin, non pollué, égal en tous points au sol pollué hormis les concentrations élevées en métaux. Les sols calaminaires étant contaminées en Zn, Cd et Pb, elle ne permet pas non plus d'isoler l'influence d'un seul métal. Par conséquent, nous avons choisi d'ajouter un sel de Zn à un sol à base de terreau. Nous revenons ensuite toujours aux mêmes discussions : quel sel de Zn ($ZnSO_4$ ou ZnO dans notre cas), et en quelle concentration ? Ces deux choix sont liés puisque la forme $ZnSO_4$ présente une forte solubilité, et sera donc toxique à des concentrations plus faibles que la forme ZnO. Comme nous souhaitions obtenir un résultat en un nombre réduit de générations, nous nous sommes dirigés vers la forme $ZnSO_4$, la plus soluble. Ensuite, la concentration en Zn appliquée devait répondre à plusieurs critères : (1) ne pas nous limiter en nombre total de graines viables afin d'assurer la production des générations successives et (2) générer en même temps des différences entre individus, au moins basées sur la production de graines, et éventuellement sur la survie.

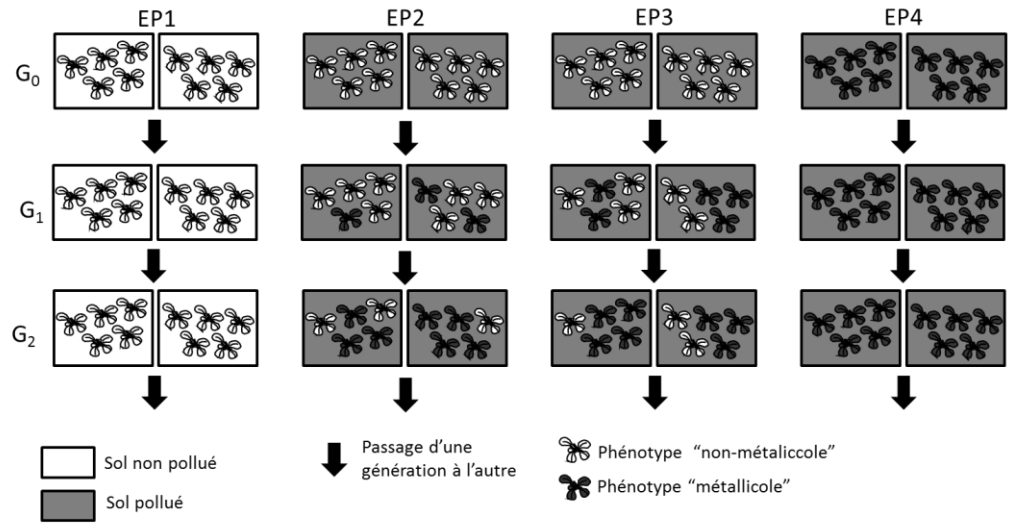

Figure 21 : Principe du dispositif d'évolution expérimentale. Ce dispositif a pour but de démontrer l'effet du zinc sur l'évolution d'une population initialement non-métallicole vers une population présentant de plus en plus de génotypes de type "métallicole" au cours des générations successives. Les génotypes sélectionnés sous l'effet du Zn devraient présenter des niveaux de tolérance au Zn plus élevés que les génotypes de départ, et des niveaux d'hyperaccumulation du Zn plus bas. La population expérimentale EP1 sert à estimer les potentiels effets de la dérive génétique. Les populations expérimentales EP2 et EP3 sont les populations-tests. La population expérimentale EP4 sert à vérifier l'absence d'une contre-sélection des génotypes métallicoles dans nos conditions expérimentales. Ce dispositif est conçu pour limiter les flux de gènes entre populations expérimentales. EP : experimental population.

Afin de prendre une décision éclairée, nous sommes passés par une étape de test de quatre concentrations : 0, 500, 1000 et 2000 $mg.kg^{-1}$ de Zn sous la forme ($ZnSO_4$, $7H_2O$). Ces quatre concentrations ont été testées en mésocosmes sur un ensemble de 49 génotypes NM provenant des trois populations du Luxembourg citées précédemment. Nous avons mesuré les traits reproducteurs nous donnant accès à la valeur sélective des individus (voir partie 1.b de ce Chapitre). A l'aide de cette mesure, nous avons ensuite constitué la génération suivante (donnant lieu à une population dérivée) pour chaque niveau de pollution, et nous avons procédé aux tests de tolérance et d'hyperaccumulation en conditions contrôlées sur un sol additionné de 500 $mg.kg^{-1}$ de Zn, permettant la comparaison entre la population ancestrale (plus exactement : une population constituée des individus frères des individus ancestraux) et les 4 populations dérivées.

La tolérance au Zn, mesurée par différents traits végétatifs et physiologiques (comme le taux de chlorophylle, évalué par un chlorophyllomètre portable) a donné des résultats intéressants mais faiblement significatifs. C'est pourquoi nous mettons en place actuellement de nouveaux projets portant sur l'estimation d'un certain nombre de biomarqueurs (activité de la Rubisco ou des enzymes de réponse au stress oxydatif, peroxydation des lipides…) et sur les tests de génotoxicité (*coll.* Bertrand Pourrut de l'Institut Supérieur d'Agriculture de Lille). En revanche, l'hyperaccumulation du Zn semble avoir suivi une tendance intéressante, et plutôt attendue (Figure 22). En effet, à la suite d'une année de sélection à 1000 et 2000 $mg.kg^{-1}$, les niveaux d'hyperaccumulation du Zn ont diminué, contrairement aux niveaux atteints à la suite d'une année de sélection à seulement 0 et 500 $mg.kg^{-1}$. Ce résultat est cohérent avec les niveaux d'hyperaccumulation du Zn moindre chez les populations M par rapport aux populations NM, et semble indiquer que la concentration en Zn du sol peut constituer une réelle pression de sélection.

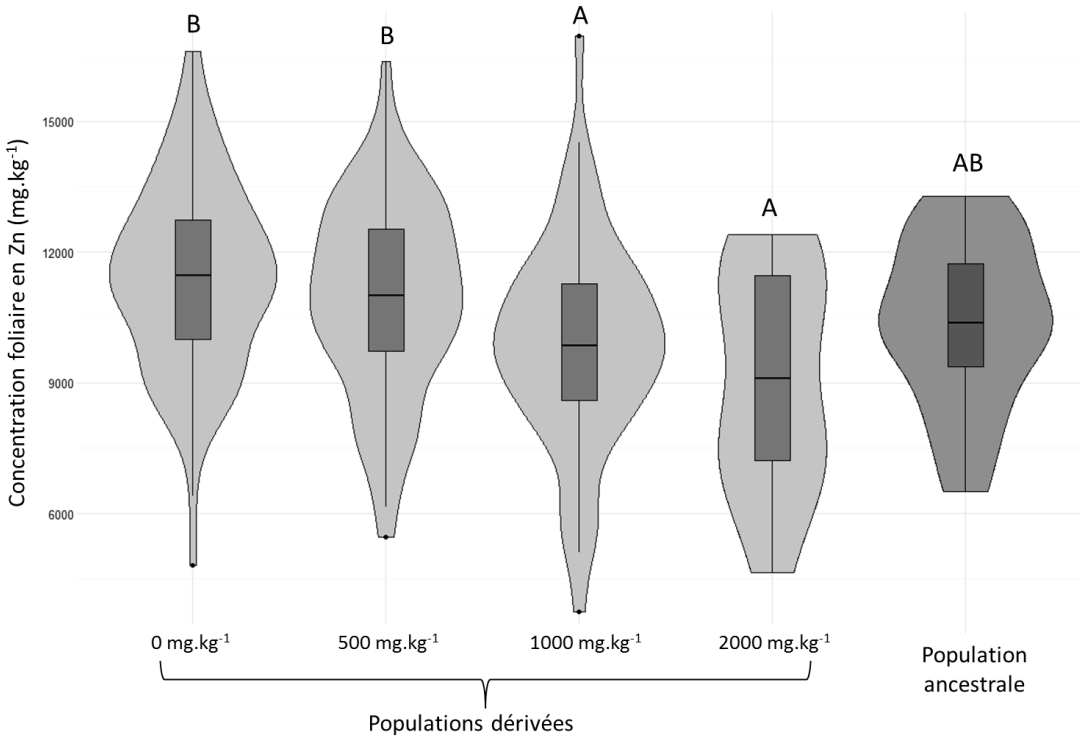

Figure 22 : Comparaison des concentrations foliaires en Zn atteintes dans la population ancestrale et les populations dérivées après un an de sélection sur des niveaux croissants de concentration en Zn. Tous les individus ont été cultivés en conditions contrôlées sur un sol additionné de 500 $mg.kg^{-1}$ de Zn sous la forme $ZnSO_4$, $7H_20$. Les lettres représentent les différences significatives au seuil 5% (test de comparaison non-paramétrique). (Figure fournie par Julien Nowak).

A la suite de cette expérience, nous avions donc bon espoir que quelques années de sélection par le Zn influenceront de manière directionnelle les niveaux de tolérance au Zn et d'hyperaccumulation du Zn chez *N. caerulescens*. Cette expérience nous a permis aussi de définir la concentration du dispositif final (Figure 21). A première vue, nous aurions pu choisir 1000 $mg.kg^{-1}$, mais à cette concentration nous avons constaté que la majorité des individus de toute une population (Winseler) ne produisaient aucune graine. Par conséquent, notre choix s'est porté sur la concentration 750 $mg.kg^{-1}$. Depuis, nous cultivons nos générations successives sur un sol additionné de 750 $mg.kg^{-1}$ de Zn.

CONCLUSION GENERALE

J'ai encore peu avancé sur la question des bases génétiques et épigénétiques de l'adaptation locale proprement dite. En effet, avant d'initier les travaux portant clairement sur ce sujet (au cœur du projet ELOCANTH), je me suis d'abord intégrée aux thématiques déjà en cours dans le laboratoire, en employant en particulier des croisements inter-spécifiques entre *A. halleri* et *A. lyrata petraea*. Le niveau inter-spécifique est rassurant car il garantit *a priori* des phénotypes contrastés chez les parents et une bonne ségrégation des traits mesurés. D'ailleurs, ces croisements ont conduit à des résultats probants qui ont permis d'aller assez loin dans la description physiologique et génétique de la tolérance et de l'hyperaccumulation (je pense notamment aux gènes *HMA4*). Néanmoins, en restant à ce niveau, les mécanismes mis en évidence relèvent plutôt de la macro-évolution, et des événements liés à l'apparition de l'espèce *A. halleri*. C'est une question intéressante en soi, mais je pensais qu'elle n'était pas nécessairement en lien avec les événements micro-évolutifs contemporains de la pollution des sols par les activités humaines, ou avec la capacité de l'espèce à s'adapter rapidement. L'expérience a montré que je me trompais puisque l'étude comparée des croisements *A. halleri* accession M x *A. lyrata petraea* et *A. halleri* accession NM x *A. lyrata petraea* a mis en évidence des régions QTL non communes, potentiellement impliquées dans l'adaptation locale (Zntol-2 et Zntol-3).

Je suis maintenant impliquée dans la production et l'étude des croisements intra-spécifiques, afin de détecter plus directement les régions QTL responsables de l'adaptation locale. De manière générale, le QTL Mapping comporte une contrainte qui est celle de recommencer la carte génétique à chaque descendance. Nous avons la possibilité de contourner assez facilement cette difficulté grâce aux techniques de séquençage « nouvelle génération ». En revanche, les nouvelles technologies ne peuvent pas nous aider à résoudre les questions relatives au phénotypage. Pour la tolérance, chaque composante de la valeur sélective peut apparaître comme une mesure valable. Néanmoins, nous savons que la pertinence de ces variables dépend de l'espèce, notamment les traits reproducteurs apportent l'essentiel des informations chez *N. caerulescens* contrairement à *A. halleri*. Nous savons aussi que les traits physiologiques, comme le rendement du photosystème II que nous avons coutume de mesurer, sont le plus souvent sensibles et représentatifs du niveau de tolérance de l'individu. Cette constatation nous a encouragés à explorer la piste des biomarqueurs et de la génotoxicité. Pour l'hyperaccumulation, il s'agit notamment de savoir à quel moment il est opportun de prélever les feuilles pour le dosage des métaux, ou s'il est possible d'en prélever seulement certaines, si oui lesquelles ? Compte tenu des nombreuses interactions QTL x environnement susceptibles d'opérer, la question des conditions de culture, et en particulier de la concentration en Zn à appliquer, se pose également. Autant de points auxquels il est nécessaire de réfléchir à chaque fois, et qui entraîne toujours une certaine prise de risque. Ceci est encore plus vrai dans le cas de croisements intra-spécifiques pour lesquels beaucoup de traits pourraient soit ne pas ségréger, soit montrer une ségrégation uniforme. Nous avons déjà rencontré ce cas avec l'étude de l'hyperaccumulation du Zn dans le croisement intra-spécifique *d'A. halleri*.

Au niveau intra-spécifique, je sais déjà que s'il existe de l'adaptation locale, elle ne porte probablement pas que sur la tolérance au Zn ou l'hyperaccumulation du Zn, car les individus choisis proviennent de populations qui diffèrent entre elles par de nombreuses variables écologiques. Cette réflexion est également vraie pour les profils épigénétiques, surtout si les différences entre populations M et NM s'avèrent indépendantes de la concentration en Zn appliquée en conditions

contrôlées. Néanmoins, à l'issue de notre expérience d'évolution nous arriverons peut-être enfin à la conclusion définitive que le Zn présent dans le sol constitue effectivement une pression de sélection, même s'il s'agit d'une pression de sélection parmi d'autres. Nous savons aussi que notre démonstration s'appuiera sur une seule concentration et une constante de biodisponibilité associée, or dans les conditions naturelles, les concentrations en métaux dans les milieux pollués sont très hétérogènes spatialement, et peut-être aussi temporellement. Cela tient à plusieurs facteurs pédologiques dont les conséquences sur la biodisponibilité des métaux en un lieu donné et un moment donné sont difficiles à prédire. Il n'est pas impossible qu'une telle hétérogénéité conduise à l'évolution de traits liés à la plasticité phénotypique, et que le « talent » des individus métallicoles provienne davantage de leur capacité à supporter des conditions environnementales très changeantes (une forme de canalisation). Je ne peux pas tester cette hypothèse pour le moment car elle nécessite de se concentrer sur un seul type de matériel, qu'il serait possible de cultiver sur plusieurs niveaux de concentrations en Zn. Ce matériel, je l'aurai probablement dans quelques années grâce aux lignées de *N. caerulescens* que nous produisons actuellement.

BIBLIOGRAPHIE

Anderson JT, Willis JH, Mitchell-Olds T (2011). Evolutionary genetics of plant adaptation. *Trends in Genetics* **27**(7)**:** 258-266.

Antonovics J, Bradshaw AD, Turner RG (1971). Heavy metal tolerance in plants. *Advances in Ecological Research* **7:** 1-85.

Assunção AGL, Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO (2003a). Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* **159:** 411-419.

Assunção AGL, Pieper B, Vromans J, Lindhout P, Aarts MGM, Schat H (2006). Construction of a genetic linkage map of *Thlaspi caerulescens* and quantitative trait loci analysis of zinc accumulation. *New Phytologist* **170:** 21-32.

Assunção AGL, Schat H, Aarts MGM (2003b). *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* **159:** 351-360.

Assunção AGL, Ten Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO (2003c). A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **159:** 383-390.

Baker A (1987). Metal Tolerance. *New Phytologist* **106:** 93 - 111.

Baker AJ (1981). Accumulators and excluders strategies in the response of plants to heavy metals. *Journal of Plant Nutrition* **8:** 643-654.

Baythavong BS, Stanton ML (2010). Characterizing selection on phenotypic plasticity in response to natural environmental heterogeneity. *Evolution* **64**(10)**:** 2904-2920.

Becher M, Talke IN, Krall L, Krämer U (2004). Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *The Plant Journal* **37:** 251-268.

Bernard C, Roosens N, Czernic P, Lebrun M, Verbruggen N (2004). A novel CPx-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. *FEBS Letters* **569**(1-3)**:** 140-148.

Bert V, Bonnin I, Saumitou-Laprade P, de Laguérie P, Petit D (2002). Do *Arabidopsis halleri* from nonmetalicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **155:** 47-57.

Bierne N, Roze D, Welch JJ (2013). Pervasive selection or is it...? Why are Fst outliers sometimes so frequent? *Molecular Ecology* **22:** 2061-2064.

Bishop J (1972). An experimental study of the clines of industrial melanism in Biston betularia (L.) (Lepidoptera) between urban Liverpool and rural North Wales. *J Anim Ecol* **4:** 209-243.

Bonin A, Paris M, Frérot H, Bianco E, Tetreau G, Desprès L (2015). The genetic architecture of a complex trait: Resistance to multiple toxins produced by *Bacillus thuringiensis israelensis* in the dengue and yellow fever vector, the mosquito *Aedes aegypti*. *Infection, Genetics and Evolution* **35:** 204-213.

Borovik AJ (1990). Characteristics of metals in biological systems. In: Shaw AJ (ed) *Heavy Metal Tolerance in Plants: Evolutionary Aspects*. CRC Press: Boca Raton, Floride, USA, pp 3-5.

Bossdorf O, Richards CL, Pigliucci M (2008). Epigenetics for ecologists. *Ecology Letters* **11:** 106-115.

Boyd RS (2007). The defense hypothesis of elemental hyperaccumulation: status, challenges and new directions. *Plant and Soil* **293:** 153-176.

Boyd RS, Martens SN (1992). The *raison d'être* for metal hyperaccumulation by plants. In: Baker AJM, Proctor J and Reeves RD (eds) *The Ecology of Ultramafic (Serpentine) Soils*. Intercept: Andover, UK.

Boyko A, Kovalchuk I (2011). Genome instability and epigenetic modification - heritable responses to environmental stress? *Current Opinion in Plant Biology* **14:** 260-266.

Brachi B, Aimé C, Glorieux C, Cuguen J, Roux F (2012). Adaptive Value of Phenological Traits in Stressful Environments: Predictions Based on Seed Production and Laboratory Natural Selection. *PLoS ONE* **7**(3)**:** e32069.

Brachi B, Faure N, Horton M, Flahauw E, Vazquez A, Nordborg M *et al* (2010). Linkage and Association Mapping of *Arabidopsis thaliana* Flowering Time in Nature. *PLoS Genetics* **6**(5)**:** e1000940.

Brachi B, Villoutreix R, Faure N, Hautekèete N, Piquot Y, Pauwels M *et al* (2013). Investigation of the geographical scale of adaptive phenological variation and its underlying genetics in *Arabidopsis thaliana*. *Molecular Ecology* **22**(16)**:** 4222-4240.

Bradl HB (2005). *Heavy metals in the environment: origin, interaction and remediation*, Vol 6. Elsevier Academic Press: Neubrucke, Germany.

Bratteler M, Lexer C, Widmer A (2006). Genetic architecture of traits associated with serpentine adaptation of *Silene vulgaris*. *Journal of Evolutionary Biology* **19:** 1149-1156.

Carlson SM, Cunningham CJ, Westley PAH (2014). Evolutionary rescue in a changing world. *Trends in Ecology & Evolution* **29**(9)**:** 521-530.

Clemens S (2001). Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* **212:** 475-486.

Colautti RI, Lee C-R, Mitchell-Olds T (2012). Origin, fate and architecture of ecologically relevant genetic variation. *Current Opinion in Plant Biology* **15:** 199-204.

Courbot M, Willems G, Motte P, Arvidsson S, Roosens N, Saumitou-Laprade P *et al* (2007). A Major Quantitative Trait Locus for Cadmium Tolerance in *Arabidopsis halleri* Colocalizes with HMA4, a Gene Encoding a Heavy Metal ATPase1[OA]. *Plant Physiology* **144:** 1052-1065.

Craig GC (1977). A method of measuring heavy metal tolerance in grasses. *Trans Rhod Sci Assoc* **58:** 9-16.

de Bello F, lavorel S, Lavergne S, Albert CH, Boulangeat I, Mazel F *et al* (2013). Hierarchical effets of environmental filters on the functional structure of plant communities: a case study in the French Alps. *Ecogeography* **36:** 393-402.

Dechamps C, Elvinger N, Meerts P, Lefèbvre C, Escarré J, Colling G *et al* (2011). Life history traits of the pseudometallophyte *Thlaspi caerulescens* in natural populations from Northern Europe. *Plant Biology* **13**(Suppl.1)**:** 125-135.

Dechamps C, Lefèbvre C, Noret N, Meerts P (2007). Reaction norms of life history traits in response to zinc in *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites. *New Phytologist* **173**(1)**:** 191-198.

Dechamps C, Noret N, Mozek R, Escarré J, Lefèbvre C, Gruber W (2008). Cost of adaptation to a metalliferous environment for *Thlaspi caerulescens*: a field reciprocal transplantation approach. *New Phytologist* **177:** 167-177.

Decombeix I (2011). Etude de l'adaptation aux milieux calaminaires chez *Arabidopsis halleri* : approche écologique, génétique et phénotypique. Université de Lille - Sciences et Technologies, Lille.

Decombeix I, Pauwels M, Hautekeete N-C, Piquot Y, Frérot H. *In situ* natural variation of zinc and cadmium hyperaccumulation in *Arabidopsis halleri* is explained by potential abiotic selective pressures. (en préparation)

Deniau AX, Pieper B, Ten Bookum WM, Lindhout P, Aarts MGM, Schat H (2006). QTL analysis of cadmium and zinc accumulation int he heavy metal hyperaccumulator *Thlaspi caerulescens*. *Theoritical and Applied Genetics* **113:** 907-920.

Dräger DB, Desbrosses-Fonrouge A-G, Krach C, Chardonnens AN, Meyer RC, Saumitou-Laprade P *et al* (2004). Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high MTP1 transcript levels. *The Plant Journal* **39**(3)**:** 425-439.

Duffus JH (2002). "Heavy metals" - a meaningless term? (International Union of Pure and Applied Chemistry Technical Report). *Pure and Applied Chemistry* **74**(5)**:** 793-807.

Ehrenreich IM, Purugganan MD (2006). The molecular genetic basis of plant adaptation. *American Journal of Botany* **93**(7)**:** 953-962.

Escarré J, Lefèbvre C, Frérot H, Mahieu S, Noret N (2013). Metal concentration and metal mass of metallicolous, non metallicolous and serpentine *Noccaea caerulescens* populations, cultivated in different growth media. *Plant and Soil* **370:** 197-221.

Escarré J, Lefèbvre C, Gruber W, Leblanc M, Lepart J, Rivière Y *et al* (2000). Zinc and Cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implications for phytoremediation. *New Phytologist* **145:** 429-437.

Escarré J, Lefèbvre C, Raboyeau S, Dossantos A, Gruber W, Cleyet-Marel JC *et al* (2011). Heavy Metal Concentration Survey in Soils and Plants of the Les Malines Mining District (Southern France): Implications for Soil Restoration. *Water Air & Soil Pollution* **216:** 485–504.

Feil R, Fraga MF (2012). Epigenetics and the environment: emerging patterns and implications. *Nature Reviews Genetics* **13:** 97-109.

Frérot H, Faucon M-P, Willems G, Godé C, Courseaux A, Darracq A *et al* (2010). Genetic architecture of zinc hyperaccumulation in *Arabidopsis halleri*: the essential role of QTL x environment interactions. *New Phytologist* **187**(2)**:** 355–367.

Frérot H, Lefèbvre C, Petit C, Collin C, Dos Santos A, Escarré J (2005). Zinc tolerance and hyperaccumulation in F1 and F2 offspring from intra and interecotype crosses of *Thlaspi caerulescens*. *New Phytologist* **165**(1)**:** 111-119.

Frérot H, Petit C, Lefèbvre C, Gruber W, Collin C, Escarré J (2003). Zinc and cadmium accumulation in controlled crosses between metallicolous and nonmetallicolous populations of *Thlaspi caerulescens*. *New Phytologist* **157:** 643-648.

Friedland AJ (1990). Movement of metals through soils and ecosystems. In: Shaw eA (ed) *Heavy metal tolerance in plants: evolutionary aspects*. CRC Press: Boca Raton, Floride, USA.

Gardner KM, Latta RG (2007). Shared quantitative trait loci underlying the genetic correlation between continuous traits. *Molecular Ecology* **16:** 4195-4209.

Geyer C, Wagenius S, Shaw R (2007). Aster models for life history analysis. *Biometrika* **94:** 415-426.

Ghalambor CK, McKay JK, Carroll SP, Reznick DN (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology* **21:** 394-407.

Gonneau C, Genevois N, Frérot H, Sirguey C, Sterckeman T (2014). Variation of trace metal accumulation, major nutrient uptake and growth parameters and their correlations in 22 populations of *Noccaea caerulescens*. *Plant and Soil* **384:** 271-287.

Gould SJ, Vbra ES (1982). Exaptation - A missing term in the science of form. *Paleobiology* **8**(1)**:** 4-15.

Halimaa P, Lin Y-F, Ahonen V, Blande D, Clemens S, Gyenesei A *et al* (2014). Gene Expression Differences between *Noccaea caerulescens* Ecotypes Help to Identify Candidate Genes for Metal Phytoremediation. *Environmental Science & Technology* **48**(6)**:** 3344–3353.

Hall MC, Basten C, Willis JH (2006). Pleiotropic Quantitative Trait Loci contribute to Population Divergence in Traits Associated With Life-History Variation in *Mimulus guttatus*. *Genetics* **172:** 1829-1844.

Hall MC, Lowry DB, Willis JH (2010). Is local adaptation in *Mimulus guttatus* caused by trade-offs at individual loci? *Molecular Ecology* **19:** 2739-2753.

Hammond JP, Bowen HC, White PJ, Mills V, Pyke KA, Baker AJM *et al* (2006). A comparison of the *Thlaspi caerulescens* and *Thlaspi arvense* shoot transcriptomes. *New Phytologist* **170**(2)**:** 239-260.

Hancock AM, Brachi B, Faure N, Horton MW, Jarymowycz LB, Sperone FG *et al* (2011). Adaptation to Climate Across the *Arabidopsis thaliana* Genome. *Science* **334:** 83-86.

Hanikenne M, Kroymann J, Trampczynska A, Bernal MP, Motte P, Clemens S *et al* (2013). Hard Selective Sweep and Ectopic Gene Conversion in a Gene Cluster Affording Environmental Adaptation. *Plos Genetics* **9**(8)**:** e1003707.

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P *et al* (2008). Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. *Nature* **453:** 391-396.

Hodson ME (2004). Heavy metals - geochemical bogey men? *Environmental Pollution* **129:** 341-343.

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J *et al* (2004). P-Type ATPase Heavy Metal Transporters with Roles in Essential Zinc Homeostasis in Arabidopsis. *The Plant Cell* **16:** 1327-1339.

Jimenez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri I, Ronce O (2007). Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* **173**(1)**:** 199-215.

Johnson LJ, Tricker PJ (2010). Epigenomic plasticity within populations: its evolutionary significance and potential. *Heredity* **105:** 113-121.

Kawecki TJ (2000). The evolution of genetic canalization under fluctuating selection. *Evolution* **54**(1)**:** 1-12.

Kawecki TJ, Ebert D (2004). Conceptual issues in local adaptation. *Ecology Letters* **7:** 1225-1241.

Kostecka AA (2009). Adaptations of *Arabidopsis halleri* to habitats rich in heavy metals in Southern Poland. Université de Lille - Sciences et Technologies / Polish Academy of Sciences, Lille / Krakow.

Kozhevnikova AD, Seregin IV, Erlikh NT, Shevyreva TA, Andreev IM, Verweij R *et al* (2014). Histidine-mediated xylem loading of zinc is a species-wide character in *Noccaea caerulescens*. *New Phytologist* **203**(2)**:** 508-519.

Krämer U (2010). Metal hyperaccumulation in plants. *Annual Review of Plant Biology* **61:** 517-534.

Küpper H, Lombi E, Zhao FJ, McGrath SP (2000). Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta* **212**(1)**:** 75-84.

Lande R, Arnold SJ (1983). The Measurement of Selection on Correlated Characters. *Evolution* **37**(6)**:** 1210-1226.

Latta RG, Gardner KM (2009). Natural selection on pleiotropic quantitative trait loci affecting a life-history trade-off in *Avena barbata*. *Evolution* **63**(8)**:** 2153-2163.

Latta RG, Gardner KM, Staples DA (2010). Quantitative Trait Locus Mapping of Genes Under Selection Across Multiple Years and Sites in *Avena barbata*: Epistasis, Pleiotropy, and Genotype-by-environment Interactions. *Genetics* **185:** 375-385.

Lenormand T, Guillemaud T, Bourguet D, Raymond M (1998). Evaluating gene flow using selected markers : a case study. *Genetics* **149:** 1383-1392.

Lin Y-F, Aarts MGM (2012). The molecular mechanism of zinc and cadmium stress response in plants. *Cellular and Molecular Life Sciences* **69:** 3187-3206.

Linhart YB, Grant MC (1996). Evolutionary significance of local genetic differentiation in plants. *Annual Review of Ecology and Systematics* **27:** 237-277.

Lovy L, Latt D, Sterckeman T (2013). Cadmium uptake and partitioning in the hyperaccumulator *Noccaea caerulescens* exposed to constant Cd concentrations throughout complete growth cycles. *Plant and Soil* **362**(1-2)**:** 345-354.

Macnair MR (1983). The genetic control of copper tolerance in the yellow monkey flower, *Mimulus guttatus*. *Heredity* **50:** 283-293.

Macnair MR (1990). The genetics of metal tolerance in natural populations. In: Shaw AJ (ed) *Heavy metal tolerance in plants: evolutionary aspects*. CRC Press: Boca Raton, Floride, USA, pp 235-253.

Macnair MR (2002). Within and between population genetic variation for zinc accumulation in *Arabidopsis halleri*. *New Phytologist* **155:** 59-66.

Macnair MR, Baker AJM (1994). Metal-tolerant plants: An evolutionary perspective. In: Farago ME (ed) *Plant and the Chemical Elements. Biochemistry, Uptake, Tolerance and Toxicity*. VCH, pp 68-83.

Macnair MR, Bert V, Huitson SB, Saumitou-Laprade P, Petit D (1999). Zinc tolerance and hyperaccumulation are genetically independant characters. *Proceedings of The Royal Society of London, Biological Series* **266:** 2175-2179.

Meerts P, Duchêne P, Gruber W, Lefèbvre C (2003). Metal accumulation and competitive ability in metallicolous and non-metallicolous *Thlaspi caerulescens* fed with different Zn salts. *Plant and Soil* **249:** 1-8.

Meerts P, Van Isacker N (1997). Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* **133:** 221-231.

Merilä J, Sheldon BC, Kruuk LEB (2001). Explaining stasis: microevolutionary studies in natural populations. *Genetica* **112-113:** 199-222.

Meyer C-L, Juraniec M, Huguet Sp, Chaves-Rodriguez E, Salis P, Isaure M-P *et al* (2015). Intraspecific variability of cadmium tolerance and accumulation, and cadmium-induced cell wall modifications in the metal hyperaccumulator *Arabidopsis halleri*. *Journal of Experimental Botany* **66**(11)**:** 3215-3227.

Meyer C-L, Kostecka AA, Saumitou-Laprade P, Créach A, Castric V, Pauwels M *et al* (2010). Variability of zinc tolerance among and within populations of the pseudometallophyte *Arabidopsis halleri* and possible role of directional selection. *New Phytologist* **185**(1)**:** 130-142.

Meyer C-L, Pauwels M, Briset L, Godé C, Salis P, Bourceaux A *et al* Potential preadaptation to anthropogenic pollution: evidence from a common QTL for zinc and cadmium tolerance in metallicolous and non-metallicolous accessions of *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz. *New Phytologist*. (in press)

Meyer C-L, Vitalis R, Saumitou-Laprade P, Castric V (2009). Genomic pattern of adaptive divergence in *Arabidopsis halleri*, a model species for tolerance to heavy metal. *Molecular Ecology* **18**(9)**:** 2050-2062.

Millennium Ecosystem Assessment (2005). *Ecosystems and Human Well-being: Synthesis*. Island Press: Washington, DC.

Mills RF, Krijger GC, Baccarini PJ, Hall JL, Williams LE (2003). Functional expression of AtHMA4, a P_{1B}-type ATP ase of the Zn/Co/Cd/Pb subclass. *The Plant Journal* **35:** 164-176.

Mirouze M, Paszkowski J (2011). Epigenetic contribution to stress adaptation in plants. *Current Opinion in Plant Biology* **14:** 267-274.

Mousset M, David P, Petit C, Pouzadoux J, Hatt C, Flaven E *et al* (2016). Lower selfing rates in metallicolous populations than in non-metallicolous populations of the pseudometallophyte *Noccaea caerulescens* (Brassicaceae) in Southern France. *Annals of Botany* **doi: 10.1093/aob/mcv191**.

Nicholls MK, McNeilly T (1979). Sensitivity of rooting and tolerance to copper in *Agrostis tenuis* sibth. *New Phytologist* **83:** 653-664.

Papoyan A, Kochian LV (2004). Identification of *Thlaspi caerulescens* Genes That May Be Involved in Heavy Metal Hyperaccumulation and Tolerance. Characterization of a Novel Heavy Metal Transporting ATPase. *Plant Physiology* **136**(3)**:** 3814-3823.

Pauwels M, Frérot H, Bonnin I, Saumitou-Laprade P (2006). A broad-scale study of population differentiation for Zn-tolerance in an emerging model species for tolerance study: Arabidopsis halleri (Brassicaceae). *Journal of Evolutionary Biology* **19**(6)**:** 1838-1850.

Pauwels M, Saumitou-Laprade P, Holl AC, Petit D, Bonnin I (2005). Multiple origin of metallicolous populations of the pseudometallophyte *Arabidopsis halleri* (Brassicaceae) in central Europe: the cpDNA testimony. *Molecular Ecology* **14**(14)**:** 4403-4414.

Pauwels M, Vekemans X, Godé C, Frérot H, Castric V, Saumitou-Laprade P (2012). Nuclear and chloroplast DNA phylogeography reveals vicariance among European populations of the model species for the study of metal tolerance, *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **193**(4)**:** 916-928.

Peralta-Videa JR, Lopez ML, Narayan M, Saupe G, Gardea-Torresdey J (2009). The biochemistry of environmental heavy metal uptake by plants: implications for the food chain. *International Journal of Biochemistry and Cell Biology* **41**(8-9)**:** 1655-1677.

Pigliucci M (2005). Evolution of phenotypic plasticity: where are we going now? *Trends in Ecology and Evolution* **20**(9)**:** 481-486.

Pollard AJ, Baker AJM (1996). Quantitative genetics of zinc hyperaccumulation in *Thlaspi caerulescens*. *New Phytologist* **132:** 113-118.

Pollard AJ, Reeves RD, Baker AJM (2014). Facultative hyperaccumulation of heavy metals and metalloids. *Plant Science* **217-218:** 8-17.

Purugganan MD, Gibson G (2003). Merging ecology, molecular evolution, and functional genetics. *Molecular Ecology* **12:** 1109-1112.

Reznick DN, Ghalambor CK (2001). The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. *Genetica* **112-113:** 183-198.

Rigola D, Fiers M, Vurro E, Aarts MGM (2006). The heavy metal hyperaccumulator *Thlaspi caerulescens* expresses many species-specific genes, as identified by comparative expressed sequence tag analysis. *New Phytologist* **170**(4)**:** 753-765.

Roosens N, Verbruggen N, Meerts P, Ximénez-Embun P, Smith JA (2003). Natural variation in cadmium tolerance and its relationship to metal accumulation for seven populations of *Thlaspi caerulescens* from western Europe. *Plant, Cell and Environment* **26**(10)**:** 1657-1673.

Roosens N, Willems G, Godé C, Courseaux A, Saumitou-Laprade P (2008a). The use of comparative genome analysis and syntenic relationships allows extrapolating the position of Zn tolerance QTL regions from *Arabidopsis halleri* into *Arabidopsis thaliana*. *Plant and Soil* **306:** 105-116.

Roosens N, Willems G, Saumitou-Laprade P (2008b). Using Arabidopsis to explore zinc tolerance and hyperaccumulation. *Trends in Plant Sciences* **13**(5)**:** 208-215.

Roux C, Castric V, Pauwels M, Wright SI, Saumitou-Laprade P, Vekemans X (2011). Does Speciation between *Arabidopsis halleri* and *Arabidopsis lyrata* Coincide with Major Changes in a Molecular Target of Adaptation? *PLoS ONE* **6**(e26872).

Sarret G, Saumitou-Laprade P, Bert V, Proux O, Hazemann JL, Traverse A *et al* (2002). Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* **130**(4)**:** 1815-1826.

Sarret G, Willems G, Isaure M-P, Marcus MA, Fakra SC, Frérot H *et al* (2009). Zinc distribution and speciation in *Arabidopsis halleri × Arabidopsis lyrata* progenies presenting various zinc accumulation capacities. *New Phytologist* **184**(3)**:** 581-595.

Savolainen O, Lascoux M, Merilä J (2013). Ecological genomics of local adaptation. *Nature Reviews Genetics* **14:** 807-820.

Schat H, Ten Bookum WM (1992). Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68:** 219-229.

Shahzad Z, Gosti F, Frérot H, Lacombe E, Roosens N, Saumitou-Laprade P *et al* (2010). The Five AhMTP1 Zinc Transporters Undergo Different Evolutionary Fates towards Adaptive Evolution to Zinc Tolerance in *Arabidopsis halleri*. *PLoS Genetics* **6(4): e1000911. doi:10.1371/journal.pgen.1000911**.

Shaw R, Geyer C, Wagenius S, Hangelbroek H, Etterson J (2008). Unifying life-history analyses for interference of fitness and population growth. *The American Naturalist* **172:** E35-E47.

Stinchcombe JR, Hoekstra HE (2007). Combining population genomics and quantitative genetics: finding the genes underlying ecologically important traits. *Heredity***:** 1-13.

Stockwell CA, Hendry AP, Kinninson MT (2003). Contemporary evolution meets conservation biology. *Trends in Ecology & Evolution* **18:** 94-101.

Takeda S, Paszkowski J (2006). DNA methylation and epigenetic inheritance during plant gametogenesis. *Chromosoma* **115:** 27-35.

Talke IN, Hanikenne M, Krämer U (2006). Zinc-dependent Global Transcriptional Control, Transcriptional Deregulation, and Higher Gene Copy Numer for Genes in Metal Homeostasis of the Hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* **142:** 148-167.

Tardieu F, Tuberosa R (2010). Dissection and modelling of abiotic stress tolerance in plants. *Current Opinion in Plant Biology* **13:** 206-212.

The Arabidopsis Genome Initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408:** 796-815.

Turner TL, Bourne EC, Von Wettberg EJ, Hu TT, Nuzhdin SV (2010). Population resequencing reveals local adaptation of *Arabidopsis lyrata* to serpentine soils. *Nature Genetics* **42**(3)**:** 260-263.

Ungerer M, Johnson L, Herman M (2007). Ecological genomics: understanding gene and genome function in the natural environment. *Heredity***:** 1-6.

van de Mortel JE, Schat H, Moerland PD, Ver Loren van Themaat E, van der Ent S, Blankestijn H *et al* (2008). Expression differences for genes involved in lignin, gluthatione and sulphate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell and Environment* **31:** 301-324.

van de Mortel JE, Villanueva LA, Schat H, Kwekkeboom J, Coughlan S, Moerland PD *et al* (2006). Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **142:** 1127-1147.

van der Ent A, Baker AJM, Reeves RD, Pollard AJ, Schat H (2013). Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. *Plant and Soil* **362**(1-2)**:** 319-334.

van Kleunen M, Fischer M (2005). Constraints on the evolution of adaptive phenotypic plasticity in plants. *New Phytologist* **166:** 49-60.

Van Rossum F, Bonnin I, Fénart S, Pauwels M, Petit D, Saumitou-Laprade P (2004). Spatial genetic structure within a metallicolous population of *Arabidopsis halleri*, a clonal, self-incompatible and heavy-metal-tolerant species. *Molecular Ecology* **13**(10)**:** 2959-2967.

Verbruggen N, Hermans C, Schat H (2009). Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist* **181**(4)**:** 759-776.

Weber M, Harada E, Vess C, Roepenack-Lahaye E, Clemens S (2004). Comparative microarray analysis of Arabidopsis thaliana and Arabidopsis halleri roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *The Plant Journal* **37:** 269 - 281.

Weber M, Trampczynska A, Clemens S (2006). Comparative transcriptome analysis of toxic metal response in *Arabidopsis thaliana* and the Cd^{2+}-hypertolerant facultative metallophyte *Arabidopsis halleri*. *Plant, Cell and Environment* **29:** 950-963.

Wilkins DA (1978). The measurement of tolerance to edaphic factors by means of root growth. *New Phytologist* **80:** 623-634.

Willems G, Dräger D, Courbot M, Godé C, Verbruggen N, Saumitou-Laprade P (2007). The Genetic Basis of Zinc Tolerance in the Metallophyte *Arabidopsis halleri ssp. halleri* (Brassicaceae): An Analysis of Quantitative Trait Loci. *Genetics* **176:** 659-674.

Willems G, Frérot H, Gennen J, Salis P, Saumitou-Laprade P, Verbruggen N (2010). Quantitative trait loci analysis of mineral element concentrations in an *Arabidopsis halleri* x *Arabidopsis lyrata petraea* F2 progeny grown on cadmium- contaminated soil. *New Phytologist* **187**(2)**:** 368–379.

Yeaman S, Whitlock MC (2011). The Genetic Architecture of Adaptation under Migration-Selection Balance. *Evolution* **65**(7)**:** 1897-1911.

ANNEXES : CINQ PUBLICATIONS REPRESENTATIVES

1. Meyer CL, Pauwels M, Briset J, Godé C, Salis P, Bourceaux A, Souleman D, **Frérot H***, Verbruggen N*. Potential preadaptation to anthropogenic pollution: evidence from a common QTL for zinc and cadmium tolerance in metallicolous and non-metallicolous accessions of *Arabidopsis halleri*. *New Phytologist*. (in press). ***contributions équivalentes**
2. Gonneau C, Genevois N, **Frérot H**, Sirguey C, Sterckeman T. (2014). Variation of trace metal accumulation, major nutrient uptake and growth parameters and their correlations in 22 populations of *Noccaea caerulescens*. *Plant and Soil*. 384(1-2): 271-287.
3. Escarré J, Lefèbvre C, **Frérot H**, Mahieu S, Noret N. (2013). Metal concentration and metal mass of metallicolous, non metallicolous and serpentine *Noccaea caerulescens* populations cultivated in different growth media. *Plant and Soil*. 370(1-2): 197-221.
4. **Frérot H**, Faucon MP, Willems G, Godé C, Courseaux, Darracq A, Verbruggen N, Saumitou-Laprade P. (2010). Genetic architecture of zinc hyperaccumulation in *Arabidopsis halleri*: the essential role of QTL x environment interactions. *New Phytologist* 187(2): 355-367.
5. Meyer CL, Kostecka AA, Saumitou-Laprade P, Créach A, Castric V, Pauwels M, **Frérot H**. (2010). Variability of zinc tolerance among and within populations of the pseudometallophyte species *Arabidopsis halleri* and possible role of directional selection. *New Phytologist* 185(1): 130-142.

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Research

Potential preadaptation to anthropogenic pollution: evidence from a common quantitative trait locus for zinc and cadmium tolerance in metallicolous and nonmetallicolous accessions of *Arabidopsis halleri*

Claire-Lise Meyer[1], Maxime Pauwels[2], Loic Briset[1], Cécile Godé[2], Piétro Salis[1], Angélique Bourceaux[2], Dima Souleman[2], Hélène Frérot[2]* and Nathalie Verbruggen[1]*

[1]Laboratoire Physiologie et Génétique Moléculaire des Plantes, Université Libre de Bruxelles, Boulevard du Triomphe, Campus Plaine, B-1050 Brussels, Belgium; [2]Université de Lille – Sciences et Technologies, CNRS, UMR 8198 Evo-Eco-Paleo, F-59655 Villeneuve d'Ascq, France

Author for correspondence:
Nathalie Verbruggen
Tel: +32 2 650 21 28
Email: nverbru@ulb.ac.be

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Summary

- As a drastic environmental change, metal pollution may promote the rapid evolution of genetic adaptations contributing to metal tolerance. In *Arabidopsis halleri*, genetic bases of zinc (Zn) and cadmium (Cd) tolerance have been uncovered only in a metallicolous accession, although tolerance is species-wide. The genetic determinants of Zn and Cd tolerance in a nonmetallicolous accession were thus investigated for the first time.
- The genetic architecture of tolerance was investigated in a nonmetallicolous population (SK2) by using first backcross progeny obtained from crosses between SK2 and *Arabidopsis lyrata petraea*, a nonmetallophyte species.
- Only one significant and common quantitative trait locus (QTL) region was identified explaining 22.6% and 31.2% of the phenotypic variation for Zn and Cd tolerance, respectively. This QTL co-localized with *HEAVY METAL ATPASE 4 (AhHMA4)*, which was previously validated as a determinant of Zn and Cd tolerance in a metallicolous accession. Triplication and high expression of *HMA4* were confirmed in SK2. In contrast, gene duplication and high expression of *METAL TOLERANT PROTEIN 1A* (*MTP1A*), which was previously associated with Zn tolerance in a metallicolous accession, were not observed in SK2.
- Overall, the results support the role of *HMA4* in tolerance capacities of *A. halleri* that may have pre-existed in nonmetallicolous populations before colonization of metal-polluted habitats. Preadaptation to metal-contaminated sites is thus discussed.

Introduction

Global change, namely the impact of human activities on the Earth, has become a major concern. In particular, the way in which habitat disturbances affect species diversity is now increasingly studied. Anthropogenic disturbances resulting in abrupt changes towards hostile environmental conditions (e.g. use of insecticides or herbicides, or soil, air and water pollution) are expected to locally reduce species diversity because they challenge the maintenance of species populations in local communities. Theoretically, population persistence may rely on the levels of phenotypic plasticity, dispersal or genetic adaptations. However, environmental changes are often so drastic that phenotypic plasticity and migration are not sufficient to avoid extinction. Accordingly, many examples suggest that population persistence, following human-induced environmental disturbances, implies the rapid evolution of genetic adaptations (Reznick & Ghalambor, 2001). Thus, whereas strong selective pressures resulting from habitat modifications should cause population decline, adaptive evolutionary changes must occur quickly enough to restore population growth before extinction, a phenomenon known as evolutionary rescue (Gomulkiewicz & Holt, 1995; Gonzalez *et al.*, 2013; Carlson *et al.*, 2014). Whether populations can rapidly adapt – or undergo evolutionary rescue – actually depends on several extrinsic and intrinsic factors (Carlson *et al.*, 2014). Among them, intrinsic factors, such as population size, the initial levels of standing variation, and the initial degree of maladaptation, seem essential (Gomulkiewicz & Holt, 1995; Bell & Gonzalez, 2009; Carlson *et al.*, 2014).

Metal pollution is one of the major sources of anthropogenic disturbances inducing toxic environmental conditions for many plant populations, and is mainly caused by the use of agricultural fertilizers or the accumulation of residues from mining or smelting industries. Before the molecular biology era, several studies

*These authors contributed equally to this work.

revealed that the maintenance of plant populations facing metal pollution and the associated selective pressures implied the rapid evolution of an adaptive trait called 'metal tolerance' (Wu *et al.*, 1975; Al-Hiyaly *et al.*, 1988). It was also shown that the evolution of metal tolerance in metallicolous (M) populations has occurred in pseudometallophyte species which displayed low frequencies of metal-tolerant individuals in nonmetallicolous (NM) populations growing in nonpolluted areas (Ingram, 1988, cited in Baker & Proctor, 1990). Therefore, the maintenance of M populations in those species suggested that they have undergone evolutionary rescue thanks to the rapid selection of favorable alleles from initial standing genetic variation (Bradshaw, 1991).

Such rapid evolution of metal tolerance supported the assumption that it may be controlled by a few major genes, although minor modifier genes could be secondarily selected (Macnair, 1983, 1997; Schat & Ten Bookum, 1992). Using quantitative trait locus (QTL) mapping, genetic studies have investigated zinc (Zn) and cadmium (Cd) tolerance in *Arabidopsis halleri*, a pseudometallophyte model species that develops on metal-polluted sites where pollution is only of anthropogenic origin. From an interspecific cross (BC1) between a Zn/Cd-tolerant M accession (Auby, France) and its nontolerant close relative *Arabidopsis lyrata petraea*, QTL analyses revealed that the genetic architecture of Zn and Cd tolerance traits involved a limited number of genomic regions accounting for a substantial part of phenotypic variance (Courbot *et al.*, 2007; Willems *et al.*, 2007). Two candidate genes co-localizing with these major QTLs were mainly detected. First, *HEAVY METAL ATPASE 4* (*HMA4*), encoding a plasma membrane metal pump involved in root-to-shoot metal translocation and cellular metal detoxification (Hanikenne *et al.*, 2008), co-localized with major QTLs for Zn and Cd tolerance. Second, paralogs of the *METAL TOLERANT PROTEIN 1* gene (*AhMTP1A1* and *AhMTP1A2* in tandem repeat and *AhMTP1B*), encoding Zn transporters involved in vacuolar sequestration (Dräger *et al.*, 2004; Shahzad *et al.*, 2010), co-localized with QTLs for Zn tolerance. More recently, *CATION EXCHANGER 1* (*CAX1*), a gene co-localizing with the second major QTL for Cd tolerance, was identified after fine-mapping. *CAX1* encodes a vacuolar Ca^{++}/H^{+} exchanger that was proposed to limit Cd-induced reactive oxygen species (ROS) accumulation under low-Ca conditions (Baliardini *et al.*, 2015). To date, only the contribution of *AhHMA4* has been validated by RNA interference (RNAi)-mediated silencing (Hanikenne *et al.*, 2008).

As QTLs were identified in an M accession, they might have been selected during the rapid adaptation of *A. halleri* to anthropogenic metal-polluted sites. However, it was demonstrated that NM populations of *A. halleri* exhibited (1) significant standing genetic variation (Meyer *et al.*, 2009) as well as (2) a high degree of Zn and Cd tolerance, so that metal tolerance in this pseudometallophyte species can be considered as species-wide or 'constitutive' (Pauwels *et al.*, 2006; Meyer *et al.*, 2010, 2015). Considering that M populations were probably founded from NM populations nearby (Pauwels *et al.*, 2005), it can be reasonably assumed that (1) initial (i.e. present in NM populations) levels of standing variation were sufficient and (2) the initial degree of maladaptation of *A. halleri* to anthropogenic metal-polluted habitats was limited enough to promote the maintenance of M populations on metal-polluted soils. Nevertheless, significant differences in average tolerance levels between M and NM populations have been observed (Pauwels *et al.*, 2006; Meyer *et al.*, 2010, 2015). This means that the maintenance of M populations also has required specific adaptive changes, as expected in an evolutionary rescue scenario. However, it remains unclear whether *AhHMA4*, *AhMTP1A1/A2/B* and/or *AhCAX1* would participate in the local adaptation of M populations to the impacted environment or whether they would be involved in constitutive tolerance capacities shared by M and NM populations.

To examine whether the genetic determinants of Zn and Cd tolerance that have been identified in the M accession of *A. halleri* were selected during adaptation to anthropogenic metal-polluted habitats or pre-existed in NM populations, the genetic architecture of Zn and Cd tolerance was explored for the first time using an NM *A. halleri* accession. For this purpose, QTL mapping of a BC1 progeny (called BC1 SK2) generated from a cross between one *A. halleri* individual from an NM Slovakian population (SK2) and *A. lyrata petraea* was performed. To allow comparison with the previous QTL mapping of the BC1 generated with the Auby accession (BC1 AU), the same phenotyping protocol as in Willems *et al.* (2007) and Courbot *et al.* (2007) was used. In addition, the genomic copy number and transcript levels of the candidate genes *HMA4* and *MTP1A* that were identified in BC1 AU were determined in BC1 SK2 and in the parental populations. By comparison with previous studies only involving M individuals, we discuss the possibility that the identified mechanisms are putative preadaptations to anthropogenic metalliferous soils.

Materials and Methods

Plant material

The NM SK2 population is a Slovakian population located in the Tatras Mountains that was initially referred to as Sl2 (Bert *et al.*, 2002). It was chosen for this study because of its particularly low Zn and Cd tolerance levels compared with other studied *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz populations (Pauwels *et al.*, 2006; Meyer *et al.*, 2010, 2015). A single cross was performed between one individual from the SK2 population and one individual from the nontolerant species *Arabidopsis lyrata petraea* (L.) O'Kane & Al-Shehbaz (from Unhošt', Central Bohemia: Macnair *et al.*, 1999). Both species are self-incompatible and usually outcrossing. Therefore, to avoid any inbreeding depression effect, one randomly selected F1 individual was used as the pollen donor to fertilize a second *A. lyrata petraea* genotype, generating the interspecific backcross progeny (BC1). The BC1 population used for linkage map construction consisted of 335 individuals of which 129 and 70 were phenotyped for Zn and Cd tolerance, respectively. The different sample sizes resulted from difficulties in maintaining some genotypes by cutting.

Evaluation of Zn and Cd tolerance

Zn and Cd tolerance were measured in two separate sequential growth tests as described in Willems *et al.* (2007) and Courbot *et al.* (2007). Genotypes were propagated by cutting and after 5 wk of growth on sand, three cuttings per BC1 genotype were transferred to vessels filled with nutrient solution. The Zn tolerance test was performed at the University of Lille (France) in a growth chamber with the following parameters: 13 h light d^{-1}, 80 μmol photons m^{-2} s^{-1} irradiance, 20°C : 18°C, day : night, and hygrometry of 65%. To minimize microenvironmental effects, vessels were randomly distributed in the chamber on a turntable (Rotoplan system; Strader, Saint Sylvain d'Anjou, France). Cd tolerance was measured under controlled glasshouse conditions (13 h light d^{-1}, 100 μmol photons m^{-2} s^{-1} irradiance, and 20°C : 17°C, day : night) at the Free University of Brussels. Vessels were randomly distributed in the chamber and moved around once a week during the change of nutrient solution. After 3 wk in nutrient solution, plants were sequentially transferred to increasing concentrations of Zn (10–3000 μM $ZnSO_4$) or Cd (10–250 μM $CdSO_4$). Individual tolerance levels were determined as the lowest concentration at which no increase in root length (for Zn tolerance) or fresh biomass (for Cd tolerance) was observed. This concentration is currently known as the effective concentration for 100% growth inhibition (EC_{100}).

Statistical analysis

For Zn tolerance and Cd tolerance, a one-way analysis of variance (ANOVA) was performed in SAS (GLM procedure; SAS Institute, Cary, NC) considering 'genotype' as a random effect. The normality of residuals was tested using the Kolmogorov–Smirnov D test and a normal quantile-quantile (Q-Q) plot. Departure from normality was low for both traits ($D = 0.15$ and $D = 0.14$, respectively). For these reasons, and because normality was also difficult to improve, data sets were not transformed before parametric tests. The broad-sense heritability (H^2) of Zn and Cd tolerance was then calculated by dividing the genetic variance (which includes additive genetic variance and other sources of genetic variance) by the total phenotypic variance. Variance components were calculated using the REML method of the VARCOMP procedure of SAS. The arithmetic mean of Zn and Cd tolerance for each genotype was then calculated for the three replicates. The correlation between Zn and Cd tolerances was estimated from genotype values using the Spearman nonparametric coefficient.

Marker analysis

The genomic DNA of the four parental genotypes and 335 individuals of the BC1 progeny was extracted using a Kit NucleoSpin 96 Plant (Macherey-Nagel, Hoerdt, France). Sixty single nucleotide polymorphism (SNP) or microsatellite markers were selected for genotyping (Supporting Information Table S1). Thirty-nine new microsatellite markers were developed as described in Frérot *et al.* (2010). Briefly, the markers were selected from a microsatellite-enriched genomic library developed from an enrichment procedure with Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA) (Glenn & Schable, 2005). Primer sequences were designed in flanking regions of *A. halleri* microsatellites using PRIMER3 software (http://frodo.wi.mit.edu/). In order to allow multiplexing of markers, primer combinations were selected according to a 60°C (± 5°C) melting temperature and compatibility of PCR product sizes (100–150, 150–250 and 250–350 bp). The microsatellite markers were combined in 12 multiplexes labeled with FAM, PET, NED or VIC fluorescent dyes. Multiplex PCR was carried out in 10-μl reactions containing 1× PCR buffer II (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$, 150 nM dNTP (Euromedex, Souffelweyersheim, France), 0.075 μM of the forward-M13 primer, 0.375 μM of the reverse-PIG primer, 1.5 μM of fluorescent dye-labeled M13 (Applied Biosystems), 0.5 U of Qiagen Multiplex PCR kit, and 5 μl of DNA (20–60 ng). The cycling conditions consisted of one initial denaturation step of 15 min at 95°C, followed by two touchdowns of five cycles each: denaturation for 45 s at 95°C, annealing at 68°C (−2°C/cycle) for 5 min for the first touchdown and at 58°C (−2°C/cycle) for 1 min for the second, and extension for 60 s at 72°C; then 27 cycles of 45 s at 95°C, 30 s at 47°C, and 60 s at 72°C; and a final extension of 10 min at 72°C. Amplification products were separated on an ABI Prism 3130 DNA sequencer (Applied Biosystems). Alleles at microsatellite loci were scored using GENEMAPPER software version 3.7 (Applied Biosystems).

In addition, re-sequencing data sets were used to design seven SNP markers. Illumina paired-end sequencing reads from six *A. halleri* populations (P. Saumitou-Laprade, unpublished data) were aligned to the Araly1 assembly of the *A. lyrata lyrata* genome (http://genome.jgi.doe.gov/Araly1/Araly1.info.html). Within regions of interest, the criteria used for the selection of SNPs were: interspecific polymorphism; fixed polymorphism within *A. halleri* or *A. lyrata*; 50-base flanking regions with low polymorphism within *A. halleri* and between *A. halleri* and *A. lyrata*; read depth > 20. The SNPs were genotyped by KBioscience competitive allele-specific polymerase chain reaction (KASP) (LGC Genomics, Teddington, UK). KASP assays were performed in a final reaction volume of 8 μl containing 4 μl of KASP master mix V2 low ROX (LGC Genomics), 0.125 μl of KASP mix assay (LGC Genomics) and *c.* 100 ng of genomic DNA. The PikoReal real-time PCR system (Thermo Scientific, Breda, the Netherlands) was used with the following cycling conditions: 15 min at 94°C; 10 touchdown cycles of 20 s at 94°C and 60 s at 61–55°C (the annealing temperature for each cycle being reduced by 0.6°C per cycle); and 26 cycles of 20 s at 94°C and 60 s at 55°C. Fluorescence detection was performed at the end of each cycle and the data sets were analyzed using the allelic discrimination RFU-based method of the PIKOREAL software 2.1.

Linkage map construction

The *A. halleri* SK2 × *A. lyrata petraea* linkage map was constructed with the JOINMAP 3.0 program (Van Ooijen & Voorrips, 2001). The grouping of loci is based on a test for independence translated into a logarithm of odds (LOD) score. Linkage groups

were obtained at an LOD of 4. Markers along each linkage group were then ordered using the sequential method implemented in JOINMAP 3.0 in which loci are added one by one starting from the two most strongly linked loci. For each added locus, the best position is determined by comparing the goodness of fit of the resulting maps for each tested position. Kosambi's mapping function was used to translate recombination frequencies into map distances (Kosambi, 1944). When dealing with an interspecific cross, segregation distortion frequently occurs. Deviations from Mendelian ratios were assessed using a χ^2 test implemented in JOINMAP 3.0 at a locus-by-locus significance level of $\alpha = 0.05$.

QTL mapping

Detection of QTLs was performed using the MAPQTL 4.0 software (Van Ooijen *et al.*, 2002). A Kruskall–Wallis rank test was first performed on each locus separately to find potential regions of QTLs. Interval mapping (IM) analysis then allowed finer detection by determining whether a QTL occurred and computing an LOD score for every centiMorgan (cM) along the linkage groups. The LOD score represents the 10-base logarithm of the quotient of two likelihoods: the likelihood of the presence of a segregating QTL (alternative hypothesis) divided by the likelihood of no segregating QTL (null hypothesis). To establish the occurrence of a QTL, the calculated LOD scores were compared with an LOD score threshold obtained by a permutation test (1000 permutations), which corresponds to a genome-wide empirical significance threshold at the 5% level (Churchill & Doerge, 1994). A multiple-QTL model (MQM) analysis was finally performed every cM, in which markers close to detected QTLs (by IM mapping) were selected as cofactors to take over the role of the nearby QTLs in the approximate multiple-QTL models used in the subsequent MQM analysis. This method reduces the residual variance and enhances the power of searching for other segregating QTLs. It also improves the precision of QTL positions. After manual selection of cofactors, an automatic selection of cofactors was executed to keep a restricted set of significant cofactors. The LOD score profiles showing QTLs with their one- and two-LOD support intervals were obtained using MAPCHART 2.1 (Voorrips, 2002).

Additionally, the power of analysis to detect the QTLs identified by Courbot *et al.* (2007) and Willems *et al.* (2007) was calculated using the R/QTLDESIGN software (Sen *et al.*, 2007).

HMA4 and *MTP1A* relative transcript levels and gene copy numbers

Eighteen BC1 progenies with different allelic combinations (*A. lyrata*/*A. lyrata* or *A. lyrata*/*A. halleri*) in the genomic region of *HMA4* (markers Chr2-06046 to Chr2-08800) and *MTP1A* (markers Chr2-17890 to Chr2-19598) were grown on soil in a controlled growth chamber (16 h light d^{-1}, 100 μmol photons m^{-2} s^{-1} irradiance, 20°C : 18°C, day : night and 70% humidity). Nine leaves of each genotype were harvested, immediately frozen in liquid nitrogen, homogenized and stored at −80°C. Total RNA was extracted with TRI Reagent (Sigma, St Louis, MO, USA) and cDNA was synthesized from 1 μg of DNaseI-treated (Promega, Fitchburg, WI, USA) total RNA using oligo-dT and the Goscript reverse transcription system (Promega). Real-time quantitative PCRs were conducted in 96-well plates with the PikoReal real-time PCR system using Takion no ROX SYBR Mastermix (Eurogentec, Cologne, Germany). Volumes of 2.5 μl of cDNA were used for PCR in a 10-μl mix containing 5 μl of SYBR mastermix, 2 μl of H_2O and 0.5 μM of each primer. A total of three technical repeats were run per cDNA and primer pair combination. Relative transcript levels of *HMA4* and *MTP1A* were calculated by normalization to *ELONGATION FACTOR 1 ALPHA* (*EF1α*) as a constitutively expressed reference gene (Talke *et al.* 2006). *HMA4, MTP1A* and *EF1α* primers were described previously (Talke *et al.*, 2006; Shazhad *et al.*, 2010; Hanikenne *et al.*, 2013). In the study by Shazhad *et al.* (2010), the *MTP1A* primers were designed for amplification on *A. halleri* DNA. By sequencing, we checked the specificity of these primers on *A. lyrata* DNA and then the PCR efficiency was evaluated from the analysis of 1 : 1, 1 : 3, 1 : 12 and 1 : 48 dilution series. Genomic copy numbers of *HMA4* and *MTP1A* were evaluated on genomic DNA of the BC1 SK2 parents. The single-copy gene *SHORT ROOT* (*SHR*) was used as a reference gene and a total of 15 technical repeats were run per DNA and primer pair combination according to the protocol previously described. All primers have been described previously (Baliardini *et al.*, 2015).

Results

Zn and Cd tolerance in BC1 SK2

For both Zn and Cd tolerance, the broad-sense heritability (H^2) was moderate: 0.54 and 0.50, respectively. The phenotypic distribution of these traits was examined in the BC1 population (Fig. 1). A slight departure from normality was observed for both traits ($D = 0.15$ and $D = 0.14$, respectively; P-value < 0.01), with skewness (−0.13 and −0.15, respectively) and kurtosis (3.07 and 3.07, respectively) values barely different from those of a normal distribution (0 and 3, respectively). Seven individuals showed higher Cd tolerance values (EC_{100} from 150 to 225 μM $CdSO_4$) than the average tolerance in the SK2 population ($EC_{100} = 133$ μM $CdSO_4$; Meyer *et al.*, 2015). This result has to be treated with caution because parent representatives are not the original parents and some individuals from this population were able to survive up to 250 μM $CdSO_4$ in the study of Meyer *et al.* (2015). For both Zn and Cd tolerance, the phenotypic distributions were rather different between the BC1 SK2 and the BC1 AU progenies, suggesting different genetic architectures (Fig. 1). The correlation between Zn and Cd was positive and significant ($N = 50$; $r = 0.29$; $P = 0.039$).

Linkage map

The 60 markers were assigned to the eight linkage groups of the *A. halleri* × *A. lyrata petraea* genetic map (Willems *et al.*, 2007; Frérot *et al.*, 2010). The length of each linkage group varied from 52.1 to 85.2 cM, while the marker number varied from five to

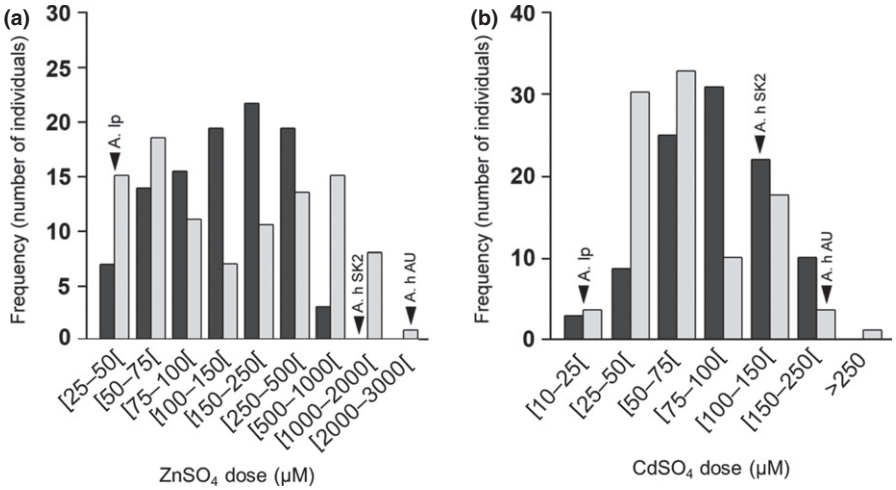

Fig. 1 Segregation profile for Zn (a) and Cd (b) tolerance in backcross progenies generated either using one SK2 individual (dark gray bars) or one Auby individual (light gray bars; from Courbot *et al.* (2007) and Willems *et al.* (2007)). Parents' representatives are noted above the arrows. A.lp, *Arabidopsis lyrata* ssp *petraea*; A.h SK2, *A. halleri* of population SK2; A.h AU, *A. halleri* from population AU (Auby).

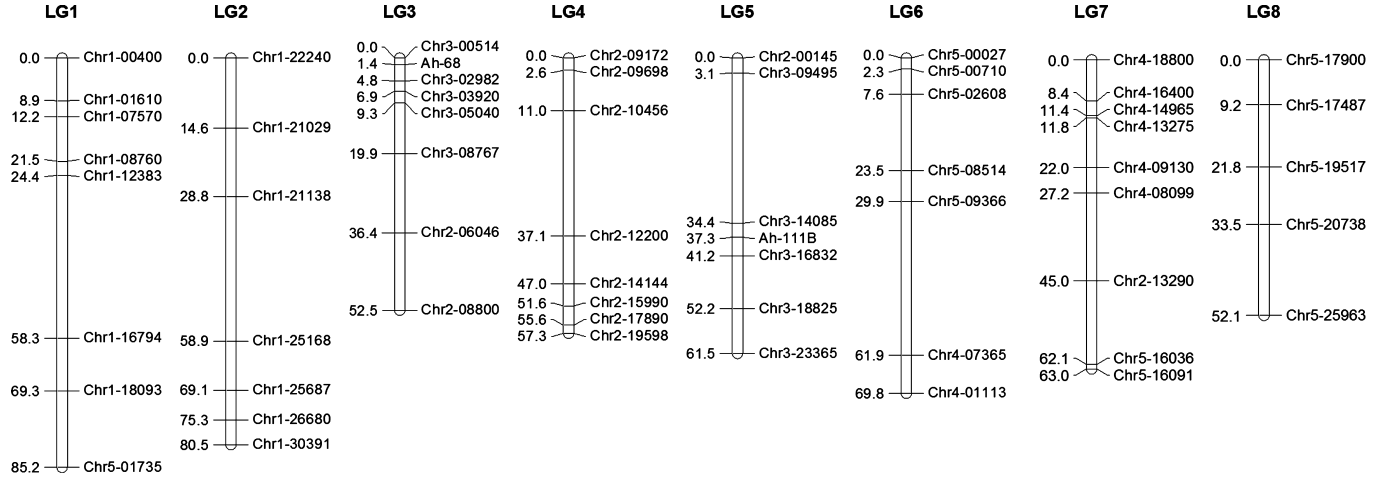

Fig. 2 Linkage map of the *Arabidopsis halleri* SK2 × *A. lyrata petraea* BC1 progeny. Markers are labeled to the right of linkage groups by their position in the *A. thaliana* genome (chromosome number – position in kb) except for some markers with unknown positions. Distances on the genetic map are expressed in cM. Markers in segregation distortion are underlined. LG, linkage group.

nine by linkage group (Fig. 2). The total length of the map (522 cM) was very similar to the previous estimations (567 and 526 cM; Willems *et al.*, 2007; Frérot *et al.*, 2010). The order of the markers in the present genetic map was very similar to that in *A. thaliana* and *A. lyrata* physical maps (Fig. 2; Table S1). Discrepancies among these three maps were only observed for one marker in linkage group 7 (Chr2-13290) and three markers at the beginning of linkage group 2 (markers Chr1-22240, Chr1-21029 and Chr1-21138). The order in this group of three markers was similar between the *A. lyrata* physical map and the present genetic map but the localization was different (end of scaffold 1 for *A. lyrata*; data not shown). At a significance threshold of 5%, 32 markers (i.e. 53% of markers) showed significant departure from the expected Mendelian ratio (Fig. 2). These markers were located mainly in LG1, LG2 and LG4 and showed distortion in the same direction (excess of the homospecific *A. l. petraea*/*A. l. petraea* allelic combination) as was observed in the BC1 AU progeny (Willems *et al.*, 2007).

QTLs of Zn and Cd tolerance

Only one significant QTL was detected for Zn tolerance and one for Cd tolerance (called SK2_ZnTol1 and SK2_CdTol1, respectively) with LOD scores higher than the threshold obtained by permutation (i.e. 2.3). These two QTLs overlapped and were located at the end of linkage group 3 between markers Chr2-08800 and Chr2-06046. They explained 22.6% and 31.2% of the total phenotypic variance for Zn and Cd tolerance, respectively (Table 1; Fig. 3). Negative additive effects were measured for both traits, meaning that the *A. halleri* alleles increased tolerance compared with the *A. lyrata petraea* alleles (Table 1). Interestingly, confidence intervals of SK2_ZnTol1 and SK2_CdTol1

Table 1 Summary characteristics of quantitative trait loci (QTLs) detected for zinc (Zn) and cadmium (Cd) tolerance in the *Arabidopsis halleri* SK2 × *A. lyrata petraea* BC1 progeny

Tolerance to	QTL[a]	LG[b]	Marker[c]	LOD score[d]	R^2 [e]	*a*[f]
Zn	SK2_ZnTol1	3	Chr2-08800/ Chr2-06046	6.85	22.6	−178.50
Zn	*SK2_ZnTol2*	*6*	*Chr5-08514*	*1.65*	*4.6*	*−79.61*
Zn	*SK2_ZnTol3*	*8*	*Chr5-17487*	*1.29*	*3.9*	*−75.00*
Cd	SK2_CdTol1	3	Chr2-08800/ Chr2-06046	4.33	31.2	−48.74
Cd	*SK2_CdTol2*	*1*	*Chr5-01735*	*1.55*	*10.5*	*−27.71*
Cd	*SK2_CdTol3*	*5*	*Chr3-14085*	*1.39*	*7.3*	*−23.30*

[a]QTLs are named according to the trait and ordered according to their significance level. Suggestive QTLs are indicated in italics.
[b]Linkage groups (LGs) where the QTLs were detected.
[c]Marker closest to the higher logarithm of odds (LOD) score.
[d]Maximum LOD score for the linkage group obtained by the multiple-QTL model (MQM) mapping method.
[e]Percentage of variance explained by the QTL.
[f]Additive effect of the QTL.

overlapped with the position of the *HMA4* gene (Chr2-08280 in the *Arabidopsis thaliana* genome). Additionally, two peaks near the LOD threshold were observed in linkage groups 6 and 8, and 1 and 5 for Zn and Cd tolerance, respectively (Fig. 3; Table 1) which may indicate minor-effect QTLs. These putative QTLs did not co-localize with the QTLs previously identified in BC1 AU. Together, they explained 8.5% and 17.8% of the Zn and Cd phenotypic variance, respectively.

Using 129 BC1 individuals, markers spaced *c.* 7 cM apart, broad-sense heritability of 0.89 and an LOD threshold of 2.3 for declaring significance, 99% power was found to detect QTLs with additive effects similar to those of ZnTol2 and ZnTol3 (Willems *et al.*, 2007). With the same parameters, 70 BC1 individuals and a broad-sense heritability of 0.79, 75% power to detect a QTL similar to CdTol2 was obtained and 50% power for a QTL similar to CdTol3 (Courbot *et al.*, 2007). These results indicated that the absence of QTLs similar to those of BC1 AU in the present study is not attributable to a lack of statistical power.

Transcript levels and genomic copy numbers of *HMA4* and *MTP1A*

In order to confirm *HMA4* as a candidate gene for the overlapping QTLs SK2_ZnTol1 and SK2_CdTol1, relative expression and genomic copy numbers were examined in some BC1 SK2

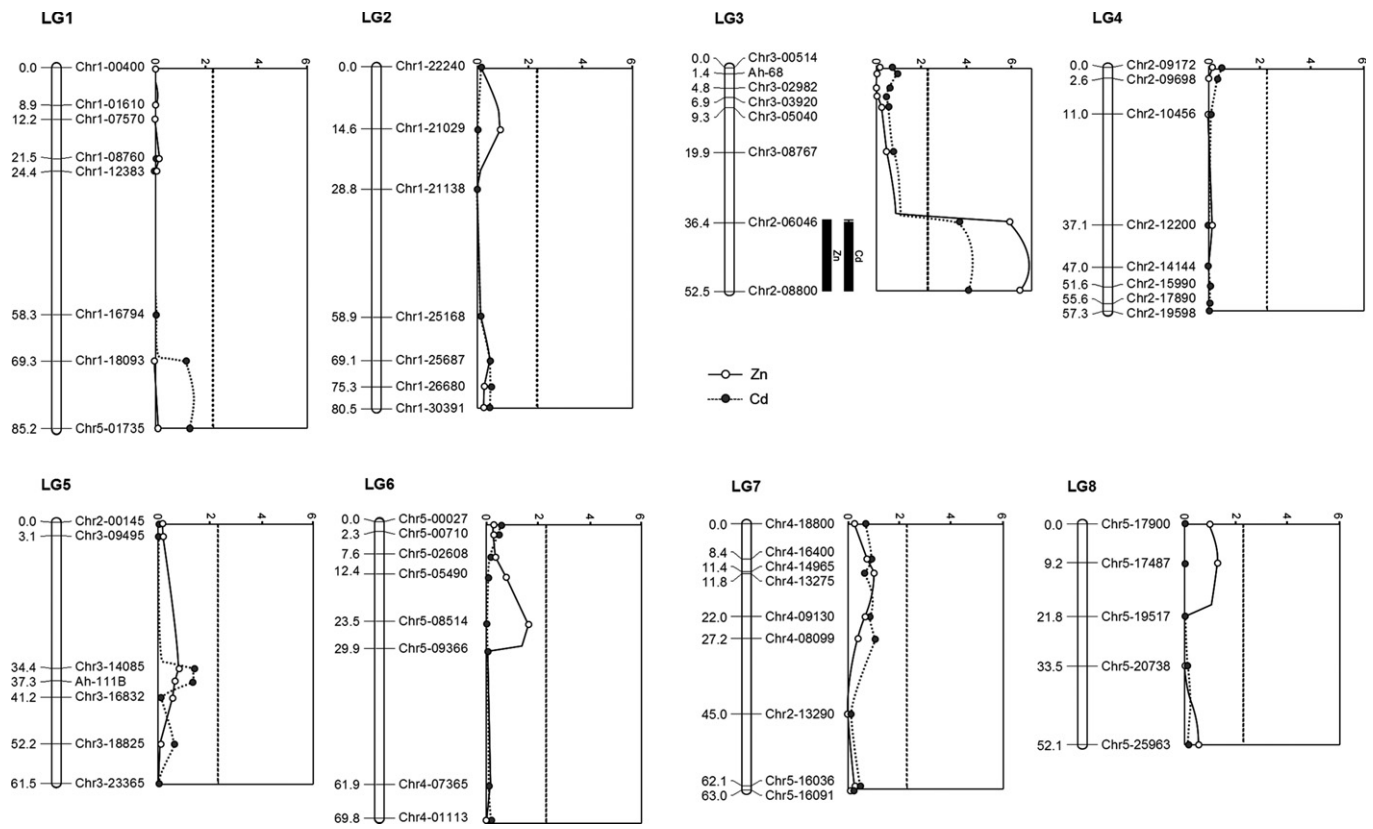

Fig. 3 Quantitative trait locus (QTL) mapping for zinc (Zn) and cadmium (Cd) tolerance in the *Arabidopsis halleri* SK2 × *A. lyrata petraea* progeny obtained by the multiple QTL model (MQM) mapping method. Marker names are designated by the position on *A. thaliana* chromosomes except for some markers with unknown positions. Logarithm of odds (LOD) scores are indicated on the horizontal axes. The vertical dashed lines represent the LOD score threshold (2.3) at a 5% error level for QTL detection. The positions of QTLs are indicated by bars representing the one-LOD support intervals (one LOD score unit on either side of the QTL peak) and whiskers representing the two-LOD support intervals (two LOD score unit on either side of the QTL peak). Black line, LOD scores for Zn tolerance; dotted line, LOD scores for Cd tolerance.

individuals and in the parents of the cross. The same analysis was performed on *MTP1A* to further confirm that *MTP1* was not a candidate gene for Zn tolerance in SK2. Relative expression of *HMA4* and *MTP1A* was studied in the shoots of 18 BC1 individuals representing the two genotypic classes (*A. l. petraea*/*A. l. petraea* and *A. l. petraea*/*A. halleri*) in each genomic region. On average, 10-fold higher *HMA4* transcript levels were observed in the BC1 genotypes harboring the *A. halleri* allele at the QTLs SK2_ZnTol1 and SK2_CdTol1 than in the individuals with two *A. lyrata petraea* alleles in this interval (Fig. 4a). By contrast, *MTP1A* was similarly expressed in the different BC1 individuals independently of the presence of the *A. halleri* allele in the QTL region (Fig. 4b). Using quantitative PCR on genomic DNA, the copy number in the parents of the BC1 SK2 was estimated. As expected, three *HMA4* gene copies were found for *A. halleri* and two for *A. lyrata petraea* (Fig. 5a). It should be noted that one of the two copies detected in *A. lyrata* is a truncated pseudogene (Hanikenne *et al.*, 2013). Only one copy of *MTP1A* was detected in the parent *A. halleri* SK2 and in *A. lyrata petraea* (Fig. 5b).

Discussion

Genetic architecture of Zn and Cd tolerance in an NM *A. halleri* accession

The genetic architecture of Zn and Cd tolerance was investigated for the first time in progeny from an interspecific cross between an NM accession of *A. halleri* (SK2) and *A. lyrata petraea*, a non-metallophyte close relative species. Previous QTL analyses suggested a genetic architecture involving six major genetic determinants for Zn and Cd tolerance, called Zntol-1, Zntol-2, Zntol-3, Cdtol-1, Cdtol-2 and Cdtol-3 (Courbot *et al.*, 2007; Willems *et al.*, 2007). Associated percentages of explained variance were moderate for Zn tolerance (12.2%, 11.2% and 5.6%, respectively) and higher for Cd tolerance (42.9%, 23.7% and 15.9%, respectively). By contrast, the genetic architecture detected here was apparently simpler, as only one significant QTL region was detected for each trait (SK2_ZnTol1 and SK2_CdTol1, respectively). The associated explained variances were reasonably high (22.6% and 31.2%, respectively), even though they may be overestimated as a result of sample size effects (Beavis, 1994). Interestingly, however, SK2_ZnTol1 co-localized with Zntol-1 in Willems *et al.* (2007) and SK2_CdTol1 co-localized with Cdtol-1 in Courbot *et al.* (2007). Moreover, SK2_ZnTol1 and SK2_CdTol1 co-localized with each other and represented the only QTL region that was shared between Zn and Cd tolerance in previous studies (Fig. 3). Additionally, two peaks near the LOD threshold which may indicate minor-effect QTLs explained 8.5% and 17.8% of the Zn and Cd phenotypic variance, respectively. These putative QTLs did not co-localize with the QTLs previously identified with BC1 AU.

The phenotypic distributions in BC1 SK2 were unimodal with an intermediate mode between the values of the parents' representatives (Fig. 1). By contrast, bimodal distributions of phenotypic values were observed for Zn and Cd tolerance of the BC1 AU progeny (Courbot *et al.*, 2007; Willems *et al.*, 2007). A bimodal distribution is commonly interpreted as evidence for one major effect locus in association with a few minor effect loci. Nevertheless, a unimodal – even normal – distribution is not evidence of genetic control by more than a single major gene (Lynch & Walsh, 1998). The phenotypic distributions in BC1 SK2 thus remained in accordance with the detection of one major-effect QTL, even though a contribution of additional weak-effect genes for Zn and Cd tolerance in *A. halleri* is very likely.

Putative role of *AhHMA4* in constitutive tolerance in *A. halleri*

SK2 showed among the lowest levels of Zn and Cd tolerance compared with other NM and M *A. halleri* accessions (Pauwels

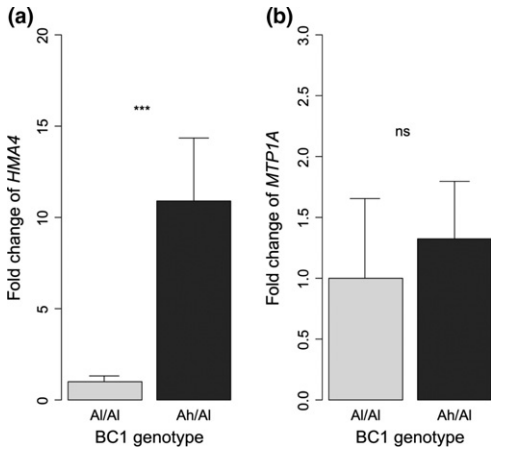

Fig. 4 Leaf transcript levels of with *HEAVY METAL ATPASE 4* (*HMA4*) and *METAL TOLERANT PROTEIN 1A* (*MTP1A*) in BC1 SK2 individuals displaying the *Arabidopsis lyrata petraea* or *A. halleri* alleles (Al and Ah, respectively). Values (mean + SE; $n = 9$) are given relative to transcript levels of the allelic class Al/Al. Asterisks indicate significant differences: ***, $P < 0.001$; ns, nonsignificant.

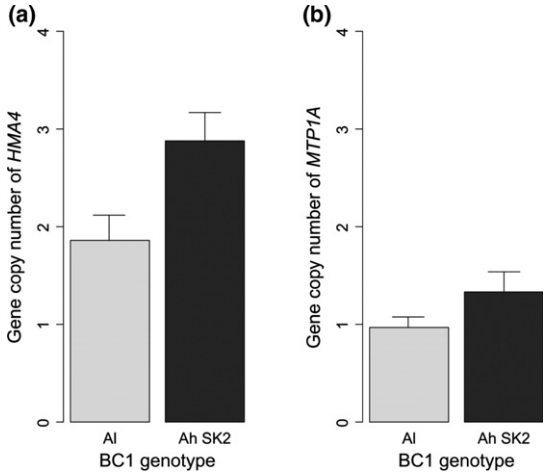

Fig. 5 Gene copy number in the parents of the *Arabidopsis halleri* SK2 × *A. lyrata petraea* BC1 progeny for (a) *HEAVY METAL ATPASE 4* (*HMA4*) and (b) *METAL TOLERANT PROTEIN 1A* (*MTP1A*). Values (mean + SE; $n = 15$ technical replicates) are given relative to the single-copy gene *SHORT ROOT* (*SHR*). *Arabidopsis lyrata petraea* (Al) genomic DNA served as a calibrator. Note that, among the *AlHMA4* copies, one is a truncated pseudogene (Hanikenne *et al.*, 2013).

et al., 2006; Meyer *et al.*, 2010, 2015). This suggests that the tolerance capacities of SK2 are representative of the constitutive capacities that are shared by the whole species and must have evolved very early after the species emergence (Bert *et al.*, 2000; Pauwels *et al.*, 2006). Accordingly, we assume that genetic bases of constitutive background tolerance are species-wide and, *a fortiori*, shared among M and NM populations. As SK2_ZnTol1 and SK2_CdTol1 (the overlapping QTLs) corresponded to the common QTL region identified for BC1 AU and BC1 SK2, this region may be involved in constitutive tolerance in *A. halleri*.

Thus, considering the co-localization of SK2_ZnTol1 and SK2_CdTol1 with Zntol-1 (Willems *et al.*, 2007) and Cdtol-1 (Courbot *et al.*, 2007), *AhHMA4* can be considered as a relevant candidate gene for constitutive tolerance. Although the wide confidence intervals of the QTL regions mean that we cannot rule out the possibility that one or more genes in linkage disequilibrium may also be valid candidates, strong evidence supports *AhHMA4* as a candidate gene for constitutive tolerance. First, *AhHMA4* was already identified as a candidate gene underlying Zntol-1 and Cdtol-1 in the BC1 AU studied by Willems *et al.* (2007) and Courbot *et al.* (2007). A contribution of *HMA4* to Zn and Cd tolerance traits was further demonstrated by RNAi-mediated silencing in the German M accession of Langelsheim (Hanikenne *et al.*, 2008). In addition, high *HMA4* expression was associated with tandem triplication of *AhHMA4*, whereas the gene is present as a single copy in nontolerant relatives (Hanikenne *et al.*, 2008). A combination of demographic and molecular evolution approaches suggested that *AhHMA4* triplication occurred well before the establishment and colonization of anthropogenic metal-polluted habitats (Roux *et al.*, 2011). Actually, the first duplication event of *AhHMA4* could have occurred very close to the time of the split between *A. lyrata* and *A. halleri*, and was subsequently followed by the second *AhHMA4* duplication (Roux *et al.*, 2011). More recently, a population survey suggested that triplication was shared among M and NM accessions from Germany (Hanikenne *et al.*, 2013). Our results confirmed that *AhHMA4* was also triplicated in SK2, a population that can be considered to be genetically isolated from M accessions than have been characterized so far (Pauwels *et al.*, 2012). Taken together, previous findings and the results of the present study strongly support the assumption that the tandem triplication and the consecutive overexpression of *AhHMA4* may be a major determinant of constitutive Zn and Cd tolerance in *A. halleri*.

Role of standing genetic variation in adaptive evolution to anthropogenic metal-polluted habitats

The subtractive comparison of QTL regions detected for BC1 AU and BC1 SK2 should be informative about the genetic mechanisms involved in the local adaptation to anthropogenic metal-polluted habitats. It is remarkable that Zntol-2 and Zntol-3, which are commonly associated with *AhMTP1A* and *AhMTP1B*, respectively (Willems *et al.*, 2007), Cdtol-2, which is associated with *AhCAX1* (Baliardini *et al.*, 2015), and Cdtol-3 were not detected in BC1 SK2. It is also remarkable that *AhMTP1A* was not overexpressed in SK2 compared with control sensitive species (Fig. 4b). This might indicate that those candidate genes may rather contribute to enhanced metal tolerance observed in M populations instead of constitutive tolerance. We suggest that the maintenance of M populations in anthropogenic polluted sites (i.e. their evolutionary rescue) might have involved selection of particular variants of those genes, either in the coding or in the promoting region. Considering *AhMTP1*, this would suggest that an improvement of Zn detoxification mechanisms, through enhanced sequestration in leaf cell vacuoles, would have been advantageous for plants exposed to higher concentrations of Zn in soils. In contrast, effective Zn detoxification is not necessary on nonmetalliferous soils, and basic activity of *AhMTP1* should be sufficient to ensure Zn homeostasis.

More generally, most adaptive genetic changes are expected to result from standing variation, that is, the selection of neutral or mildly deleterious alleles present at variable frequency in populations (Matuszewski *et al.*, 2015). Based on investigations of within-population genetic variation, it has already been suggested that M populations of *A. halleri* may have evolved from large standing variation existing in NM populations (including SK2) rather than from new mutations (Pauwels *et al.*, 2006; Meyer *et al.*, 2010). Several authors came to the same conclusion regarding tolerance polymorphism within NM populations of *Noccaea caerulescens* (Meerts and Van Isacker, 1997; Escarré *et al.*, 2000).

Preadaptation of *A. halleri* to anthropogenic metal-polluted habitats

Apart from selection of standing variation, local adaptation may also benefit from the existence of advantageous alleles that are already at high frequency or even fixed in populations. In this case, individuals can be considered as 'preadapted'. In some pseudometallophytes, it has already been suggested that adaptation to metal-polluted soils may have benefited from preadapting processes. For example, preadaptation to high soil Zn concentrations was reported for Canadian populations of *Deschampsia cespitosa* as all NM individuals tested revealed quite high Zn tolerance levels (Cox & Hutchinson, 1981). Similarly, the widespread tolerance to high magnesium : calcium ratios and nickel concentrations covered by a granite outcrop population of *Phacelia dubia* suggested that this species showed some degree of preadaptation to serpentine habitats (Taylor & Levy, 2002). In *A. halleri*, the pre-existence of constitutive tolerance and of *AhHMA4* copy number expansion (Roux *et al.*, 2011; Hanikenne *et al.*, 2013) suggests that the species was somehow preadapted to anthropogenic metal-polluted habitats. It also interrogates the evolutionary 'raison d'être' of constitutive metal tolerance in a pseudometallophyte. Evidence of positive selection in the *AhHMA4* genomic region in M as well as NM populations implies that *AhHMA4* paralogs may have played a role in a nonmetalliferous environment (Hanikenne *et al.*, 2013). However, the selective pressure that may have favored the selection of *AhHMA4* overexpression remains unclear.

A preadaptation may occur when a trait that is currently associated with a particular adaptation was previously selected for another function in response to a different selective pressure

(Gould & Vbra, 1982). The current trait is thus considered as an 'exaptation', in comparison with an 'adaptation' which is an original trait that evolved for its present function. A preadaptation is thus a potential exaptation if it becomes selected for a different function. Considering *AhHMA4* paralogs, available data indicate that they may not only be involved in tolerance of toxic metals in soils. In particular, *AhHMA4* gene copies have been shown to participate to Zn and Cd hyperaccumulation (Hanikenne *et al.*, 2008; Frérot *et al.*, 2010; Willems *et al.*, 2010), that is, the abnormal concentrations of these trace elements in aerial plant parts (for recent reviews, see Krämer, 2010; van der Ent *et al.*, 2013; Verbruggen *et al.*, 2009). Indeed, *HMA4* is a plasma membrane pump that plays a role in Zn and Cd xylem loading and cellular detoxification of Zn and Cd excess (Hanikenne *et al.*, 2008). The selective pressures that promote the evolution of hyperaccumulation have been, and are still, a subject of debate (Boyd & Martens, 1992; Pollard *et al.*, 2014). Interestingly, some of these putative selective pressures could act in a nonpolluted environment. For example, elemental defense against herbivores and pathogens or elemental allelopathy have been suggested as potential roles for metal hyperaccumulation (Boyd & Martens, 1992, 1998).

Finally, our study provides consistent arguments in favor of some degree of preadaptation to Zn and Cd tolerance in *A. halleri*. These arguments will be strengthened when the occurrence of *AhHMA4* copies, associated with significant Zn and Cd tolerance capacities, is demonstrated in several NM populations. Undoubtedly, the more integrated NM accessions are in future molecular studies, the greater will be the potential to elucidate the evolutionary origin of tolerance capacities in *A. halleri*. Other important implications of our work relate to investigations into mechanisms of local adaptation to metal-polluted soils. In this regard, it should be enlightening (1) to pursue the identification of candidate genes associated with the QTL regions specific to BC1 AU, (2) to explore the association between their expression and quantitative variations for Zn and Cd tolerance in natural populations of *A. halleri*, and (3) to examine the consequence of local adaptation to metal-polluted sites for the population structure of genetic diversity at those genes.

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Author contributions

C-L.M., H.F. and N.V. planned and designed the research, analyzed data and wrote the manuscript; C-L.M., L.B. and C.G. performed experiments; M.P. helped with the *MTP1* genotyping and revised the manuscript; A.B. and P.S. maintained plant collections; D.S. performed preliminary experiments.

References

Al-Hiyaly SA, McNeilly T, Bradshaw AD. 1988. The effect of zinc contamination from electricity pylons – evolution in a replicated situation. *New Phytologist* **110**: 571–580.

Baker AJM, Proctor J. 1990. The influence of cadmium, copper, lead, and zinc on the distribution and evolution of metallophytes in the British Isles. *Plant Systematics and Evolution* **173**: 91–108.

Baliardini C, Meyer C-L, Salis P, Saumitou-Laprade P, Verbruggen N. 2015. CAX1 co-segregates with Cd tolerance in the metal hyperaccumulator *Arabidopsis halleri* and plays a role in limiting oxidative stress in Arabidopsis. *Plant Physiology* **169**: 549–559.

Beavis WD. 1994. The power and deceit of QTL experiments: lessons from comparative QTL studies. *Proceedings of the 49th Annual Corn & Sorghum Industry Res. Conf. Am Seed Trade Assoc.*, Chicago, IL, USA, 250–266.

Bell G, Gonzalez A. 2009. Evolutionary rescue can prevent extinction following environmental change. *Ecology Letters* **12**: 942–948.

Bert V, Bonnin I, Saumitou-Laprade P, de Laguérie P, Petit D. 2002. Do *Arabidopsis halleri* from nonmetalicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **155**: 47–57.

Bert V, Macnair MR, De Laguérie P, Saumitou-Laprade P, Petit D. 2000. Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **146**: 225–233.

Boyd RS, Martens SN. 1992. The *raison d'être* for metal hyperaccumulation by plants. In: Baker AJM, Proctor J, Reeves RD, eds. *The ecology of ultramafic (Serpentine) soils*. Andover, UK: Intercept, 279–289.

Boyd RS, Martens SN. 1998. Nickel hyperaccumulation by *Thlaspi montanum* var. *montanum* (Brassicaceae): a constitutive trait. *American Journal of Botany* **85**: 259–265.

Bradshaw AD. 1991. Genostasis and the limit to evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences* **333**: 289–305.

Carlson SM, Cunningham CJ, Westley PAH. 2014. Evolutionary rescue in a changing world. *Trends in Ecology & Evolution* **29**: 521–530.

Churchill GA, Doerge RW. 1994. Empirical threshold values for quantitative trait mapping. *Genetics* **138**: 963–971.

Courbot M, Willems G, Motte P, Arvidsson S, Roosens N, Saumitou-Laprade P, Verbruggen N. 2007. A major quantitative trait locus for cadmium tolerance in *Arabidopsis halleri* colocalizes with HMA4, a gene encoding a heavy metal ATPase. *Plant Physiology* **144**: 1052–1065.

Cox RM, Hutchinson TC. 1981. Multiple and co-tolerance to metal in the grass *Deschampsia cespitosa*: adaptation, preadaptation and the cost. *Journal of Plant Nutrition* **3**: 731–741.

Dräger DB, Desbrosses-Fonrouge A-G, Krach C, Chardonnens AN, Meyer RC, Saumitou-Laprade P, Krämer U. 2004. Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high MTP1 transcript levels. *Plant Journal* **39**: 425–439.

van der Ent A, Baker AJM, Reeves RD, Pollard AJ, Schat H. 2013. Hyperaccumulators of metal and metalloid trace elements: facts and fiction. *Plant and Soil* **362**: 319–334.

Frérot H, Faucon M-P, Willems G, Godé C, Courseaux A, Darracq A, Verbruggen N, Saumitou-Laprade P. 2010. Genetic architecture of zinc hyperaccumulation in *Arabidopsis halleri*: the essential role of QTL × environment interactions. *New Phytologist* **187**: 355–367.

Glenn TC, Schable NA. 2005. Isolating microsatellite DNA loci. *Methods in Enzymology* **395**: 202–222.

Gomulkiewicz R, Holt RD. 1995. When does evolution by natural selection prevent extinction? *Evolution* **49**: 201–207.

Gonzalez A, Ronce O, Ferriere R, Hochberg ME. 2013. Evolutionary rescue: an emerging focus at the intersection between ecology and evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences* **368**: 20120404.

Gould SJ, Vbra ES. 1982. Exaptation – a missing term in the science of form. *Paleobiology* **8**: 4–15.

Hanikenne M, Kroymann J, Trampczynska A, Bernal MP, Motte P, Clemens S, Krämer U. 2013. Hard selective sweep and ectopic gene conversion in a gene cluster affording environmental adaptation. *PLoS Genetics* **9**: e1003707.

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Krämer U. 2008. Evolution of metal hyperaccumulation required *cis*-regulatory changes and triplication of HMA4. *Nature* **453**: 391–396.

Kosambi D. 1944. The estimation of map distances from the recombination values. *Annals of Eugenics* **12**: 172–175.

Krämer U. 2010. Metal hyperaccumulation in plants. *Annual Review of Plant Biology* **61**: 517–534.

Lynch M, Walsh B. 1998. *Genetics and analysis of quantitative traits*. Sunderland, MA, USA: Sinauer Associates.

Macnair MR. 1983. The genetic control of copper tolerance in the yellow monkey flower, *Mimulus guttatus*. *Heredity* **50**: 283–293.

Macnair MR. 1997. The evolution of plants in metal-contaminated environments. In: Bijlsma R, Loeschcke V, eds. *Environmental stress, adaptation and evolution*. Basel, Switzerland: Birkhäuser-Verlag, 3–24.

Macnair MR, Bert V, Huitson SB, Saumitou-Laprade P, Petit D. 1999. Zinc tolerance and hyperaccumulation are genetically independant characters. *Proceedings of the Royal Society of London B: Biological Sciences* **266**: 2175–2179.

Matuszewski S, Hermisson J, Kopp M. 2015. Catch me if you can: adaptation from standing genetic variation to a moving phenotypic optimum. *Genetics* **200**: 1255–1274.

Meyer C-L, Juraniec M, Huguet SP, Chaves-Rodriguez E, Salis P, Isaure M-P, Goormaghtigh E, Verbruggen N. 2015. Intraspecific variability of cadmium tolerance and accumulation, and cadmium-induced cell wall modifications in the metal hyperaccumulator *Arabidopsis halleri*. *Journal of Experimental Botany* **66**: 3215–3227.

Meyer C-L, Kostecka AA, Saumitou-Laprade P, Créach A, Castric V, Pauwels M, Frérot H. 2010. Variability of zinc tolerance among and within populations of the pseudometallophyte *Arabidopsis halleri* and possible role of directional selection. *New Phytologist* **185**: 130–142.

Meyer C-L, Vitalis R, Saumitou-Laprade P, Castric V. 2009. Genomic pattern of adaptive divergence in *Arabidopsis halleri*, a model species for tolerance to heavy metal. *Molecular Ecology* **18**: 2050–2062.

Pauwels M, Frérot H, Bonnin I, Saumitou-Laprade P. 2006. A broad-scale study of population differentiation for Zn-tolerance in an emerging model species for tolerance study: *Arabidopsis halleri* (Brassicaceae). *Journal of Evolutionary Biology* **19**: 1838–1850.

Pauwels M, Saumitou-Laprade P, Holl AC, Petit D, Bonnin I. 2005. Multiple origin of metallicolous populations of the pseudometallophyte *Arabidopsis halleri* (Brassicaceae) in central Europe: the cpDNA testimony. *Molecular Ecology* **14**: 4403–4414.

Pauwels M, Vekemans X, Godé C, Frérot H, Castric V, Saumitou-Laprade P. 2012. Nuclear and chloroplast DNA phylogeography reveals vicariance among European populations of the model species for the study of metal tolerance, *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **193**: 916–928.

Pollard AJ, Reeves RD, Baker AJM. 2014. Facultative hyperaccumulation of heavy metals and metalloids. *Plant Science* **217–218**: 8–17.

Reznick DN, Ghalambor CK. 2001. The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. *Genetica* **112–113**: 183–198.

Roux C, Castric V, Pauwels M, Wright SI, Saumitou-Laprade P, Vekemans X. 2011. Does speciation between *Arabidopsis halleri* and *Arabidopsis lyrata* coincide with major changes in a molecular target of adaptation? *PLoS ONE* **6**: e26872.

Schat H, Ten Bookum WM. 1992. Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68**: 219–229.

Sen S, Satagopan JM, Broman KW, Churchill GA. 2007. R/qtlDesign: inbred line cross experimental design. *Mammalian Genome* **18**: 87–93.

Shahzad Z, Gosti F, Frérot H, Lacombe E, Roosens N, Saumitou-Laprade P, Berthomieu P. 2010. The five AhMTP1 zinc transporters undergo different evolutionary fates towards adaptive evolution to zinc tolerance in *Arabidopsis halleri*. *PLoS Genetics* **6**: e1000911.

Taylor SI, Levy F. 2002. Responses to soils and a test for preadaptation to serpentine in *Phacelia dubia* (Hydrophyllaceae). *New Phytologist* **155**: 437–447.

Talke IN, Hanikenne M, Krämer U. 2006. Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* **142**: 148–167.

Van Ooijen JW, Boer MP, Jansen RC, Maliepaard C. 2002. *MapQTL 4.0: software for the calculation of QTL positions on genetic maps*. Wageningen, the Netherlands: Plant Research International.

Van Ooijen JW, Voorrips RE. 2001. *Joinmap 3.0: software for the calculation of genetic linkage maps*. Wageningen, the Netherlands: Plant Research International.

Verbruggen N, Hermans C, Schat H. 2009. Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist* **181**: 759–776.

Voorrips RE. 2002. Mapchart: software for the graphical presentation of linkage maps and QTLs. *Heredity* **93**: 77–78.

Willems G, Dräger D, Courbot M, Godé C, Verbruggen N, Saumitou-Laprade P. 2007. The genetic basis of zinc tolerance in the metallophyte *Arabidopsis halleri* ssp. *halleri* (Brassicaceae): an analysis of quantitative trait loci. *Genetics* **176**: 659–674.

Willems G, Frérot H, Gennen J, Salis P, Saumitou-Laprade P, Verbruggen N. 2010. Quantitative trait loci analysis of mineral element concentrations in an *Arabidopsis halleri* × *Arabidopsis lyrata petraea* F_2 progeny grown on cadmium-contaminated soil. *New Phytologist* **187**: 368–379.

Wu L, Bradshaw AD, Thurman DA. 1975. The potential for evolution of heavy metal tolerance in plants. III. The rapid evolution of copper tolerance in *Agrostis stolonifera*. *Heredity* **34**: 165–187.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Table S1 List of markers used in linkage map construction

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REGULAR ARTICLE

Variation of trace metal accumulation, major nutrient uptake and growth parameters and their correlations in 22 populations of *Noccaea caerulescens*

Cédric Gonneau · Nicolas Genevois · Hélène Frérot · Catherine Sirguey · Thibault Sterckeman

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Abstract
Background and aims *Noccaea caerulescens* is a model plant for the understanding of trace metal accumulation and a source of cultivars for phytoextraction. The aim of this study was to investigate natural variation for trace metal accumulation, major nutrient uptake and growth parameters in 22 populations. The correlations among these traits were particularly examined to better understand the eco-physiology and the phytoextraction potential of the species.
Methods Populations from three edaphic groups, i.e. calamine (CAL), serpentine (SERP) and non metalliferous (NMET) sites were grown in hydroponics for seven weeks at moderate trace metal exposure. Growth indicators, element contents and correlations between these variables were compared.
Results All the phenotypic characteristics showed a wide variability among groups and populations. The SERP populations showed a smaller plant size, higher cation contents and strong correlations between all element concentrations. NMET populations did not differ in plant size from the CAL ones, but had higher Zn and Ni contents. The CAL populations showed higher Cd and Mn accumulations and lower Ca contents. The trade-off between biomass production and Cd, Ni and Zn accumulation was high in SERP populations and low in the CAL and NMET ones.
Conclusions *N. caerulescens* is a genetically diverse species, showing specific features depending on the group and the population. These features may reflect the wide adaptive capacities of the species, and also reveal promising potential for phytoextraction of Cd, Ni and Zn.

Keywords Hyperaccumulation · Phenotyping · Genetic variability · Major element · Trace metal

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C. Gonneau · N. Genevois · C. Sirguey · T. Sterckeman
Université de Lorraine, LSE, UMR1120,
Vandœuvre-lès-Nancy cedex F-54518, France

C. Gonneau · N. Genevois · C. Sirguey (✉) · T. Sterckeman
INRA, LSE, UMR1120,
Vandœuvre-lès-Nancy cedex F-54518, France
e-mail: catherine.sirguey@univ-lorraine.fr

H. Frérot
Laboratoire Génétique et Evolution des Populations Végétales, CNRS UMR 8198, Université Lille1,
F-59655 Villeneuve d'Ascq cedex, France

Introduction

Noccaea caerulescens (formerly *Thlaspi caerulescens*) is a pseudo-metallophyte growing both on unpolluted and trace metal enriched soils. This species is particularly known to accumulate some trace metals in its aboveground parts. These include zinc (Zn), cadmium (Cd) and nickel (Ni) (Meerts and Van Isacker 1997; Escarré et al. 2000; Escarré et al. 2013; Assunção et al. 2003; Visioli et al. 2011). In situ, the plant can

concentrate in its shoots up to 53,450 mg Zn kg^{-1}, 2,890 mg Cd kg^{-1} and 12,880 mg Ni kg^{-1} (Reeves et al. 2001). Previous studies have shown that this wild species has a good potential for remediation, through phytoextraction, of soils moderately contaminated with Cd (Schwartz et al. 2003; Zhao et al. 2003; Maxted et al. 2007; Koopmans et al. 2008). Feasible trace metal phytoextraction implies simultaneous improvement of several plant characteristics, such as metal uptake, biomass production and growth speed. The achievement of phytoextraction may thus imply genetic engineering, necessarily based on existing variation for parameters of interest in candidate species. To date, natural variation of Zn, Cd or Ni hyperaccumulation has been largely investigated in *N. caerulescens* (Meerts and Van Isacker 1997; Escarré et al. 2000; Escarré et al. 2013; Assunção et al. 2003; Keller et al. 2006; Visioli et al. 2011). In the field, the various studied populations have shown contrasting accumulation abilities, some of them being able to hyperaccumulate Cd, Ni and Zn in their shoots at higher concentrations than the others (Reeves et al. 2001; Molitor et al. 2005; Basic et al. 2006). In general, the highest concentrations of Cd and Zn have been measured in calamine sites and those of Ni in serpentine ones (Reeves et al. 2001; Chardot et al. 2007), leading to the idea that hyperaccumulation of trace elements could be mainly attributed to their high bioavailability in soils. However, when cultivated in controlled conditions, the calamine populations of *N. caerulescens* exhibited a lower ability to hyperaccumulate Zn in their shoots than the non-metallicolous ones (Escarré et al. 2000; Escarré et al. 2013). For Cd the reverse was found, with the calamine populations exhibiting the highest concentrations (Escarré et al. 2000), except in Belgian calamine populations which show no group difference with non-metallicolous populations from Luxembourg (Dechamps et al. 2005).

Although the exploration of natural variation of trace metal hyperaccumulation provides interesting phenotypic data, the underlying genetic mechanisms are still far from being uncovered, as hyperaccumulation would be a multigenic process (Clemens 2001; Verbruggen et al. 2009; Hanikenne and Nouet 2011), involving numerous genes from the homeostasis network (Van de Mortel et al. 2006; Van de Mortel et al. 2008; Halimaa et al. 2014; Milner et al. 2014). This renders highly improbable, on the medium term, the creation of transgenic cultivars for phytoextraction, for example by introducing Cd hyperaccumulation ability into a high dry mass yield crop. That is why it seems more reasonable in a first phase to investigate natural variations both for trace metal accumulation and for nutritional and growth parameters, in order to exploit original genetic combinations potentially created in the wild. Therefore, what now appears essential is to estimate on a large scale the natural variability not only of trace metal accumulation, nutrient uptake, or plant growth taken separately, but also of the correlations between these parameters. A trade-off between plant dry biomass and Zn hyperaccumulation has already been demonstrated in some populations of *N. caerulescens* from Southern France, Belgium and Luxembourg (Dechamps et al. 2005; Escarré et al. 2000; Meerts and Van Isacker 1997). In these studies, metallicolous populations displayed higher plant biomass and lower Zn hyperaccumulation levels than non-metallicolous populations cultivated on metal-contaminated soils. On the contrary, a positive correlation between Cd hyperaccumulation and plant dry biomass was evidenced by Escarré et al. (2000), since metallicolous populations had higher Cd hyperaccumulation than non-metallicolous ones. Using F1 progenies within the same edaphic origin (i.e. among metallicolous or among non-metallicolous populations) or among different edaphic origins, Frérot et al. (2005) showed that a negative relationship between biomass production and Zn hyperaccumulation clearly appeared when plants expressed high levels of hyperaccumulation, that is to say, mostly among progenies from non-metallicolous origin. Therefore, the correlation between growth parameters and hyperaccumulation seems to be origin and metal-dependent, even metal concentration-dependent. This suggests the possibility of a large natural variation for correlation among these traits, to be exploited for agronomic purposes. On the other hand, very few studies reported the correlation between plant growth, metal hyperaccumulation and plant nutritional status. Meerts and Van Isacker (1997) estimated calcium, phosphorus, magnesium, and potassium shoot concentrations, and found no clear correlation between foliar concentrations of these elements and plant growth, but a highly positive correlation between Mg and Zn accumulation in non-metallicolous populations of *N. caerulescens*. In the present study, we investigated all the possible correlations among several nutritional and growth parameters in a large sampling of population to better understand eco-physiology and phytoextraction potential of the species. In Western Europe, and particularly in France, *N. caerulescens* is present in numerous

locations both on metalliferous and non-metalliferous sites (Basic et al. 2006; Molitor et al. 2005; Peer et al. 2006; Reeves et al. 2001). The various populations of *N. caerulescens* are usually classified into three main groups, corresponding to the three contrasting edaphic environments in which they live. Two edaphic groups from metalliferous soils can be distinguished. The calamine group (hereafter CAL) is composed of populations which grow in soils highly enriched with Zn, Cd, and lead (Pb), such as those of mining or smelting sites (Reeves et al. 2001; Escarré et al. 2011). These plants tolerate high amounts of Zn and Cd and hyperaccumulate these metals. The serpentine group (hereafter SERP) corresponds to populations which seem to be adapted to soils naturally enriched with Ni, such as those originating from ultramafic rocks, i.e. mainly rocks with serpentine in Western Europe. These populations are known to tolerate and accumulate higher amounts of Ni than the others (Assunção et al. 2003; Escarré et al. 2013). The last edaphic group corresponds to the populations developing in non-metalliferous soils (hereafter NMET), which have been generally found in mountain areas such as the Ardennes, Jura, Alps and Cévennes (Reeves et al. 2001; Molitor et al. 2005; Basic et al. 2006). However, some NMET populations also develop in the Vosges and Massif Central mountains, but these have yet to be investigated.

This is why this work was designed to characterize the populations of the three groups of *N. caerulescens* on a wider scale than previously undertaken. We assessed for the first time nine new NMET populations of *N. caerulescens* from the Massif Central mountains and one new SERP population from the Alps, thus extending knowledge about the species' abilities to accumulate trace metal and to grow in trace metal-contaminated environments. In particular, our main objectives are:

- To assess phenotypic variability for growth parameters, major nutrient uptake and trace metal accumulation among edaphic groups, among populations and within populations in controlled conditions, on a large number of populations (22) exposed to moderate metal concentrations,
- To search for significant correlations among nutritional traits, among growth traits, and between these two categories of traits,
- To detect those populations with a highest potential for use in Cd phytoextraction.

Materials and methods

Plant material

During the summer of 2011, seeds from three maternal plants (=family) of 22 populations of *N. caerulescens*, were collected by cutting the base of the inflorescence. Seeds were first stored in paper bags at room temperature for 2 months and then at 4 °C. The accessions studied included nine calamine (CAL) and 10 non-metallicolous (NMET) populations from the Massif Central (9) and Luxembourg (1) and three serpentine (SERP) populations from the Massif Central, the Vosges and the Alps (Table 1). Two species were used as controls, namely *Arabidopsis thaliana* (Col-0) and a population of *Noccaea montana* from an ultramafic soil in the Massif Central. *A. thaliana* was chosen because its ionic composition has been extensively studied (e.g. McDowell et al. 2013). *N. montana* was chosen because it belongs to the *Noccaea* genus as a non hyperaccumulating species (mean Ni concentration in shoots in situ: 334 mg kg^{-1}, standard deviation: 226 mg kg^{-1}; C. Gonneau, unpublished data). These controls allowed the toxicity of the nutrient solution to be tested and the hyperaccumulating status of the individuals in the studied populations to be assessed.

Plant cultivation

For each *Noccaea ssp* population, twelve plants (4 plants x 3 families) were randomly distributed in 12 polyethylene trays (400×300×220 mm) so that each tray contained 24 plants (i.e. one plant per population). They were grown hydroponically in a growth chamber for 49 days, each tray containing 17 L of nutrient solution. In each tray, a 2 cm thick polystyrene plate, floating on the nutrient solution was used to support the plants. Each plate contained 24 conical holes filled with 7 gL^{-1} agar as a stand for the seeds and the young seedlings after germination.

The composition of the nutrient solution was adapted from Küpper et al. (2007): 1 mM KNO_3, 1 mM Ca $(NO_3)_2$, 0.5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 50 μM KCl, 20 μM NaFe (III) EDTA, 10 μM H_3BO_3, 10 μM $ZnSO_4$, 1 μM Cd $(NO_3)_2$, 1 μM $MnCl_2$, 0.7 μM $NiSO_4$, 0.2 μM Na_2MoO_4, 0.2 μM $CuSO_4$ in deionized water. The nutrient solution also contained 2 mM 2-(N-morpholino) ethanesulfonic acid (MES) to buffer the pH at 5.5, which was adjusted through the addition of

Table 1 Location and coding of the 22 *Noccaea caerulescens* populations and the two control species (*Arabidopsis thaliana* and *N. montana*) used in the experiments

Species	Edaphic group	Department (except for Luxembourg)	Location	Altitude (m)	Coordinate	Abbreviation
Noccaea caerulescens	CAL	Saône-et-Loire	Auxy	500	46°57′44.13″N 04°23′47.05″E	AUX
		Southern Ardèche	Largentière	264	44°32′26.33″N 04°18′18.44″E	LAR
		Southern Ardèche	Sainte Marguerite	741	44°27′29.13″N 04°00′31.32″E	SML
		Lozère	Ramponenche	698	44°20′15.85″N 03°40′00.31″E	RAM
		Gard	Saint Felix	349	44°02′36.21″N 03°56′11.27″E	SFP
		Gard	Durfort	195	43°59′56.70″N 03°57′08.54″E	DUR
		Gard	Malines	436	43°55′20.67″N 03°37′14.57″E	MAL
		Gard	Ganges	175	43°56′10.98″N 03°40′19.88″E	GA
		Aveyron	Viviez	202	44°33′34.16″N 02°13′01.39″E	VIV
	NMET	Southern Ardèche	Pic de Chenavari	460	44°35′58.80″N 04°41′04.20″E	PC
		Northern Ardèche	Mezilhac	1118	44°48′28.00″N 04°20′44.80″E	MEZ
		Northern Ardèche	Sainte Eulalie	1270	44°48′13.10″N 04°12′47.10″E	SEU
		Haute-loire	L'Herm	1111	44°54′24.20″N 03°49′10.10″E	HER
		Cantal	Nouvialle	1016	45°02′49.00″N 02°56′45.40″E	NOU
		Cantal	Salesses	1132	45°17′44.80″N 02°51′35.20″E	SAL
		Puy-de-Dôme	Chavignée	954	45°23′48.30″N 02°43′59.00″E	CHA
		Puy-de-Dôme	Besse	1036	45°30′25.50″N 02°56′26.30″E	BES
		Puy-de-Dôme	Laschamp	964	45°44′36.00″N 02°58′08.90″E	LAS
		Wiltz (Luxembourg)	Winseler	325	49°57′N 5°53′E	WIN
	SERP	Vosges	Bergenbach	810	47°54′22.90″N 06°57′25.50″E	BER
		Aveyron	Puy de Wolf	472	44°33′23.80″N 02°18′21.07″E	PW
		Hautes-Alpes	Montgenèvre	1860	44°55′33.49″N 06°43′08.02″E	MON
Arabidopsis thaliana	Controls	-	-	-	-	At
Noccaea montana		Northern Ardèche	Clava	586	45°18′39.10″N 04°38′46.00″E	Nm

CAL, Calamine; *NMET*, non-metalliferous; *SERP*, serpentine

20 ml of 0.34 mM KOH. The Cd concentration (1 μM) can be regarded as moderate, although it is higher than the majority of the concentrations of contaminated soil solutions which are generally around 0.1 μM. However, Cd concentrations above 4 μM can be found in highly contaminated soils (Maxted et al. 2007). In addition, the value of 1 μM is a bit above the *Km* value determined through Cd absorption kinetics with *N. caerulescens* (Redjala et al. 2009). The nutrient solution might therefore enable a high (even close to the maximum) root influx, without excessive root exposure and toxicity for the plant.

Six capillary tubes were placed at regular intervals through each tray and sunk into the nutrient solution to allow its oxygenation by bubbling with filtered air. The nutrient solutions were renewed once a week at the beginning of the cultivation, then twice a week. Cadmium concentration in the solution was monitored by sampling and analysing the nutrient solution before and after its renewal (Online Resource, Fig. S1). The photoperiod was set at 16 h day and 8 h night, with a light intensity of 196 μmol photon m^{-2} s^{-1}. The temperature was maintained at 23 °C during the day and 18 °C during the night and the relative humidity was set at 70 %. Twice a week, the trays were translated to the neighbouring position and individually rotated at 180°, in such a way that each of them thus held at least once, each of the positions available. This was done to reduce the effect of environmental differences that may exist in different points of the growth chamber.

Harvest and analyses

At the end of the cultivation, namely 49 days after germination for each plant, the roots were separated from the leaves at the crown. Leaf surface area (LA, cm^2) was measured by scanning using WinRhizo® software. The total number of leaves (NL) and the largest diameter of the rosette (RD, mm) were also measured. Root and leaf dry biomass (RDM and LDM respectively, g) were weighed after drying for 24 h at 70 °C.

The plant material was finely ground using an agate mortar before successive digestion of 0.5 g with 8 ml 65 % HNO_3 for 12 h and 4 ml 30 % H_2O_2 during 3.5 h at 95 °C using the DigiPREP® system (SCP Science, Baie-d'Urfé, QC, Canada). After filtration of the extract at 0.45 μm, the samples were adjusted to 25 mL with ultrapure water (18 MΩ).

Major elements (Ca, Fe, K, Mg, Na) and minor elements (Cd, Cu, Mn, Ni and Zn) in the nutrient solution and plant extracts were determined by induced coupled plasma emission spectrometry (ICP-AES, iCAP 6000 Series, Thermo Scientific, Cambridge, UK). Control material from *N. caerulescens* with known compositions (internal analyses carried out by INRA-USRAVE, Villenave d'Ornon, France), as well as a certified solution (EU-H_2, SCP Science, Courtaboeuf, France) were included in all analyses for quantitative verification of the results. All elements concentrations were expressed in mmol kg^{-1}.

Data processing

The translocation factor was calculated as $[Element]_{shoot}/[Element]_{root}$. The allocation factor corresponded to the ratio of the element quantity in the shoot to its quantity in root of each plant: $([Element]_{shoot}$ x LDM)/$([Element]_{root}$ x RDM).

All statistical analyses were performed using R v2.10.14. Before analysis, all data were log-transformed in order to improve normality and variance homogeneity of residues. Variance analyses for biomass production and element content in *N. caerulescens* were analysed by a hierarchical mixed model analysis using the lmer function inside the lme4 package. For this analysis, the edaphic group was treated as a fixed factor, whereas the population nested within the group and the family nested within the population nested within the group were treated as random factors. The significance of the fixed effect was determined using type III *F*-statistics. Log-likelihood ratios corresponding to comparisons between the likelihoods of the models with and without the random effects were calculated. For significance tests of random effects, log-likelihood ratios were compared to a χ^2 distribution. Then, within each edaphic group, variance components were estimated for each random factor using the restricted maximum likelihood (REML) method. Multiple correlation tests were performed using the Pearson method and FDR control for type I errors.

Results

Growth parameters and their correlations

At the end of cultivation, that is 49 days after seed germination, *N. caerulescens* and *N. montana* were at the rosette stage whereas *A. thaliana* was at the flowering stage which started after 20 days of cultivation. No plants presented any symptoms of toxicity (e.g. chlorosis). Mean values and variation coefficients for LDM, RDM, LDM/RDM, LA, LN and RD at the population level are given in Table S1 (Online Resource).

Only LN and the LDM/RDM ratio were not significantly different between groups (Table 2). Considering LDM and RDM, mean values showed no significant differences between CAL and NMET, whereas SERP exhibited significantly lower mean values by a factor from 1.5 to 1.9 (Fig. 1; Table S1 in Online Resource). As for dry mass, CAL and NMET showed similar mean LN and LA. Unlike LN, LA was significantly lower in SERP populations. Finally, mean RD per group varied from 93.4 mm to 132 mm and was significantly different between groups in the order CAL > NMET > SERP. Among populations within groups, the variation factors for both LDM and RDM reached 2.5, 3 and 2 for CAL, NMET and SERP respectively (Online Resource, Table S1). Among individuals, LDM and RDM ranged respectively from 32 to 860 mg and from 10 to 873 mg. The highest and lowest total biomasses were found respectively in a non metallicolous (BES) population and a serpentine (MON) one (Fig. 1). Populations of LAR and VIV showed the highest LDM and RDM among calamine populations (Fig. 1). Within the CAL and NMET groups, a factor of two was found between maximum and minimum values for LDM/RDM.

Most of the biomass parameters were significantly positively correlated (p=0.05). In order to describe the strongest correlations, only |r| values above 0.57 were

Table 2 Analyses of variance for growth parameters accounted for edaphic group (group), population within group (pop (group)) and family within population within group (fam {pop (group)})

Source	ddl	LDM[a]	RDM[b]	LDM/RDM	LA[c]	LN[d]	RD[e]
group	2	3.49*	7.97***	0.58	3.45*	1.12	4.49*
pop (group)	19	69.1 ***	8.48*	4.50	42.4***	9.50**	10.4**
fam {pop (group)} residues	44	32.3 ***	6.61*	1.78	70.7***	40.2***	35.1***
	187						

[a] Leaf dry matter (g); [b] Root dry matter (g); [c] Leaf area (cm²); [d] Number of leaves from the rosette; [e] Diameter of the rosette (mm)

The table shows test statistics with *F*-ratios for fixed effects and $\chi 2$ for random effects. Group is considered as a fixed factor, whereas pop (group) and fam {pop (group)} are random factors. Significant level: *$p<0.05$; **$p<0.01$; ****$p<0.001$

taken into account. On this basis, Table S2 (Online Resource) shows very strong positive correlations between LDM and LA ($r>0.9$), between RD and LA ($0.63>r>0.76$), and between LDM and RD ($0.59<r<0.73$) whatever the group considered. The LDM/RDM ratio was also strongly negatively correlated ($r<-0.62$) to RDM for NMET and SERP. Lastly, some correlations were observed only in the NMET group, namely RDM, which was positively correlated to LDM and LA ($r=0.66$).

Major element contents and their correlations

Only Ca and Na contents in shoots showed some significant differences between groups (Table 3; Online Resource, Table S3). Indeed, the CAL group had a significantly lower mean shoot concentration and translocation factor of Ca than the NMET and SERP ones, the highest values being observed for the SERP group. A quite similar behaviour was found for Na with the CAL and NMET groups showing significantly lower mean shoot concentrations and translocation factors than the SERP one. At the population level, there were significant differences for all major elements when considering their concentrations in shoots and their translocation factors, except for the shoot Ca concentration (Table 3). However in roots, the population effect was significant only for K. At this level, the variation coefficients for major element concentrations in shoots and in roots were similar for a given element, but differed between elements. These coefficients varied from 7.3 % for Ca to 149.0 % for Na (Online Resource, Table S3). The MON serpentine population clearly showed higher concentrations in major elements than the other populations did, with however a high variation coefficient (Online Resource, Table S3). The family factor caused significant differences in shoots for Ca, Fe, K and Mg but none in roots. The translocation factor of Ca only was significant at the family level (Table 3). In each edaphic group, most of the phenotypic variance was residual (from 36.9 to 85.7 %), particularly

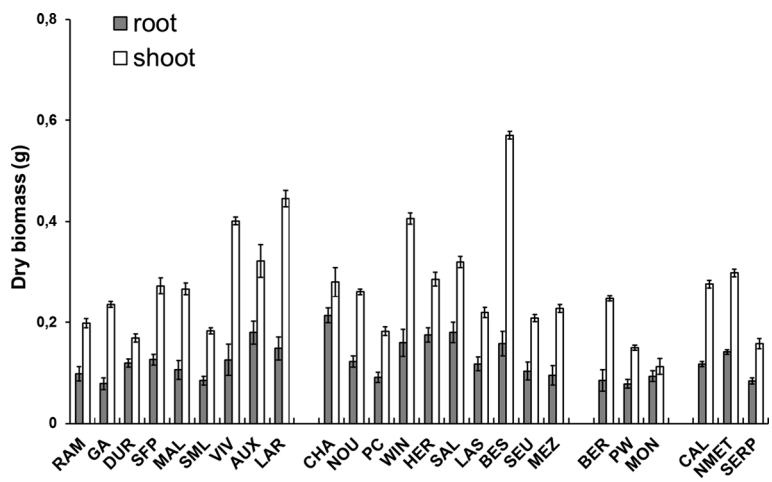

Fig. 1 Roots (*grey bars*) and shoot (*white bars*) mean biomass in 22 populations of *Noccaea caerulescens* after 49 days of hydroponic cultivation. Mean per edaphic group (CAL: calamine; NMET: non metalliferous; SERP: serpentine). *Vertical bars* represent standard errors. For population code, see Table 1

Table 3 Analyses of variance for element concentrations in shoot, in roots and translocation factor accounted for edaphic group (group), population within group (pop (group)) and family within population within group (fam {pop (group)})

		Major elements					Trace elements				
Source	ddl	Ca	Fe	K	Mg	Na	Cd	Cu	Mn	Ni	Zn
Shoot											
group	2	5.00**	1.19	0.839	0.516	4.12*	9.61***	5.31*	20.0***	4.97*	7.65***
pop (group)	19	10.6**	22.1***	62.4***	37.6***	19.0***	114***	61.9***	98.9***	154***	40.1***
fam {pop (group)}	44	5.03*	10.2**	14.2***	11.9***	0.317	10.1**	24.7***	13.5***	28.9***	20.0***
residues	187										
Roots											
group	2	0.318	0.202	0.315	0.401	0.384	7.69***	3.14*	4.60*	3.19*	4.43*
pop (group)	19	0	0.478	3.51*	0.044	0.081	19.9***	7.96*	24.8***	41.4***	60.6***
fam {pop (group)}	44	0	0.485	0.03	0.042	0.071	9.87**	0.298	0	6.34*	2.29
residues	187										
Translocation factor											
group	2	1.38	0.405	0.740	0.584	1.80	1.37	5.68**	2.22	3.14*	6.95**
pop (group)	19	49.3***	3.67*	5.08*	3.79*	6.64*	43.7***	19.2***	11.1**	24.7***	68.5***
fam {pop (group)}	44	44.4***	0	1.49	0.514	2.37	17.9***	0.039	1.10	0.072	2.25
residues	187										

The table shows test statistics with *F*-ratios for fixed effects and $\chi 2$ for random effects. Group is considered as a fixed factor, whereas pop (group) and fam {pop (group)} are random factors. Significant level: *$p<0.05$; **$p<0.01$; ****$p<0.001$

in the SERP group (Table 4). In CAL and NMET groups, the population factor explained 40.3 and 44.4 % of the variability for K, respectively, and 48.7 and 20.1 % for Mg. The family factor was mainly explicative only for Mg in the NMET group (41 %). All major elements, except Na, were positively correlated to each other ($p=0.05$, Table S2 in Online Resource). As previously indicated, to better describe the correlations between the

Table 4 Percentage of total variance of major and trace elements concentrations in shoots for each edaphic group accounted for population within population (pop) and family within population (fam (pop)

Edaphic group	Source	Major elements					Trace elements				
		Ca	Fe	K	Mg	Na	Cd	Cu	Mn	Ni	Zn
CAL	pop	31.0	14.2	40.3	48.7	0	42.3	12.0	44.3	0	1.1
	fam (pop)	11.4	22.6	12.0	0	17.2	8.1	29.7	21.0	41.3	62.1
	residues	57.6	63.1	47.7	51.3	82.8	49.6	58.2	34.7	58.7	36.8
NMET	pop	10.6	22.2	44.4	20.1	25.9	63.9	39.2	41.1	76.9	41.0
	fam (pop)	17.6	3.1	18.7	41.0	0	8.6	15.9	9.9	11.4	0.1
	residues	71.8	74.7	36.9	38.6	74.1	27.5	44.8	49.0	11.7	52.9
SERP	pop	0	0	0	0	25.0	14.3	0	22.0	32.4	0
	fam (pop)	14.3	38.5	29.0	30.1	16.3	27.3	44.4	22.0	6.13	16.0
	residues	85.7	61.5	71.0	69.9	58.7	58.3	55.6	56.0	61.4	84.0

major elements in *N. caerulescens*, only |r| values above 0.57 have been considered. On this basis, Table S2 (Online Resource) showed an increasing number of significant correlations in the order NMET (1:2:2) < CAL (1:5:1) < SERP (9:6:4), with values in parentheses corresponding respectively to the number of correlations for the concentrations of major elements in shoots and in roots and for the translocation factors (shoot/root). The most significant correlations were thus found in roots of the CAL group and in shoots of the SERP group. Moreover, the strength of the correlations decreased in the order SERP > CAL > NMET. For instance the correlation between Ca and Mg was the only one found for all groups and all parameters with the correlation coefficient ranges: 0.75-0.95 for SERP, 0.81-0.83 for CAL and 0.57-0.80 for NMET.

Trace element contents and their correlations

The mean shoot concentrations of Cd exceeded the hyperaccumulation threshold (Van der Ent et al. 2013) in all *N. caerulescens* populations and in *A. thaliana*. Significant differences were observed between groups with increasing mean Cd concentrations as follows (Fig. 2, Table 3, Table S4 in Online Resource): SERP (4.4 mmol kg^{-1}) < NMET (5.7 mmol kg^{-1}) < CAL (11.5 mmol kg^{-1}). A similar pattern was observed for Cd concentrations in roots. Furthermore, Cd concentrations in shoots appeared as a continuum among populations, with six populations from the NMET group showing Cd concentrations close to those of the three lowest CAL populations (Cd range: 6 to 9 mmol kg^{-1}, Fig. 2). The variation factors for Cd concentrations in shoots reached 6.4 among populations and 2.2, 3.2 and 1.4 within groups for CAL, NMET and SERP respectively. The population factor mainly explained a large part of the total variance with 42.3, 63.9 % within CAL and NMET groups respectively, but only 14.3 % in SERP groups since most of the variance was residual in this group (Table 4).

With 0.113 mmol kg^{-1} on average (Online Resource, Table S4), Cu was not accumulated in the shoots of *N. caerulescens*. Considering the new threshold value for Cu hyperaccumulation (4.72 mmol kg^{-1}; Van der Ent et al. 2013), all populations had Cu concentrations in shoots from 30 to 67 times below this level. However, significant differences were found at the group level, the NMET group having Cu concentrations lower in shoots but higher in roots, than the two others (Table 3, Table S4 in Online Resource). For this element, a large part of total variance was residual whatever the group (58.2, 44.8 and 55.6 % for CAL, NMET and SERP respectively). In SERP group, 44.4 % of total variance was nevertheless attributed to families within population, with no variance left for the population factor. Manganese concentrations in shoots and roots showed the same pattern as Cd (Fig. 2, Table S4 in Online Resource). The Mn concentrations in shoots were more than twice as high in the CAL group (4.26 mmol kg^{-1}) than in the NMET (1.53 mmol kg^{-1}) and SERP (2.06 mmol kg^{-1}). The variation factor for Mn concentrations in shoots was similar to that observed for Cd. It reached 6.4 among populations and about 2 within the group, whatever the group considered. Concerning the control *A. thaliana*, Mn shoot concentrations fell between those of CAL and NMET (Fig. 2). For Mn, most of the variance was attributed to populations (from 22.0 to 44.3 %) and/or to residues (from 34.7 to 56.0 %) whatever the group.

No *N. caerulescens* population reached the hyperaccumulation threshold for Ni (17 mmol kg^{-1}) with an average of 1.73 mmol kg^{-1} in the shoots. Significant differences were observed at the group level (Table 3), with increasing Ni concentrations in shoots as follows: CAL (1.13 mmol kg^{-1}) < SERP (1.86 mmol kg^{-1}) ≤ NMET (2.25 mmol kg^{-1}). Thus, three NMET populations showed higher Ni shoot concentrations than PW, a typical SERP population (Fig. 2, Table S4 in Online Resource). Some CAL and other NMET populations also showed similar Ni concentrations to the two SERP populations BER and MON. In the roots, the concentrations of Ni were relatively close for the three groups. However, Ni root concentration was higher for NMET than that of SERP, the CAL group being intermediate (Online Resource, Table S4). For this element, phenotypic variance was mainly residual in CAL and SERP groups (58.7 and 61.4 %, respectively), but was largely due to populations in NMET group (76.9 %).

For Zn concentrations in shoots, twelve populations exceeded the hyperaccumulation threshold (46 mmol kg^{-1}), including nine NMET and two SERP (BER and MON) populations (Fig. 2, Table S4 in Online Resource). Significant differences were observed between groups (Table 3) with increasing mean Zn concentrations in shoots as follows: CAL (38.2 mmol kg^{-1}) < SERP (55.5 mmol kg^{-1}) < NMET (63.7 mmol kg^{-1}). An opposite trend was observed for Zn concentrations in roots, the CAL group having a significantly higher value than those of NMET and SERP, which were close together (Online Resource, Table S4). Phenotypic variance was mainly

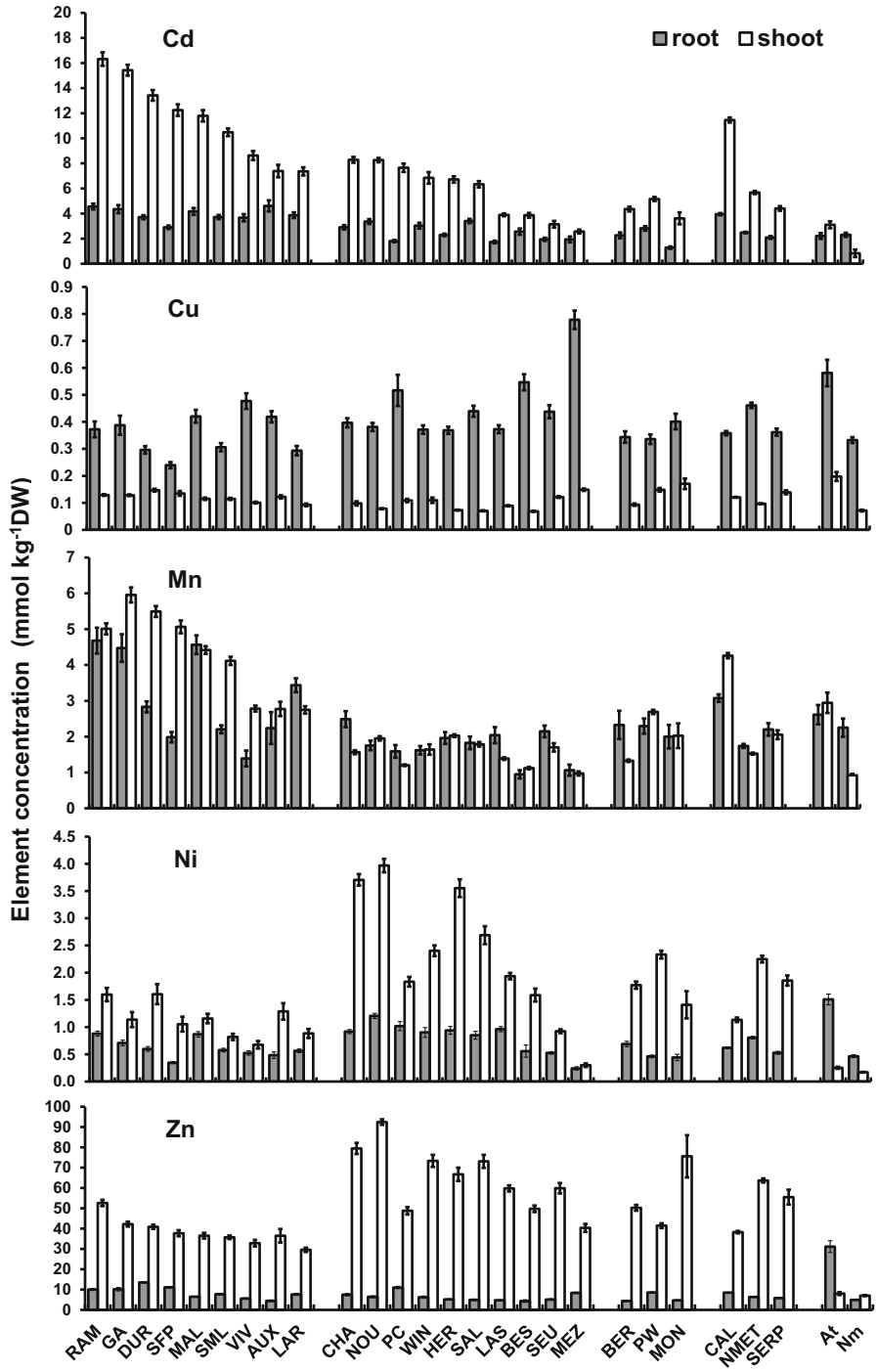

Fig. 2 Cadmium, Cu, Mn, Ni and Zn mean concentrations in roots (*grey bars*) and shoot (*white bars*) in 22 populations of *Noccaea caerulescens* after 49 days of hydroponic cultivation. Mean per edaphic group (*CAL*, calamine; *NMET*, non metalliferous; *SERP*: serpentine) and control plant (At: *Arabidopsis thaliana* Col-0; Nm: *N. montana*). *Vertical bars* represent standard errors. For population code, see Table 1

explained by the population factor for the NMET group (41.0 %), whereas it was largely explained by families within population for the CAL group (62.1 %). For SERP group, most of the variance was residual (84.0 %).

All *N. caerulescens* populations presented Cd, Ni and Zn translocation factors greater than 1, as is consistent with one of the hyperaccumulation criteria (Fig. 2, Table S4 in Online Resource). All populations combined, the mean values reached 3 for Cd and Ni and 13 for Zn. For Cu, an inverse pattern was found with concentrations in roots about three times higher than those in shoots, whereas concentrations in shoots and in roots were close for Mn. Even if *A. thaliana* is not a hyperaccumulator, it exhibited a translocation factor of 2.29 for Cd and < 1 for Zn. Inversely, *N. montana* showed a translocation factor <1 for Cd and of 1.60 for Zn. At the group level, the translocation factors of Cd, Mn and Zn were significantly greater in CAL than in NMET and similar to SERP (Online Resource, Table S4). For Ni, the translocation factor significantly increased from 2.1 to 5.9 in the order CAL < NMET < SERP. In the case of Cu, the lowest translocation factor was found in NMET, while it was similar for CAL and SERP.

Correlations between trace elements varied widely between groups (Online Resource, Table S2). When considering only |r| values above 0.57, there was an increasing number of significant correlations in the order NMET (4:0:0) < CAL (5:0:0) < SERP (9:9:1), with values in parentheses corresponding respectively to the number of correlations for the concentrations of trace elements in shoots and in roots and for the translocation factors. In contrast to major elements, the most significant correlations were always found in shoots, whatever the group. Moreover, as observed with the major elements, the strength of the correlations was higher in the SERP group. This group had a particular behaviour, as all the elements were strongly correlated both in the shoots and in the roots. In the shoots, three strong positive correlations were found for all groups: Cd/Mn ($0.56<r<0.86$), Mn/Ni ($0.58<r<0.88$) and Ni/Zn ($0.63<r<0.69$). Three other weaker correlations were also outstanding: Cd/Ni ($0.46<r<0.85$), Cd/Zn ($0.50<r<0.81$) and Mn/Zn ($0.41<r<0.77$).

Ratios between elements in shoots presented high coefficients of variation at both group (from 37.9 to 47.3 %) and population levels (from 9.3 to 127.0 %; Online Resource, Table S5). All *N. caerulescens* plants showed higher average trace element ratios in shoots than those of the hydroponic solution, except for Zn/Cd ratio which was the same in shoot as in the nutrient solution (Online Resource, Table S5). The CAL group showed lower Zn/Cd and Zn/Mn ratios and higher Cd/Ni and Mn/Ni ratios (by a factor of about four) than SERP and NMET groups, which presented similar values. Only Cd/Mn and Ni/Zn ratios did not vary between groups.

Correlations between growth parameters and element contents

In the principal component analysis performed on all the plant parameters, the first axis explained 23.1 % of the variation; it was positively correlated to Cd and Mn concentrations in shoots and negatively to Zn and Na concentrations in shoots (Fig. 3a). This axis allowed separating populations into two sets: all CAL populations had positive values, whereas all NMET + SERP populations all had negative values (Fig. 3b). The second axis, which opposed dry mass production to major element concentrations (in particular shoot Na and Mg concentrations), explained 19.3 % of the variance (Fig. 3a), and mostly discriminated the MON population from the others due to its low dry mass production. In general, the interpretation of these two first axes showed that growth parameters were negatively correlated to major element uptake but mostly uncorrelated to trace element accumulation. The third axis explained 14.2 % of the variance (data not shown) and was linked to Ni concentrations in shoots and roots. This last axis did not discriminate any set of populations.

When considering the relationships between trace and major elements in the shoots and the biomass production, a general trend in negative correlations was observed, whatever the element and the growth parameter considered (Online Resource, Table S2 and Fig. S2). Although mostly weak, the strength of the correlations was higher for major elements than for trace elements. Only K in CAL and NMET groups and Cu in SERP group were strongly correlated with LDM ($|r|>0.57$). In the roots, the significant correlations between dry mass and element content were also negative. They were stronger in the roots of SERP than in those of the two other groups (Online Resource, Table S2).

Correlations between major and trace elements

The two first axes of the PCA showed that accumulation of major and trace elements were mostly uncorrelated traits (Fig. 3a). However, when looking into detail, these correlations seemed to depend on the group and the plant part (Online Resource, Table S2). As a general trend, a decrease in the strength of correlations was observed

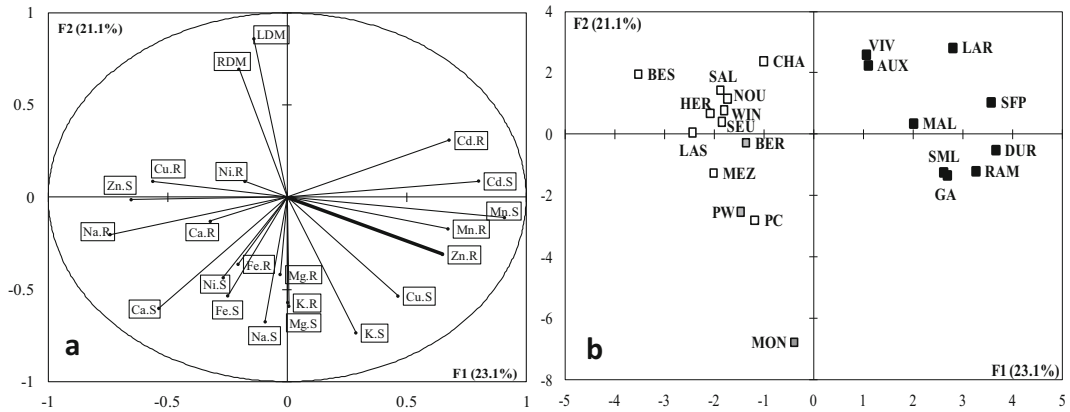

Fig. 3 Principal component analysis on all data including biomass production, elemental concentrations in shoots and in roots: **a** Correlation circle on the axis F1 and F2; **b** distribution of the population according to the correlation circle on axis F1 and F2 with the 22 *Noccaea caerulescens* populations. For population code, see Table 1

whatever the element considered both at the group (SERP > CAL > NMET) and the plant part (shoots > roots) levels. In the SERP group, the trace element concentrations in shoots were strongly positively correlated (i.e. $|r|>0.57$) with those of all the measured major elements except Na. In the CAL group, trace elements (Cd, Cu, Ni, Zn) concentrations in shoots were significantly, positively but weakly correlated with those of Ca, K and Mg (Online Resource, Table S2). Only three close correlations (i.e. $|r|>0.57$) were found in CAL shoots: Mn/Ca, Cu/Fe and Mn/K. In NMET, the only relevant correlation was Zn/Ca. Furthermore, even if the correlations were weak, Fe and Na appeared negatively correlated with Cd, Mn, Ni and Zn, but positively correlated with Cu in the shoots of NMET and CAL groups. A similar pattern was observed in the roots.

Sum and proportions of cations

Sum of cation concentrations in whole plants differed between groups, populations and families and also between the three species (Table 5, Table S6 in Online Resource). Plants from the SERP populations showed a higher cation content than the other two groups, which were not significantly different on this point. The same ranking applied when comparing the major cation contents, while the NMET population appeared to accumulate more trace cations than CAL populations, the SERP ones being intermediate (Online Resource, Table S6).

Major elements represented from 97.5 to 98.0 % of the whole plant cation content in the three edaphic groups. In the shoots, values were slightly lower (96.4 to 97.1 %), because of much lower Fe concentrations than in the roots. The variation of the Ca proportion was compensated by that of K, the proportions of the other major cations (Fe, Mg, Na) being only slightly variable between groups. This was particularly visible in the CAL group, which had the lowest Ca proportion (Online Resource, Table S7).

The proportion of trace elements was very low compared to that of the major elements, as it varied between 1.97 % (SERP) to 2.55 % (NMET) for the whole plant. However, it was higher in *N. caerulescens* than in *A. thaliana* (1.05 %) and *N. montana* (0.66 %).

Quantity of trace elements taken up

The quantities of accumulated Cd, Cu and Mn (concentration x dry mass) decreased significantly in the order

Table 5 Analysis of variance of the sum of cations and percentage of total variance accounted for by sink, edaphic group (group), population within group (pop (group)) and family within population within group (fam {pop (group)})

Source	Sum of cations	Sum of major cations	Sum of trace cations
group	3.28*	4.08*	9.36
pop (eco)	14.3***	13.7**	48.3***
fam {pop (group)} residues	14.1***	4.54*	15.2***

The table showed test statistics with *F*-ratios for fixed effects and $\chi 2$ for random effects. Group is considered as a fixed factor, whereas pop (group) and fam {pop (group)} are random factors. Significant level: *$p<0.05$; **$p<0.01$; ****$p<0.001$

CAL > NMET > SERP, whereas Ni and Zn amounts were significantly higher in the NMET group compared than in CAL and SERP, which had similar values (Fig. 4, Table S8 in Online Resource). Six CAL populations showed the greatest quantities of Cd in shoots. This was explained either by a high biomass production for VIV and LAR populations, or a high shoot concentration for RAM, GA, MAL and SML. Then, similar quantities were observed in the other CAL populations and the six NMET populations with the highest Cd uptake (Fig. c4). These NMET populations also took up the highest Ni and Zn quantities. In contrast, two NMET populations, SEU and MEZ, geographically very close, extracted the lowest quantities for all elements (Online Resource, Table S8). Lastly, two serpentine populations showed similar Ni quantities, but with opposing behaviours i.e. one by producing higher biomass (BER) and the other by a higher shoot Ni concentration (PW).

Discussion

Particular phenotypic characteristics observed in groups and populations

First, our results highlighted specific characteristics of *N. caerulescens* populations from the SERP group. They had lower shoot and root dry mass, and smaller leaf surface area and rosette diameter compared to the two other groups, while CAL and NMET groups were not clearly different in terms of biomass production. SERP populations also exhibited the highest cation contents, while they were similar in the CAL and NMET populations. The strongest differences among groups were the number and the strength of positive correlations between major element contents: the SERP populations clearly showed many strongly positive correlations between major and trace element contents in roots and shoots and also between shoot/root concentration ratios. Such strong correlations between major elements in shoots was also reported by Lee et al. (1977) in the Ni hyperaccumulating plant *Homalium kanaliense* developed on a serpentine soil of New Caledonia. In contrast, the Ni hyperaccumulator *Hybanthus austrocaledonicus* developed on less hostile soils (higher Ca, K and P content) of the same region did not show such correlations. Later, Kazakou et al. (2010) compared the plant composition of 21 species that developed on both serpentine and non-serpentine soils from Lesbos Island (Greece). They showed that serpentine populations exhibited much more strong positive correlations between major and trace elements in shoots than non-serpentine ones. Moreover, in both studies, plants from serpentine soils showed both high K content and Ca/Mg ratio despite the very great deficiency of K and Ca in soils. These characteristics may emphasize the particular homeostasis of SERP populations, which grow in soils with particularly low cation contents and Ca/Mg ratios (Brady et al. 2005; Kazakou et al. 2008). It can be hypothesised that these plants have adapted to nutritionally poor environments by increasing their cation uptake, allocating more energy to cation absorption (see below) and Mg detoxification, the excess of this element being toxic in serpentine soils (Kruckeberg 1954; Walker 1954; Proctor 1971). As it is well established that the energy cost of ion uptake is high (Tinker and Nye 2000), the supplementary uptake of cations could cause a trade-off with plant growth. This is also consistent with the study of Adamidis et al. (2014) on the variation of leaf traits of 17 species, all of them occurring on both serpentine and non-serpentine soils. They determined that non-serpentine populations are associated with a resource exploitative strategy where plants tend to acquire resources rapidly with high relative growth and photosynthesis rates whereas serpentine populations are associated with a resource conservative strategy where plants tend to invest more resources to structural compounds and have a low relative growth. In addition, Cd concentration in roots and shoots clearly discriminated the groups, as it increased according the following order: SERP < NMET < CAL. This result was also found in previous studies, but with higher Cd concentrations in solutions (Roosens et al. 2003). However, if Cd shoot concentrations were clearly higher in the CAL group than in the two other groups, this was not true for all the CAL populations. Indeed, only few of them showed remarkably high Cd concentrations: the Ganges (GA) population and the other geographically close populations (RAM, DUR, SFP, MAL, SML). More distant CAL populations from Ganges (VIV, AUX, LAR) accumulated Cd at the same level as the NMET did. These results confirm the specific trait of *N. caerulescens* from the Ganges region, i.e. the highest ability to accumulate Cd, which had already been observed (Escarré et al. 2000; Escarré et al. 2013; Roosens et al. 2003). It is interesting to note that manganese contents varied in a similar way to that of Cd, the

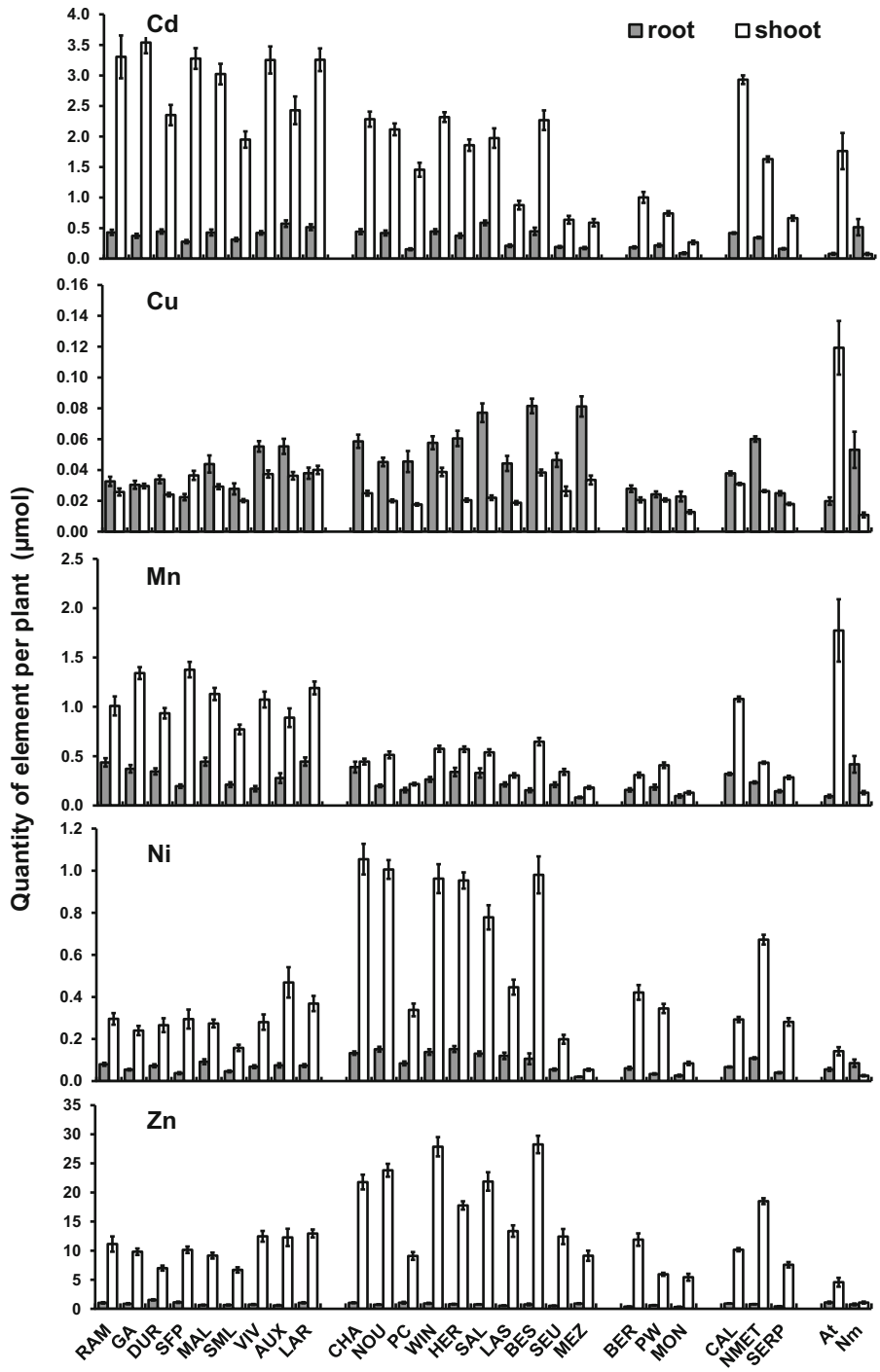

Fig. 4 Cadmium, Cu, Mn, Ni and Zn mean quantities in roots (*grey bars*) and shoot (*white bars*) in 22 populations of *Noccaea caerulescens* after 49 days of hydroponic cultivation. Mean per edaphic group (*CAL*, calamine; *NMET*, non metalliferous; *SERP*, serpentine) and control plant (At: *Arabidopsis thaliana* Col-0; Nm: *N. montana*). *Vertical bars* represent standard errors. For population code, see Table 1

CAL populations showing much higher Mn contents than the NMET and the SERP ones.

The NMET populations showed the highest Ni contents and were followed by the SERP and CAL

populations, even though no population exceeded the Ni hyperaccumulation threshold (17 mmol kg^{-1}), probably because of a too low Ni content in the nutrient solution (0.7 μM). Ni hyperaccumulation by serpentine soils populations is well known (Assuncão et al. 2008; Escarré et al. 2013), but this work shows for the first time that NMET populations can accumulate more Ni than the SERP ones. This is a surprising result, as in the field (Reeves et al. 2001; Visioli et al. 2011) and in controlled conditions (soil and hydroponic cultivations) (Assunção et al. 2003; Escarré et al. 2013), SERP populations have always shown the highest Ni shoots concentrations. Assuncão et al. (2008) studied the effect of different ratios between Cd, Ni and Zn in hydroponics solutions on shoot trace element accumulation in four populations including two CAL and one NMET and SERP; they showed that Cd and Zn in solution inhibited shoots' Ni accumulation in each of three edaphic group, with the exception of the calamine population from La Calamine (Belgium). These results suggest that root absorption is controlled by a low-affinity transport system with Cd and Zn preference over Ni. It seems that in our results, the competition between Cd, Zn and Ni for root absorption is weaker in the NMET populations than in the two other groups.

Zinc content also enabled a clear discrimination between the three groups: CAL populations had lower Zn contents in shoots than SERP and NMET did, while the opposite was true for roots. A majority of the NMET populations exceeded the recently suggested hyperaccumulation threshold (3 000 mg kg^{-1}, i.e. 46 mmol kg^{-1}) (Van der Ent et al. 2013), as did two serpentines populations. These results were consistent with the high shoot concentrations measured in situ, mainly in NMET accessions growing in uncontaminated soils, with low Zn concentrations in soil solution (Basic et al. 2006).

Correlations between major and trace elements

According to the results presented in Meerts and Van Isacker (1997), major elements concentrations were poorly correlated to trace elements concentrations in *N. caerulescens*. However, these authors did not examine serpentine populations, which, in our study, displayed the most remarkable characteristics. Indeed, in the SERP group, as indicated above, trace element concentrations in shoots were strongly positively correlated with those of all the major elements measured, except Na. It is not conceivable that the different cations are taken up by a unique poorly selective transporter. Therefore, these correlations suggest that SERP populations demonstrate a stronger sink for all cations, although they have a more or less specific transport system for each of them. This sink could result from an increase in the activity of proton pumps, particularly of the plasma membrane bound H^+-ATPase, which is responsible for the electrochemical gradient which drives root absorption and the vacuolization of most cations. Other ATPases are involved in cation uptake, such as HMA4, which controls the loading of xylem with trace metals (Verbruggen et al. 2009).

In CAL group, trace elements (Cd, Cu, Ni, Zn) concentrations in shoots were weakly correlated with those of Ca, K and Mg (Online Resource, Table S2). Only three close correlations were found in CAL shoots: Mn *vs* Ca, Cu *vs* Fe and Mn *vs* K. These indicate that, apart the specific transport system (e.g. ZIP, CAX, NRAMP transport systems), the trace metals might enter *N. caerulescens* taking the route of several major cations, which might be more or less specific according to the population.

In NMET, the only relevant correlation was Zn *vs* Ca, which suggests that Zn could take the Ca pathway to accumulate in the plant (Cheng et al. 2002; Cheng et al. 2005). The correlation between shoot Mg and Zn accumulation pointed in Meerts and Van Isacker (1997) was significant but quite weak in our study (r= 0.367). Furthermore, even if the correlations were weak, Fe and Na were negatively correlated with Cd, Mn, Ni and Zn, which indicates that transporter(s) of Fe and Zn would also carry the listed trace elements, but with a lower affinity.

Sources of phenotypic variability

Our results showed a wide variability in biomass production and elemental composition among *N. caerulescens* plants. Nevertheless, most of the variability was attributed to residues and thus remained unexplained. This was particularly true for major elements (except for Mg and K concentrations in CAL and NMET groups). The same pattern was found for trace elements in the SERP group. On the contrary, in CAL and NMET groups, the population level was often a significant source of variability. An interesting exception was for Zn hyperaccumulation in CAL group, in which the responses of the different populations seemed

almost uniform. All these results suggest that in NMET populations, local selective pressures may act on (mainly trace) element shoot concentrations, creating strong among-population differentiation but low within-population variability. Among CAL populations, some shared selective pressures seem to widely act on Zn hyperaccumulation levels, creating low among-population differentiation but locally leaving the possibility of substantial within-population genetic variability.

Which populations for phytoextraction?

N. caerulescens is one of the plants which could be used for phytoextraction of Cd in moderately contaminated soils (Schwartz et al. 2003; Zhao et al. 2003; Maxted et al. 2007; Koopmans et al. 2008). To this end and based on the results obtained here, the CAL populations are the best candidates as they should extract more Cd than the others due to their concentrations and/or biomasses being among the highest. More particularly, the Ganges population, which shows Cd contents among the highest, also has the advantage of being highly tolerant of Cd and Zn (Assunção et al. 2003), thus allowing its use in more toxic soils. However, the use of NMET populations in phytoremediation strategy should not be excluded, as some of them can extract large amounts of Zn, while others can accumulate more Ni than the SERP populations. Some NMET populations can simultaneously extract significant amounts of Cd, Ni and Zn and this could be an advantage in case of polymetallic soil contamination, with the purpose of economic metal valorisation (phytomining). Moreover, NMET populations might be cultivated in climate and soil conditions to which the CAL populations from Southern France would not be well-adapted. However, as shown by Escarré et al. (2013), hydroponic cultivation might give different results to soil cultivation. As a consequence, the results found here have to be verified in soils. Noteworthy, it seems that SERP populations are not suitable for phytoextraction since they exhibited low biomass production and largely unexplained source of genetic variation. .

Finally, our results showed weak ($r<0.25$) but negative correlations between shoot or root biomass and cation contents. Consequently, they indicate a trend towards a trade-off between biomass production and cation accumulation, which can be explained by the energetic cost of cation uptake (see above). This trade-off can be viewed as an obstacle in the search for naturally "high trace-metal- enriched biomass" genotypes. However, the fact that these correlations are weak and often non-significant for Cd, Ni and Zn in the shoots of the CAL and NMET populations indicates possibilities for associating by crossbreeding a high biomass yield with a high accumulation ability in a given cultivar.

Conclusions

Hydroponics enabled a characterisation of the biomass production and the cationic composition of 22 populations of *N. caerulescens*, with a realistic and controlled root exposure to trace metals. Our results suggest a wide genetic variability of the species, favouring their adaptation to specific environments. The populations from ultramafic soils showed particular features (low biomass, high major and trace element contents, strong correlations between all elements), suggesting a homeostasis adapted to their nutrient-poor environment. Calamine populations showed adaptive abilities (high biomass, high Cd and Mn accumulation) to their metal-contaminated environment. In particular, the strong accumulation of Cd of some populations from the calamine soils, particularly in the area of Ganges, confirms the potential of *N. caerulescens* for the production of phytoextraction cultivars. Non-metallicolous populations also showed interesting abilities (high biomass, high Ni and Zn accumulation) mostly related to each local condition. Our study showed for the first time, that the populations from non-metalliferous soils accumulated more Ni than those from Ni naturally enriched soils, when moderately exposed to this metal. Finally, the high genotypic variability of the species and the weak correlation between biomass production and Cd, Ni or Zn accumulation could be used to combine those traits favourable to the remediation of contaminated soils in a given cultivar.

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References

Adamidis GC, Kazakou E, Fyllas NM, Dimitrakopoulos PG (2014) Species adaptive strategies and leaf economic relationships across serpentine and Non-serpentine habitats on Lesbos, eastern Mediterranean. PLoS One 9:e96034. doi:10.1371/journal.pone.0096034

Assunção AGL, Bookum WM, Nelissen HJ et al (2003) Differential metal-specific tolerance and accumulation patterns among Thlaspi caerulescens populations originating from different soil types. New Phytol 159:411–419

Assuncão AGL, Bleeker P, Wilma M et al (2008) Intraspecific variation of metal preference patterns for hyperaccumulation in Thlaspi caerulescens: evidence from binary metal exposures. Plant Soil 303:289–299

Basic N, Keller C, Fontanillas P et al (2006) Cadmium hyperaccumulation and reproductive traits in natural Thlaspi caerulescens populations. Plant Biol Stuttg 8:64–72

Brady KU, Kruckeberg AR, Bradshaw HD Jr (2005) Evolutionary ecology of plant adaptation to serpentine soils. Annu Rev Ecol Evol Syst 36:243–266

Chardot V, Echevarria G, Gury M et al (2007) Nickel bioavailability in an ultramafic toposequence in the Vosges mountains (France). Plant Soil 293:7–21

Cheng S-H, Willmann MR, Chen H-C, Sheen J (2002) Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. Plant Physiol 129:469–485. doi:10.1104/pp. 005645

Cheng N-H, Pittman JK, Shigaki T et al (2005) Functional association of Arabidopsis CAX1 and CAX3 is required for normal growth and ion homeostasis. Plant Physiol 138:2048–2060

Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212:475–486

Dechamps C, Roosens NH, Hotte C, Meerts P (2005) Growth and mineral element composition in two ecotypes of Thlaspi caerulescens on Cd contaminated soil. Plant Soil 273:327–335

Escarré J, Lefèbvre C, Gruber W et al (2000) Zinc and cadmium hyperaccumulation by Thlaspi caerulescens from metalliferous and nonmetalliferous sites in the Mediterranean area: implications for phytoremediation. New Phytol 145:429–437

Escarré J, Lefèbvre C, Raboyeau S et al (2011) Heavy metal concentration survey in soils and plants of the Les Malines mining district (Southern France): implications for soil restoration. Water Air Soil Pollut 216:485–504. doi:10.1007/s11270-010-0547-1

Escarré J, Lefèbvre C, Frérot H, et al. (2013) Metal concentration and metal mass of metallicolous, non metallicolous and serpentine Noccaea caerulescens populations, cultivated in different growth media. Plant Soil 1–25. doi: 10.1007/s11104-013-1618-z

Frérot H, Lefèbvre C, Petit C, Collin C, Dos Santos A, Escarré J (2005) Zinc tolerance and hyperaccumulation in F1 and F2 offspring from intra and interecotype crosses of Thlaspi caerulescens. New Phytol 165:111–119

Halimaa P, Lin Y-F, Ahonen VH et al (2014) Gene expression differences between Noccaea caerulescens ecotypes help to identify candidate genes for metal phytoremediation. Environ Sci Technol 48:3344–3353. doi:10.1021/es4042995

Hanikenne M, Nouet C (2011) Metal hyperaccumulation and hypertolerance: a model for plant evolutionary genomics. Curr Opin Plant Biol 14:252–259

Kazakou E, Dimitrakopoulos PG, Baker AJM et al (2008) Hypotheses, mechanisms and trade-offs of tolerance and adaptation to serpentine soils: from species to ecosystem level. Biol Rev 83:495–508

Kazakou E, Adamidis GC, Baker AJM et al (2010) Species adaptation in serpentine soils in Lesbos Island (Greece): metal hyperaccumulation and tolerance. Plant Soil 332:369–385. doi:10.1007/s11104-010-0302-9

Keller C, Diallo S, Cosio C et al (2006) Cadmium tolerance and hyperaccumulation by Thlaspi caerulescens populations grown in hydroponics are related to plant uptake characteristics in the field. Funct Plant Biol 33:673–684

Koopmans GF, Römkens P, Fokkema MJ et al (2008) Feasibility of phytoextraction to remediate cadmium and zinc contaminated soils. Environ Pollut 156:905–914

Kruckeberg AR (1954) The ecology of serpentine soils. III. Plant species in relation to serpentine soils. Ecology 267–274.

Küpper H, Parameswaran A, Leitenmaier B et al (2007) Cadmium-induced inhibition of photosynthesis and long-term acclimation to cadmium stress in the hyperaccumulator Thlaspi caerulescens. New Phytol 175:655–674

Lee J, Brooks RR, Reeves RD et al (1977) Plant-soil relationships in a new Caledonian serpentine flora. Plant Soil 46:675–680

Maxted AP, Black CR, West HM et al (2007) Phytoextraction of cadmium and zinc from arable soils amended with sewage sludge using Thlaspi caerulescens: development of a predictive model. Environ Pollut 150:363–372. doi:10.1016/j.envpol.2007.01.021

McDowell SC, Akmakjian G, Sladek C, Mendoza-Cozatl D, Morrissey JB, Saini N, Mittler R, Baxter I, Salt DE, Ward JM, Schroeder JI, Guerinot ML, Harper JF (2013) Elemental concentrations in the seed of mutants and natural variants of Arabidopsis thaliana grown under varying soil conditions. Plos One 8. doi:10.1371/journal.pone.0063014

Meerts P, Van Isacker N (1997) Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of Thlaspi caerulescens from continental Europe. Plant Ecol 133:221–231

Milner MJ, Mitani-Ueno N, Yamaji N, et al. (2014) Root and shoot transcriptome analysis of two ecotypes of Noccaea caerulescens uncovers the role of NcNramp1 in Cd hyperaccumulation. Plant J n/a–n/a. doi: 10.1111/tpj.12480

Molitor M, Dechamps C, Gruber W, Meerts P (2005) Thlaspi caerulescens on nonmetalliferous soil in Luxembourg: ecological niche and genetic variation in mineral element composition. New Phytol 165:503–512

Peer WA, Mahmoudian M, Freeman JL, Lahner B, Richards EL, Reeves RD, Murphy AS, Salt DE (2006) Assessment of plants from the Brassicaceae family as genetic models for the study of nickel and zinc hyperaccumulation. New Phytol 172:248–260

Proctor J (1971) The plant ecology of serpentine: II. Plant response to serpentine soils. The Journal of Ecology 59:397. doi:10.2307/2258320

Redjala T, Sterckeman T, Morel JL (2009) Cadmium uptake by roots: contribution of apoplast and of high-and low-affinity membrane transport systems. Environ Exp Bot 67:235–242

Reeves RD, Schwartz C, Morel JL, Edmondson J (2001) Distribution and metal-accumulating behavior of Thlaspi caerulescens and associated metallophytes in France. Int J Phytoremediation 3:145–172

Roosens N, Verbruggen N, Meerts P et al (2003) Natural variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of Thlaspi caerulescens from western Europe. Plant Cell Environ 26: 1657–1672

Schwartz C, Echevarria G, Morel JL (2003) Phytoextraction of cadmium with Thlaspi caerulescens. Plant Soil 249:27–35. doi:10.1023/A:1022584220411

Tinker PB, Nye PH (2000) Solute movement in the rhizosphere. Oxford University Press

Van de Mortel JE, Almar Villanueva L, Schat H et al (2006) Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of Arabidopsis thaliana and the related metal Hyperaccumulator Thlaspi caerulescens. Plant Physiol 142: 1127–1147. doi:10.1104/pp. 106.082073

Van de Mortel JE, Schat H, Moerland PD et al (2008) Expression differences for genes involved in lignin, glutathione and sulphate metabolism in response to cadmium in Arabidopsis thaliana and the related Zn/Cd-hyperaccumulator Thlaspi caerulescens. Plant Cell Environ 31:301–324. doi:10.1111/j.1365-3040.2007.01764.x

Van der Ent A, Baker AJ, Reeves RD et al (2013) Hyperaccumulators of metal and metalloid trace elements: facts and fiction. Plant Soil 1–16:319–334

Verbruggen N, Hermans C, Schat H (2009) Molecular mechanisms of metal hyperaccumulation in plants. New Phytol 181:759–776

Visioli G, Vincenzi S, Marmiroli M, Marmiroli N (2011) Correlation between phenotype and proteome in the Ni hyperaccumulator Noccaea caerulescens subsp. caerulescens. Environ. Exp. Bot.

Walker RB (1954) The ecology of serpentine soils. II. Factors affecting plant growth on serpentine soils. Ecology 259–266.

Zhao FJ, Lombi E, McGrath SP (2003) Assessing the potential for zinc and cadmium phytoremediation with the hyperaccumulator Thlaspi caerulescens. Plant Soil 249:37–43

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REGULAR ARTICLE

Metal concentration and metal mass of metallicolous, non metallicolous and serpentine *Noccaea caerulescens* populations, cultivated in different growth media

J. Escarré · C. Lefèbvre · H. Frérot · S. Mahieu · N. Noret

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Abstract
Aims Evaluate the genetic and environmental variability of metal concentration and metal mass of *Noccaea caerulescens*, from metalliferous (MET), non metalliferous (NMET) and serpentine (SERP) soils.
Methods 18 populations were cultivated in 18 different growth conditions, such as a soil mine tailing, soils amended with zinc (Zn), cadmium (Cd) and nickel (Ni) salts (in mixtures or in monometallic salts) and a hydroponic solution with two Zn concentrations.
Results MET populations had Zn concentrations lower than NMET and SERP in the different soils but higher Cd mass (the product of aerial biomass and foliar metal concentration). SERP had the highest Ni concentration and Ni mass values. The addition of Cd or Ni to a Zn-contaminated soil significantly decreases Zn concentration. In hydroponics, MET and NMET had equivalent Zn concentrations but these were three times higher than those obtained in soil experiments. Zn mass of NMET was significantly lower than MET with the latter having Zn mass values largely above those obtained in mine soil.
Conclusions Results showed a large heterogeneity of responses among populations depending on the substrate used, and it was not possible to correctly assign a single population to its accurate origin with only one experiment. Finally, data on metal concentration obtained in culture soils are closer to those in field soils than those from hydroponics so that they could give a more accurate information on the accumulating capacity of *Noccaea caerulescens* and its use in phytoextraction of metals in field conditions.

Keywords *Thlaspi caerulescens* · Mine soil · Plant populations · Phytoremediation · Zn/Cd/Ni hyperaccumulation · Metal tolerance

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J. Escarré (✉) · S. Mahieu
Centre d'Ecologie Fonctionnelle et Evolutive
(CNRS) – UMR 5175,
Route de Mende 1919,
34293 Montpellier Cedex 05, France
e-mail: jose.escarre@cefe.cnrs.fr

C. Lefèbvre · N. Noret
Laboratoire d'Ecologie végétale et Biogéochimie,
Université Libre de Bruxelles,
ULB-Campus de la Plaine, CP244. Boulevard du Triomphe,
1050 Bruxelles, Belgium

H. Frérot
Laboratoire de Génétique et Evolution des Populations
Végétales, UMR CNRS 8198, Université Lille 1,
Bâtiment SN2,
59655 Villeneuve d'Ascq Cedex, France

Introduction

Natural soils with high metal concentrations, such as nickel-rich ultramafic soils or anthropogenic metal-contaminated soils from the mining industry, are the theatre of evolutionary processes resulting in genetic and physiological adaptations that enable organisms to

colonise these extreme environments (Antonovics et al. 1971). Most plant species growing on these sites show a tolerance to high metal concentrations due to a restriction of metal transport to the aerial tissues; however a limited number of these species, called hyperaccumulators, are able to accumulate metals at very high concentrations (e.g. 1 % of Zn) in aerial dry parts (Baker and Walker 1990; van der Ent et al. 2012).

Among these species, *Noccaea* (formerly *Thlaspi*) *caerulescens,* a Brassicaceae that hyperaccumulates Zn, Cd and Ni, has been widely studied (Assunção et al. 2003b). The species shows a large climatic and geographical range in Europe. Populations are found from the lowlands up to 2000 m (Alpine climate) and from the North of Spain to Scandinavia (Tutin et al. 1964–1993).

N. caerulescens grows on soils contaminated by heavy metals (Zn, Cd, Pb), on serpentine soils (Ni) and is also present on non-contaminated soils (Reeves et al. 2001; Meyer 2006). Studies comparing plants from metal-contaminated soils (metallicolous populations, MET) and non-contaminated soils (non-metallicolous populations, NMET) showed that NMET plants often have a lower biomass than those in metal-contaminated soils. Interestingly, NMET individuals usually accumulate more Zn than MET plants when grown in the same soil (e.g. Meerts and Van Isacker 1997; Escarré et al. 2000; Meerts et al. 2003; Dechamps et al. 2007). Serpentine populations (SERP), that have been less studied, can also accumulate Zn even with low extractable soil Zn concentrations (Peer et al. 2003).

Despite the high number of papers concerning *N. caerulescens,* most experimental studies investigating metal concentration patterns have compared a very low number (usually 1 or 2) of MET populations (with the exception of Peer et al. 2003 and Roosens et al. 2003). Most experiments were performed with nutrient solutions (e.g. Brown et al. 1995; Assunção et al. 2003a) or with non-contaminated soils supplemented with metal salts (e.g. Meerts et al. 2003). Only a very few number included soils from contaminated sites (e.g. Meerts and Van Isacker 1997; Escarré et al. 2000; Lombi et al. 2001a). In addition, experimental studies comparing MET with NMET populations simultaneously are scarce and were mostly conducted by Belgian and French teams (Meerts and Van Isacker 1997; Escarré et al. 2000; Meerts et al. 2003; Noret et al. 2005, 2007; Dechamps et al. 2007, 2008b), and even less common are studies that compared populations from metal-contaminated and serpentine soils (Peer et al. 2003; Roosens et al. 2003). Only two studies (Assunção et al. 2003a; 2008) compared one population of each of the three origins. However, when only one population per origin is used, generalisation is hazardous as population replicates are missing. To our knowledge there are no published experiments comparing several populations of plants from each origin, i.e. non-contaminated, metal-contaminated and serpentine soils.

If *N. caerulescens* must be used for the phytoremediation of moderate contaminated soils (Zhao et al. 2003), it would be necessary to conduct a large screening process to evaluate the among-population variability of metal accumulation with different metal concentrations and growth media, including contaminated soils from the field.

Here, several experiments are presented that compare leaf metal concentrations and metal masses (leaf metal concentration times aerial biomass, i.e. the amount of metal in the aerial parts) of *N. caerulescens* plants from the three origins that were cultivated in different substrata.

The following questions were addressed:

1) Do metallicolous, non-metallicolous and serpentine populations exhibit contrasting patterns of biomass, metal concentration and metal mass?
2) Do these differences vary according the characteristics of culture media, of metallic salts and of the composition of their mixtures?
3) Is there evidence of interactions among metals, particularly between Zn and Cd or between Zn and Ni?

Material and methods

Plant and soil material

N. caerulescens seeds were collected from 7 metalliferous sites, from 2 serpentine soils and from 9 non-metalliferous soils in Belgium, the Grand Duchy of Luxembourg and France (Table 1). In each population, seeds from 20–30 flowering plants were collected. To analyse the extractable Zn, Cd, Pb and Ni concentrations of soil, bulk samples were air-dried, ground and the <2 mm fractions were separated from coarse rock

Table 1 Characteristics of metallicolous, non-metallicolous and serpentine sites of *Noccaea caerulescens* populations. Soil concentrations are expressed as $mgkg^{-1}$ ammonium acetate-EDTA extractable element. ABBR: Abbreviations of localities. Abbreviations of treatments: AS (Les Avinières soil), BM (Binary metallic salt mixtures), MS (Monometallic salts), HY (Hydroponics)

	Localities	ABBR.	Treatments	Altitude (m)	Latitude (N)	Longitude (E)	Soil Zn	Soil Ni	Soil Cd	Environment
Metallicolous	Avinières mine	AV	AS-BM-MS-HY	168	43°55′56″	003°39′58″	29279[a]	22	360[a]	Calcareous waste grassland
	Durfort mine	DU	AS-BM	203	43°59′59″	003°57′08″	19348	<10	69	calcareous waste grassland
	Prayon smelter	PR	AS-BM	160	50°35′03″	005°40′24″	16510(2)	<10[b]	429[b]	limestone and shale grassland
	St Felix mine	SF	AS-BM-MS-HY	337	44°01′57″	003°56′21″	8051	<10	36	calcareous waste grassland
	Treves mine	TR	AS-BM-MS	648	44°04′17″	003°23″50"	6887	<10	41	calcareous waste grassland
	Vernissiere mine	VE	AS-BM	266	43°59′53″	003°56′24″	5716	<10	28	calcareous waste shrubland
	Viviez smelter	VI	AS-BM	262	44°33′07″	002°13′21″	3098–99500[c]	–	28–578[c]	micaschist grassland
Non metallicolous	Baraquette	BA	AS-BM-MS-HY	745	43°55″06″	003°36′57″	60	<10	<10	calcareous shrubland
	Buege	BU	AS-BM	120	43°50′27″	003°40′18″	–	–	–	calcareous shrubland
	Navacelles	NA	AS-BM	610	43°53′15″	003°30′31″	18	<10	<10	calcareous shrubland
	Ranquas	RA	AS-BM	669	43°50′14″	003°31′58″	–	–	–	calcareous shrubland
	St Baudille	SB	AS-BM	805	43°44′45″	003°29′12″	59	<10	<10	calcareous shrubland
	St Come	SC	AS-BM-MS	982	44°18′14″	003°23′27″	114	<10	<10	calcareous shrubland
	St Michel	SM	AS-BM	691	43°52′00″	003°25′41″	50	<10	<10	calcareous shrubland
	Seranne	SE	AS-BM-MS-HY	902	43°52′11″	003°38′30″	<10	<10	<10	calcareous shrubland
	Wilwerwitz	WI	AS-BM	350	49°59′04″	005°59′54″	15(2)	<10[b]	<10[b]	shale grassland
Serpentine	Bergenbach	BE	AS-BM-MS	829	47°54′39	006°57′15″	25	116[d]	<10	serpentine grassland
	Puy wolf[e]	PW	AS-BM-MS	479	44°32′56″	002°18′34″	69	371[e]	<10	serpentine shrubland

[a] (Frérot et al. 2006) Total Zn 126126±17620 mg/kg; total Cd 899±200 n=3 (Escarré et al. 2011)

[b] (Dechamps et al. 2008a,b; Molitor et al. 2005)

[c] Total Zn concentration (Reeves et al. 2001)

[d] Total Ni concentration: 146 to 2500 mg/kg (Chardot et al. 2007)

[e] Total Ni concentration:784–1984 mg/kg (Reeves et al. 2001)

fragments by sieving. All chemical analyses were performed on the <2 mm soil fractions. Mineral elements were extracted with ammonium acetate-EDTA 1 N (pH4.65) for 30 min (10 g dry soil in 50 ml of extractant) (Cottenie et al. 1982). The supernatant was filtered and analysed by inductively-coupled plasma optical emission spectroscopy (ICP-OES; Varian Vista MPX). This method is known to extract the 'labile' and 'less labile' pools of trace elements (Fangueiro et al. 2005; Labanowski et al. 2008), i.e. the mineral fraction potentially available to plants via root absorption. In previous experiments, this fraction showed a good correlation with the concentrations of Zn and Cd in the aerial tissues of *Noccaea caerulescens* (Robinson et al. 1998). Extractable Ni concentrations were analysed only for the ultramafic soils from Bergenbach and Puy de Wolf. A sampling of the other soils from Southern France was also analysed but the Ni EDTA concentrations were all lower than 10 mg/kg.

Plant culture in soils

Germinated seedlings were transferred into 0.5 L containers and assigned to different treatments for 3 months (from May to July) in a glasshouse with natural day: night regime and watered with distilled water at the CEFE–CNRS experimental field Station in Montpellier (France). Containers were completely randomised in the glasshouse.

The following treatments were used:

1) Contaminated soil.

   This was collected in a tailing pond at Les Avinières mine. The soil had high metal content (Table 1), low organic matter and a shortage of major plant nutrients. It was mixed with commercial compost (90 % soil-10 % compost) to facilitate plant growth. Initially, 10 plants per population were used, but the mortality in some populations reduced the number of surviving plants at the end of the experiment. Only plant Zn and Cd concentrations and mass were analysed because the Ni concentration values in this soil are very low. We used Les Avinières soil because in previous experiments (Escarré et al. 2000) it allowed to discriminate MET and NMET origins with low mortality. In addition it is a "true soil" used as a control to compare with the other treatments.

2) Garden soil with metallic (Zn, Cd, Ni) salts added.

   a) BINARY MIXTURES. The soil was a mixture of commercial garden compost and soil from the CEFE-CNRS experimental station with a very low organic C content (3.8 %) and a low C:N ratio (16) (see Escarré et al. 2000 for other characteristics of this soil). Zn was supplied to soil as $ZnSO_4.7H_2O$ and mixed with two salts ($CdSO_4$ or $NiCl_2.6H_2O$) in six treatments: 1) 250 mg/kg Zn – 100 mg/kg Cd (hereafter 250Zn100Cd; id for the following treatments); 2) 100 mg/kg Zn – 250 mg/kg Cd; 3) 250 mg/kg Zn – 250 mg/kg Cd; 4) 100 mg/kg Zn – 250 mg/kg Ni; 5) 250 mg/kg Zn – 250 mg/kg Ni; 6) 250 mg/kg Zn – 100 mg/kg Ni .

   b) MONOMETALLIC ZN, CD AND NI SALTS. Different Zn, Cd and Ni salts with contrasted solubility in water (20 °C) were used: 250 and 1500 mg/kg $ZnSO_4.7H_2O$ (solubility 96.5 g/100 ml); 1500 mg/kg ZnO (solubility 0.00016 g/100 ml); 250 and 500 mg/kg $CdSO_4$ (solubility 76.4 g/100 ml); 500 mg/kg $CdCl_2$ (solubility 135 g/100 ml); 250 and 1000 mg/kg $NiCl_2.6H_2O$ (solubility 254 g/100 ml); 1000 mg/kg $NiSO_4.6H_2O$ (solubility 65 g/100 ml).

Five plants per population and per treatment were used in the experiments with metallic salts.

Soil metal concentrations of the mixtures were relatively low compared to those of soils from Les Avinières mine (Table 1) and in the range of ultramafic soils for Ni (Table 1). Our goal was to allow survival and optimal growth for populations from the three origins. Nevertheless, six of nine treatments with monometallic salts (Zn, Cd and Ni) had very high concentrations of metals with the purpose to test the tolerance of populations and the aerial metal concentration of the plants from three origins in extreme soil conditions.

Plant culture in hydroponics

Individuals of four populations (2 MET: AV, SF; 2 NMET: BA, SE) were randomly placed into 3.9 L containers with a capacity for 16 plants, in a growth room with artificial light, as detailed in Garnier (1992), and then filled in with a nutritive solution

described by Koch et al. (1987). The photoperiod was set at 16:8 h (day:night), the air temperature was 22:18 °C and the relative humidity was maintained above 60 %. The Photosynthetically Active Radiation flux (PAR) at seedling height was 515±8 μmol photons $m^{-2}s^{-1}$ (n=100). The solution was renewed twice a week, the pH was adjusted to 5.5 with HCl every 2 days, and the containers were periodically moved to homogenize light distribution. Nine containers received 1.5 μM of zinc, and nine received 2000 μM ($ZnSO_4.7H_2O$ form). Plants were harvested at 7, 20, 40, 60, 80, 100 and 125 days after transplanting. The number of plants at each harvest was variable depending on the mortality. Two or three plants were collected per population and treatment at the first harvest, and five for the other harvests, while for the last harvest all survivors were collected (4–7 individuals) for a total of 265 plants. At each harvest, only one plant per population and per container was taken at random and the containers were also randomly selected.

Plant analysis

Aerial biomass of all plants grown in soil was harvested after 3 months, rinsed in deionised water and dried at 60 °C for 3d. Due to the very large number of elemental analyses, only a subset of MET and NMET populations were analysed for Cd and Ni in the experiments with salt mixtures and monometallic salts, whereas the two SERP were consistently analysed (Table 1). Some plants with too low biomass were excluded from Cd/Ni analyses. Dried samples were ground and a subset was mineralised in a mixture of nitric and perchloric acid with a Tecator digestor, and their individual cadmium and nickel contents determined by ICP-OES (Varian Vista MPX). The rest of the ground material was used to measure the individual Zn concentration by the zincon method (see hereafter).

The totality of individuals grown in soil and in hydroponics was analysed for Zn concentrations with the Zincon method developed for *Arabidopsis halleri* (Macnair and Smirnoff 1999). This method is based on UV-visible spectrophotometry using zincon as a coloured Zn-chelating agent and is less expensive than ICP. The Zincon method allows the analysis of individuals with low biomass. This method has been previously validated with *Noccaea caerulescens* (Frérot et al. 2005).

Metal mass was calculated as the product of metal concentration, times the above-ground biomass.

Despite the fact that metal mass is concentration-dependent, unlike biomass, this measure was also selected because it provides a good idea of the potential of each *Noccaea caerulescens* population to extract metals from soil.

Statistical methods

First, dry aerial metal concentrations and metal masses were analysed for all soil treatments by 6 three-way partially nested ANOVAs (SAS 2004) with the following factors: origins (MET, NMET and SERP), treatments (10 for Zn, 7 for Cd and 6 for Ni) and populations nested within origins and treatments. The population factor was considered as random. Type III sums of squares and the Satterthwaite approximation were used. Means were compared using least square means tests. These analyses were made to compare both the mean values in the different treatments and the performances of the three origins.

Secondly, because of the complex interactions between populations and treatments (see results), each treatment was also analysed independently by a nested ANOVA with the three origins and the populations nested within the origins. Population was considered as random. The ANOVAs for metal concentrations and masses were as follows: i) one for the experiment with the mine substrate, ii) 6 for each of the treatments with Zn binary salt mixtures (3 with Cd and 3 with Ni), and iii) 9 for the monometallic salt treatments (3 for each metal).

Lastly, differences in metal concentrations or metal masses among salt mixtures and monometallic salts for a population were performed by one-way ANOVAs followed by *a posteriori* contrasts.

Data from hydroponics were analysed independently because the experimental conditions were not the same as for the other experiments. Zn concentration and mass were analysed by partially-nested ANOVAs with the following factors and their interactions: dates (7), origins (3), treatments (2) and populations (4) nested within origins. Values were log-transformed prior to statistical analyses. Therefore, the results of the ANOVAs are given for log-transformed values, but means (±SE) are in arithmetical values for the sake of clarity.

Survival differences between origins and treatments were tested using Fisher's exact test (Statistix 2003).

Results

Comparisons among soil treatments and origins (populations nested within origin and treatment)

a) *Biomass and survival.* Plant cultivated only with Cd or Ni monometallic salts had lower biomass (Fig. 1) than those grown with mixtures (ANOVA's Treatment effect significant). In most treatments MET had higher biomass than NMET and SERP (ANOVA's Origin effect significant). The latter had on average the lowest biomass (see the three histograms at the right end of the graph). The only exception was in the 250$ZnSO_4$ treatment and in the treatments with Ni monometallic salts where SERP biomass was equivalent to that of MET individuals.

As for biomass, survival values (Table 2) were lowest for plants grown without Zn but with Cd salts (including 500 $CdCl_2$: 43 % survival) and to a lesser extent with Ni salts of (1000 $NiSO_4$: 63 % survival). Overall survival of plants growing with Zn salts (in mixtures or with monometallic salts) was significantly higher (97.6 % 645/660) than plants without Zn salts (69.6 % 167/240). There was no difference in survival between origins for mixtures. On the other hand NM survival (66.6 % 90/135) was significantly lower than that of MET (79.2 % 90/135) and that of the SERP (82.2 % 74/90) when plants were cultivated in Zn, Cd and Ni monometallic salts. Survivals of individuals grown in Les Avinières soil was 76 % (137/180) similar to that of monometallic salts but significantly lower than that of mixtures. There were no significant survival differences among origins in this soil.

b) *Zinc*. Plants cultivated in monometallic salts or in Les Avinières soil had the highest Zn concentration values per plant (significant treatment effect, capital letters above bars in Fig. 2a) and plants from Zn-Cd mixtures had the lowest Zn concentration values (Fig. 2a). Overall, plants from MET origin had the lowest mean Zn concentration (MEAN, last three bars at the right of each graph) but a high Zn mean mass (with NMET) because the high biomass of MET individuals compensated for the low Zn concentration. SERP individuals had overall the highest Zn concentration and the

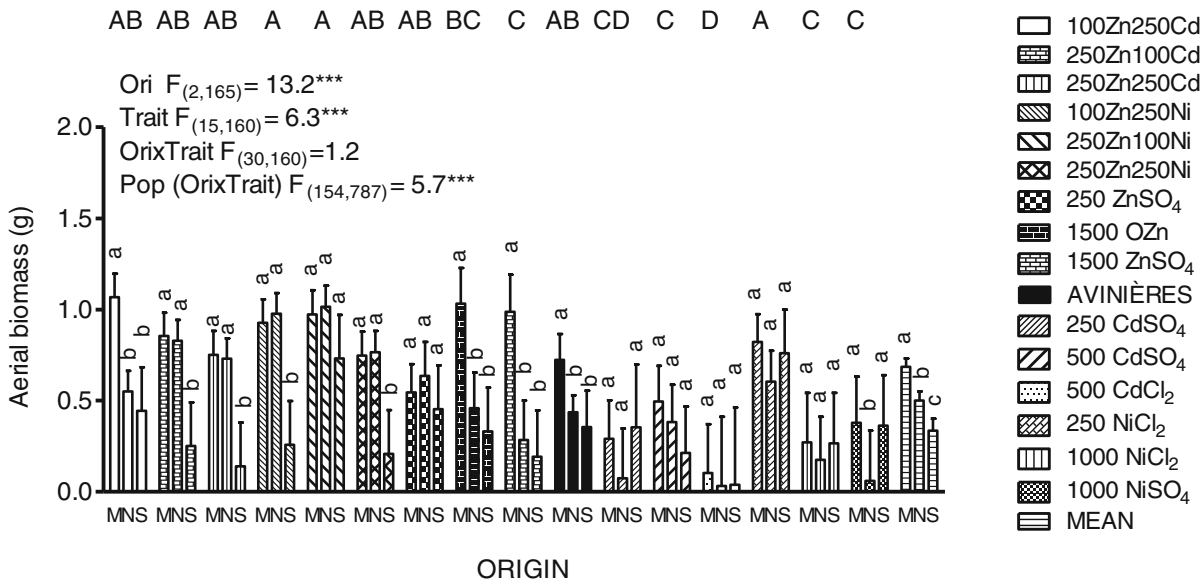


Fig. 1 Mean (±SE) aerial biomass value per individual of metallicolous (M), non-metallicolous (N) and serpentine (S) *Noccaea caerulescens* plants cultivated in a garden soil contaminated with different metal concentrations and in Les Avinières mine soil. In binary mixtures, Zn was provided as $ZnSO_4$, Cd as $CdSO_4$ and Ni as $NiCl_2$. In monometallic salts, Zn was provided as $ZnSO_4$ and ZnO, Cd as $CdSO_4$ and $CdCl_2$ and Ni as $NiCl_2$ and $NiSO_4$. For each treatment, bars topped with the same lower letter are not significantly different with a least squares means test (SAS). Results of the mixed nested ANOVA including all treatments are shown. Treatments with the same capital letter are not significantly different with a Tukey test (SAS). Means for origins across treatments are shown as the last three bars

Table 2 Survival (percentage number of living individuals/total number) of metallicolous, non-metallicolous and serpentine populations of *Noccaea caerulescens* cultivated in 3 Zn/Cd and 3 Zn/Ni salt mixtures, in Zn, Cd and Ni monometallic salts and in a contaminated soil from the tailing basins of Les Avinières mine. For salt mixtures and monometallic salt treatments, survival values with the same letter are not significantly different with a Fisher's exact test

Survival (%)											
Salt mixtures											
	100Zn250Cd	250Zn100Cd	250Zn250Cd	100Zn250Ni	250Zn100Ni	250Zn250Ni				AVINIÈRES SOIL	
Metallicolous	97 (34/35)	100 (35/35)	97 (34/35)	100 (35/35)	100 (35/35)	97 (34/35)				73 (51/70)	
Non-metallicolous	97 (44/45)	97 (44/45)	100 (45/45)	100 (45/45)	97 (44/45)	95 (43/45)				80 (71/90)	
Serpentine	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)				75 (15/20)	
Total	98 (88/90) a	99 (89/90) a	99 (89/90) a	100 (90/90) a	99 (89/90) a	97 (87/90) a				76 (137/180) b	
Monometallic salts											
	250$ZnSO_4$	1500$ZnSO_4$	1500ZnO	250$CdSO_4$	500$CdSO_4$	500$CdCl_2$	250$NiCl_2$	1000$NiCl_2$	1000$NiSO_4$		
Metallicolous	93 (14/15)	93 (14/15)	100 (15/15)	60 (9/15)	100 (15/15)	53 (8/15)	100 (15/15)	53 (8/15)	60 (9/15)		
Non-metallicolous	86 (13/15)	86 (13/15)	100 (15/15)	40 (6/15)	93 (14/15)	26 (4/15)	100 (15/15)	73 (11/15)	53 (8/15)		
Serpentine	100 (10/10)	90 (9/10)	100 (10/10)	50 (5/10)	90 (9/10)	50 (5/10)	100 (10/10)	80 (8/10)	80 (8/10)		
Total	93 (37/40) a	90 (36/40) a	100 (40/40) a	50 (20/40) bc	95 (38/40) a	43 (17/40) c	100 (40/40) a	68 (27/40) b	63 (25/40) bc		

lowest Zn mass due to their small biomass, particularly in some Zn-Cd treatments and Cd monometallic salts, probably due to the low Cd tolerance of these populations. NMET individuals had intermediate Zn mean concentration. In the 1500 $ZnSO_4$ treatment (the most soluble salt) plants from SERP had values above 1,0000 mg Zn/kg (the admitted threshold for Zn hyperaccumulation).

Zn concentrations were significantly lower in metallic salt mixtures (Zn-Cd or Zn-Ni) than in monometallic salts (250$ZnSO_4$) at equivalent Zn concentration (250 mg/kg) (Fig. 2a). Zn mass was significantly lower for Zn-Cd (but not for Zn-Ni) mixtures (250Zn100Cd and 250Zn250Cd), than in monometallic salts (Fig. 2b).

c) *Cadmium*. The highest Cd concentrations were measured in plants grown with monometallic Cd salts (Fig. 2c), whereas plants grown in Les Avinières soil had the lowest Cd concentrations and Cd mass (Fig. 2c, d). No differences among the origins were found for Cd concentration, but MET populations had a significantly higher Cd mass overall than the others. In all treatments and origins Cd concentrations were above the Cd hyperaccumulation threshold.

Cd concentrations (but not Cd mass) were significantly lower in salt mixtures (100Zn250Cd and 250Zn250Cd) than in monometallic salt (250 $CdSO_4$) at equivalent Cd concentrations (250 mg/kg) (Fig. 2c, d). Even at high Cd concentrations (especially in 500 mg/kg $CdCl_2$) Cd mass was very low because Cd (particularly in the most soluble salt $CdCl_2$) decreased biomass and therefore Cd mass.

d) *Nickel.* Plants cultivated with the highest concentration of Ni monometallic salts (1,000 $NiCl_2$ or 1000 $NiSO_4$) had the highest Ni concentrations, and those with Zn-Ni binary mixtures had the lowest (Fig. 2e). Overall, SERP populations had significantly higher mean Ni concentrations and masss; while MET had the lowest concentrations but similar masses as NMET (Fig. 2e, f). In 1000 mg Ni/kg treatments, SERP populations had mean Ni concentration values above the Ni hyperaccumulation threshold (1000 mg Ni/kg plant dry mass).

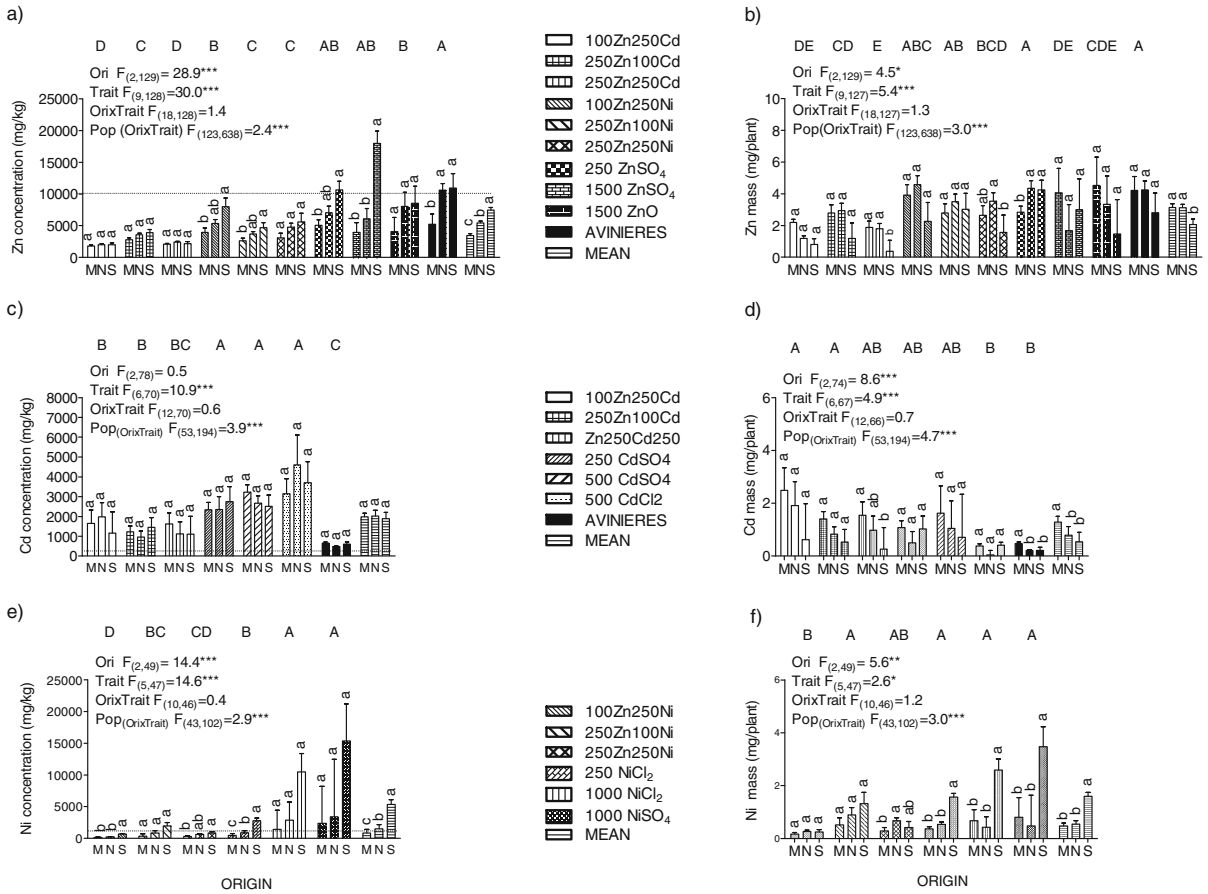


Fig. 2 Mean (±SE) Zn (**a**), Cd (**c**) and Ni (**e**) concentrations (mg/kg) and mean (±SE) Zn (**b**), Cd (**d**) and Ni (**f**) masses (mg/plant) per individual in *Noccaea caerulescens* aerial parts of metallicolous (M), non-metallicolous (N) and serpentine (S) *Noccaea caerulescens* plants cultivated in a garden soil contaminated with different metal concentrations and in Les Avinières mine soil. In binary mixtures, Zn was provided as $ZnSO_4$, Cd as $CdSO_4$ and Ni as $NiCl_2$. In monometallic salts, Zn was provided as $ZnSO_4$ and ZnO, Cd as $CdSO_4$ and $CdCl_2$ and Ni as $NiCl_2$ and $NiSO_4$. For each treatment, bars topped with the same lower letter are not significantly different with a least squares means test (SAS). Treatments with the same capital letter are not significantly different with a least squares means test (SAS). Means for origins across treatments are shown as the last three bars in each graph. Hyperaccumulation thresholds for Zn (**a**), Cd (**c**) and Ni (**e**) are indicated with a *dotted horizontal line*. Results of the mixed nested ANOVA including all treatments are shown

The comparison of Ni concentration and mass between Zn-Ni mixtures and monometallic salts ($250NiCl_2$) at the same soil Ni concentration (250 mg/kg) did show a significant decrease in Ni concentration but not in Ni mass (Fig. 2e, f).

In all analyses there was a significant interaction effect for Pop (Ori x Trait), indicating that metal concentration and metal mass among populations within origins and treatments were heterogeneous. For this reason, data were reanalysed treatment by treatment.

Comparisons for each soil treatment (populations nested within origin)

Biomass, survival, Zn and Cd concentration and mass in Les Avinières soil (Fig. 3)

Overall, MET populations (but Prayon) had high mean biomass values (Fig. 3e). NM and SERP had lower but similar values. The two SERP populations had very different biomass with BE having high and PW having low values (similar to PR and WI). Survival was very

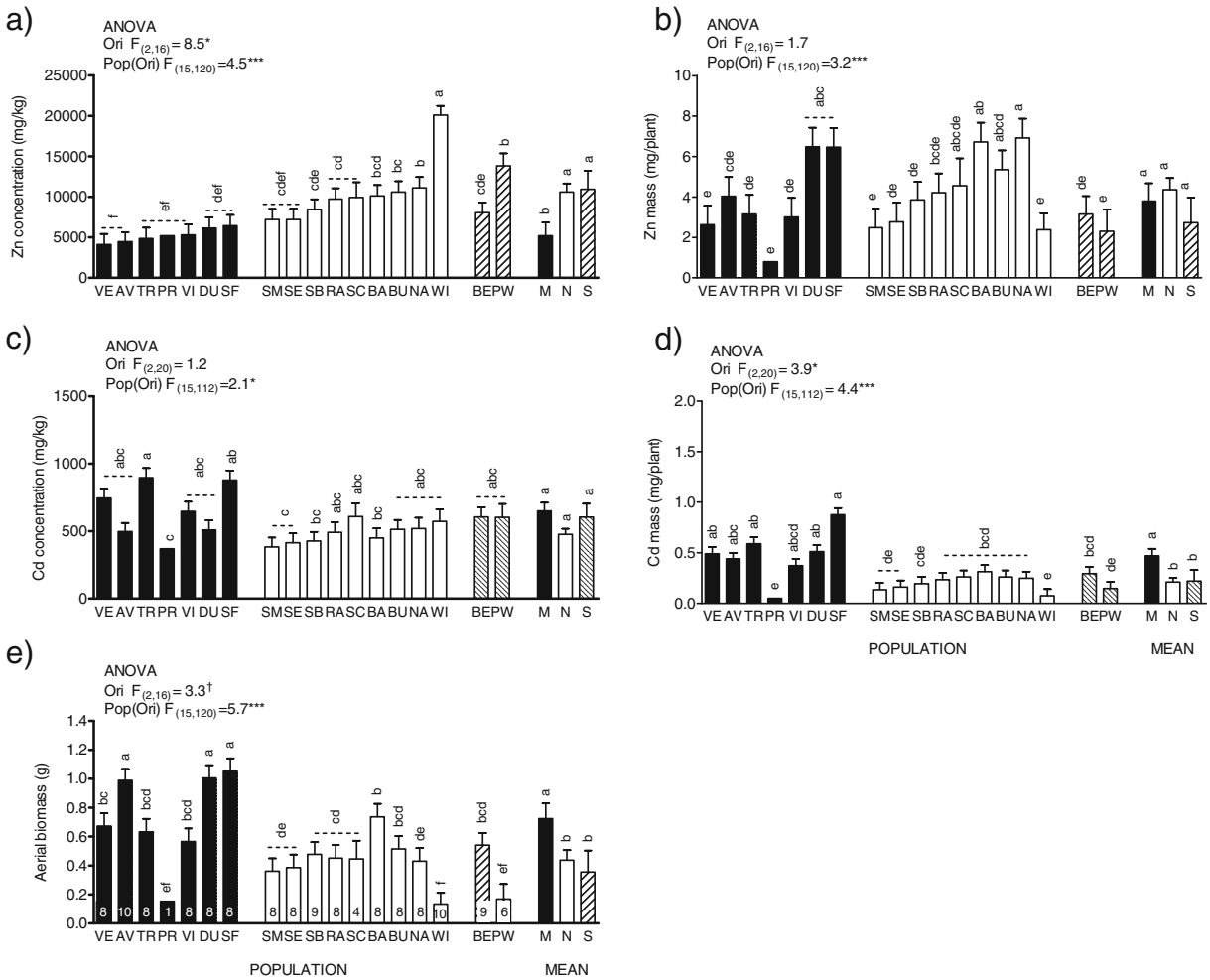


Fig. 3 Mean (±SE) Zn (**a**) and Cd (**b**) concentration (mg/kg), mean (±SE) Zn (**c**) and Cd (**d**) mass (mg/plant) and mean (±SE) biomass (g) (**e**) per individual of metallicolous (*black bars*), non-metallicolous (*white bars*) and serpentine (*hatched bars*) populations of *Noccaea caerulescens* cultivated in Les Avinières soil. For population names please see Table 1. Population means with the same letter are not significantly different with a least squares means test (SAS). *M* metallicolous, *N* non-metallicolous, *S* serpentine. Results of the nested ANOVA are shown

low for PR (1/10), SC (4/10) and PW (6/10). Surprisingly, all WI individuals were alive despite their low biomass.

MET populations had Zn concentrations lower than NMET and SERP (significant differences among origins). There was a high heterogeneity within origins: individuals from WI had two times higher zinc concentrations than the other NMET populations and the PW (SERP) accumulated 80 % more Zn than BE (SERP). There were no differences among the origins for Zn mass (Fig. 3c), but the northern populations PR (MET), WI (NMET) and PW (SERP) had the lowest zinc mass (due to low biomass), and the southern populations DU and SF (MET), NA and BA (NMET) had the highest [significant population (origin)].

There were no significant differences among origins for Cd concentration (Fig. 3b), but a great heterogeneity occurred, particularly among MET populations [significant population (origin)]. Overall, MET populations had higher Cd masses than NMET or SERP (Fig. 3d). However, there was a high heterogeneity within MET, with SF showing the highest values and the northern PR the lowest.

Biomass, survival, Zn concentration and Zn mass in binary mixtures and in Zn monometallic salts (Table 3)

In 4 of 9 treatments there were significant differences among origins for plant biomass with MET having higher values than NMET and SERP. In 5 of 6 mixture treatments, NM populations had the highest biomass (exception: MET PR in the 100Zn250Cd). This highlights the high tolerance of NM to moderate levels of metals. In 6 of 9 treatments, there were significant differences among populations. This shows that within origins population respond differently to metals. Survival of the different populations was very high (see also Table 2) in mixtures as in treatments with monometallic salts (two of which had very high concentrations of Zn). Despite that SERP had low biomass there was no mortality in any treatment.

Zn salt mixtures (Zn-Cd and Zn-Ni) generated contrasting responses among populations for Zn concentration and mass (Table 3a). Thus, for the three Zn-Cd treatments there was only one significant difference ($p<0.10$) in the Zn concentration among origins and among populations (250Zn100Cd). The mixture with 250Zn250Cd showed significant differences for zinc mass among origins (SERP had the lowest values, see also Fig. 2b) and among populations, with the northern populations WI (NMET) and PW (SERP) having the lowest Zn mass values (Table 3b).

Out of the three Zn-Ni treatments, two (100Zn250Ni and 250Zn100Ni) showed significant Zn concentration differences among origins (Table 3a) with SERP showing high values (Table 3b & Fig. 2a). However, all of the Zn-Ni treatments had a significant population (origin) effect for Zn concentration and Zn mass. Unexpectedly, some populations had contrasted differences in Zn mass for the same soil Zn concentration (250 mg/kg). For instance, WI (NMET) had the highest Zn mass values in the 250Zn250Ni treatment (7.3 mg/plant) and the lowest in the 250Zn100Ni treatment (0.7 mg/plant).

The two zinc sulphate treatments (monometallic salts) showed significant differences for Zn concentration among the origins (Table 3a) with the two SERP populations having the highest values (Table 3b). There were large variations for some populations among the three treatments with monometallic salts (e.g. Zn concentration values among BE (SERP) individuals did vary from 4,000 to nearly 20000 mg/kg), and the treatment with the lowest Zn concentration in the soil (250 mg/kg) in other populations (BA, TR, SF) gave the highest values of Zn concentration in plants. Both 1500 Zn treatments showed significant differences among populations, particularly for the two SERP populations. Thus, PW had a higher Zn concentration than BE for zinc oxide, but had the lowest zinc mass. However, for zinc sulphate at 1500 mg/kg, BE had the highest Zn concentration.

Previously (see Fig. 2a), it was shown that Zn concentrations were significantly lower in metallic salt mixtures (Zn-Cd or Zn-Ni) than in monometallic salts ($250ZnSO_4$) at equivalent Zn concentrations (250 mg/kg). We checked whether this occurred in the 8 populations tested or if only some populations showed such a decrease. The one-way ANOVAs followed by *a posteriori* contrasts showed that the three MET populations (AV, SF and TR), two NMET (BA and SC) and the two SERP (BE and PW) populations had significantly ($p<0.05$) lower Zn concentrations values in the Zn-Cd mixtures compared to monometallic salts. Only one NMET (SE) showed non-significant Zn concentration differences ($p>0.05$). For the Zn-Ni TR (MET), BA and SC (NMET) and PW (SERP) showed a significant ($p<0.05$) decrease of Zn concentration values in mixtures, whereas the other populations AV, SF (MET), SE (NMET) and BE (SERP) did not show any significant differences.

The results of the treatments with Zn salts clearly showed that the highest Zn concentrations and masses were achieved by different populations in different treatments (Table 3b). Thus, the maximum Zn concentration in each treatment was achieved by 6 different populations (4 NMET and the 2 SERP), and the maximum Zn mass in each treatment was achieved by 8 different populations (2 MET, 4 NMET, 1 SERP) (Table 3).

Biomass, survival, Cd concentration and Cd mass in binary mixtures and monometallic salts (Table 4)

There were no significant biomass differences among origins for any of the treatments, but for all treatments, there were significant differences among populations within origin. Some populations had an important mortality at high Cd concentrations. This was the case of SF (MET), BA and SC (NMET) and PW (SERP). The latter population had only one survivor in the 500 $CdCl_2$ treatment.

Table 3 Results of nested ANOVAs for aerial biomass, Zn concentration and Zn mass (a) and means (±SE) for aerial biomass (g) (bold), Zn concentration (mg/kg) (normal) and Zn shoot mass (mg/plant) (*italics*) per individual (b) of metallicolous, non-metallicolous **(bold)** and serpentine (*italics*) populations of *Noccaea caerulescens* cultivated in six Zn salt mixtures ($ZnSO_4$) and in three Zn monometallic salts ($ZnSO_4$, ZnO). For each salt treatment, population means with the same letter (**bold for biomass,** normal for Zn concentration and *italics for Zn mass*) are not significantly different with a least squares means test (SAS). Maximal biomass, Zn concentration and mass values for each treatment are underlined. Sampling number is enclosed in parentheses after the mean aerial biomass

	Zinc salt mixtures						Zinc monometallic salts		
	100Zn 250Cd	250Zn 100Cd	250Zn 250Cd	100Zn 250Ni	250Zn 100Ni	250Zn 250Ni	250$ZnSO_4$	1500$ZnSO_4$	1500ZnO
a ANOVAS									
Shoot biomass	**Ori $F_{2,15}$=3.1†**	**Ori $F_{2,15}$ =1.6 ns**	**Ori $F_{2,15}$=4.0***	**Ori $F_{2,15}$=4.2***	**Ori $F_{2,15}$=0.1 ns**	**Ori $F_{2,15}$=5.7***	**Ori $F_{2,5}$=0.8 ns**	**Ori $F_{2,5}$ =1.0 ns**	**Ori $F_{2,5}$=1.2 ns**
	Pop(Ori) $F_{15,70}$=1.6 ns	**Pop(Ori) $F_{5,70}$=4.6*****	**Pop(Ori) $F_{15,70}$=4.7*****	**Pop(Ori) $F_{15,72}$=3.2****	**Pop(Ori) $F_{15,68}$=6.6****	**Pop(Ori) $F_{15,67}$=2.4****	**Pop(Ori) $F_{5,28}$=1.7 ns**	**Pop(Ori) $F_{5,28}$=8.9 *****	**Pop(Ori) $F_{5,32}$=16.9*****
Shoot zinc concentration	Ori $F_{2,15}$=1.2 ns	Ori $F_{2,15}$=2.9†	Ori $F_{2,15}$=1.6 ns	Ori $F_{2,15}$=3.7*	Ori $F_{2,15}$=3.1†	Ori $F_{2,15}$=2.0 ns	Ori $F_{2,5}$=6.8*	Ori $F_{2,5}$=7.4*	Ori $F_{2,5}$=1.1 ns
	Pop(Ori) $F_{5,70}$=1.3 ns	Pop(Ori) $F_{15,70}$=1.7†	Pop(Ori) $F_{15,70}$=1.0 ns	Pop(Ori) $F_{15,72}$=3.5**	Pop(Ori) $F_{15,68}$=5.2**	Pop(Ori) $F_{15,67}$=2.0*	Pop(Ori) $F_{5,28}$=0.7 ns	Pop(Ori) $F_{5,28}$=2.2†	Pop(Ori) $F_{5,32}$ =6.5***
Shoot zinc mass	*Ori $F_{2,15}$= 0.9 ns*	*Ori $F_{2,15}$= 1.3 ns*	*Ori $F_{2,15}$= 3.8**	*Ori $F_{2,15}$= 1.7 ns*	*Ori $F_{2,15}$= 0.7 ns*	*Ori $F_{2,15}$= 2.6†*	*Ori $F_{2,5}$= 3.2†*	*Ori $F_{2,5}$= 0.5 ns*	*Ori $F_{2,5}$= 0.8 ns*
	Pop(Ori) $F_{15,70}$= 1.4 ns	*Pop(Ori) $F_{15,70}$= 3.1*****	*Pop(Ori) $F_{15,70}$= 2.5***	*Pop(Ori) $F_{15,72}$= 1.9**	*Pop(Ori) $F_{15,68}$= 5.1*****	*Pop(Ori) $F_{15,67}$= 1.6†*	*Pop(Ori) $F_{5,28}$= 0.7 ns*	*Pop(Ori) $F_{5,28}$= 8.3*****	*Pop(Ori) $F_{5,32}$= 9.2*****
b **Aerial biomass (g)**/Zn Concentration (mg/kg)/*Zn MASS (mg/plant)*									
Avinières (AV)	**0.90±0.34ab (5)**	**1.34±0.14 a (5)**	**0.61±0.23 abc (4)**	**1.08±0.24 a (5)**	**0.78±0.11 ab (5)**	**0.38±0.21 abcd (4)**	**0.76±0.09 a (5)**	**1.58±0.23 a (5)**	**1.29±0.26 a (5)**
	1289±384 a	2804±300 ab	1763±673 a	4934±926 defg	3428±122 cdef	3987±224 bcd	4429±966 b	4615±299 cd	4701±756 bc
	1.6±0.7 a	*3.7±0.4 abc*	*1.6±1 abc*	*5.3±1.4 ab*	*3.0±0.2 abc*	*2.1±1.7 b*	*3.6±0.7 a*	***7.2±0.9 a***	*6.3±1.6 a*
Durfort (DU)	**1.04±0.42 ab (5)**	**1.19±0.26 abc (5)**	**0.71±0.15 abc (5)**	**0.81±0.17 ab (5)**	**1.10±0.16 ab (5)**	**0.71±0.22 abcd (5)**	–	–	–
	1455±306 a	2813±575 ab	2178±249 a	6165±659 cd	3725±289 bcde	3052±851 cde			
	1.8±0.8 a	*3.8±0.9 abc*	*1.7±0.5 abc*	*5.3±1.4 a*	*4.2±0.9 ab*	*2.9±1.2 ab*			
Prayon (PR)	**1.37±0.24 a (5)**	**0.37±0.22 abcd (5)**	**0.97±0.32 abc (5)**	**0.71±0.17 ab (5)**	**0.28±0.07 bc (5)**	**0.63±0.22 abcd (5)**	–	–	–
	2456±319 a	1585±235 b	2572±415 a	3045±591 efgh	2310±447 efg	2810±992 cde			
	3.3±0.6 a	*0.7±0.5 de*	*3.3±0.7 a*	*2.6±0.8 b*	*0.8±0.3 cd7*	*1.80±0.7b*			
St Felix (SF)	**1.16±0.36 ab (5)**	**0.99±0.22 abc (5)**	**0.99±0.38 abc (5)**	**1.14±0.29 a (5)**	**1.44±0.22 a (5)**	**1.05±0.26 abc (5)**	**0.35±0.13 a (4)**	**1.04±0.33 ab (5)**	**1.33±0.24 a (5)**
	1902±240 a	4356±384 a	2034±485 a	4812±834 defg	3718±590 bcde	4443±453 bcd	6209±2010 b	3026±1875 cd	3938±558 c
	2.4±0.8 a	*4.6±1.2 ab*	*2.6±1.2 ab*	*6.2±2.3 a*	*5.7±1.6 a*	*4.8±1.2 ab*	*2.9±1.1 a*	*3.3±1.1 ab*	*5.4±1.5 a*
Treves (TR)	**1.08±0.57 ab (4)**	**0.62±0.28 abcd (5)**	**0.21±0.08 bcde (5)**	**1.34±0.34 a (5)**	**1.07±0.43 ab (5)**	**0.62±0.29 abcd (5)**	**0.58±0.17 a (5)**	**0.34±0.3 bc (4)**	**0.48±0.20 b (5)**
	2222±482 a	2763±723 ab	2284±511 a	3990±759 defg	1594±232 g	2121±383 e	5129±623 b	4195±2096 d	3575±444 c
	2.5±1.2 a	*1.9±0.8 cd*	*0.5±02 abc*	*4.6±0.7 ab*	*1.9±0.8 bcd*	*1.6±0.8 b*	*2.9±1.0 a*	*1.7±1.1 bc*	*1.9±0.9 bc*
Vernissiere (VE)	**0.87±0.3 ab (5)**	**0.34±0.14 cd (5)**	**1.18±0.3ab (5)**	**0.62±0.21 ab (5)**	**0.95±0.35 ab (5)**	**1.12±0.14 ab (5)**	–	–	–
	1343±417 a	2258±594 ab	1793±188 a	1832±344 h	1475±138 g	3113±796 cde			
	1.6±0.7 a	*1.2±0.5 de*	*2.3±0.6 abc*	*1.3±0.5 b*	*1.4±0.6 abcd*	*3.8±1.5 ab*			
Viviez (VI)	**1.03±0.22 ab (5)**	**1.12±0.16 ab (5)**	**0.60±0.18 abc (5)**	**0.79±0.12 ab (5)**	**1.18±0.08 a (5)**	**0.70±0.04 abcd (5)**	–	–	–
	1912±317 a	3103±424 ab	1794±306 a	2792±450 efgh	2128±202 fg	2083±782 e			
	2.1±0.6 a	*3.6±0.8 abc*	*1.2±0.5 abc*	*2.2±1.2 b*	*2.5±0.2 abc*	*1.5±0.6 b*			
Baraquette (BA)	**0.84±0.18 ab (5)**	**1.54±0.13 a (5)**	**1.81±0.16 a (5)**	**1.67±0.20 a (5)**	**1.18±0.17 a (5)**	**1.60±0.21 a (5)**	**0.52±0.14 a (5)**	**0.05±0.01 c (5)**	**0.13±0.07 c (5)**
	2541±656 a	2611±222 ab	2078±555 a	2876±612 gh	3179±266 defg	3676±606 cde	7539±774 b	4288±2563 cd	4510±1328 ab

Table 3 (continued)

	Zinc salt mixtures						Zinc monometallic salts		
	100Zn 250Cd	250Zn 100Cd	250Zn 250Cd	100Zn 250Ni	250Zn 100Ni	250Zn 250Ni	$250ZnSO_4$	$1500ZnSO_4$	1500ZnO
	2.0±0.6 a	*4.0±0.5 abc*	*3.6±0.8 a*	*4.5±0.9 ab*	*3.6±0.4 ab*	*5.6±0.6 ab*	*4.3±1.6 a*	*0.1±0.1 c*	*0.6±0.4 bc*
Buege (BU)	**0.65±0.16 ab (5)**	**1.01±0.19 abc (5)**	**0.85±0.13 abc (5)**	**0.93±0.23 a (5)**	**1.13±0.11 ab (5)**	**0.87±0.14 abcd (5)**	–	–	–
	2270±225 a	4561±495 a	2714±213 a	5412±756 cde	4195±537 bcd	3458±260 cde			
	1.6±0.6 a	*4.6±0.9 ab*	*2.4±0.5 ab*	*5.0±1.4 ab*	*4.7±0.6 a*	*3.0±0.6 ab*			
Navacelles (NA)	**0.61±0.21 ab (5)**	**0.72±0.21 abc (5)**	**0.48±0.17 abcd (5)**	**0.92±0.10 a (5)**	**1.17±0.17 ab (5)**	**0.53±0.13 abcd (5)**	–	–	–
	2338±332 a	3670±616 ab	2795±257 a	4847±748 defg	4606±492 bc	4961±833 bc			
	1.2±0.3 a	*2.8±0.7 abcd*	*1.4±0.6 abc*	*4.7±1 ab*	*5.3±0.8 a*	*2.9±0.7 ab*			
Ranquas (RA)	**0.15±0.03 b (4)**	**0.47±0.13 abcd (5)**	**0.48±0.13 abcd (5)**	**0.56±0.10 ab (5)**	**1.01±0.05 ab (5)**	**0.26±0.07 bcd (5)**	–	–	–
	1482±178 a	2740±523 ab	1415±298 a	5997±409 cd	2911±287 defg	2649±780 de			
	0.2±0.1 a	*1.5±0.5 de*	*0.7±0.7 abc*	*3.5±0.8 ab*	*3.0±0.3 abc*	*0.8±0.3 b*			
Seranne (SE)	**0.74±0.27 ab (5)**	**0.66±0.23 abc (5)**	**0.84±0.15 abc (5)**	**1.78±0.12 a (5)**	**1.1±0.13 ab (5)**	**1.15±0.27 ab (5)**	**0.87±0.08 a (3)**	**0.74±0.17 ab (5)**	**1.13±0.09 a (5)**
	1862±356 a	3327±555 ab	2525±325 a	4501±544 defg	2849±407 defg	2821±667 de	4116±445 b	4472±1875 cd	7392±742 c
	1.4±0.7 a	*2.5±1 bcde*	*2.2±0.5 ab*	*8.0±0.9 a*	*3.1±0.4 abc*	*3.5±1.1 ab*	*3.6±0.6 a*	*3.7±1.1 ab*	*8.1±0.3 a*
St Baudille (SB)	**0.61±0.12 ab (5)**	**1.3±0.31 ab (5)**	**0.99±0.15 ab (5)**	**0.94±0.2 a (5)**	**0.92±0.06 ab (4)**	**0.91±0.17 abcd (5)**	–	–	–
	1787±137 a	3607±333 ab	2750±337 a	4577±958 defg	3478±401 cde	3947±863 cde			
	1.1±0.2 a	*4.7±1.3 a*	*2.9±0.7 a*	*5.0±1.6 ab*	*3.4±0.4 abc*	*3.9±0.9 ab*			
St Come (SC)	**0.70±0.29 ab (5)**	**1.11±0.37 abc (5)**	**0.57±0.17 abc (5)**	**1.12±0.19 a (5)**	**1.57±0.20 a (5)**	**0.69±0.20 abcd (5)**	**0.48±0.03 a (5)**	**0.063±0.04 c (3)**	**0.12±0.09 cd (5)**
	1980±349 a	3530±552 ab	2885±397 a	4745±516 defg	2941±336 defg	4871±770 bc	6477±537 b	11695±4639 abc	12286±2716 a
	1.5±0.7 a	*3.7±1 abc*	*1.6±0.7 abc*	*5.0±0.7 ab*	*4.4±0.2 a*	*3.7±1.3 ab*	*3.1±0.3 a*	*1.1±1.0 bc*	*1.3±0.9 bc*
St Michel (SM)	**0.41±0.10 ab (5)**	**0.55±0.09 abc (5)**	**0.49±0.06 abc (5)**	**0.69±0.20 ab (5)**	**0.92±0.23 ab (5)**	**0.29±0.03 abcd (5)**	–	–	–
	1921±388 a	4365±372 a	2599±313 a	4283±1245 defg	4307±600 bcd	3631±1259 cde			
	0.8±0.3 a	*2.3±0.4 cde*	*1.2±0.1 abc*	*3.2±1.2 ab*	*3.5±0.6 abc*	*1.2±0.5 b*			
Wilwerwiltz (WI)	**0.23±0.16 b (5)**	**0.08±0.03 d (4)**	**0.06±0.01 e (5)**	**0.17±0.06 b (5)**	**0.12±0.04 c (5)**	**0.58±0.14 abcd (3)**	–	–	–
	2351±600 a	3723±897 ab	1930±329 a	10836±2317 a	4953±945 b	12660±568 a			
	0.9±0.7 a	*0.3±0.2 e*	*0.1±0.04 c*	*2.1±0.9 b*	*0.7±0.3 d*	*7.3±1.9 a*			
Bergenbach (BE)	**0.43±0.16 ab (5)**	**0.28±0.15 abcd (5)**	**0.18±0.06 cde (5)**	**0.35±0.15 ab (5)**	**0.91±0.05 ab (5)**	**0.17±0.06 d (5)**	**0.56±0.10 a (5)**	**0.36±0.14 abc (5)**	**0.65±0.19 ab (5)**
	2 349±429 a	3992±1091 ab	2337±845 a	8737±663 ab	2722±653 efg	6579±1487 b	8301±2345 ab	19748±3852 a	4010±605 c
	0.8±0.2 a	*1.4±0.8 cde*	*0.5±0.3 abc*	*3.2±1.3 ab*	*2.6±0.7 abcd*	*1.4±0.5 b*	*4.4±1.1 a*	*5.9±1.9 ab*	*2.7±0.8 ab*
Puy de Wolf (PW)	**0.45±0.05 ab (5)**	**0.22±0.06 abcd (5)**	**0.10±0.04 de (5)**	**0.16±0.05 b (5)**	**0.55±0.08 ab (5)**	**0.24±0.12 cd (5)**	**0.35±0.08 a (5)**	**0.03±0.005 c (4)**	**0.01±0.003 d (5)**
	1647±225 a	3832±533 ab	2093±212 a	7281±893 bc	6696±1074 a	4629±1589 bcd	13068±3633 a	15801±3744 ab	13076±2428 a
	0.8±0.2 a	*1.0±0.4 de*	*0.2±0.1 bc*	*1.3±0.4 b*	*3.5±0.6 abc*	*1.8±1.2 b*	*4.1±1.5 a*	*0.4±0.1 bc*	*0.2±0.1 c*

*** $p<0.001$; ** $p<0.01$; * $p<0.05$; † $p<0.10$; ns $p>0.10$

Table 4 Results of nested ANOVAs for aerial biomass, Cd concentration and mass (a) and mean (±SE) aerial biomass (g) (**bold**), Cd concentration (mg/kg) (normal) and mean (±SE) Cd shoot mass (mg/plant) (*italics*) per individual (b) from metallicolous (normal), non-metallicolous **(bold)** and serpentine (*italics*) populations of *Noccaea caerulescens* cultivated in three Cd salt mixtures ($CdSO_4$) and in three Cd monometallic salts ($CdSO_4$, $CdCl_2$). For each salt treatment, population means with the same letter **(bold for biomass,** normal for Cd concentration and *italics for Cd mass*) are not significantly different with a least squares means test (SAS). Maximal Cd concentration and mass values for each treatment are underlined. Sampling number is enclosed in parentheses after the mean aerial biomass. Degrees of freedom differ among biomass and Cd analyses because sampling number was not the same

	Cadmium salt mixtures			Cadmium monometallic salts		
	100Zn 250Cd	250Zn 100Cd	250Zn 250Cd	$250CdSO_4$	$500CdSO_4$	$500CdCl_2$
a **ANOVAS**						
Shoot biomass	**Ori $F_{2,9}$=1.9 ns**	**Ori $F_{2,9}$=1.5 ns**	**Ori $F_{2,9}$=2.3 ns**	**Ori $F_{2,5}$=0.1 ns**	**Ori $F_{2,5}$=0.14 ns**	**Ori $F_{2,2}$=1.0 ns**
	Pop(Ori) $F_{9,47}$=2.0†	**Pop(Ori) $F_{9,47}$=7.0*****	**Pop(Ori) $F_{9,47}$=6.3*****	**Pop(Ori) $F_{5,12}$=5.6****	**Pop(Ori) $F_{5,30}$=28.5*****	**Pop(Ori) $F_{2,12}$=15.8*****
Shoot cadmium concentration	Ori $F_{2,9}$=0.2 ns	Ori $F_{2,9}$=0.3 ns	Ori $F_{2,9}$=1.4 ns	Ori $F_{2,3}$=0.2 ns	Ori $F_{2,4}$=0.9 ns	Ori $F_{2,3}$=3.2 ns
	Pop(Ori) $F_{9,23}$=3.6**	Pop(Ori)$F_{9,21}$=6.6***	Pop(Ori) $F_{9,19}$=4.2**	Pop(Ori) $F_{3,4}$=15.8**	Pop(Ori) $F_{4,7}$=0.7 ns	Pop(Ori) $F_{1,3}$=0.1 ns
Shoot cadmium mass	*Ori $F_{2,9}$= 0.7 ns*	*Ori $F_{2,9}$= 0.8 ns*	*Ori $F_{2,9}$= 3.2 ns*	*Ori $F_{2,3}$= 0.4 ns*	*Ori $F_{2,4}$= 0.2 ns*	*Ori $F_{2,3}$= 8.7 ns*
	Pop(Ori) $F_{9,23}$= 2.2†	*Pop(Ori) $F_{9,21}$= 3.6***	*Pop(Ori) $F_{9,19}$= 4.8***	*Pop(Ori) $F_{3,4}$= 13.6***	*Pop(Ori) $F_{4,7}$= 7.5**	*Pop(Ori) $F_{1,3}$= 1.4 ns*
b **Aerial biomass (g)**/Cd Concentration (mg/kg)/*Cd MASS (mg/plant)*						
Avinières (AV)	**0.90±0.34 ab (5)**	**1.34±0.14 a (5)**	**0.61±0.23 abc (4)**	**0.34±0.24 ab (4)**	**0.78±0.30 a (5)**	**0.13±0.01 a (4)**
	575±94 cd	1131±353 bcd	803±92 cd	1991±199 bc	3547±387 a	3199±1105 a
	0.8±0.1 b	*1.5±0.6 ab*	*0.8±0.3 cd*	*1.4±0.9 a*	*1.3±0.1 ab*	*0.5±0.2 a*
Prayon (PR)	**1.37±0.24 a (5)**	**0.37±0.22 abcd (5)**	**0.97±0.32 abc (5)**	–	–	–
	635±312 cd	300±56 d	673±92 cd			
	1.0±0.5 b	*0.2±0.2 d*	*0.9±0.2 cd*			
St Felix (SF)	**1.16±0.36 ab (5)**	**0.99±0.22 abc (5)**	**0.99±0.38 abc (5)**	**0.012 b (1)**	**0.67±0.22 a (5)**	dead
	3152±621 ab	1644±91 ab	2369±64 b	–	3497±674 a	
	5.2±0.6 a	*2.1±0.5 a*	*3.6±0.8 a*	–	*3.5±0.7 a*	
Treves (TR)	**1.08±0.57 ab (4)**	**0.62±0.28 abcd (5)**	**0.21±0.08 bcde (5)**	**0.18±0.04 ab (4)**	**0.03±0.01 b (5)**	**0.07±0.02 ab (4)**
	3417±1594 ab	1570±478 ab	2092±941 b	3406±383 ab	2660 a [a]	3097±456 a
	4.7±2.2 a	*1.3±0.4 ab*	*0.8±0.6 cd*	*0.7±0.1 ab*	*0.1 c*	*0.3±0.1 a*
Viviez (VI)	**1.03±0.22 ab (5)**	**1.12±0.16 ab (5)**	**0.60±0.18 abc (5)**	–	–	–
	432±37 d	1447±142 b	2181±32 b			
	0.6±0.1 b	*1.9±0.3 a*	*1.7±0.4 bc*			
Baraquette (BA)	**0.84±0.18 ab (5)**	**1.54±0.13 a (5)**	**1.81±0.16 a (5)**	**0.02 ab (1)**	**0.07±0.04 b (5)**	**0.03±0.01 b (4)**
	890±410 bcd	566±160 cd	479±100 cd	–	1844±316 a	4603 a[a]
	0.9±0.4 b	*0.9±0.3 abc*	*0.8±0.1 cd*	–	*0.3±0.2 c*	*0.1 a*
Buege (BU)	**0.65±0.16 ab (5)**	**1.01±0.19 abc (5)**	**0.85±0.13 abc (5)**	–	–	–
	629±116 cd	402±49 d	550±95 cd			
	0.6±0.2 b	*0.5±0.1 bcd*	*0.6±0.2 cd*			

Table 4 (continued)

	Cadmium salt mixtures			Cadmium monometallic salts		
	100Zn 250Cd	250Zn 100Cd	250Zn 250Cd	$250CdSO_4$	$500CdSO_4$	$500CdCl_2$
Seranne (SE)	**0.74±0.27 ab (5)** 1567±514 abc *1.5±0.3 b*	**0.66±0.23 abc (5)** 581±91 cd *0.6±0.2 bcd*	**0.84±0.15 abc (5)** 543±7 cd *0.6±0.1 cd*	**0.05±0.01 ab (3)** *915 c* [a] *0.1 ab*	**1.1±0.2 a (5)** 2602±681 a *2.8±1.1 ab*	dead
St Come (SC)	**0.70±0.29 ab (5)** 4759±1210 a *4.9±2.4 a*	**1.11±0.37 abc (5)** 1334±475 bc *1.9±0.8 a*	**0.57±0.17 abc (5)** 3921±236 a *2.9±0.7 ab*	**0.16±0.13 ab (2)** 3871 a[a] *1.1 a*	**0.02±0.004 b (4)** 3565 a[a] *0.1 c*	dead
Wilwerwiltz (WI)	**0.23±0.16 b (5)** 2050±1796 abcd *1.6±1.6 b*	**0.08±0.03 d (4)** 1969±113 ab *0.2±0.1 cd*	**0.06±0.01 e (5)** 148 d[a] *0.1 d*	–	–	–
Bergenbach (BE)	**0.43±0.16 ab (5)** 461±29 cd *0.3±0.1 b*	**0.28±0.15 abcd (5)** 576±122 cd *0.3±0.1 cd*	**0.18±0.06 cde (5)** 1510±633 bc *0.4±0.2 cd*	**0.70±0.18 a (2)** 2823±251 ab *2.0±0.6 a*	**0.40±0.16 a (4)** 2503±632 a *0.7±0.2 bc*	**0.08±0.02 ab (4)** 3700±441 a *0.4±0.1 a*
Puy de Wolf (PW)	**0.45±0.05 ab (5)** 1847±231 abc *0.9±0.2 b*	**0.22±0.06 abcd (5)** 2312±352 a *0.8±0.3 abc*	**0.10±0.04 de (5)** 687±659 cd *0.2±0.1 d*	**0.01±0.002 b (3)** 2682 ab [a] *0.2 b*	**0.025±0.008 b (5)** – –	**0.002 c (1)** – –

[a] $n=1$

*** $p<0.001$; ** $p<0.01$; * $p<0.05$; † $p<0.10$; ns $p>0.10$

We did not found statistical differences in Cd concentrations among origins when plants were cultivated in mixtures of Zn-Cd, but large significant differences were found among populations of the same origin (Table 4a). For instance, SC individuals (NMET) had high Cd concentration values (3921 mg/kg) in the treatment 250Zn250Cd, whereas the other NMET populations had values below 550 mg/kg (Table 4b). Similar contrasting results were obtained between this SC population and the other NMET in the treatment 100Zn250Cd.

Likewise, the MET populations SF and TR had values above 3000 mg/kg in the 100Zn250Cd treatment, whereas the other MET populations had values lower than 650 mg/kg. For Cd mass in the same treatment, SC (NMET), SF and TR (MET) showed values of around 5 mg/plant whereas SERP individuals had Cd values lower than 1 mg/plant (Table 4b). In monometallic salts, there was a high mortality (80-100 %) for SF (MET), BA, SE and SC (NMET), and PW (SERP). Plants in 500$CdCl_2$ soil showed low Cd mass despite high concentration values, probably because of the high solubility of this salt decreased biomass (4 of 8 populations died in this treatment).

Physiological studies investigating Cd and Zn influx in the root apoplast suggested that the Cd transport from root to shoot was not Zn suppressible and mediated by the same transporter with a higher affinity for Cd than for Zn in the MET AV (called "Ganges ecotype"; Lombi et al. 2001b; Zhao et al. 2002). Roosens et al. (2003) further showed that the addition of Zn (10 or 100 μM) in hydroponic solutions containing Cd (30 μM) did not decrease leaf Cd concentration in SF population (also called "Ganges ecotype"), confirming the idea that Cd is accumulated independently of Zn in these populations. We tested this hypothesis for the AV population and two others by comparing the Cd concentration between the two salt mixtures (100Zn250Cd and 250Zn250Cd) and the monometallic salt 250 $CdSO_4$ at the same Cd concentration (250 mg Cd/kg). Cd concentrations and mass differences were tested with one-way ANOVAs followed by *a posteriori* contrasts for each population (it was not possible to test SF, BA, SE, SC and PW because of high mortality (≥80 %) in the 250 $CdSO_4$ treatment. In contrast to precedent studies we showed a significant decrease of leaf Cd concentrations in MET AV ($p<0.05$) and in SERP BE ($p<0.10$) in the two salt mixtures compared with monometallic salts. For TR (MET), the mean Cd concentrations and mass values were not significantly different between monometallic salts and salt mixtures (see Table 4b for mean values).

Compared to Zn, only a reduced number of populations achieved the highest Cd concentrations and mass values in the different treatments (Table 4). Thus, SC (NMET) had the highest concentration values in 4 treatments and BA (NMET) and PW (SERP) in the other two treatments. The SF (MET) population showed the highest Cd mass values for 4 treatments and AV (MET) and BE (SERP) for the other two treatments.

Biomass, survival, Ni concentration and Ni mass in binary mixtures and Ni monometallic salts (Table 5)

Only 2 of the 6 treatments showed significant differences among origins. None of them involved monometallic salts. On the other hand, 5 of 6 treatments showed significant differences among populations within an origin.

Population survival was higher in mixtures since all plants had survived until the end of the experiment. Again plants cultivated in monometallic salts with high concentrations of Ni (1000 $NiCl_2$ and 1000 $NiSO_4$), had a high mortality e.g. MET (SF, TR), NM (SE, SC) and to a lesser extent in the SERP (PW). BE was the only population which showed no mortality.

There were significant differences among origins for shoot Ni concentrations in treatments with 250 mg Ni/kg only (binary mixtures and monometallic salts) (Table 5a). In addition, significant differences among populations were found in all treatments for Ni concentrations and masses, with the northern PR (MET) having less Ni than the others (Table 5b). Unexpectedly, the highest Ni concentration in mixtures was obtained for PW (SERP) at the lowest soil Ni concentration (250Zn100Ni). Similar results were obtained with TR (MET), SE and WI (NMET). The PW population had the highest Ni concentration with monometallic salts (25451 mg Ni/kg with 1000 $NiSO_4$).

Previous results (see Fig. 2e) showed that the Ni concentrations were significantly lower in metallic salt mixtures (Zn-Ni) than in monometallic salts (250$NiCl_2$) at equivalent Ni concentrations (250 mg/kg). As for Zn and Cd, we checked whether the 8 populations tested all showed such a decrease. One of the three MET

Table 5 Results of nested ANOVAs for aerial biomass, Ni concentration and mass (a) and mean (±SE) aerial biomass (g) (**bold**), Ni concentration (mg/kg) (normal) and mean (±SE) Ni shoot mass (*italics*) (mg/plant) per individual (b) from metallicolous (normal), non-metallicolous (**bold**) and serpentine (*italics*) populations of *Noccaea caerulescens* cultivated in three Ni salt mixtures ($NiCl_2$) and in three Ni monometallic salts ($NiCl_2$, $NiSO_4$). For each salt treatment, population means with the same letter (**bold for biomass,** normal for Ni concentration and *italics for Ni mass*) are not significantly different with a least squares means test (SAS). Maximal biomass, Ni concentration and mass values for each treatment are underlined. Sampling number is enclosed in parentheses after the mean aerial biomass. Degrees of freedom differ among biomass and Ni analyses because sampling number was not the same

	Nickel salt mixtures			Nickel monometallic salts		
	100Zn 250Ni	250Zn 100Ni	250Zn 250Ni	250$NiCl_2$	1000$NiCl_2$	1000$NiSO_4$
a ANOVAS						
Shoot biomass	**Ori $F_{2,9}$=3.4†**	**Ori $F_{2,9}$=0.1 ns**	**Ori $F_{2,9}$=12.5*** **	**Ori $F_{2,5}$=0.4 ns**	**Ori $F_{2,5}$=0.3 ns**	**Ori $F_{2,3}$=1.4 ns**
	Pop(Ori) $F_{9,48}$=5.2* **	**Pop(Ori) $F_{9,48}$=8.0*** **	**Pop(Ori) $F_{9,45}$=1.3 ns**	**Pop(Ori) $F_{5,32}$=6.4*** **	**Pop(Ori) $F_{5,8}$=3.8***	**Pop(Ori) $F_{3,19}$=8.7*** **
Shoot nickel concentration	Ori $F_{2,9}$=3.6†	Ori $F_{2,9}$=1.2 ns	Ori $F_{2,9}$=3.2†	Ori $F_{2,8}$=10.6**	Ori $F_{2,5}$=2.9 ns	Ori $F_{2,3}$=2.8 ns
	Pop(Ori) $F_{9,22}$=7.4***	Pop(Ori) $F_{9,23}$=2.3*	Pop(Ori) $F_{9,21}$=3.2*	Pop(Ori) $F_{8,22}$=2.2†	Pop(Ori) $F_{5,8}$=10.4**	Pop(Ori) $F_{3,6}$=8.1*
Shoot nickel mass	*Ori $F_{2,9}$= 0.4 ns*	*Ori $F_{2,9}$= 0.6 ns*	*Ori $F_{2,9}$= 3.5†*	*Ori $F_{2,8}$= 6.0**	*Ori $F_{2,5}$= 3.0 ns*	*Ori $F_{2,3}$= 2.4 ns*
	*Pop(Ori) $F_{9,22}$= 7.3*** *	*Pop(Ori) $F_{9,23}$= 2.7**	*Pop(Ori) $F_{9,21}$= 3.2**	*Pop(Ori) $F_{8,22}$= 1.3 ns*	*Pop(Ori) $F_{5,8}$= 3.1†*	*Pop(Ori) $F_{3,6}$= 2.3 ns*
b **Aerial biomass (g)**/Ni Concentration (mg/g)/*Ni MASS (mg/plant)*						
Avinières (AV)	**1.08±0.24 a (5)**	**0.78±0.11 ab (5)**	**0.38±0.21 abcd (4)**	**0.99±0.14 a (5)**	**0.09±0.04 bc (3)**	**0.64±0.09 a (5)**
	330±78 b	417±158 bc	345±28 bc	432±119 b	857±369 d	1994±501 c
	0.3±0.01 ab	*0.3±0.01 cd*	*0.2±0.01 cd*	*0.4±0.1 a*	*0.1±0.01 d*	*1.1±0.4 ab*
Prayon (PR)	**0.71±0.17 ab (5)**	**0.28±0.07 bc (5)**	**0.63±0.22 abcd (5)**	–	–	–
	25±9 d	76±18 c	124±28 c			
	0.02±0.005 c	*0.03±0.004 d*	*0.1±0.007 d*			
St Felix (SF)	**1.14±0.29 a (5)**	**1.44±0.22 a (5)**	**1.05±0.26 abc (5)**	**0.91±0.09 a (5)**	**0.65±0.33 a (3)**	dead
	124±37 c	638±122 bc	621±89 abc	428±140 b	1793±379 bcd	
	0.2±0.01 bc	*1.1±0.2 bc*	*0.8±0.1 a*	*0.4±0.2 a*	*1.8±0.9 ab*	
Treves (TR)	**1.34±0.34 a (5)**	**1.07±0.43 ab (5)**	**0.62±0.29 abcd (5)**	**0.66±0.11 ab (5)**	**0.07±0.005 b (2)**	**0.12±0.07 c (4)**
	110±19 c	433±30 bc	157±24 c	573±34 b	1663 cd[a]	2738±177 bc
	0.2±0.01 bc	*0.8±0.1 bcd*	*0.1±0.03 d*	*0.4±0.1 a*	*0.1 cd*	*0.5±0.4 ab*
Viviez (VI)	**0.79±0.12 ab (5)**	**1.18±0.08 a (5)**	**0.70±0.04 abcd (5)**	–	–	–
	132±28 c	255±23 bc	161±15 c			
	0.1±0.03 bc	*0.3±0.05 cd*	*0.1±0.01 d*			
Baraquette (BA)	**1.67±0.20 a (5)**	**1.18±0.17 a (5)**	**1.60±0.21 a (5)**	**0.33±0.09 c (5)**	**0.05±0.04 c (3)**	**0.04±0.007 c (5)**
	157±40 c	612±74 bc	524±83 abc	963±208 b	3353 bc [a]	2035 bc [a]
	0.3±0.1 ab	*0.9±0.2 bc*	*0.9±0.2 a*	*0.3±0.04 a*	*0.07 cd*	0.02 b
Buege (BU)	**0.93±0.23 a (5)**	**1.13±0.11 ab (5)**	**0.87±0.14 abcd (5)**	–	–	–
	364±76 ab	521±90 bc	563±80 abc			

Table 5 (continued)

	Nickel salt mixtures			Nickel monometallic salts		
	100Zn 250Ni	250Zn 100Ni	250Zn 250Ni	$250NiCl_2$	$1000NiCl_2$	$1000NiSO_4$
	0.5±0.1 a	*0.7±0.1 bcd*	*0.5±0.1 abc*			
Seranne (SE)	**1.78±0.12 a (5)**	**1.1±0.13 ab (5)**	**1.15±0.27 ab (5)**	**0.74±0.06 ab (5)**	**0.31±0.06 ab (5)**	dead
	105±10 cd	1097±87 bc	449±116 bc	748±185 b	744±227 d	
	0.2±0.01 bc	*1.4±0.1 b*	*0.7±0.1 ab*	*0.6±0.2 a*	*0.3±0.1 bcd*	
St Come (SC)	**1.15±0.19 a (5)**	**1.57±0.20 a (5)**	**0.69±0.20 abcd (5)**	**0.87±0.06 a (5)**	**0.16±0.11 abc (3)**	**0.08±0.06 c (3)**
	222±23 bc	619±394 bc	866±70 ab	1020±110 ab	4495±828 b	4751 bc [a]
	0.3±0.05 ab	*1.2±0.7 b*	*0.8±0.2 a*	*0.8±0.1 a*	*0.9±0.6 abc*	*0.9 ab*
Wilwerwiltz (WI)	**0.17±0.06 b (5)**	**0.12±0.04 c (5)**	**0.58±0.14 abcd (3)**	–	–	–
	115±3 c	1333±121 b	716±34 abc			
	0.03±0.01 c	*0.3±0.05 cd*	*0.4±0.1 bcd*			
Bergenbach (BE)	**0.35±0.15 ab (5)**	**0.91±0.05 ab (5)**	**0.17±0.06 d (5)**	**1.1±0.12 a (5)**	**0.38±0.13 ab (5)**	**0.63±0.22 ab (5)**
	522±263 ab	323±81 bc	474±446 bc	1486±637 ab	4360±273 b	5231±1107 b
	0.2±0.1 b	*0.3±0.1 cd*	*0.2±0.1 cd*	*1.4±0.7 a*	*2.5±0.7 a*	*4.7±2 a*
Puy de Wolf (PW)	**0.16±0.05 b (5)**	**0.55±0.08 ab (5)**	**0.24±0.12 cd (5)**	**0.46±0.12 bc (5)**	**0.15±0.07 abc (3)**	**0.09±0.03 bc (3)**
	789±146 a	3549±495 a	1201±422 a	4025±823 a	16632±2611 a	25451±1797 a
	0.2±0.02 b	*2.3±0.3 a*	*0.7±0.2 ab*	*1.7±0.5 a*	*2.7±1.3 a*	*2.2±1 a*

[a] $n=1$

***$p<0.001$; **$p<0.01$; *$p<0.05$; † $p<0.10$; ns $p>0.10$

populations (TR), the three NMET (BA, SE and SC) and the two SERP populations showed significantly ($p<0.05$) lower Ni concentrations values in the Zn-Ni mixtures compared to the monometallic salts (one-way ANOVAs followed by *a posteriori* contrasts). These populations were the same (except SE) as those that showed a decrease of Zn mass in Zn-Ni mixtures. PW (SERP) was the only population that showed a decrease of Ni mass between monometallic Ni salts and salt mixtures.

As for Cd, only a reduced number of populations achieved the highest Ni concentrations and mass values in the different treatments (Table 5). Thus, PW (SERP) had the highest concentration values for all treatments. The SERP populations also showed the highest Ni mass values in 4 treatments and BA and BU (NMET) in the other 2 treatments.

Biomass, Zinc concentration and Zn mass in hydroponics (Fig. 4)

Differences in aerial biomass (Fig. 4a, b) were logically significant for all of the dates of harvest for the two treatments. For the treatment at 2,000 μM (but not for the other treatment at 1.5 μM) there were significant differences between origins with the MET having higher biomass than NMET in the last three harvests. In the 1.5 μM treatment MET had higher biomass than NMET only in the last harvest.

The two treatments did not show significant differences in Zn concentration between origins (Fig. 4c, d). There were significant differences between populations (within origin) in the two last harvests in 2,000 μM with SER populations having the highest Zn values. In the low Zn treatment there were also significant differences between populations (within origin) but in this treatment the two metallicolous populations had the highest values in the last harvest. By way of comparison, when the 4 populations used in hydroponics were cultivated in Les Avinières soil, the two MET populations (AV and SF) had significantly lower Zn concentrations (mean±SE: 5,337±654, $n=18$) than NMET (BA and SE; 8,674 ±694, $n=16$).

There were significant differences in Zn mass between origins (Fig. 4e, f) in the two treatments with the MET populations having values largely higher than those of NMET because of their greater aerial biomass in high Zn solution and higher Zn concentration in low Zn solution. However, in low Zn solution the differences were significant in the last harvest only. Zn mass values of NMET in high Zn solution were close to those of MET in the low Zn solution due to the low biomass produced by NMET in the treatment with high Zn concentration.

For comparison, in Les Avinières soil, Zn mass was not significantly different between the two origins (MET: 5.12±0.60, mean±SE, $n=18$; NMET: 4.75±0.64, $n=16$).

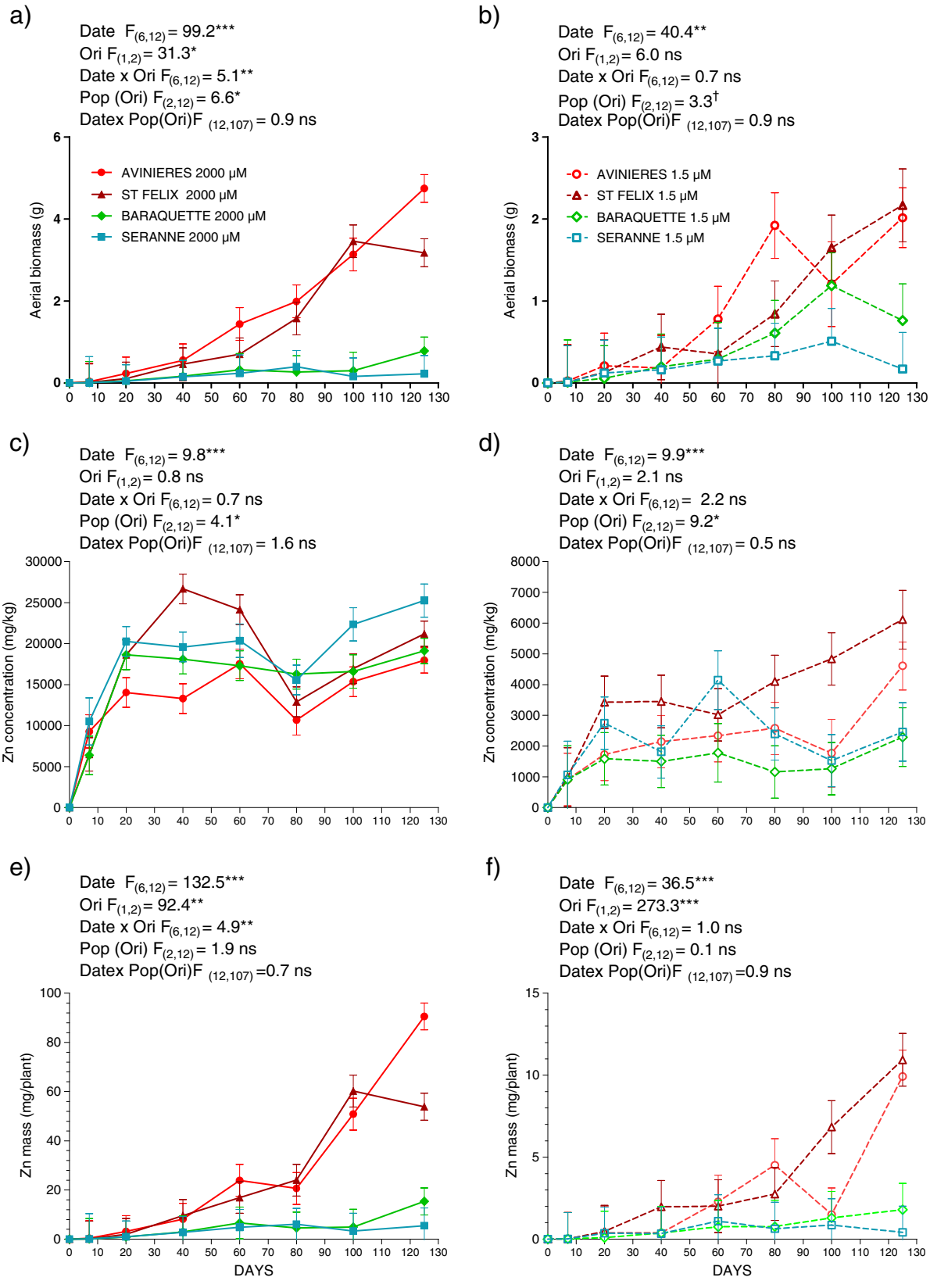
Fig. 4 Mean (±SE) biomass (g) (**a**, **b**),mean (±SE) Zn concentration (mg/kg) (**c**, **d**) and mean (±SE) Zn mass (mg/plant) per individual (**e**, **f**) in aerial parts of two metallicolous (Avinières, St Félix) and two non-metallicolous (Baraquette, Séranne) populations of *Noccaea caerulescens* cultivated in hydroponic solutions with two Zn ($ZnSO_4.7\ H_2O$ form) concentrations: 1.5 μM of zinc (*open symbols*: **b**, **d**, **f**), and 2000 μM (*black symbols*: **a**,**c**,**e**). Results of mixed nested ANOVAs are shown

Discussion

For the first time, Zn, Cd and Ni concentrations and mass of 18 *Noccaea caerulescens* populations from MET, NMET and SERP origins were compared. The analyses with the totality of treatments showed significant differences between origins for Zn and Ni concentration and mass and for Cd mass. However, there was a large heterogeneity of responses among populations depending on the substrate used in the experiments. For this reason, the origin factor was often non-significant when data were analysed treatment by treatment. Thus, even if it is possible to find *a posteriori* statistical differences in metal content or metal mass among origins (when the edaphic origin of each population is known) with many experiments, it is not possible to correctly assign a single population to its accurate origin on the basis of only one experiment.

Zinc concentration and Zinc mass

Variations among origins The present study is the first to assess metal concentration and mass of SERP populations in soil conditions. Overall, SERP populations (and particularly PW) had low biomass values but the highest Zn concentrations, followed by NMET and MET. Several studies comparing MET and NMET populations showed that NMET have higher Zn concentration capacities than MET (Meerts and Van Isacker 1997; Escarré et al. 2000; Meerts et al. 2003; Dechamps et al. 2007), which was accounted for by the need for *N. caerulescens* growing on Zn-poor soil



to compensate for the low Zn availability in their natural environment (Meerts and Van Isacker 1997). The results of this study suggest that SERP populations behave like NMET with huge capacities of foliar Zn concentration. This is in agreement with Reeves et al. (2001), who showed that field-collected *N. caerulescens* of PW accumulated up to 6000 mg/kg, despite the fact that the soil Zn concentration was below 70 mg/kg (Table 1). In hydroponic experiments, SERP populations had higher (Roosens et al. 2003) or similar (Assunção et al. 2003a; 2008) values of foliar Zn concentration compared to MET populations.

SERP populations had the lowest biomass and the lowest Zn mass, showing that a high Zn concentration was associated with a low biomass and therefore a low metal tolerance. Such an inverse relationship had already been suggested by comparisons between NMET and MET, with NMET populations accumulating more Zn but being less tolerant (i.e. lower biomass) to this metal (Escarré et al. 2000). Assunção et al. (2003c) also showed a negative correlation between metal tolerance and concentration among individuals from crosses between metallicolous and non-metallicolous populations of *Noccaea caerulescens*. The high Zn concentration of SERP individuals did not result in a mortality increase because survival values were similar to M and higher than NM individuals.

Variations among populations In the present study, the use of several replicate populations within each origin allowed us to highlight the huge variability of foliar Zn concentration among populations of the same origin. For instance, when MET and NMET populations were cultivated in Les Avinières soil, a continuous distribution of Zn concentration values was observed, but there was still a significant mean difference between origins overall (with NMET showing higher values than MET, Fig. 2) which confirms previous results (Escarré et al. 2000; Frérot et al. 2005).

Mass values, which combine concentration and tolerance (assessed by biomass and survival), were also highly variable among populations, mainly for SERP and NMET. For instance, in 1500Zn, the SERP PW had Zn mass levels 10 times lower than the SERP BE, and the NMET BA had Zn mass levels 13–30 times lower than the NMET SER.

Interactions between elements The results of this study showed that 7 out of 8 populations had lower Zn concentration values in Zn-Cd salt mixtures than in monometallic salts at the same Zn concentration (250 mg Zn/kg). Similarly, Roosens et al. (2003) showed that exposing two French MET populations (among which SF) to high Cd decreased their Zn concentrations. Zn and Cd are classed in the transition-elements subgroup of the Periodic Table. The addition of Cd to the soil resulted in a decrease in the leaf Zn concentration either by inhibiting the root Zn uptake or because they share a common transport, such as those of the ZIP family (Hart et al. 2005). For instance, ZIP2 is a Zn transporter but also has a good affinity for Cd and Cu (Grotz et al. 1998).

Overall, in Zn-Ni mixtures plants had Zn concentrations lower than in monometallic salts. However, only 4 of 8 populations had significantly lower Zn concentration values in Zn-Ni mixtures. There were non-significant differences for the 4 others. This suggests that Zn-Ni interaction is weaker than Zn-Cd interaction. Peer et al. (2003) analysed the Zn-Ni interference with many plant species from MET and SERP sites, including some *N. caerulescens* populations from this study. They showed that PW individuals had higher Ni and Zn concentration in mixtures (Ni $(NO_3)_2 \cdot 6H_2O$ and $Zn(NO_3)_2 \cdot 6H_2O$ with 100 μg Ni g^{-1} dry weight and 100 μg Zn g^{-1} dry weight) than in a single metal soil, whereas individuals from SF did not show significant differences in Zn concentration in either treatments. The results from SF are in agreement with the current results, whereas those of PW are the opposite because PW individuals showed a significant decrease in Zn concentration in mixtures. These differences illustrate the difficulty of comparing experiments performed with different salts and concentrations.

Perspectives from field data Four populations, two NMET (SC, WI) and the two SERP populations, exceeded the hyperaccumulation threshold for Zn in a few treatments. Even in Les Avinières soil, only WI (NMET) showed Zn values above 10000 mg/kg. According to these results, MET populations from southern France are not zinc hyperaccumulators on average according to the "classic" concentration treshold (Baker and Brooks 1989). AV individuals harvested in situ had Zn mean values of 7310 mg/kg (±646) (Escarré et al. 2011). However, *Noccaea caerulescens* from PR (MET) (Zn in soil 18360 mg/kg) had mean leaf Zn concentrations up to 13400 mg/kg (Faucon 2004).

In 9 NMET soils in Switzerland (<120 mg/kg of Zn in soil), Zn leaf concentrations values averaged 4857 mg/kg (Basic et al. 2006), and in 15 NMET populations from Luxembourg (soil=8.6 mg/kg Zn) mean Zn leaf values were 7300 mg/kg (Molitor et al. 2005). Banásová et al. (2008) found mean Zn concentrations of 13650 mg/kg and of 10729 mg/kg in MET and NMET populations, respectively, in central Slovakia. However, the SE populations as well as other NM populations from the Larzac plateau had concentrations of up to 2510 mg/kg Zn (Noret et al. 2005; Lefèbvre and Escarré, unpublished results) for the same soil Zn levels as in Luxembourg.

The numerous Zn treatments used in this study showed that the highest Zn concentrations and masses were achieved by different populations. This suggests that any Zn phytoremediation program should first allow a preliminary experiment to select the best accessions in those particular conditions. Of particular note, a large within-population variation also exists in some accessions, showing that artificial selection of efficient tolerant and hyperaccumulating genotypes might be possible.

Cd concentration and mass

Variations among origins There were no differences in Cd concentration among origins, nor were there differences between MET and NMET from southern France as found previously by Escarré et al. (2000) in Les Avinières soil with few populations. Similarly, Dechamps et al. (2005) did not find any differences in Cd concentration between MET populations from Belgium (including PR) and NMET populations from Luxembourg (including WI). However, in the current study, MET had overall a higher mean Cd mass than NMET and SERP populations, which can be accounted for by a higher tolerance (estimated here by overall mean biomass and a slightly higher survival) of MET on high soil Cd concentration (MET:71 %; NMET:53 %; SERP: 63 %). Hydroponic experiments (Assunção et al. 2003a; Roosens et al. 2003) had shown that SERP populations are particularly intolerant to Cd.

Variations among populations The results of this study highlight important variations within origins, i.e. among populations of the same origin. The SF (MET) had the greatest Cd values (in particular for the Cd mass), which was in agreement with the hydroponic results of Roosens et al. (2003). This high Cd mass capacity does not seem to be related to the soil Cd concentration of the SF site (36 mg Cd/kg; Table 1) as similar levels were measured in sites of populations that did not accumulate as much Cd (e.g. TR 41 mg Cd/kg). Some populations considered to be high Cd accumulators such as "Ganges ecotype" (here AV) can show extraordinary fluctuations in their shoot concentrations depending on treatments used whereas other populations such as SC (NMET) and BE (SERP) showed high Cd concentrations in many treatments. Therefore, the use of the term "ecotype", which refers to "an intraspecific product of environmental selection arising as a result of genotypic response to a particular habitat" (Gregor and Watson 1961) should be avoided to refer to AV and SF populations for two reasons. First, the adaptive function of Cd accumulation has not been shown yet, and secondly, the high Cd foliar concentration is not only limited to these populations, but also to other NM and SERP populations.

This study also showed an important mortality in the 3 monometallic Cd treatments, even at 250 mg Cd/kg (e.g. 5 of 8 populations had at least 80 % mortality, among which was the SF population). As there was almost no mortality in binary mixtures with the same Cd concentration, this suggests that the lack of Zn in the presence of Cd decreases survival.

Interactions between elements The addition of Zn in the soil significantly decreased the shoot Cd concentration in the 2 salt mixtures compared with monometallic salts at the same Cd concentration, suggesting that they probably share a common transporter. However, some studies have shown that the influx of Cd in root apoplast was not Zn-suppressible in some MET populations, such as AV or SF (Lombi et al. 2001b; Roosens et al. 2003), suggesting that the accumulation of Cd was mediated by a transporter with a higher affinity for Cd than for Zn. Here, after 3 months of growth in soil a significant decrease in leaf Cd concentration in salt mixtures was found in AV, as well as in BE (SERP), compared with those obtained in monometallic salts at the same concentration (250 mg Cd/kg). This indicates that the leaf Cd concentration was lowered by the addition of Zn in soil. However, for TR (MET), the mean Cd concentration was not significantly different among monometallic salts and salt mixtures, and the Cd mass was even

higher in a salt mixture (100Zn250Cd) compared to monometallic salts. In the current study, the Cd mass difference is linked to differences in biomass because the Cd concentrations were very similar between a salt mixture (100Zn250Cd) and the monometallic salt ($250CdSO_4$).

Perspectives with field data All populations were found to hyperaccumulate Cd over the threshold (100 mg/kg), even in Les Avinières soil, where the Cd concentration of plants was the lowest. NMET plants collected in situ in southern France accumulate very small amounts of Cd (mean 29 mg/kg Cd, n=5; Lefèbvre and Escarré, unpublished results) compared with plants from mine sites around Saint-Laurent-le-Minier (mean 1280 mg/kg, *n*=24; Escarré et al. 2011). Molitor et al. (2005) obtained an average of 31 mg/kg (*n*=15) for the NMET from Luxembourg. Swiss and Slovak NMET populations had higher Cd concentrations (263 and 127 mg/kg, respectively; Basic et al. 2006; Banásová et al. 2008).

The Cd concentrations of plants cultivated in Les Avinières soil were largely lower than those obtained when using metal salts. With monometallic salts, most populations showed Cd values higher than those found in plants collected in situ and may provide inaccurate information on the phytoremediation performances of the species. However, the values obtained with salts were still largely below the level of 14000 mg/kg obtained in hydroponics by Lombi et al. (2001b).

Ni concentration and mass

Variations among origins SERP plants generally showed the highest values of Ni concentration and mass with Ni salts, while MET and NMET had rather similar lower values. A similar ranking among origins was obtained in hydroponics by Assunção et al. (2003a; 2008). In soil experiments, Dechamps et al. (2008a) showed that a NMET population accumulated more than 15 times more Ni than the MET PR. This suggests that SERP *Noccaea* plants are better adapted to Ni availability than MET and NMET. The adaptation of plant species to serpentine soil is frequent. For instance, in *Helianthus exilis* populations from serpentine and normal soils, growth was significantly higher in their respective soils, showing ecotypical differentiation (Sambatti and Rice 2006). The same result was found for *Collinsia sparsiflora* (Wright et al. 2006) and *Cerastium alpinum* (Berglund et al. 2004). An exception is the case of *Thlaspi goesingense*, where plants from serpentine and normal soils had a similar growth on both soils (Reeves and Baker 1984) showing that tolerance to Ni is constitutive in this species.

Variations among populations There were significant variations for biomass, Ni concentrations and mass among populations for almost all Ni treatments. In particular, the two SERP populations often showed different responses, with PW having lower biomass values and higher Ni concentrations than BE, which was in agreement with field concentration results (BE 1,882-5945 mg Ni/kg; *n*=4; PW 3170–8550 mg Ni/kg *n*=4, Reeves et al. 2001). PW was the population which showed the highest Ni concentration values for all of the Ni treatments and the highest Ni mass values in 3 of 6 treatments. The 1000 $NiSO_4$ treatment decreased survival as 4 of 8 populations had a mortality of ≥80 %.

Interactions between elements Cataldo et al. (1978) showed that the transfer of Ni from root to shoot in soybean was inhibited by the presence of Zn^{2+}. In the current study, the antagonism between Zn and Ni appeared at the concentration of 250mgNi/kg, particularly for serpentine populations. The latter had Ni concentration values that were four times lower, and Ni mass values two to seven times lower in binary mixtures than in monometallic salts at equivalent concentrations. Surprisingly, in salt mixtures, the lowest concentrations of Ni (100 mg/kg) produced the highest Ni concentration values for TR (MET), SE and WI (NMET) and PW (SERP). A similar phenomenon was reported by Assunção et al. (2008), where the highest concentration of a metal (in our case 250Zn) inhibited the concentration of another metal less than a lower concentration (100Zn250Ni). Taylor and Macnair (2006) showed an inhibition of Ni translocation from roots to shoots in presence of Zn in two serpentine endemic species, *Thlaspi pindicum* (*Noccaea tymphaea*) and *T. alpinum* var. *sylvium* (*Noccaea sylvia*), and concluded that Ni transport was achieved by acting on a pre-existing Zn transporters and therefore that Zn accumulation may have evolved first.

Perspectives with field data The SERP PW was the only one to almost always reach the Ni hyperaccumulation threshold of 1000 mg/kg, even in the 250Zn100Ni

treatment. Nickel can be highly accumulated in leafs of field-growing NMET plants (178 mg Ni/kg with a soil Ni concentration of 1 mg/kg, $n=15$; Molitor et al. 2005). It is therefore obvious that any phytoextraction program intending to improve Ni extraction should use serpentine populations.

Comparisons of Zn concentration and mass between soils and hydroponics

The results of Zn concentrations in hydroponics are close to those of Shen et al. (2000; Zn concentration in solution of 500μM), Zhao et al. (1998; 1000μM) or Brown et al. (1995; 3600μM Zn in solution) after approximately 1 month of culture when using the metallicolous PR population. Brown et al. (1995) showed values of Zn concentration of 26,000 mg/kg and Zn mass of 35 mg/plant with 3600 μM Zn after 30 days of growth. In the present study, about 22000 mg/kg was obtained in 2 mM Zn concentrations for MET and NMET plants, and masses of around 70 and 10 mg/plant for MET and NMET, respectively after 3 months of growth. These values are considerably higher than those obtained with the soil from Les Avinières mine which, nevertheless, had a very high Zn concentration. When AV and SF (MET) populations, were cultivated in soils, they had a Zn concentration of about 1/4 and a Zn mass of 1/10 of those obtained in hydroponics. In addition, when MET and NMET populations were cultivated in Les Avinières soil, they showed significantly different mean Zn concentrations, which was not the case in high Zn hydroponic solution. In addition, the results between the two Zn treatments in hydroponics were very different. In the low Zn solution differences between origins for biomass, Zn concentration and Zn mass were significant only in the last harvest after 125 days of growth. If we had stopped the experiment after 90 days of growth (as for the experiments with soil) results obtained in the low Zn concentrations would not have shown a clear pattern between the populations of the two origins. In the high Zn solution, MET had higher biomass and Zn mass than NMET in the last three harvests.

In mine soil, there was no Zn mass differences between MET and NMET compared to the large differences in hydroponics. Thus, the results obtained in hydroponics and in Les Avinières mine soil are contradictory.

We suggest that these contradictory results may be due to the fact that in hydroponic culture, Zn is directly available because of the low pH of the nutrient solution and there are no complex molecules like organic matter or clay, which may interact with Zn absorption. Similarly, Assunção et al. (2003a) compared Zn concentrations in MET and NMET in hydroponics but did not find significant differences between the Ganges population (named here AV) and the non-metallicolous population from Lellingen (close to WI).

Conclusion

This study shows that differences in concentrations and masses occur between MET, NMET and SERP origins when taking into account all treatments. However, there was also a large heterogeneity of responses in terms of metal concentration among populations of the same origin. The numerous treatments used in this study clearly show that the highest Zn concentrations and Zn masses were achieved by different populations. MET populations have higher Cd masses, mainly due to higher Cd tolerance in Cd-contaminated soil. It was also shown that the addition of Cd or Ni to a Zn-contaminated soil significantly decreased the shoot Zn concentration. Ni experiments showed that serpentine populations were particularly adapted to Ni-rich soils as they had the highest values of Ni concentration, but they were also able to have high Zn concentrations in Les Avinières mine soil. Finally, the experiments in hydroponics give Zn concentrations and Zn mass far higher and contradictory with those found in a mine soil. Data on metal concentration obtained in culture soils are closer to those in field soils than those from hydroponics so that they could give a more accurate information on the accumulating capacity of *Noccaea caerulescens* and its use in phytoextraction of metals in field conditions.

References

Antonovics J, Bradshaw AD, Turner RG (1971) Heavy metal tolerance in plants. Adv Ecol Res 7:1–85

Assunção AGL, Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO (2003a) Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens*

populations originating from different soil types. New Phytol 159:411–419

Assunção AGL, Schat H, Aarts MGM (2003b) *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. New Phytol 159:351–360

Assunção AGL, Ten Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO (2003c) A co-segregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. New Phytol 159:383–390

Assunção AGL, Bleeker P, ten Bookum WM, Vooijs R, Schat H (2008) Intra-specific variation of metal preference patterns for hyperaccumulation in *Thlaspi caerulescens*: evidence from binary metal exposures. Plant Soil 303:289–299

Baker AJM, Brooks RR (1989) Terrestrial higher plants which hyperaccumulate metallic elements—a review of their distribution, ecology and phytochemistry. Biorecovery 1:81–126

Baker AJM, Walker PL (1990) Ecophysiology of metal mass by tolerant plants. In: Shaw AJ (ed) Heavy metal tolerance in plants: evolutionary aspects. CRC Press, Boca Raton, pp 155–177

Banásová V, Horak O, Nadubinská M, Čiamporová M, Lichtscheidl I (2008) Heavy metal content in *Thlaspi caerulescens* J. et C. Presl growing on metalliferous and non-metalliferous soils in Central Slovakia. Int J Environ Pollut 33:133–145

Basic N, Keller C, Fontanillas P, Vittoz P, Besnard G, Galland N (2006) Cadmium hyperaccumulation and reproductive traits in natural *Thlaspi caerulescens* populations. Plant Biology 8:64–72

Berglund ABN, Dahlgren S, Westerbergh A (2004) Evidence for parallel evolution and site-specific selection of serpentine tolerance in *Cerastium alpinum* during the colonization of Scandinavia. New Phytol 161:199–209

Brown SL, Chaney RL, Angle JS, Baker AJM (1995) Zinc and cadmium mass by hyperaccumulator *Thlaspi caerulescens* grown in nutrient solution. Soil Sci Soc Am Proc 59:125–133

Cataldo DA, Garland TR, Wildung RE (1978) Nickel in Plants. I. Uptake kinetics using intact soybean seedlings. Plant Physiol 62:563–565

Chardot V, Echevarria G, Gury M, Massoura S, Morel JL (2007) Nickel bioavailability in an ultramafic toposequence in the Vosges Mountains (France). Plant Soil 293:7–21

Cottenie A, Verloo M, Kiekens L, Velghe G, Camerlynck R (1982) Chemical analysis of plants and soils. State University Ghent, Belgium, Laboratory of Analytical and Agrochemistry, 63p

Dechamps C, Roosens NH, Hotte C, Meerts P (2005) Growth and mineral element composition in two ecotypes of *Thlaspi caerulescens* on Cd contaminated soil. Plant Soil 273:327–335

Dechamps C, Lefèbvre C, Noret N, Meerts P (2007) Reaction norms of life history traits in response to zinc in *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites. New Phytol 173:191–198

Dechamps C, Noret N, Mozek R, Draye X, Meerts P (2008a) Root allocation in metal-rich patch by *Thlaspi caerulescens* from normal and metalliferous soil - new insights into the rhizobox approach. Plant Soil 310:211–224

Dechamps C, Noret N, Mozek R, Escarré J, Lefèbvre C, Gruber W, Meerts P (2008b) Cost of adaptation to a metalliferous environment for *Thlaspi caerulescens*: a field reciprocal transplantation approach. New Phytol 177:167–177

Escarré J, Lefèbvre C, Gruber W, Leblanc M, Lepart J, Rivière Y, Delay B (2000) Zinc and cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implications for phytoremediation. New Phytol 145:429–437

Escarré J, Lefèbvre C, Raboyeau S, Dossantos A, Gruber W, Cleyet-Marel JC, Frérot H, Noret N, Mahieu S, Collin C, van Oort F (2011) Heavy metal concentration survey in soils and plants of the Les Malines mining district (Southern France): Implications for soil restoration. Water Air Soil Pollut 216:485–504

Fangueiro D, Bermond A, Santos E, Carapuça H, Duarte A (2005) Kinetic approach to heavy metal mobilization assessment in sediments: choose of kinetic equations and models to achieve maximum information. Talanta 66:844–857

Faucon MP (2004) Adaptation des plantes aux sites métallifères. I. Réponse à l'hétérogénéité du substrat (calcaire et schisteux) chez *Thlaspi caerulescens*. II. Propriétés allélopathiques chez *Armeria maritima*. Mémoire de Licence. Université Libre de Bruxelles, Brussels (Belgium)

Frérot H, Lefèbvre C, Petit C, Collin C, Dos Santos A, Escarré J (2005) Zinc tolerance and hyperaccumulation in F_1 and F_2 offspring from intra- and inter-ecotype crosses of *Thlaspi caerulescens*. New Phytol 165:111–119

Frérot H, Lefèbvre C, Gruber W, Collin C, Dos Santos A, Escarré J (2006) Specific interactions between local metallicolous plants improve the phytostabilization of mine soils. Plant Soil 282:53–65

Garnier E (1992) Growth analysis of congeneric annual and perennial grass species. J Ecol 80:665–675

Gregor JW, Watson PJ (1961) Ecotypic differentiation: Observations and reflections. Evolution 15:166–173

Grotz N, Fox T, Connolly E, Park W, Guerinot ML, Eide D (1998) Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. Proc Natl Acad Sci SA 95:7220–7224

Hart J, Welch R, Norvell W, Clarke J, Kochian L (2005) Zinc effects on cadmium accumulation and partitioning in near-isogenic lines of durum wheat that differ in grain cadmium concentration. New Phytol 167:391–401

Koch GW, Winner WE, Nardone A, Mooney HA (1987) A system for controlling the root and shoot environment for plant growth studies. Environ Exp Bot 27:365–377

Labanowski J, Monna F, Bermond A, Cambier P, Fernandez C, Lamy I, van Oort F (2008) Kinetic extractions to assess mobilization of Zn, Pb, Cu, and Cd in a metal-contaminated soil: EDTA vs. citrate. Environ Pollut 153:693–701

Lombi E, Zhao FJ, Dunham SJ, McGrath SP (2001a) Phytoremediation of heavy metal-contaminated soils: Natural hyperaccumulation versus chemically enhanced phytoextraction. J Environ Qual 30:1919–1926

Lombi E, Zhao FJ, McGrath SP, Young SD, Sacchi GA (2001b) Physiological evidence for a high-affinity cadmium transporter highly expressed in a *Thlaspi caerulescens* ecotype. New Phytol 149:53–60

Macnair MR, Smirnoff N (1999) Use of zincon to study mass and accumulation of zinc by zinc tolerant and

hyperaccumulating plants. Commun Soil Sci Plant Anal 30:1127–1136

Meerts P, Van Isacker N (1997) Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. Plant Ecology 133:221–231

Meerts P, Duchêne P, Gruber W, Lefèbvre C (2003) Metal accumulation and competitive ability in metallicolous and non-metallicolous *Thlaspi caerulescens* fed with different Zn salts. Plant Soil 249:1–8

Meyer FK (2006) Kritische Revision der "*Thlaspi*"-Arten Europas, Afrikas und Vorderasiens. Spezieller Teil. IX. *Noccaea* MOENCH. Thüringische Botanische Gesellschaft, Haussknechtia Suppl.12. 343p.

Molitor M, Dechamps C, Gruber W, Meerts P (2005) *Thlaspi caerulescens* on nonmetalliferous soil in Luxembourg: ecological niche and genetic variation in mineral element composition. New Phytol 165:503–512

Noret N, Meerts P, Tolrà R, Poschenrieder C, Barceló J, Escarré J (2005) Palatability of *Thlaspi caerulescens* for snails: influence of zinc and glucosinolates. New Phytol 165:763–772

Noret N, Meerts P, Vanhaelen M, Dos Santos A, Escarré J (2007) Do metal-rich plants deter herbivores? A field test of the defence hypothesis. Oecologia 152:92–100

Peer WA, Mamoudian M, Lahner B, Reeves RD, Murphy AS, Salt DE (2003) Identifying model metal hyperaccumulating plants: germplasm analysis of 20 *Brassicaceae* accessions from a wide geographical area. New Phytol 159:421–430

Reeves RD, Baker AJM (1984) Studies on metal mass by plants from serpentine and non-serpentine populations of *Thlaspi goesingense* Hálácsy (Cruciferae). New Phytol 98:191–204

Reeves RD, Schwartz C, Morel JL, Edmondson J (2001) Distribution and metal-accumulating behaviour of *Thlaspi caerulescens* and associated metallophytes in France. Int J Phytoremediation 3:145–172

Robinson BH, Leblanc M, Petit D, Brooks RR, Kirkman JH, Gregg PEH (1998) The potential of *Thlaspi caerulescens* for phytoremediation of contaminated soils. Plant Soil 203:47–56

Roosens N, Verbruggen N, Meerts P, Ximénez-Embún P, Smith JAC (2003) Natural variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of *Thlaspi caerulescens* from western Europe. Plant Cell Environ 26:1657–1672

Sambatti JBM, Rice KJ (2006) Local adaptation, patterns of selection, and gene flow in the Californian serpentine sunflower (*Helianthus exilis)*. Evolution 60:696–710

SAS (2004) SAS-STAT® 9.1 User's guide. In: SAS Institute Inc, Cary, NC, USA

Shen ZG, Li XD, Chen HM (2000) Comparison of elemental composition and solubility in the zinc hyperaccumulator *Thlaspi caerulescens* with the non-hyperaccumulator *Thlaspi ochroleucum*. Bull Environ Contam Toxicol 65:343–350

STATISTIX (2003) STATISTIX 8. User's manual. Analytical Software. Tallahassee, FL. USA

Taylor SI, Macnair MR (2006) Within and between population variation for zinc and nickel accumulation in two species of *Thlaspi* (Brassicaceae). New Phytol 169:505–513

Tutin TG, Burges NA, Chater AO, Edmondson JR, Heywood VH, Moore DM, Valentine DH, Walters SM, Webb DA (1964–1993) Flora Europaea. Cambridge University Press, Cambridge, UK

van der Ent A, Baker AJM, Reeves RD, Pollard AJ, Schat H (2012) Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. Plant Soil 362:319–334

Wright JW, Stanton ML, Scherson R (2006) Local adaptation to serpentine and non-serpentine soils in *Collinsia sparsiflora*. Evol Ecol Res 8:1–21

Zhao FJ, Shen ZG, McGrath SP (1998) Solubility of zinc and interactions between zinc and phosphorus in the hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ 21:108–114

Zhao FJ, Hamon RE, Lombi E, McLaughlin MJ, McGrath SP (2002) Characteristics of cadmium mass in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens*. J Exp Bot 53:535–543

Zhao FJ, Lombi E, McGrath SP (2003) Assessing the potential for zinc and cadmium phytoremediation with the hyperaccumulator *Thlaspi caerulescens*. Plant Soil 249:37–43

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Research

Genetic architecture of zinc hyperaccumulation in *Arabidopsis halleri*: the essential role of QTL × environment interactions

Hélène Frérot[1], Michel-Pierre Faucon[1], Glenda Willems[1], Cécile Godé[1], Adeline Courseaux[1], Aude Darracq[1], Nathalie Verbruggen[2] and Pierre Saumitou-Laprade[1]

[1]Laboratoire de Génétique et Evolution des Populations Végétales, UMR CNRS 8016, Université des Sciences et Technologies de Lille - Lille1, F-59655 Villeneuve d'Ascq Cedex, France; [2]Laboratoire de Physiologie et de Génétique Moléculaire des Plantes, Université Libre de Bruxelles, Campus de la Plaine, CP242, boulevard du Triomphe, 1050 Bruxelles, Belgium

Author for correspondence:
Hélène Frérot
Tel: +33 3 20 43 40 33
Email: helene.frerot@univ-lille1.fr

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Summary

- This study sought to determine the main genomic regions that control zinc (Zn) hyperaccumulation in *Arabidopsis halleri* and to examine genotype × environment effects on phenotypic variance. To do so, quantitative trait loci (QTLs) were mapped using an interspecific *A. halleri* × *Arabidopsis lyrata petraea* F_2 population.
- The F_2 progeny as well as representatives of the parental populations were cultivated on soils at two different Zn concentrations. A linkage map was constructed using 70 markers.
- In both low and high pollution treatments, zinc hyperaccumulation showed high broad-sense heritability (81.9 and 74.7%, respectively). Five significant QTLs were detected: two QTLs specific to the low pollution treatment (chromosomes 1 and 4), and three QTLs identified at both treatments (chromosomes 3, 6 and 7). These QTLs explained 50.1 and 36.5% of the phenotypic variance in low and high pollution treatments, respectively. Two QTLs identified at both treatments (chromosomes 3 and 6) showed significant QTL × environment interactions.
- The QTL on chromosome 3 largely colocalized with a major QTL previously identified for Zn and cadmium (Cd) tolerance. This suggests that Zn tolerance and hyperaccumulation share, at least partially, a common genetic basis and may have simultaneously evolved on heavy metal-contaminated soils.

Introduction

Metal hyperaccumulation in plants is the capacity to concentrate high amounts of certain metals in the shoots without suffering from toxicity symptoms (Baker, 1981; Baker & Brooks, 1989; Reeves & Baker, 2000). In metallophyte or pseudometallophyte species, all or some populations can successfully grow and reproduce on heavy metal-contaminated soils (the so-called 'metal tolerance'; Antonovics *et al.*, 1971; Macnair, 1983). Only a few species are able to accumulate metals in their aerial parts (Baker & Brooks, 1989; Baker *et al.*, 2000; Broadley *et al.*, 2001). Whether hyperaccumulation is an adaptive trait to extreme metallic environments is still elusive, as many hypotheses of adaptive function have been proposed. Hyperaccumulation may be a mechanism of metal detoxification and tolerance, metal removal from the rhizosphere for further elimination by defoliation or rainfall action, drought resistance, allelopathy, the by-product of a mechanism that has another adaptive function (often called 'inadvertent uptake hypothesis'), or protection against herbivores or pathogens (summarized in Boyd & Martens, 1992). To date, however, none of these hypotheses have been able to completely elucidate the origin of metal hyperaccumulation (Whiting *et al.*, 2003; Noret *et al.*, 2005). Hyperaccumulation is also interesting from an environmental or agronomic point of view. In mining or industrial sites and their surroundings, heavy metals are responsible for severe and unhealthy soil contamination. In these cases, hyperaccumulating plants could be used as biological tools for phytoremediation techniques as they may

help remove metals from soils (Salt *et al.*, 1995, 1998; Pilon-Smits, 2005). Since some heavy metals are also essential minerals that can be deficient in staple food crops, genetic determinants of hyperaccumulation could be utilized in biofortification with the aim of improving the nutritional value of these crops (Cakmak, 2008; Jeong & Guerinot, 2008; Mayer *et al.*, 2008; Palmgren *et al.*, 2008). Since metal hyperaccumulation is of both fundamental and applied interest, the mechanisms underlying this trait thus deserve to be understood, from root uptake to vacuolar sequestration, via xylem loading, translocation to shoots, and xylem unloading to leaf cells (for review, see Clemens, 2001, 2006; Verbruggen *et al.*, 2009). Over the past few years, the genetics of metal accumulation has benefited from the development of molecular tools (Verbruggen *et al.*, 2009). Transcriptomic analyses comparing hyperaccumulator and nonaccumulator species has revealed that many genes are involved in hyperaccumulation and show different expression profiles or regulation-level modifications (Becher *et al.*, 2004; Weber *et al.*, 2004; Filatov *et al.*, 2006; van de Mortel *et al.*, 2006, 2008; Talke *et al.*, 2006).

Arabidopsis halleri is a pseudometallophyte of the Brassicaceae family. This species has been described as cadmium (Cd)-tolerant, constitutively zinc (Zn)-tolerant as well as Zn-hyperaccumulating (Bert *et al.*, 2000, 2002, 2003; Macnair, 2002; Pauwels *et al.*, 2006). While metallicolous populations tend to show higher Zn tolerance than nonmetallicolous populations (Pauwels *et al.*, 2006), they also display lower hyperaccumulation capacities in controlled conditions than nonmetallicolous populations (Bert *et al.*, 2000). Being a close relative of *Arabidopsis thaliana*, with which it shows roughly 94% nucleotide identity within coding regions (Becher *et al.*, 2004), *A. halleri* is an excellent model species for studying metal tolerance and hyperaccumulation (Pauwels *et al.*, 2008a,b; Roosens *et al.*, 2008a,b). Using first-generation backcross progeny (BC1) from an interspecific cross between *A. halleri* and *Arabidopsis lyrata petraea*, a nontolerant and nonaccumulator relative, quantitative trait locus (QTL) analyses have been performed for Zn (Willems *et al.*, 2007) and Cd tolerance (Courbot *et al.*, 2007). A major QTL region common to Zn and Cd tolerance has been identified, colocalizing with *HMA4*, a gene encoding a heavy metal-transporting ATPase. The importance of this gene was recently confirmed with functional studies using RNAi-mediated silencing (Hanikenne *et al.*, 2008). *A. halleri* plants with reduced expression of *HMA4* translocate less Zn from roots to shoots, while *A. thaliana* plants expressing *AhHMA4* show an increase in shoot Zn accumulation. However, because these transgenic *A. thaliana* plants showed signs of Zn hypersensitivity in shoots (Hanikenne *et al.*, 2008), *AhHMA4* expression alone is not sufficient to detoxify Zn. Additional genes are thus required to completely understand Zn hyperaccumulation in *A. halleri*.

In this study, to identify the main genomic regions responsible for Zn hyperaccumulation in *A. halleri*, QTL mapping was performed on F_2 progeny from an *A. halleri* and *A. lyrata petraea* cross. In *A. halleri*, Zn hyperaccumulation variability depends on external Zn concentrations (Macnair, 2002), which implies that variation in Zn hyperaccumulation may – in part – correspond to a genotype × environment interaction. This type of interaction was tested in this study by cultivating the same F_2 progeny at different Zn concentrations. Since it has been demonstrated that interactions among genes (i.e. epistasis) are also involved in genetic architecture of adaptive traits (Malmberg & Mauricio, 2005), epistasis was also evaluated here. Additionally, based on a large set of markers common to the *A. halleri* × *A. lyrata petraea* BC1 map and the F_2 linkage map, the genomic regions detected for Zn : Cd tolerance and Zn (this paper) or Cd (Willems *et al.*, 2010) hyperaccumulation were compared. Finally, taking advantage of the high synteny between the *A. halleri* and the *A. thaliana* genomes (Roosens *et al.*, 2008a,b), putative candidate genes for Zn hyperaccumulation were proposed.

Materials and Methods

Plant material

Arabidopsis halleri (L.) O'Kane et Al-Shehbaz individuals originated from an industrial calamine site in the north of France contaminated with Zn, Cd and lead (Auby, France) (Van Rossum *et al.*, 2004), and *A. lyrata petraea* individuals came from a nonpolluted site in the Czech Republic (Unhošť, Central Bohemia) (Macnair *et al.*, 1999). Both species have a haploid set of eight chromosomes. They are self-incompatible. Hence, to avoid inbreeding depression effect, two randomly selected *A. halleri* (pollen donor) individuals and two randomly selected *A. lyrata petraea* (pollen recipient) individuals were necessary to produce two independent F_1 progeny. Two randomly selected F_1 plants from each cross were used to generate F_2 populations, and the F_2 progeny presenting the largest seed number (roughly 300) was selected. The F_2 population used for linkage map construction consisted of 288 individuals, of which 208 were analyzed for Zn accumulation. F_2 genotypes were duplicated and maintained in time by cuttings. As cuttings of parental and F_1 plants died before they could be analyzed, individuals from the same population (n = 4 for *A. halleri* and n = 3 for *A. lyrata petraea*) or generation (n = 8 for F_1) were used in this study.

Plant cultivation and evaluation of Zn hyperaccumulation

All plants were grown individually in 1 l pots containing compost, in a glasshouse environment (temperature, 20°C

day : 15°C night; photoperiod, 14 h day : 10 h night). The photoperiod was adjusted by 400 W high-pressure sodium lamps, photosynthetically active radiation (PAR) of 90 μM photons m^{-2} s^{-1} over the wavelength range 400–700 nm; the lamps were automatically switched off when daylight was sufficiently intense. Plants were watered every 2 d with deionized water. After 6 months of cultivation, leaves were collected for DNA extraction.

Several cuttings of each genotype were produced so as to obtain six replicates of the same genotype. Three replicates were grown in a 'low pollution' (LP) treatment consisting of compost with no added Zn. Total Zn concentration in an LP treatment corresponded to 15 mg kg^{-1} dry matter, while available Zn concentration corresponded to 8 mg kg^{-1} dry matter. The three other replicates were grown in a 'high pollution' (HP) treatment consisting of a soil contaminated with 50 ml of a 100 mM ($ZnSO_4$, $7H_2O$) solution added to 650 g of fresh compost, 2 d before transplantation to allow restoration of equilibrium between soil elements. Total Zn concentration was, in this case, 1500 mg kg^{-1} dry matter, while available Zn concentration was 1200 mg kg^{-1} dry matter. This concentration was selected because it ensured complete survival and sufficient growth of all plants, as it corresponded to a low amount of pollution in comparison to that in metalliferous sites (Bert *et al.*, 2002; Meyer *et al.*, 2010). Environmental heterogeneity was controlled by a weekly rotation of the 1248 pots. After 6 wk of cultivation, the whole rosette of each replicate was harvested, washed twice in demineralized water, dried at 55°C for 72 h, weighted and ground. A 25 mg aliquot was digested in 750 μl of a 2% sulfosalicylic acid solution. After 24 h of digestion, 4 μl of this solution was mixed with 40 μl of 0.03% zincon solution, the colorimetric reagent, and 156 μl of a buffer at pH 9.6 (Macnair & Smirnoff, 1999). Absorbance values were measured at 606 nm on a microplate absorbance reader (SUNRISE Tecan V 3.17, Grödig, Austria). Shoot Zn concentrations were then expressed as mg kg^{-1} shoot dry weight.

Statistical analysis

The arithmetic mean of Zn accumulation for each parental and F_1 representative and each F_2 genotype was calculated from the three replicates in each treatment. On the base of mean value for each F_2 genotype, the Spearman rank correlation between the two treatments (CORR procedure of SAS Institute 2002) and the comparison of means between the two treatments using a paired Student's *t*-test (TTEST procedure of SAS Institute 2002) were computed. A normality test based on the Kolmogorov–Smirnov D statistics (UNIVARIATE procedure of SAS Institute 2002) was also performed (H_0: the distribution data corresponds to a normal distribution). Because of a significant but quite low departure from normality ($D = 0.09$), mainly as a result of extreme values, data were not transformed before analysis of variance (ANOVA). A two-way ANOVA using the GLM procedure in SAS Institute (2002) was performed to determine the treatment and genotype effects and their interaction. The main factor 'genotype' was considered as a random effect, because the F_2 individuals tested for Zn accumulation represent a random sampling of the total F_2 population. Type III sums of squares were used because of unbalanced data sets. Variance components were also estimated using the TYPE 1 method of the VARCOMP procedure of SAS Institute (2002). The broad-sense heritability of Zn accumulation was estimated for each environment using variance components as $H^2 = V_G/V_P$ (Wu & Stettler, 1997; Juenger *et al.*, 2005). V_G is the total genetic variance, which included additive genetic variance and other genetic sources of variance (dominance and epistasis). V_P is the total phenotypic variance calculated as ($V_G + V_{error}$). These two variance components were estimated using the TYPE 1 method of the VARCOMP procedure of SAS Institute (2002), with the 'genotype' factor considered as a random effect.

Quantitative trait locus × environment interactions were tested by two-way analyses of variance by using the GLM procedure of SAS Institute (2002). The models involved two main fixed factors represented by one of the markers 1-04488, 2-08286, 2-15997, 4-17540 (instead of 4-17202 because this marker displayed only two genotype classes), 5-05494 or 5-04824, at QTL positions or closest to the QTL positions, and Zn treatment (HP or LP). In addition, between-QTLs interactions at the corresponding markers were tested using exact nonparametric Kruskall–Wallis tests (StatXact v.7 Cytel Studio 2005, Cambridge, Massachusetts, USA) because sample sizes were very unbalanced (see Supporting Information Table S3).

Development of the markers

The genomic DNA of the four parental genotypes, the two F_1 individuals that were crossed out, and 288 individuals of the F_2 progeny, was extracted using a Kit NucleoSpin 96 Plant (Macherey-Nagel, Hoerdt, France). Seventy markers were selected for genotyping (Table S1). Some of them are derived from genes directly implied in metal homeostasis in either *A. halleri* or *Thlaspi caerulescens* (*Ah-NAS2*, *Ah-CCH*, *Ah-HMA4*, *Ah-NRAMP3*, *Ah-ZIP6*, *Ah-CAX1*, *Ah-MHX1*, *Ah-HMADP2*, *Ah-YSL3*, *Ah-NRAMP4*, and *Tc-UP2*). Details on 44 of these markers (primer sequence and PCR conditions) were described in Willems *et al.* (2007), Courbot *et al.* (2007), Roosens *et al.* (2008a) and Ruggiero *et al.* (2008), as they were previously mapped in the BC1 progeny of *A. halleri* and *A. lyrata petraea*. Two new markers were developed to complete the genetic map in uncovered regions or to replace previously mapped markers that were not polymorphic in the F_2. Marker Chr.5-21773/*Ah-YSL3*, belonging to the Yellow-Stripe-like transporter

family (nicotianamine complex) and chosen for its position on linkage group 8, and marker Chr.3-05134/Lyr133, a microsatellite locus defined in *A. lyrata petraea*, were kindly provided by Dr Tom Mitchell-Olds. These markers were amplified and scored as described in Willems *et al.* (2007) with the specific PCR condition: 50°C annealing temperature for 1 min and 50°C for 45 s, respectively. Twenty-two new microsatellite markers were also produced in this work to increase the coverage of the *A. halleri* genetic map. One microsatellite marker was defined within exon 10 from the *HMA4* sequence (Hanikenne *et al.*, 2008) and included a simple sequence repeat (SSR) motif corresponding to $(CCA)_6$. The other new microsatellite markers were selected from a microsatellite-enriched genomic library developed in the GEPV laboratory following an enrichment procedure with Dynabeads (Glenn & Schable, 2005). *In silico* analysis on sequenced microsatellites were conducted with an in-house program using ClustalW (Larkin *et al.*, 2007) to eliminate sequence redundancy and MREPS (Kolpakov *et al.*, 2003) to find microsatellite patterns. Primer sequences were designed in flanking regions of *A. halleri* microsatellites using Primer3 software (http://frodo.wi.mit.edu/). In order to allow polymerase chain reaction (PCR) multiplexing of markers, primer combinations were chosen with a 60°C (± 5°C) melting temperature, and a PIG-tail (GTGTCTT) was added to the 5′-end region of the reverse primer to facilitate adenylation and avoid stutter bands (Brownstein *et al.*, 1996). To help combination into four fluorescently labelled multiplex groups, allowing the loading of multiplexes, a universal M13 tail (CACGACGTTGTAAAACGAC) was added to the 5′-region of the forward primers.

Multiplex PCR was carried out in 10 μl reactions containing 1× PCR buffer II (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$, 150 nM dNTP (Euromedex, France), 0.075 μM of the Forward-M13 primer, 0.375 μM of the reverse-PIG primer, 1.5 μM of fluorescently dye labelled M13 (Applied Biosystems), 0.5 U of Qiagen Multiplex PCR kit, and 5 μl DNA (20–60 ng). PCRs were conducted on a Mastercycler pro S (Eppendorf, UK). In order to improve the specificity and the quality of amplification, parameters for thermal cycling included two touchdown phases in which the annealing temperature was decreased by 2°C every cycle as follows: 94°C for 5 min followed by first touchdown over five cycles of 45 s at 95°C, 5 min at 68–60°C, 1 min at 72°C, followed by second touchdown over five cycles of 45 s at 95°C, 1 min at 58–50°C, 1 min at 72°C, then 27 cycles of 45 s at 95°C, 30 s at 47°C, 1 min at 72°C, and a final elongation cycle of 10 min at 72°C. Allele sizing of microsatellite multiplex amplified products was performed using an ABI Prism® 3100 Genetic Analyzer 16-capillary array system (Applied Biosystems). The four PCR products labelled with different dyes were mixed and 1.5 μl of the mixture was added to 0.25 μl GeneScan-500 LIZ Size standard and 9.95 μl of HiDi formamide (both products from Applied Biosystems). Fragment sizes were analyzed with GeneMapper Software version 3.7 (Applied Biosystems).

The putative position of microsatellite markers on the *A. thaliana* genome was determined by searching for sequence homology using the BLAST function of NCBI (http://www.ncbi.nlm.nih.gov/). For Ah75 and Ah77 markers, the expected position was not consistent with the observed position on the *A. halleri* map, so that no position was indicated (Table S1).

Linkage analysis and map construction

The *A. halleri* × *A. lyrata petraea* linkage map was constructed using the software package JoinMap 3.0 (Van Ooijen & Voorrips, 2001). Markers that were polymorphic in the parents and in the F_1 displayed from two to four different alleles that could be identified in F_2 progeny. For population type CP ('cross-pollinators'), JoinMap software uses different segregation types corresponding to genotypes of the last parental generation, here the F_1 generation. These segregation types are <abxcd> for four alleles, <efxeg> for three alleles, and <hkxhk>, <lmxll> and <nnxnp> for two alleles. These genotypes are also associated with their linkage phases for both parents during the estimation of recombination frequencies.

The grouping of loci is based upon a test for independence translated into a logarithm of odds (LOD) score. Loci determined to be significantly associated at a reasonable LOD threshold will be in the same group. Then, to order loci on linkage groups, a mapping procedure was used, which is based on a sequential method adding loci one by one and starting from the most informative pair of loci. For each added locus, the best position is sought by comparing the goodness-of-fit of the resulting map for each tested position. The goodness-of-fit measure is a G^2 likelihood ratio statistic that compares all direct recombination frequencies (i.e. the pairwise data based on the original genotype data of the two loci involved) with the map-derived combination frequencies (i.e. calculated with an inverse mapping function). Kosambi's mapping function (Kosambi, 1944) was used to convert recombination frequencies into map distances (cM).

Marker segregation

According to Mendelian inheritance, the allele segregation in the F_2 progeny is expected to fit different ratios depending on the segregation type, that is, 1 : 1 : 1 : 1 for <abxcd> and <efxeg>, 1 : 2 : 1 for <hkxhk>, and 1 : 1 for <lmxll> and <nnxnp>. Deviations from Mendelian expected ratios were performed in JoinMap 3.0 using a chi-square test at a locus-by-locus significance threshold of 5% (Van Ooijen & Voorrips, 2001).

Detection of QTLs

Potential QTLs for each trait (ZnAcLP for 'Zn accumulation in LP treatment' and ZnAcHP for 'Zn accumulation in HP treatment') were detected using the MapQTL 4.0 software (Van Ooijen *et al.*, 2002). A Kruskall–Wallis rank test was first performed on each locus separately to find potential regions of QTL. A segregating QTL closely linked to the tested marker results in a large difference in average rank of the marker genotype classes. Interval mapping (IM) analysis then allowed a finer detection by testing the occurrence of a QTL and computing a LOD score for every centiMorgan (cM) along the linkage groups. The LOD score represents the 10-base logarithm of the quotient of two likelihoods: the likelihood of the presence of a segregating QTL (alternative hypothesis) divided by the likelihood of no segregating QTL (null hypothesis). To declare the occurrence of a QTL, the calculated LOD scores were compared with a LOD score threshold obtained by a permutation test (1000 permutations), which corresponds to a genome-wide empirical significance threshold at the 5% level (Churchill & Doerge, 1994). In this way the threshold value was set at 4.0 for each trait. A Multiple-QTL Model (MQM) analysis was finally performed every cM, in which markers close to detected QTLs (by IM mapping) were selected as cofactors to take over the role of the nearby QTLs in the approximate multiple-QTL models used in the subsequent MQM analysis. This method reduces the residual variance and enhances the power of searching for other segregating QTLs. It also improves the precision of QTL positions. After manual selection of cofactors, an automatic selection of cofactors was executed to keep a restricted set of significant cofactors. MQM mapping was thus performed again using this new set of cofactors to obtain the best possible QTL positions and maximal LOD scores. The threshold LOD score was maintained at 4.0 for each trait. The LOD score profiles showing QTLs with their one- and two-LOD support intervals were obtained using MapChart 2.1 (Voorrips, 2002).

Additive effects (a) of the QTL were calculated as follows: $(\mu_{lyr1/lyr2} - \mu_{hal1/hal2})/2$, where $\mu_{hal1/hal2}$ and $\mu_{lyr1/lyr2}$ are the mean concentrations for the homospecific genotypes *halleri*-1/*halleri*-2 and *lyrata*-1/*lyrata*-2 at the markers closest to or at the QTL. Two heterospecific genotype classes (*halleri*-1/*lyrata*-2 and *halleri*-2/*lyrata*-1) can be distinguished at the QTL in this mapping population. Therefore, dominance effects (d) at the QTL correspond to either $(\mu_{hal1/lyr2} - (\mu_{hal1/hal2} + \mu_{lyr1/lyr2})/2)$ or $(\mu_{hal2/lyr1} - (\mu_{hal1/hal2} + \mu_{lyr1/lyr2})/2)$, where $\mu_{hal1/lyr2}$ and $\mu_{hal2/lyr1}$ are the mean concentrations of the genotypes that are heterospecific *halleri*-1/*lyrata*-2 and *halleri*-2/*lyrata*-1, respectively, at the QTL. All the mean concentrations were estimated by the MQM method implemented in MapQTL. The degree of dominance at the QTL is obtained by the ratio $|d/a|$. According to the degree of dominance, QTLs were classified as additive ($|d/a| < 0.2$), partially dominant ($0.2 \leq |d/a| < 0.8$), dominant ($0.8 \leq |d/a| < 1.2$), or overdominant ($|d/a| \geq 1.2$) (Stuber *et al.*, 1987).

Results

Trait heritability and segregation

In LP and HP treatments, the broad-sense heritabilities estimated as a percentage of total phenotypic variance were high: 81.9 and 74.7%, respectively.

Zinc accumulation values in the LP treatment were distributed between 176 and almost 3000 mg kg^{-1} among the F_2 progeny, while in the HP treatment they were widely distributed from almost 1000 to 9196 mg kg^{-1} (Fig. 1). All the *A. lyrata petraea* representatives fell in the lowest phenotype class, whereas the *A. halleri* representatives and F_1 progeny showed a larger distribution. Some F_1 progeny were phenotypically close to *A. lyrata petraea*, indicating that Zn accumulation was partially recessive in *A. halleri*. Because parents' representatives were not the

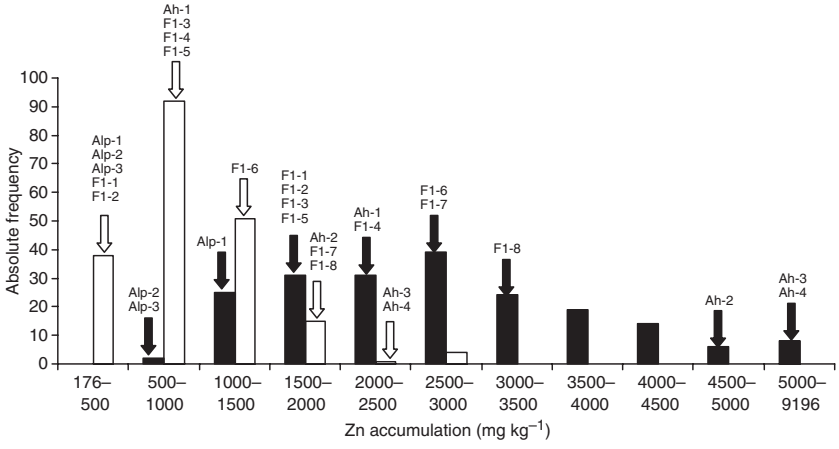

Fig. 1 Segregation profiles of Zn accumulation in the 'low pollution' treatment (white bars) and the 'high pollution' treatment (black bars). Parents' representatives are noted above the arrows to give their phenotype class. Alp, representatives of *Arabidopsis lyrata petraea*; Ah, representatives of *Arabidopsis halleri*; F_1, representatives of F_1 progeny.

original parents, it was not possible to evaluate phenotypic transgression effectively. Nevertheless, only four F_2 individuals in the LP treatment and two F_2 individuals in the HP treatment showed higher accumulation values than *A. halleri* individuals (data not shown). In addition, it is worth noting that in the two soil treatments, the *A. halleri* individuals displayed different phenotypic values for accumulation. This suggests that Zn accumulation capacity is not fixed in the species. The two segregation profiles did not correspond to a normal distribution ($D = 0.09$, $P < 0.01$ for both LP and HP). Nevertheless, the skewness (1.4) and kurtosis (3.9) values were not very dissimilar to those of a normal distribution (0 and 3, respectively). For this reason, and because normality was difficult to be significantly improved, the data were not transformed before parametric tests.

The paired *t*-test showed that mean shoot Zn concentrations were significantly different between LP and HP treatments ($t = 25.78$, df = 196, $P < 0.001$). The means ± SD were 906 ± 480 mg kg^{-1} for the LP treatment and 2805 ± 1261 mg kg^{-1} for the HP treatment. A noticeable change of ranking among genotypes between LP and HP treatments was shown by crossing reaction norms (Supporting Information, Fig. S1). Nevertheless, it seemed that most of reaction norms looked similar and simply showed an increase in Zn concentration differences among genotypes from LP to HP treatment (i.e. a change of scales). This was supported by a significant Spearman rank correlation ($r = 0.49$, $P < 0.001$) between the values of both treatments.

Linkage map

All the 288 individuals from the F_2 progeny were used for the construction of the *A. halleri* × *A. lyrata petraea* linkage map. At a LOD threshold of 4, the 70 markers were assigned to eight linkage groups (Fig. 2) corresponding to the same linkage groups as described in Willems *et al.* (2007). The lengths of each linkage group varied from 51.9 to 85.2 cM, while the marker number varied from 6 to 11 by linkage group. The total length was *c.* 526 cM. The map produced in this study covered 87% of the previous *A. halleri* map (Willems *et al.*, 2007) with an average distance of 8.5 cM between adjacent markers varying from < 1 to 34 cM. The order of the 31 markers shared with Willems *et al.* (2007) was identical in both studies.

Markers in segregation distortion

At a significance threshold of 5%, 46 markers (i.e. 66% of the markers) showed a significant departure from the expected Mendelian ratio (Fig. 2). These markers were located on the eight linkage groups, mainly on LG1, LG2, LG6 and LG7. For all markers on LG1 and half of the markers on

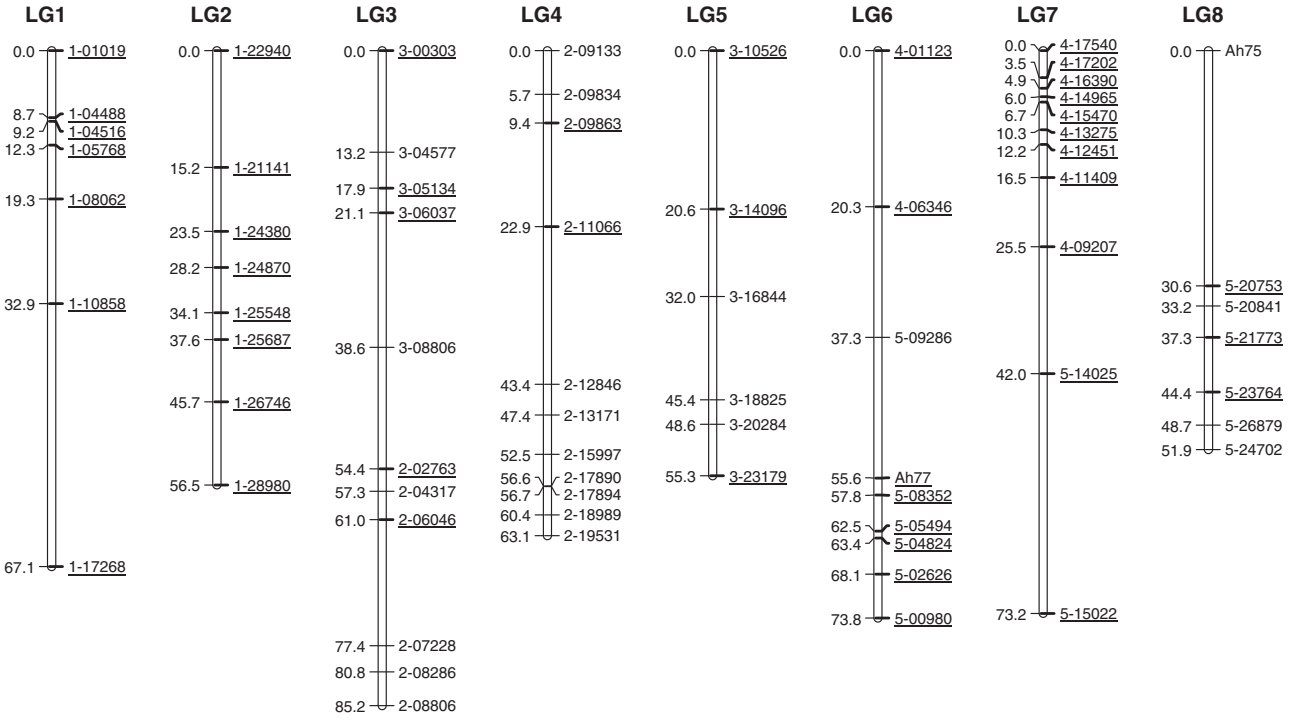

Fig. 2 Linkage map of the *Arabidopsis halleri* × *Arabidopsis lyrata petreae* F_2 progeny constructed with JoinMap 3.0. Markers are labelled on the right of bars by their approximate position on the *Arabidopsis thaliana* genome (chromosome number-position in kb) except for Ah75 and Ah77 (see text for details). Distances on the genetic map are expressed in cM on the left of the bars. Names of loci in segregation distortion are underlined. Ah, *Arabidopsis halleri* microsatellite marker; LG, linkage group.

LG6 and LG7, the progeny was deficient in homospecific *A. halleri* genotype (Table S2). By contrast, for all markers on LG2 and the other part of LG6, the progeny was deficient in homospecific *A. lyrata petraea* genotype (Table S2).

QTL mapping of Zn accumulation in LP and HP treatments

Five QTL regions, located on five linkage groups (LG1, LG3, LG4, LG6 and LG7), were detected by IM and subsequent MQM Mapping as they showed LOD score values above the LOD score threshold (Fig. 3). Five QTL regions that were associated with Zn accumulation in the LP treatment explained together 50.1% of the total phenotypic variance, with most of the variance explained by ZnAcLP-3 on linkage group 7 (Table 1). Three QTL regions were involved in Zn accumulation in the HP treatment. They explained 36.5% of the total phenotypic variance, mostly because of ZnAcHP-1 on linkage group 3 (Table 1). Three QTLs were common to both Zn treatments (ZnAcLP-1/ZnAcHP-1 on LG3, ZnAcLP-2/ZnAcHP-2 on LG6 and ZnAcLP-3/ZnAcHP-3 on LG7), while two additional QTLs were specific to the LP treatment (ZnAcLP-4 on LG1 and ZnAcLP-5 on LG4). Suggestive QTLs were also noticed on chromosomes 2, 4 and 8 as LOD score values (*c.* 3) were close to the LOD threshold (Fig. 3).

For all QTLs in each treatment negative additive effects were obtained, meaning that the *A. halleri* allele increases Zn accumulation compared with the *A. lyrata petraea* allele (Table 2). Dominance effects were either negative or positive, and in some cases variable for a given QTL depending on the parental alleles. This suggests that the parental *A. halleri* alleles can be frequently recessive ($a < 0$ and $d < 0$). Dominance degrees ranged from 0.006 to 2.23. QTLs were rarely additive (three cases among 18) but were often dominant and partially dominant (11 cases). Two QTLs (ZnAcLP-1 and ZnAcLP-3) were slightly over-dominant.

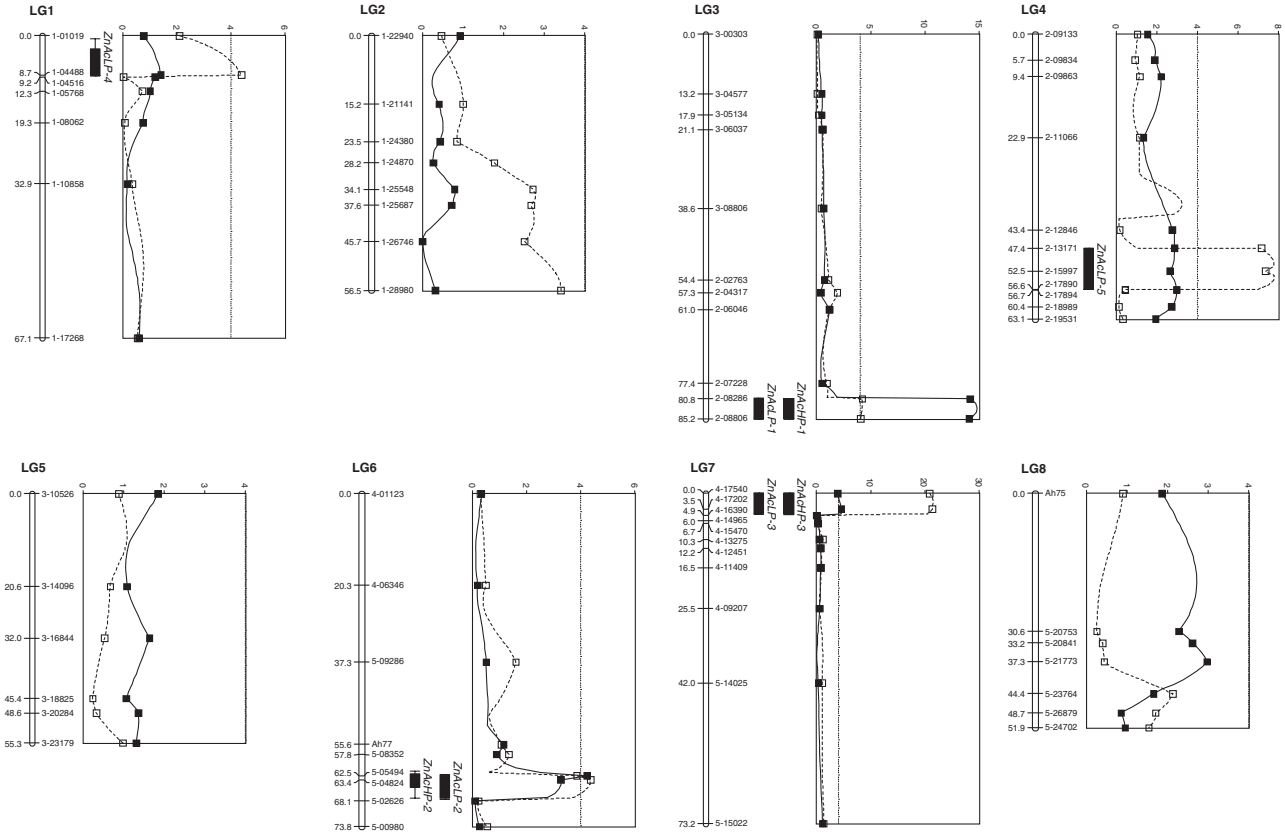

Fig. 3 Quantitative trait locus (QTL) mapping results for Zn accumulation in 'low pollution' (LP) and 'high pollution' (HP) treatments obtained by the Multiple QTL Model (MQM) Mapping method. The eight linkage groups (LG) are represented. Marker names are designated by the position on *Arabidopsis thaliana* chromosomes (except for Ah75 and Ah77 (*Arabidopsis halleri* microsatellite marker), see text for details). On the associated logarithm of odds (LOD) score profiles: horizontal axis, LOD scores; closed squares, HP treatment; open squares, LP treatment. The vertical dotted lines represent the LOD score threshold (4.0) at a 5% error level for QTL detection. The positions of QTLs ('ZnAcLP' for QTL of Zn accumulation in the LP treatment, and 'ZnAcHP' for QTL of Zn accumulation in the HP treatment) are indicated by bars representing the one-LOD support intervals (one LOD score unit on either side of the QTL peak) and whiskers representing the two-LOD support intervals (two LOD score unit on either side of the QTL peak). Bars and whiskers, 95% confidence interval.

Table 1 Summary characteristics of quantitative trait loci (QTLs) detected for zinc (Zn) accumulation in 'low pollution' (LP) and 'high pollution' (HP) treatments

LG/marker[a]	QTL designation[b]	Location[c]	QTL confidence interval[d]	LOD score[e]	R^{2f}
LG1/1-04488	ZnAcLP-4	8.7	1-01019; 1-04488	4.39	4.7
LG3/2-08286	ZnAcLP-1	81.8	2-08286; 2-08806	4.25	4.2
LG3/2-08286	ZnAcHP-1	82.8	2-08286; 2-08806	14.85	24.1
LG4/2-15997	ZnAcLP-5	50.4	2-13171; 2-17894	7.77	8.6
LG6/5-04824	ZnAcLP-2	63.4	5-02626; 5-05494	4.36	4.6
LG6/5-05494	ZnAcHP-2	62.5	5-02626; 5-05494	4.22	5.9
LG7/4-17202	ZnAcLP-3	2.0	4-16390; 4-17540	21.59	28.0
LG7/4-17202	ZnAcHP-3	3.5	4-16390; 4-17540	4.48	6.5

[a]Linkage groups (LGs) where the QTLs were detected and markers at, or closest to, the QTL positions.
[b]QTLs are designed by the trait (Zn accumulation) and the treatment (LP for 'low pollution' treatment and HP for 'high pollution' treatment).
[c]Location of the QTL on each linkage group (in centiMorgans, cM) according to the Multiple QTL Model (MQM) Mapping method.
[d]95% confidence intervals designed by positions of the markers closest to the lower and upper bounds, transferred onto the *Arabidopsis thaliana* genome and expressed as 'chromosome number-position in kb'.
[e]Maximum logarithm of odds (LOD) score value of the linkage group obtained by the MQM Mapping method.
[f]Percentage of variance explained by the QTL.

Table 2 Additive effects, dominance effects, and dominance degrees at the quantitative trait locus (QTL) for Zn accumulation

LG/marker	QTL designation	a[a]	$d_{hal1/lyr2}$[b]	$d_{hal2/lyr1}$[b]	$\|d/a\|_{hal1/lyr2}$[c]	$\|d/a\|_{hal2/lyr1}$[c]
LG1/1-04488	ZnAcLP-4	−192.93	−86.28	−29.46	0.44	0.15
LG3/2-08286	ZnAcLP-1	−115.74	145.84	−24.37	1.26	0.21
LG3/2-08286	ZnAcHP-1	−848.03	476.99	150.02	0.56	0.17
LG4/2-15997	ZnAcLP-5	−200.26	164.68	146.04	0.82	0.72
LG6/5-04824	ZnAcLP-2	−125.22	58.53	−128.74	0.46	1.02
LG6/5-05494	ZnAcHP-2	−481.08	−305.79	−309.43	0.63	0.64
LG7/4-17202	ZnAcLP-3	−179.74	−171.25	401.26	0.95	2.23
LG7/4-17202	ZnAcHP-3	−427.76	2.69	340.12	0.006	0.79

LG, linkage group.
[a]Additive effects of the QTL calculated as $(\mu_{lyr1/lyr2} - \mu_{hal1/hal2})/2$, where $\mu_{hal1/hal2}$ and $\mu_{lyr1/lyr2}$ are the mean concentrations for the homospecific genotypes *Arabidopsis halleri*-1/*halleri*-2 and *A. lyrata*-1/*lyrata*-2 at the markers closest to or at the QTL.
[b]Dominance effects at the QTL corresponding to either $(\mu_{hal1/lyr2} - (\mu_{hal1/hal2} + \mu_{lyr1/lyr2})/2)$ or $(\mu_{hal2/lyr1} - (\mu_{hal1/hal2} + \mu_{lyr1/lyr2})/2)$, where $\mu_{hal1/lyr2}$ and $\mu_{hal2/lyr1}$ are the mean concentrations of the genotypes that are heterospecific *halleri*-1/*lyrata*-2 and *halleri*-2/*lyrata*-1, respectively.
[c]Degree of dominance at the QTL: a QTL is classified as additive ($|d/a| < 0.2$), partially dominant ($0.2 \leq |d/a| < 0.8$), dominant ($0.8 \leq |d/a| < 1.2$), or overdominant ($|d/a| \geq 1.2$).

Interaction tests

Interactions between the QTLs for each treatment were assessed. Even though they were all significant (Table S3), this result has to be interpreted with caution because of very small sample sizes in several genotype classes. Nevertheless, mean phenotype values were always higher in hhxhh genotype classes (when existing) than in llxll genotype classes. Other genotype classes displayed intermediate values. In most cases, a higher number of *A. halleri* alleles resulted in a higher mean accumulation value.

Genotype × environment interactions significantly explained variations in shoot Zn concentration even though the variance component was only 13.6% (Table 3). Likewise, QTL × environment interactions were significant only for ZnAcLP-1/ZnAcHP-1 at marker 2-08286 ($F = 13.43$, df = 3, $P < 0.001$), ZnAcLP-2/ZnAcHP-2 at both close markers 5-04824 ($F = 3.48$, df = 2, $P = 0.032$) and 5-05494 ($F = 5.04$, df = 2, $P = 0.0069$), but surprisingly not for ZnAcLP-3/ZnAcHP-3 at marker 4-17540 ($F = 0.81$, df = 3, $P = 0.49$). However, the 4-17540 marker showed many missing values (see Table S2) which could bias the result, and hence the occurrence of a QTL × environment interaction could not be totally excluded for the ZnAcLP-3/ZnAcHP-3 QTL.

Discussion

Genetic architecture of Zn hyperaccumulation in *Arabidopsis halleri*

This is the first study to locate QTLs of Zn hyperaccumulation on an *A. halleri* × *A. lyrata petraea* genetic linkage map using soil with two different Zn concentrations. At both Zn

Table 3 ANOVA on Zn accumulation values in *Arabidopsis* in both low pollution and high pollution treatments

Source	df	Type III SS	MS	*F*	*P* > *F*	Variance component (%)
Treatment	1	852824650	852824650	2547.94	< 0.0001	58.8
Genotype	202	608169558	3010740	9.00	< 0.0001	16.5
Treatment × genotype	196	256682696	1309606	3.91	< 0.0001	13.6
Error	595	199152913	334711	11.1		

pollution levels, Zn accumulation was a widely segregating trait in the *A. halleri* × *A. lyrata petraea* F_2 progeny. The continuous and quite normal distribution suggests polygenic inheritance, consistent with previous reports. Indeed, in *A. halleri* as well as *T. caerulescens* metallicolous populations continuous variations for Zn hyperaccumulation were observed (Pollard & Baker, 1996; Macnair, 2002). Pollard *et al.* (2002) suggested that one or a few major genes would control the ability to hyperaccumulate metals, while multiple modifier genes probably regulated the degree of hyperaccumulation. Assunção *et al.* (2003) found a continuous segregation pattern in *T. caerulescens*, and suggested that Zn hyperaccumulation was governed by more than one gene.

The linkage map produced in this study, constructed from 70 markers, was highly congruent with the linkage map reported in Willems *et al.* (2007). It showed the eight linkage groups corresponding to the haploid chromosome number of both parental species with a reasonable number of markers by group (≥ 6) and a relevant average distance between markers (8.5 cM). Strong segregation distortion, as reported on the *A. halleri* × *A. lyrata petraea* BC1 map (Willems *et al.*, 2007), was observed in the F_2 progeny. As, on the genetic linkage map presented in this study, segregation distortion largely involved the same linkage groups as on the BC1 map, and the distorted markers were in most cases linked to markers distorted in the same direction, segregation biases were probably mainly the result of biological reasons rather than genotyping errors. In an interspecific cross, outbreeding depression can be a biological explanation. As an example, genetic combinations between *A. halleri* alleles favoring Zn accumulation and *A. lyrata petraea* alleles decreasing Zn tolerance are possible. Such combinations may have been selected against.

Using this linkage map, this study demonstrated that Zn hyperaccumulation is largely controlled by five QTL regions in the LP treatment (50.1% of the phenotypic variance) on chromosomes 1, 3, 4, 6 and 7 and three QTL regions in the HP treatment (36.5% of the phenotypic variance) on chromosomes 3, 6 and 7. With the exception of chromosome 1, these chromosomes were also reported in previous studies using *A. halleri* × *A. lyrata petraea* progenies to harbor genes involved in Zn hyperaccumulation (Filatov *et al.*, 2006, 2007). Filatov *et al.* (2006) compared the transcriptional profiles of *A. halleri* with those of *A. lyrata petraea*, and also of accumulator F_3 families with those of nonaccumulator F_3 families. They tested the cosegregation with Zn accumulation of markers significantly differing in their expression level. On chromosome 3, the QTL region identified by Filatov *et al.* (2006) was located between the markers 3-05134 and 2-02763, that is, it did not overlap with the QTL region detected in the present study (between 2-08286 and 2-08806). At any rate, on this chromosome, they had no marker located between 2-02763 and 2-08806, and were therefore not able to detect a QTL in this region. The authors also detected a QTL region on chromosome 7 between 4-09207 and 4-16390 markers, which was adjacent to the QTL identified in the present study (between 4-16390 and 4-17540). Filatov *et al.* (2007) constructed a linkage map between *A. halleri* and *A. lyrata petraea* based on 25 markers and assessed Zn hyperaccumulation using hydroponics at two Zn concentrations. They revealed three genomic regions contributing to Zn accumulation, on chromosomes 4, 6 and 7. Some of their markers significantly associated with Zn accumulation (Ahp8, Ahp20, Ahp21, Ahp22 and Ahp23) were located in or close to QTL regions identified in the present study on the same chromosomes. In addition, these authors also detected a possible interaction with metal concentration on chromosome 7, as it was also suggested here at the QTL ZnAcLP-3/ZnAcHP-3.

At all QTLs, *A. halleri* alleles provided higher Zn accumulation values, though with various degrees of dominance. Several *A. halleri* alleles were totally recessive, as suggested by the skewed distribution of F_1 phenotypes towards low Zn accumulation values. As parents' representatives showed different Zn accumulation concentrations, it was expected that several alleles, at one or a few genes, were segregating in *A. halleri* and *A. lyrata petraea*, and thus that several allelic combinations could appear in the F_2 progeny. However, transgressive F_2 phenotypes were rare, suggesting that phenomena such as epistasis or overdominance would be occasional. This was confirmed by the occurrence of only two overdominant QTLs. In addition, epistatic effects estimated through QTL × QTL interactions are still elusive because many genotype classes are poorly represented. Four of the six markers at which QTL × QTL interactions were tested showed significant segregation distortion and, more precisely, a shortage of hal/hal genotypes (Table S2). QTLs for Zn hyperaccumulation detected in this study are consequently largely additive, which is consistent with the observation that the joined effect of several *A. halleri* alleles

at the different QTLs tend to enhance Zn accumulation values. Furthermore, the genotype × environment interaction significantly affected Zn hyperaccumulation, as suggested by significant interaction terms at the QTLs ZnAcLP-1/AnAcHP-1 and ZnAcLP-2/ZnAcHP-2. Surprisingly, while the magnitude of the effect of the QTL ZnAcLP-3/ZnAcHP-3 was highly different in both environments, a significant QTL × environment interaction was not detected at this QTL. As only few reaction norms apparently crossed (Fig. S1), cross-over effects (inversion in the ranking of allelic effects across environments) seemed probable but rare in comparison to scale effects (variation in the magnitude of allelic effects without inversion of ranks) (Juenger *et al.*, 2005). Hence, not surprisingly, the three QTLs identified at both treatments were involved in scale effects, since *A. halleri* alleles increased Zn accumulation in both treatments (additive effects remained negative). No QTL × environment effect was detected at the two treatment-specific QTLs ZnAcLP-4 and ZnAcLP-5, suggesting that phenotypic values at these QTLs in both environments are not contrasted enough, perhaps because of weak statistical power (missing genotype data, segregation distortion).

The occurrence of QTLs identified at either one or both treatments supports distinct genetic models to explain genotype × environment interactions: the allelic sensitivity model (also known as the pleiotropic model) and the gene regulation model (also known as the epistatic model or conditional neutrality) (Juenger *et al.*, 2005; Lacaze *et al.*, 2009). The first model implies that constitutive genes directly exhibit differential expression across environments, while the second one implies that regulatory genes mediate expression of constitutive genes, resulting in their expression only in some environments. Therefore, it cannot be excluded that, in addition to Zn homeostasis genes, regulatory genes may be major determinants in Zn accumulation.

The putative candidate genes

Physiological and biochemical studies showed the importance of the following steps for metal hyperaccumulation: enhanced root uptake, limited storage in the root, active xylem loading, transport by ligands, efficient unloading and storage in the leaves (see Verbruggen *et al.*, 2009 for review; Sarret *et al.*, 2009). Therefore, genes controlling basic mechanisms of Zn homeostasis can be candidate genes for Zn hyperaccumulation. Such candidate genes can be identified in QTL regions according to the method described in Roosens *et al.* (2008b), which takes advantage of the synteny between the *A. halleri* × *A. lyrata petraea* linkage map and the *A. thaliana* genome. Following this method, several genes could be proposed for ZnAcLP-4 and ZnAcLP-5 that showed higher expression levels in *A. halleri* compared with *A. thaliana* (Talke *et al.*, 2006): *ZIP3* and *ZIP4* at ZnAcLP-4, and *SAMS3* and *MTP11* at ZnAcLP-5. In the ZnAcLP-3/ZnAcHP-3 QTL region, *HMA1* was a possible candidate gene, although its expression regulation under different Zn supply is still unknown. Indeed, AtHMA1 is located in chloroplast membrane and affects shoot Zn content (Kim *et al.*, 2009). The most promising candidate was *HMA4*, located in ZnAcLP-1/ZnAcHP-1 QTL. This QTL showed a major effect at high external Zn concentration, and *HMA4* is a gene whose role in Zn homeostasis becomes essential at high Zn concentrations since it encodes a Zn : Cd : Pb pump that ensures effective metal translocation to the shoot after metal uptake and radial symplastic passage through the root (Courbot *et al.*, 2007; Hanikenne *et al.*, 2008).

In addition to the candidate genes presented here, it cannot be excluded that other genes may be actually responsible for Zn hyperaccumulation in *A. halleri*. On the one hand, most of the investigated QTL regions contained several interesting genes with regard to Zn hyperaccumulation, or genes encoding proteins that are not yet characterized in *A. thaliana.* These genes could become pertinent after further functional studies. On the other hand, QTL positions may be imprecise because of genotyping and/or phenotyping errors. Therefore, identification of candidate genes in QTL regions as performed in this study based on synteny between the QTL regions on the *A. halleri* × *A. lyrata petraea* linkage map and the *A. thaliana* genome has to be cautiously interpreted.

Evolution of Zn tolerance and hyperaccumulation

Being an exceptional phenomenon, Zn hyperaccumulation raises several interesting evolutionary questions, such as the nature of the selection pressure, or the relationship with Zn tolerance, which is a clearly adaptive trait. As has been demonstrated for *T. caerulescens* (Assunção *et al.*, 2003; Frérot *et al.*, 2005), this study shows that, also in *A. halleri*, Zn tolerance and hyperaccumulation are partially correlated. Indeed, the major QTL of Zn tolerance on chromosome 3 identified by Willems *et al.* (2007) largely colocalizes with the ZnAcLP-1/ZnAcHP-1 QTL found in the present study. As for Cd and Zn tolerance (Courbot *et al.*, 2007; Willems *et al.*, 2007), *AhHMA4* remains the most likely candidate gene for Zn hyperaccumulation. Indeed, translocation from root to shoot driven by HMA4 may improve Zn tolerance by maintaining low metal concentration in cytoplasm of root cells, and, simultaneously, tends to increase hyperaccumulation in shoots. Such common genetic bases suggest that Zn tolerance and hyperaccumulation may have simultaneously evolved on heavy metal-contaminated soils.

By contrast, while two copies of the vacuolar transporter *MTP1* gene colocalized with two Zn tolerance QTLs (Willems *et al.*, 2007), no *MTP1* gene copy appeared in the QTL regions detected for Zn accumulation. As has been

evidenced in the hyperaccumulator *T. caerulescens* (Küpper *et al.*, 1999), Zn is probably also sequestered in the vacuolar compartment in *A. halleri* (Küpper *et al.*, 2000). Moreover, the role of MTP1 in Zn tolerance and accumulation was recently demonstrated in *Thlaspi goesingense* (Gustin *et al.*, 2009). One possible reason why no QTL at *MTP1* was detected is that variation of *MTP1* expression or function may be present, but is controlled by genetic variation at the locus of a MTP1 regulatory gene. Alternatively, this study may also suggest that, in *A. halleri*, mechanisms other than vacuolar sequestration might be primarily involved in Zn hyperaccumulation, without excluding a role of MTP1, although a less significant one. In support, hyperaccumulation of Zn in leaves of the hyperaccumulator *T. caerulescens* seems to be primarily dictated by root processes, as recently demonstrated by grafting experiments (Guimaraes *et al.*, 2009). Detoxification processes, including vacuolar sequestration, could have evolved only secondarily on metal-contaminated sites, while Zn hyperaccumulation could originate from nonmetalliferous sites. In this regard it would be interesting to perform QTL analyses using progenies involving *A. halleri* individuals from nonmetallicolous populations, as these could reveal original genetic determinants of Zn hyperaccumulation in *A. halleri*.

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References

Antonovics J, Bradshaw AD, Turner RG. 1971. Heavy metal tolerance in plants. *Advances in Ecological Research* **7**: 1–85.

Assunção AGL, Ten Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO. 2003. A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **159**: 1–8.

Baker AJ. 1981. Accumulators and excluders strategies in the response of plants to heavy metals. *Journal of Plant Nutrition* **8**: 643–654.

Baker AJM, Brooks RR. 1989. Terrestrial higher plants which hyperaccumulate metallic elements – a review of their distribution, ecology and phytochemistry. *Biorecovery* **1**: 81–126.

Baker AJM, McGrath SP, Reeves RD, Smith JAC. 2000. Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. In: Terry N, Bañuelos G, eds. *Phytoremediation of contaminated soil and water*. Boca Raton, FL, USA: Lewis Publishers, 85–107.

Becher M, Talke IN, Krall L, Krämer U. 2004. Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant Journal* **37**: 251–268.

Bert V, Bonnin I, Saumitou-Laprade P, de Laguérie P, Petit D. 2002. Do *Arabidopsis halleri* from nonmetalicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **155**: 47–57.

Bert V, Macnair MR, De Laguérie P, Saumitou-Laprade P, Petit D. 2000. Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **146**: 225–233.

Bert V, Meerts P, Saumitou-Laprade P, Salis P, Gruber W, Verbruggen N. 2003. Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and Soil* **249**: 9–18.

Boyd RS, Martens SN. 1992. The *raison d'être* for metal hyperaccumulation by plants. In: Baker AJM, Proctor J, Reeves RD, eds. *The ecology of ultramafic (serpentine) soils*. Andover, UK: Intercept, 279–289.

Broadley MR, Willey NJ, Wilkins JC, Baker AJ, Mead A, White PJ. 2001. Phylogenetic variation in heavy metal accumulation in angiosperms. *New Phytologist* **152**: 9–27.

Brownstein MJ, Carpten JD, Smith JR. 1996. Modulation of non-templated nucleotide addition by tag DNA polymerase: primer modifications that facilitate genotyping. *BioTechniques* **20**: 1004.

Cakmak I. 2008. Enrichment of cereal grains with zinc: agronomic or genetic biofortification? *Plant and Soil* **302**: 1–17.

Churchill GA, Doerge RW. 1994. Empirical threshold values for quantitative trait mapping. *Genetics* **138**: 963–971.

Clemens S. 2001. Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* **212**: 475–486.

Clemens S. 2006. Toxic metal accumulation, responses to exposure and mechanismes of tolerance in plants. *Biochimie* **88**: 1707–1719.

Courbot M, Willems G, Motte P, Arvidsson S, Roosens N, Saumitou-Laprade P, Verbruggen N. 2007. A major quantitative trait locus for cadmium tolerance in *Arabidopsis halleri* colocalizes with HMA4, a gene encoding a heavy metal ATPase1[OA]. *Plant Physiology* **144**: 1052–1065.

Filatov V, Dowdle J, Smirnoff N, Ford-Lloyd B, Newbury HJ, Macnair MR. 2006. Comparison of gene expression in segregating families identifies genes and genomic regions involved in a novel adaptation, zinc hyperaccumulation. *Molecular Ecology* **15**: 3045–3059.

Filatov V, Dowdle J, Smirnoff N, Ford-Lloyd B, Newbury H, Macnair MR. 2007. A quantitative trait loci analysis of zinc hyperaccumulation in *Arabidopsis halleri*. *New Phytologist* **174**: 580–590.

Frérot H, Lefèbvre C, Petit C, Collin C, Dos Santos A, Escarré J. 2005. Zinc tolerance and hyperaccumulation in F_1 and F_2 offspring from intra and interecotype crosses of *Thlaspi caerulescens*. *New Phytologist* **165**: 111–119.

Glenn TC, Schable NA. 2005. Isolating microsatellite DNA loci. *Methods in Enzymology* **395**: 202–222.

Guimaraes MD, Gustin JL, Salt DE. 2009. Reciprocal grafting separates the roles of the root and shoot in zinc hyperaccumulation in *Thlaspi caerulescens*. *New Phytologist* **184**: 323–329.

Gustin JL, Loureiro ME, Kim D, Na G, Tikhonova M, Salt DE. 2009. MTP1-dependent Zn sequestration into shoot vacuoles suggests dual roles in Zn tolerance and accumulation in Zn-hyperaccumulating plants. *Plant Journal* **57**: 1116–1127.

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Krämer U. 2008. Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. *Nature* **453**: 391–396.

Jeong J, Guerinot ML. 2008. Biofortified and bioavailable: the gold standard for plant-based diet. *Proceedings of the National Academy of Sciences, USA* **105**: 1777–1778.

Juenger TE, Sen S, Stowe KA, Simms EL. 2005. Epistasis and genotype-environment interaction for quantitative trait loci affecting flowering time in *Arabidopsis thaliana*. *Genetica* **123**: 87–105.

Kim Y-Y, Choi H, Segami S, Cho H-T, Martinoia E, Maeshima M, Lee Y. 2009. AtHMA1 contributes to the detoxification of excess Zn(II) in Arabidopsis. *Plant Journal* **58**: 737–753.

Kolpakov R, Bana G, Kucherov G. 2003. MREPS: efficient and flexible detection of tandem repeats in DNA. *Nucleic Acid Research* **31**: 3672–3678.

Kosambi D. 1944. The estimation of map distances from the recombination values. *Annals of Eugenics* **12**: 172–175.

Küpper H, Lombi E, Zhao FJ, McGrath SP. 2000. Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta* **212**: 75–84.

Küpper H, Zhao FJ, McGrath SP. 1999. Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **119**: 305–311.

Lacaze X, Hayes PM, Korol A. 2009. Genetics of phenotypic plasticity: QTL analysis in barley, *Hordeum vulgare*. *Heredity* **102**: 163–173.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R *et al.* 2007. ClustalW and ClustalX version 2. *Bioinformatics* **23**: 2947–2948.

Macnair MR. 1983. The genetic control of copper tolerance in the yellow monkey flower, *Mimulus guttatus*. *Heredity* **50**: 283–293.

Macnair MR. 2002. Within and between population genetic variation for zinc accumulation in *Arabidopsis halleri*. *New Phytologist* **155**: 59–66.

Macnair MR, Bert V, Huitson SB, Saumitou-Laprade P, Petit D. 1999. Zinc tolerance and hyperaccumulation are genetically independant characters. *Proceedings of the Royal Society of London, Biological Series* **266**: 2175–2179.

Macnair MR, Smirnoff N. 1999. Use of zincon to study uptake and accumulation of zinc by zinc tolerant and hyperaccumulating plants. *Communications in Soil Science and Plant Analysis* **30**: 1127–1136.

Malmberg RL, Mauricio R. 2005. QTL-based evidence for the role of epistasis in evolution. *Genetical Research* **86**: 89–95.

Mayer JE, Pfeiffer WH, Beyer P. 2008. Biofortified crops to alleviate micronutrient malnutrition. *Current Opinion in Plant Biology* **11**: 166–170.

Meyer C-L, Kostecka AA, Saumitou-Laprade P, Créach A, Castric V, Pauwels M, Frérot H. 2010. Variability of zinc tolerance among and within populations of the pseudometallophyte *Arabidopsis halleri* and possible role of directional selection. *New Phytologist* **185**: 130–142.

van de Mortel JE, Schat H, Moerland PD, Ver Loren van Themaat E, van der Ent S, Blankestijn H, Ghandilyan A, Tsiatsiani S, Aarts MGM. 2008. Expression differences for genes involved in lignin, gluthatione and sulphate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell & Environment* **31**: 301–324.

van de Mortel JE, Villanueva LA, Schat H, Kwekkeboom J, Coughlan S, Moerland PD, Ver Loren van Themaat E, Koornneef M, Aarts MGM. 2006. Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **142**: 1127–1147.

Noret N, Meerts P, Tolrà RP, Poschenrieder C, Barceló D, Escarré J. 2005. Palatability of *Thlaspi caerulescens* for snails: influence of Zn and glucosinolates. *New Phytologist* **165**: 763–772.

Palmgren MG, Clemens S, Williams LE, Krämer U, Borg S, Schjorring JK, Sanders D. 2008. Zinc biofortification of cereals: problems and solutions. *Trends in Plant Sciences* **13**: 464–473.

Pauwels M, Frérot H, Bonnin I, Saumitou-Laprade P. 2006. A broad-scale study of population differentiation for Zn-tolerance in an emerging model species for tolerance study: *Arabidopsis halleri* (Brassicaceae). *Journal of Evolutionary Biology* **19**: 1838–1850.

Pauwels M, Roosens N, Frérot H, Saumitou-Laprade P. 2008a. When population genetics serves genomics: putting adaptation back in a spatial and historical context. *Current Opinion in Plant Biology* **11**: 129–134.

Pauwels M, Willems G, Roosens N, Frérot H, Saumitou-Laprade P. 2008b. Merging methods in molecular and ecological genetics to study the adaptation of plants to anthropogenic metal-polluted sites: implications for phytoremediation. *Molecular Ecology* **17**: 108–119.

Pilon-Smits E. 2005. Phytoremediation. *Annual Review in Plant Biology* **56**: 15–39.

Pollard AJ, Baker AJM. 1996. Quantitative genetics of zinc hyperaccumulation in *Thlaspi caerulescens*. *New Phytologist* **132**: 113–118.

Pollard AJ, Powell KD, Harper FA, Smith JA. 2002. The genetic basis of metal hyperaccumulation in plants. *Critical Review in Plant Sciences* **21**: 539–566.

Reeves RD, Baker AJM. 2000. Metal accumulating plants. In: Raskin I, Ensley BD, eds. *Phytoremediation of toxic metals: using plants to clean up the environment*. New York, NY, USA: Wiley and Sons, 193–229.

Roosens N, Willems G, Godé C, Courseaux A, Saumitou-Laprade P. 2008a. The use of comparative genome analysis and syntenic relationshipsallows extrapolating the position of Zn tolerance QTL regions from *Arabidopsis halleri* into *Arabidopsis thaliana*. *Plant and Soil* **306**: 105–116.

Roosens N, Willems G, Saumitou-Laprade P. 2008b. Using Arabidopsis to explore zinc tolerance and hyperaccumulation. *Trends in Plant Sciences* **13**: 208–215.

Ruggiero MV, Jacquemin B, Castric V, Vekemans X. 2008. Hitch-hiking to a locus under balancing selection: high sequence diversity and low population subdivision at the S-locus genomic region in *Arabidopsis halleri*. *Genetical Research* **90**: 37–46.

Salt DE, Blaylock M, Kumar NPBA, Dushenkov V, Ensley BD. 1995. Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. *Biotechnology* **13**: 468–474.

Salt DE, Smith RD, Raskin I. 1998. Phytoremediation. *Annual Review of Plant Physiology and Molecular Biology* **49**: 643–668.

Sarret G, Willems G, Isaure MP, Marcus MA, Fakra SC, Frérot H, Pairis S, Geoffroy N, Manceau A, Saumitou-Laprade P. 2009. Zinc distribution and speciation in *Arabidopsis halleri* × *Arabidopsis lyrata* progenies presenting various zinc accumulation capacities. *New Phytologist* **184**: 581–595.

SAS Institute. 2002. *SAS user's guide: statistics. Version 9.1*. Cary, NC, USA: SAS Institute.

Stuber CW, Edwards MD, Wendel JF. 1987. Molecular marker-facilitated investigation of quantitative trait loci in maize. II. Factors influencing yields and its component traits. *Crop Science* **27**: 639–648.

Talke IN, Hanikenne M, Krämer U. 2006. Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene

copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* **142**: 148–167.

Van Ooijen JW, Boer MP, Jansen RC, Maliepaard C. 2002. MapQTL 4.0: software for the calculation of QTL positions on genetic maps. Wageningen, the Netherlands: Plant Research International.

Van Ooijen JW, Voorrips RE. 2001. Joinmap 3.0: software for the calculation of genetic linkage maps. Wageningen, the Netherlands: Plant Research International.

Van Rossum F, Bonnin I, Fénart S, Pauwels M, Petit D, Saumitou-Laprade P. 2004. Spatial genetic structure within a metallicolous population of *Arabidopsis halleri*, a clonal, self-incompatible and heavy-metal-tolerant species. *Molecular Ecology* **13**: 2959–2967.

Verbruggen N, Hermans C, Schat H. 2009. Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist* **181**: 759–776.

Voorrips RE. 2002. Mapchart: software for the graphical presentation of linkage maps and QTLs. *Heredity* **93**: 77–78.

Weber M, Harada E, Vess C, Roepenack-Lahaye E, Clemens S. 2004. Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant Journal* **37**: 269–281.

Whiting SN, Neumann PM, Baker AJM. 2003. Nickel and zinc hyperaccumulation by *Alyssum murale* and *Thlaspi caerulescens* (Brassicaceae) do not enhance survival and whole-plant growth under drought stress. *Plant, Cell & Environment* **26**: 351–360.

Willems G, Dräger D, Courbot M, Godé C, Verbruggen N, Saumitou-Laprade P. 2007. The genetic basis of zinc tolerance in the metallophyte *Arabidopsis halleri* ssp. *halleri* (Brassicaceae): an analysis of quantitative trait loci. *Genetics* **176**: 659–674.

Willems G, Frérot H, Gennen J, Salis P, Saumitou-Laprade P, Verbruggen N. 2010. Quantitative trait loci analysis of mineral element concentrations in an *Arabidopsis halleri* × *Arabidopsis lyrata petraea* F_2 progeny grown on cadmium contaminated soil. *New Phytologist*, doi: 10.1111/j.1469-8137.2010.03294.x.

Wu R, Stettler RF. 1997. Quantitative genetics of growth and development in Populus. II. The partitioning of genotype × environment interaction in stem growth. *Heredity* **78**: 124–134.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Reaction norms for Zn accumulation values in the 'high pollution' treatment (HP) in comparison to values in 'low pollution' (LP) treatment.

Table S1 List of markers used in linkage map construction.

Table S2 List of markers showing significant segregation distortion in comparison to the Mendelian segregation ratios expected in a F_2 progeny.

Table S3 Mean ± SD of Zn accumulation at the nine genotype classes corresponding to marker by marker interactions for each quantitative trait locus (QTL).

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Variability of zinc tolerance among and within populations of the pseudometallophyte species *Arabidopsis halleri* and possible role of directional selection

Claire-Lise Meyer[1,*], Alicja A. Kostecka[2,*], Pierre Saumitou-Laprade[1], Anne Créach[1], Vincent Castric[1], Maxime Pauwels[1] and Hélène Frérot[1]

[1]Laboratoire de Génétique et Évolution des Populations Végétales, UMR CNRS 8016, Université des Sciences et Technologies de Lille, Bâtiment SN2, F–59655 Villeneuve d'Ascq Cedex, France; [2]Department of Ecology, Institute of Botany Polish Academy of Sciences, Lubicz 46, PL–31 512 Cracow, Poland

Author for correspondence:
Claire-Lise Meyer
Tel: +33 320434033
Email: claire-lise.meyer@ed.univ-lille1.fr

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Summary

• We estimated the level of quantitative polymorphism for zinc (Zn) tolerance in neighboring metallicolous and nonmetallicolous populations of *Arabidopsis halleri* and tested the hypothesis that divergent selection has shaped this polymorphism.

• A short-term hydroponic test was used to capture the quantitative polymorphism present between edaphic types, among and within populations. We measured six morphological and physiological traits on shoots and roots to estimate the response of *A. halleri* to Zn. In order to assess the adaptive value of Zn tolerance polymorphism, we compared differentiation of quantitative traits with that of molecular markers.

• Zinc tolerance of metallicolous populations was, on average, higher than that of nonmetallicolous populations according to the morphological and physiological traits measured. Phenotypic variability within edaphic types was very high and mainly explained by polymorphism among individuals within populations. Genetic differentiation for photosystem II yield of leaves (a measure of photosynthetic efficiency) was greater than the differentiation for microsatellite and thus, probably shaped by divergent selection.

• Overall, these results suggest that, in the sampled populations, Zn tolerance has been increased in metallicolous populations through selection on standing genetic variation within local nonmetallicolous ancestral populations.

Introduction

Interaction between selection, gene flow and genetic drift is a key phenomenon because in the case of spatially heterogeneous selection it may result in local adaptation, a mechanism promoting speciation and maintaining adaptive variation. Therefore, a challenging goal of evolutionary biology is to evaluate the relative contribution of selection vs other forces in shaping phenotypic differentiation among populations. Among plants, attractive models to study phenotypic differentiation driven by natural selection are pseudometallophyte species, which have the ability to grow on soils contaminated by heavy metals as well as on noncontaminated soils. In highly contaminated sites, extreme environmental conditions may promote rapid differentiation between metallicolous (M) and nonmetallicolous (NM) populations (Reznick & Ghalambor, 2001; Dechamps *et al.*, 2006; Jiménez-Ambriz *et al.*, 2006). In this context, the trait typically chosen to investigate population differentiation is metal tolerance (i.e. the capacity to survive on soil with levels of metals toxic for most organisms; Antonovics *et al.*, 1971; Macnair & Baker, 1994). Previous investigations focused on this trait because it has been found to evolve rapidly following exposure to heavy metal stress (Wu *et al.*, 1975; Al-Hiyaly *et al.*, 1988) and because it showed intraspecific quantitative variation in several instances (Schat *et al.*, 1993; Meerts & Van Isaker, 1997; Smith & Macnair, 1998; Mengoni *et al.*,

*These authors contributed equally to this work.

2001; Pauwels *et al.*, 2006). In addition, evolutionary studies have shown that metal tolerance could have evolved independently in geographically distant conspecific populations (Schat *et al.*, 1996; Vekemans & Lefèbvre, 1997; Koch *et al.*, 1998; Mengoni *et al.*, 2001; Pauwels *et al.*, 2005).

Among the pseudometallophyte species, *Arabidopsis halleri* has recently emerged as a model species to study tolerance to heavy metals. This species, constitutively tolerant and hyperaccumulator of zinc (Zn) and cadmium (Cd) (Bert *et al.*, 2000, 2002; Küpper *et al.*, 2000; Pauwels *et al.*, 2006), is indeed the closest metal-tolerant relative of the model species *Arabidopsis thaliana* (Al-Shehbaz & O'Kane, 2002), with which it shows high nucleotide sequence identity and good syntheny (Roosens *et al.*, 2008a). Differentiation for Zn tolerance was investigated at a broad scale by Pauwels *et al.* (2006) in 31 European populations using a hydroponic sequential test (Schat & Ten Bookum, 1992). They showed that despite its constitutive nature, Zn tolerance is a quantitative trait in this species, with significant differences in average tolerance among populations. In particular, a tendency towards higher tolerance and less polymorphism in M populations compared with NM ones was shown, suggesting that Zn tolerance could have evolved secondarily in M populations. Nevertheless, the possible role of directional selection in shaping the variability of Zn tolerance among *A. halleri* populations has not been established. These results and those of Pauwels *et al.* (2005), which showed that the M populations have probably been founded independently by the nearby NM populations, may imply distinct genetic bases for increased Zn tolerance in unrelated M populations (Pauwels *et al.*, 2006). Therefore, the role of gene flow, drift and selection in shaping variation of tolerance between edaphic types and among *A. halleri* populations should be investigated on a homogeneous genetic background (i.e. at a local scale in a network of populations with possible gene flow).

To investigate the variability of heavy metal tolerance among and within populations, most studies used tests of tolerance on short periods in hydroponic culture (Humphreys & Nicholls, 1984; Von Frenckell-Insam & Hutchinson, 1993; Schat & Vooijs, 1997; Assunção *et al.*, 2003; Pauwels *et al.*, 2006; Galardi *et al.*, 2007). These tests typically estimate only a part of the phenotypic variability responding to heavy metals (i.e. vegetative growth), but are still very useful because they show high repeatability in space and time (Schat & Ten Bookum, 1992) and allow to control the source of stress acting on the plants (one or a combination of different metals). In addition, when combined with genetic studies such as quantitative trait loci (QTL) or linkage disequilibrium (LD) mapping on large numbers of individuals, these tests provide the opportunity to identify the genetic bases of complex quantitative traits (Courbot *et al.*, 2007; Willems *et al.*, 2007). Short-term hydroponic tests were initially based on either qualitative measures (ability of individuals to produce new roots) at a certain fixed concentration (Macnair, 1983) or quantitative measures (root growth in solutions with and without metal to estimate a tolerance index, Wilkins, 1978). Possible bias, owing to both innate root growth variation and choice of metal concentration, have led Schat & Ten Bookum (1992) to develop a sequential exposure test that uses the *EC*100 (lowest concentration for 100% growth inhibition) for root growth as an end point. This test has been successfully applied in several studies to estimate quantitative differences of tolerance between populations (Schat *et al.*,1996; Schat & Vooijs, 1997; Bert *et al.*, 2000; Pauwels *et al.*, 2006). However, tolerance is then regarded as a binary trait (presence or absence of root growth at a sequence of fixed concentrations), when tolerance may actually show quantitative variation. In addition, the history of exposure during such a sequential test may actually lead to underestimation of the variability in the more tolerant populations (Schat & Ten Bookum, 1992).

Root growth was classically used to estimate heavy metal tolerance because, on one hand, early studies of metal tolerance showed that this trait was particularly sensitive to metals (Bradshaw, 1952). On the other hand, it was considered that the first organ in contact with metals (i.e. roots) is likely to be the one in which metal toxicity will first manifest. Nevertheless, shoot performance could be a more relevant trait according to the species under study. For example, for hyperaccumulator species in which metals are translocated very efficiently towards the leaves where they are stored, we might expect the most striking manifestation of metal toxicity to be found in the leaves. This was observed for *Thlaspi caerulescens*, in which the threshold metal exposure level for leaf chlorosis seems to be a good measure of metal tolerance (Assunção *et al.*, 2003) but not for the nickel hyperaccumulator *Alyssum bertolonii*. In this species the root was more sensitive to metal than the shoot (Galardi *et al.*, 2007), possibly because of a higher fraction of cytosolic metal in root cells. Overall, these results suggest that measuring tolerance is not necessarily a trivial task, such that using a number of different end points is needed to analyse the response of hyperaccumulator to toxic metal concentrations.

In the present study, we investigated the genetic variability of Zn tolerance in *A. halleri* at a local scale in a network of genetically and geographically close M and NM populations. We captured quantitative variation using tolerance indices based on shoot and root morphological and physiological traits. In order to assess the adaptive value of Zn tolerance, we compared population differentiation for each quantitative trait (Q_{ST} statistics) with that for neutral molecular markers (F_{ST} statistics). Departure from neutral expectations ($F_{ST} \neq Q_{ST}$) can be used to distinguish between a history dominated by divergent selection

(F_{ST} < Q_{ST}) as opposed to stabilizing selection (F_{ST} > Q_{ST}) (for review see Whitlock, 2008).

Using the tolerance test and the analysis of molecular markers we addressed three questions:

- On average, is Zn tolerance in metallicolous and nonmetallicolous populations different?
- What is the degree of polymorphism within and among populations?
- Have differences of Zn tolerance among *A. halleri* populations been shaped by natural selection, thus reflecting local adaptation?

Materials and Methods

Populations studied

In June 2005, a total of 12 *A. halleri* (L.) (O'Kane & Al-Shehbaz, 2003) (syn. *Cardaminopsis halleri* (L.) Hayek) subsp. *halleri* populations were sampled in the south of Poland and in Slovakia (Fig. 1 and Table 1). A previous survey based on cpDNA variation has shown that populations in this region are genetically very similar (Pauwels *et al.*, 2005). Sampled populations were categorized as metallicolous (M) and nonmetallicolous (NM) according to the total concentration of Zn in soil (Bert *et al.*, 2002). The M populations were located in a small region of southern Poland on metallurgic sites (PL19, PL30, PL22, PL24) as well as in ancient spoil heaps of Zn smelters (PL15, PL17) or mines (PL27). In metalliferous sites, plants are rooted in natural and/or artificial substrates and other metallophytes are present in the vegetation. The distance among M populations ranged from 1 to 70 km. The NM populations were located either at low altitude in southern Poland (PL13, PL14) or at moderate altitude in the Tatra mountains (PL32), as well as in Slovakia (SK2). These populations were found in forest edges and meadows (Table 2). One NM population (PL21) was sampled near a polluted area, in close proximity (*c.* 7 km) to two sampled M populations (PL19 and PL30). This population will be referred to as 'nonmetallicolous in a polluted area' (NMp) according to Pauwels *et al.* (2006). Heavy metal contamination was extremely dissimilar between M and NM sites with total Zn concentrations in soil ranging from 1167 to 35 942 μg g^{-1} for M populations, and concentrations < 169 μg g^{-1} for NM populations (Table 1). Mature individuals collected in each population (Table 1) were grown on nonpolluted compost in a glasshouse. In order to minimize the carry-over effects of the native population environment, each individual was propagated several times via cuttings.

Zinc tolerance experiments

The experiment was performed in a controlled growth chamber under 13 h light d^{-1}, 80 μmol photons m^{-2} s^{-1} irradiance, 20°C d/18°C night and 80% humidity during the first 2 wk and 65% for the rest of the experiment. Light was generated by four metal halide lamps (Radium HRI-T 400W/N, wavelength from 400 to 700 nm, enriched in blue) and four high pressure sodium lamps (Osram Plantastar 400W, wavelength < 700 nm, enriched in red). Six cuttings from each genotype were transferred directly into hydroponic conditions for rooting. Each cutting was grown in a 1 l polyethylene pot filled with standard nutrient solution containing: 20 μM Fe-EDDHA (iron salt of ethylenediamine-di-o-hydroxyphenylacetic acid), 500 μM $MgSO_4$, 1 mM $NH_4H_2PO_4$, 0.1 μM $(NH4)_6Mo_7O_{24}$, 0.1 μM $CuSO_4$, 25 μM H_3BO_3, 2 μM $MnSO_4$, 1 μM KCl, 0.1 μM NaCl, 2 mM $Ca(NO_3)_2$, 3 mM KNO_3, and 10 μM Zn added as $ZnSO_4$. To ensure metal bioavailability, pH of the solution was buffered using 2 mM of MES (2-morpholinoethanesulphonic acid) adjusted to 5.5 with KOH. The nutrient solution was changed once per week. Pots were randomly distributed in the chamber and moved around during each change of nutrient solution. After a 2-wk period allowing plants to initiate roots and acclimatize to hydroponic conditions, the Zn tolerance experiment started. Three clones (i.e. ramets) per genotype were treated with the standard hydroponic solution added with 2000 μM of Zn (contaminated condition, C), whereas three others were grown in the control solution with 10 μM of Zn (noncontaminated condition, NC, according to Macnair *et al.*, 1999). The metal concentration in C condition was chosen on the basis of preliminary experiments (H. Schat, pers. comm.). The 2000 μM Zn concentration in solution allowed all individuals to survive and revealed variability among individuals.

Quantitative variation of Zn tolerance was investigated measuring several morphological and physiological traits expected to reflect Zn toxicity (Broadley *et al.*, 2007): length and biomass of roots as a measure of inhibition of cell division and elongation, shoot biomass and width of leaves as a measure of inhibition of growth by metal, photosystem II yield (Φ_{PSII}) as a measure of the decrease of photosynthetic efficiency and the relative content of chlorophyll as a measure of chlorosis. Measurements were performed after 6 wk of Zn treatment for each plant in NC and C conditions. The width of leaves was measured as the maximum width of the blades of the three largest leaves. Physiological traits were measured on the three youngest leaves that were large enough to be measured. Chlorophyll fluorescence was measured under ambient light (of the growth chamber) with a PAM-2100 modulated fluorometer (Walz, Effeltrich, Germany). The fiber optic of the fluorometer was mounted in the Arabidopsis Leaf Clip 2060-B (Walz) applied to the young leaves in such a way that the distance between the fiber optic and the leaves was constant and standard. Moreover, the fiber optic formed a 60° angle with the sample, avoiding awkward shading or darkening. In order

Fig. 1 Geographic locations of *Arabidopsis halleri* populations surveyed in the present study. Open squares, nonmetallicolous (NM) populations; closed squares, metallicolous (M) populations; circle, nonmetallicolous population in polluted area (NMp).

to estimate response of plants under normal growing and steady-state conditions, actinic light irradiance used to drive photosynthesis was equivalent to growth irradiance (80 μmol m^{-2} s^{-1}) and measures were performed after a minimal exposition to the actinic light of 8 h. The effective quantum yield of photosystem II (Φ_{PSII}) was calculated according to Genty *et al.* (1989) as: $(F_m' - F_t)/F_m'$ where F_t is the fluorescence steady-state level under ambient light and F_m' the maximum level of fluorescence measured during a saturating pulse (0.8 s, 8500 μmol m^{-2} s^{-1}). Relative chlorophyll content was measured using the CL-01 Chlorophyll Content Meter (Hansatech Instruments, Kings Lynn, UK), which determines the relative content using dual wavelength optical absorbance (620 nm and 940 nm). This equipment allowed us to compare the responses of the plants to Zn contamination and to highlight a potential evolution toward chlorosis. For traits measured on three leaves (leaves width, photosystem II yield and relative content of chlorophyll), the median was used to evaluate the value per ramet. To estimate dry biomass, shoots and roots were separated and dried at 60°C during 72 h.

To eliminate most of the variation in plant responses unrelated to metal treatment, we calculated a tolerance index (TI) for each trait. The TI could be calculated in two different ways: by dividing the value for a ramet in the C condition by either a randomly chosen value among one of the three ramets of the same genotype in the NC condition or by statistic (mean or median, the latter being more suitable for small samples) summarizing the set of value over the three ramets in the NC condition. Owing to the weak

Table 1 Geographic location and edaphic type of the *Arabidopsis halleri* investigated natural populations

Type	Name	Location	Altitude (m)	Latitude	Longitude	Zinc total concentration in soil (μg g^{-1})	Ni est.	*n*
M	PL22	Bukowno	339	50°16′58.08″ N	19°28′43.38″ E	3969	< 1000	6
M	PL24	Bolesław	334	50°17′00.18″ N	19°29′05.64″ E	14 964	< 1000	4
M	PL27	Galman	447	50°11′36.78″ N	19°32′15.12″ E	35 942	< 500	22
M	PL15	Wełnowiec	302	50°17′12.96″ N	19°01′32.04″ E	10 163	100–500	5
M	PL17	Wełnowiec	297	50°16′57.12″ N	19°01′46.98″ E	10 642	> 1000	8
M	PL19	Miasteczko Śląskie	308	50°30′12.84″ N	18°56′08.34″ E	1167	< 500	7
M	PL30	Miasteczko Śląskie	325	50°30′10.03″ N	18°56′20.02″ E	1481	< 1000	4
NMp	PL21	Bibiela	300	50°29′45.66″ N	18°59′00.12″ E	327	< 100	4
NM	PL13	Nieposłomice Forest	206	50°06′35.64″ N	20°21′40.26″ E	160	> 500	25
NM	PL14	Nieposłomice Forest	188	50°06′31.80″ N	20°22′02.88″ E	169	> 500	9
NM	PL32	Western Tatra Mts.	970	49°16′26.94″ N	19°52′41.76″ E	125	> 500	11
NM	SK2	Kosicka Bela	690	48°46′10.20″ N	21° 7′48.60″ E	51*	> 500	17

M, metallicolous; NM, nonmetallicolous; NMp, nonmetallicolous in polluted area; Ni est., approximate population size; *n*, number of phenotyped individuals; * in Bert *et al.* (2002).
Populations are all located in Poland (PL) except one (SK2 is located in Slovakia)

Table 2 Ecological background of the 12 sampled sites.

				Substrate		Vegetation*	
Type	Name	Habitat	Origin of contamination	Natural	Artificial	Dominant species	Vegetative cover (%)
M	PL22	Woody area	Mining activities and metallurgic activities since 13th and 19th century, respectively	X	X	*Agrostis gigantea*	100
M	PL24	Meadow		X	X	*Achillea millefolium, Lotus corniculatus*	100
M	PL27	Forest	Mining activities during the 19th century	X		*Silene vulgaris, Carex hirta*	100
M	PL15	Meager grassland	Waste heap from Zn smelter built during the 19th and 20th century		X	*Festuca ovina*	60
M	PL17	Woody area			X	*Festuca ovina*	100
M	PL19	Degraded woody area	Metallurgic activities since 1966		X	*Arabidopsis arenosa*	60
M	PL30	Degraded woody area			X	*Arabidopsis halleri*	80
NMp	PL21	Ditch along a roadside	/	X		*Calamagrostis epigejos*	100
NM	PL13	Wet forest edge	/	X		*Aepodium podagraria*	100
NM	PL14	Wet forest edge	/	X		*Aegopodium podagraria*	100
NM	PL32	Forest edge and meadow	/	X		*Picea abies, Sanicula europaea*	100
NM	SK2	Meadow	/	X		NA	100

*Krystyna Grodzińska pers. comm.; M, metallicolous; NM, nonmetallicolous; X, presence of this substrate; NA, not available

between-ramet variance in the NC condition (*c.* 10% of the total variance), we found identical results using both procedures. As a consequence, only results using the median of the three ramets in the NC condition for TI estimates are presented in the paper.

Analysis of phenotypic differentiation

Because of the unbalanced sample (from 4 to 25 individuals/population), we performed nonparametric exact tests (StatXact v.8 Cytel Studio 2007, MA, USA) to analyse phenotypic differentiation among edaphic types, among populations within edaphic type and within populations. These tests make no assumptions about distributions and are suitable for small and/or unbalanced samples. Wilcoxon–Mann–Whitney exact tests for two independent groups were used to analyse phenotypic differences between NM and M groups of populations in NC and C conditions, and differences between NM and M groups of populations for tolerance indices. Differences within each edaphic type were investigated with Kruskal–Wallis exact test for *k* independent groups. Monte Carlo approximations for *P*-values were obtained using 10 000 permutation tests. In order to test for differences between pairs of population within edaphic type we used a nonparametric *post-hoc* test for multiple comparisons, according to Siegel &

Castellan (1988). Within edaphic type, we also estimated the partial variances explained by differences among populations and within population using the VARCOMP procedure (method TYPE 1 which does not assume normality of data) of the SAS program v. 9.1 (SAS InstituteCary, NC USA). Correlations between tolerance indices were examined using the Spearman coefficient estimated with the CORR procedure (SAS Institute).

The variability observed for tolerance indices was summarized using a canonical discriminant analysis on tolerance indices and populations using the CANDISC procedure (SAS Institute). This linear combination maximizes differentiation between populations and allows one to test if populations group according to their edaphic type. This analysis was performed on genotypes to respect assumption of independent data of discriminant analysis (Mundry & Sommer, 2007). All data were square-elevated before the canonical analysis to fit a normal distribution.

We estimated the broad sense heritability or clonal repeatability of the tolerance indices as $H^2 = V_G/V_P$ (V_G is total genetic variance, which includes additive genetic variance (V_A) and other sources of genetic variance (dominance and epistasis); V_P is the total phenotypic variance). Both V_G and V_P were estimated using the TYPE 1 method of the VARCOMP procedure (SAS Institute, 2002). The model considered the edaphic type (NM or M) as fixed factor and the population within the edaphic type and the genotype within the population as random factors.

Comparisons of genetic differentiation for quantitative traits and molecular markers

Owing to an imbalance in our data (see Table 1), measures of differentiation for quantitative traits (Q_{ST}) were computed with a Bayesian analysis following the procedure described by Waldmann *et al.* (2005). The Q_{ST} statistics was estimated as $V_b/(V_b + 2V_w)$ where V_b is the component of genetic variance between populations and V_w the component within populations. The model considers edaphic type as a fixed factor and population and genotype as random factors. We used a Gamma distribution (0.001, 0.001) as priors for the inverse of the variance ($1/V_b$, $1/V_w$ and $1/V_{genotype}$). To calculate confidence intervals we ran two chains for 1 000 000 iterations, with a 500 000 burn-in. Four populations (PL15, PL24, PL30 and PL21) of our data set had sample sizes inferior to five individuals per population. Consequently, to avoid bias in the estimation of genetic differentiation we excluded these populations from the comparison of Q_{ST}/F_{ST}.

To estimate population differentiation at neutral molecular markers, 10 nuclear microsatellite loci were scored for a total of 203 individuals representing the eight populations studied. These samples partly overlapped with those used for phenotypic traits, *c.* 50% of the individuals genotyped were also phenotyped. We used five microsatellite (*ATH*, *GC16*, *LYR132*, *LYR133*, *LYR104*) previously described in Van Rossum *et al.* (2004) and five others (*GC22*, *NGA112*, *ICE 13*, *MDC16*, *NGA361*) recently transferred from *A. thaliana* and combined in a multiplex (Llaurens *et al.*, 2008). For each microsatellite we estimated the level of polymorphism (number of alleles and total gene diversity H_T) and the inbreeding coefficient (F_{IS}) using FSTAT 2.9.3 (Goudet, 1995). Measures of differentiation for molecular markers were calculated based on F_{ST} (Weir & Cockerham, 1984) with the software FSTAT. The 95% confidence interval for the F_{ST} was estimated using 1000 bootstrap resampling of individuals.

Results

Morphological and physiological response to zinc of M and NM populations

Under NC conditions, morphological and physiological trait values of NM populations did not differ from those of M populations (Fig. 2). By contrast, in the C conditions, trait values in M populations were all significantly higher than those in NM populations. The NM plants had less dry biomass, shorter roots, narrower leaves, lower photosystem II yields and lower relative chlorophyll contents. Variances of traits were relatively similar between NC and C conditions except for the photosystem II yield that showed higher variance in C than in NC conditions.

Tolerance indices further showed that Zn had a negative effect on most of the morphological traits measured and on all physiological traits, regardless of the edaphic type of the populations (Fig. 2). However, toxicity of Zn was, on average, significantly more severe for NM individuals than for M individuals. The NM plants showed smaller tolerance indices for dry biomass (0.48 ± 0.34 vs 0.78 ± 0.48 for shoot and 0.65 ± 0.46 vs 0.99 ± 0.59 for root), root length (0.71 ± 0.23 vs 0.82 ± 0.24), leaf width (0.73 ± 0.20 vs 0.89 ± 0.35), photosystem II yield (0.87 ± 0.16 vs 0.97 ± 0.06) and relative content of chlorophyll (0.54 ± 0.35 vs 0.74 ± 0.23). Broad sense heritabilities of the tolerance indices were high for all the traits (> 0.50; see Table 3). The highest heritabilities were found for the relative content of chlorophyll (0.78) and the width of leaves (0.69). All the morphological indices were significantly correlated among each other, with r values ranging from 0.28 to 0.68 (Table 3). The highest correlation was observed for shoot and root dry biomass. Photosystem II yield was correlated with each index, except the one for leaf width. Relative chlorophyll content was correlated only with photosystem II yield and shoot biomass. Metal contamination is variable between metalliferous sites (Table 1), but we found no correlation between

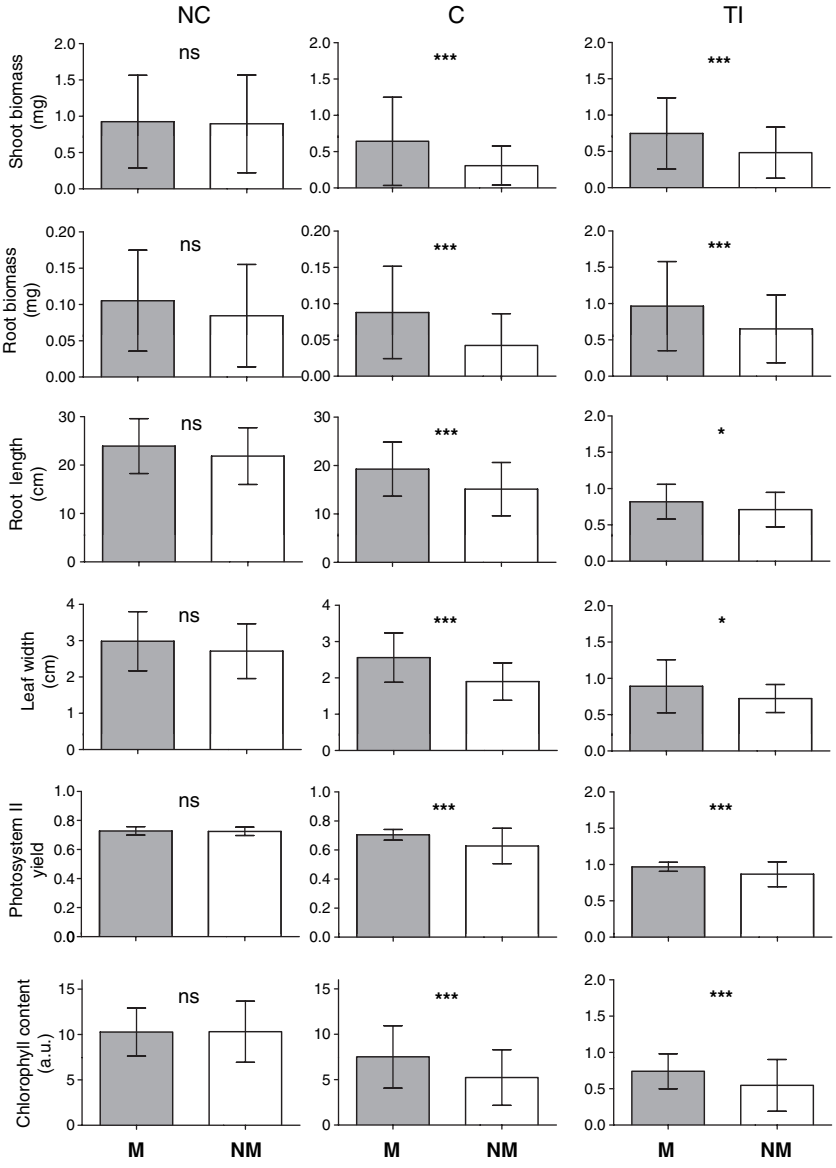

Fig. 2 Mean ± SD from measures of morphological and physiological traits in metallicolous (M, tinted bars) and nonmetallicolous (NM, open bars) populations on zinc contaminated (C) and noncontaminated conditions (NC). Tolerance indices (TI) are shown on the right side of the figure. Results from exact permutation tests are indicated as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, nonsignificant.

tolerance indices and Zn soil concentration (P-value from 0.071 for leaf width to 0.759 for root length).

Canonical discriminant analysis showed that populations were differentiated for all tolerance indices ($P < 0.0001$). Variations among populations were significantly explained by the first three axes, representing 47%, 21% and 15% of the variance. The first axis was mainly explained by the photosystem II yield and the root length, the second by the width of leaves and the third by the relative chlorophyll content (Table 4). The first axis, upon which populations are continuously distributed, separated populations according to their edaphic type (Fig. 3). The NM populations had smaller values on this axis than M populations with extreme values for populations SK2 and PL17. The second axis identified two M populations with a different behavior, PL30 and PL19; these showed high values for the width of leaves. Interestingly, the NMp population (PL21) was less associated with the NM group than with the major M group.

Variability within edaphic type and populations in response to Zn

Apart from photosystem II yield, tolerance indices for all traits showed high variance within edaphic types (see Fig. 2). Because of these high variances, we observed a large overlap of values between the two edaphic groups. Indeed, some M and NM individuals showed comparable values for all tolerance indices (Fig. 2). The high variances within edaphic type were mainly explained by variation within populations. For all tolerance indices, the part of variance explained by difference among populations was weak compared with that

Table 3 Broad-sense heritability (H^2) of six zinc tolerance indices (TI) measured on 12 *Arabidopsis halleri* populations and correlation between these indices

		Correlation				
TI	H^2	Leaf width	Root biomass	Root length	Photosystem II yield	Relative chlorophyll content
Shoot biomass	0.56	0.50***	0.68***	0.30***	0.27**	0.21*
Leaf width	0.69		0.38***	0.28**	0.09 ns	0.06 ns
Root biomass	0.57			0.40***	0.18*	0.02 ns
Root length	0.50				0.26*	0.06 ns
Photosystem II yield	0.63					0.33***
Relative Chlorophyll content	0.78					

P-values are represented as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, ns, nonsignificant.

Table 4 Canonical loadings and proportion of variance from discriminate analysis of population differentiation for zinc tolerance indices (TI)

TI	Axis 1	Axis 2	Axis 3
Shoot biomass	0.401	0.185	−0.493
Leaf width	0.129	0.896	−0.475
Root biomass	0.273	0.056	−0.053
Root length	0.538	−0.086	−0.166
Photosystem II yield	0.934	0.060	0.005
Relative chlorophyll content	0.309	0.055	0.802
Proportion of variance explained (%)	47	21	15

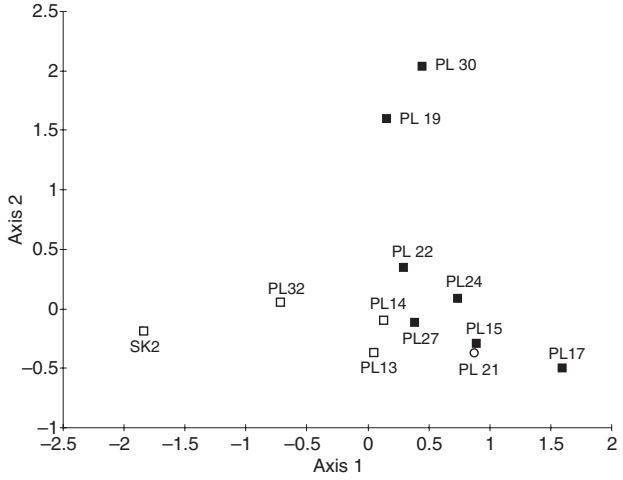

Fig. 3 Canonical discriminant analysis on six zinc tolerance indices in metallicolous (M, closed squares) and nonmetallicolous (NM, open squares) populations of *Arabidopsis halleri*. Axes 1 and 2 represent 47% and 21% of the variation between populations, respectively. The open circle corresponds to the nonmetallicolous population in polluted area (NMp).

explained by polymorphism within populations (Table 5). This demonstrates that a high variability is present within populations, irrespective of edaphic type. Variance within M populations was significantly smaller than within the NM populations for two traits: the relative chlorophyll content and the photosystem II yield ($P = 0.001$ and $P = 0.003$, respectively). We also found significant differences among populations within edaphic types. Namely, M populations showed different tolerance indices for photosystem II yield and relative chlorophyll content ($P = 0.03$ and $P = 0.012$, respectively; Fig. 4), whereas NM populations differed significantly from each other only for photosystem II yield ($P < 10^{-4}$; Fig. 4). The difference in photosystem II yield between NM and M populations was mainly explained by the populations PL32 and SK2.

Comparison of phenotypic and molecular differentiation

The 10 microsatellites showed 5–15 alleles (average = 8.5), and were polymorphic in all the populations. Statistics of population genetic diversity (H_T and F_{IS}) are presented in Table S1 for each of these loci. Total gene diversity was relatively high for all loci (mean $H_T = 0.599$) and inbreeding coefficient ranged from −0.122 to 0.269. Across all eight populations, the differentiation at microsatellite loci (F_{ST}) was, on average, 0.146 with a 95% confidence interval (CI) from 0.116 to 0.171. This estimate of genetic differentiation at molecular markers was higher than those obtained in the same area in a previous survey using AFLP (average F_{ST} of 0.066 in Meyer *et al.*, 2009).

The highest Q_{ST} was observed for photosystem II yield (average = 0.33) with a 95% CI from 0.13 to 0.67 (Fig. 5). Overlap between this CI and that of molecular markers was very weak. Hence, these results suggest that photosystem II yield has probably experienced divergent selection. Shoot biomass, root length and relative chlorophyll content also showed Q_{ST} higher than F_{ST}, although their wide CI overlapped largely with the CI of the F_{ST}. Consequently, we could not make a conclusion about the role of selection in shaping variability at these traits. On average, the differentiation levels for root biomass and leaf width were

Table 5 Part of variance explained by variability among and within populations for zinc (Zn) tolerance indices (TI)

	Variance components					
	Within M type			Within NM type		
TI	Among populations	Within populations	Total	Among populations	Within populations	Total
Shoot biomass	0.0087	0.1376	0.3660	0.0001	0.0362	0.0831
Leaf width	0.0044	0.0582	0.1171	0.0068	0.1582	0.2482
Root biomass	0.0273	0.2572	0.9251	0.0037	0.0371	0.0683
Root length	0.0081	0.0436	0.0713	0.0009	0.0229	0.0619
Photosystem II yield	0.0005	0.0021	0.0045	0.0100	0.0229	0.0328
Relative chlorophyll content	0.0169	0.0444	0.0920	0.0042	0.0526	0.1174

M, metallicolous; NM, nonmetallicolous.

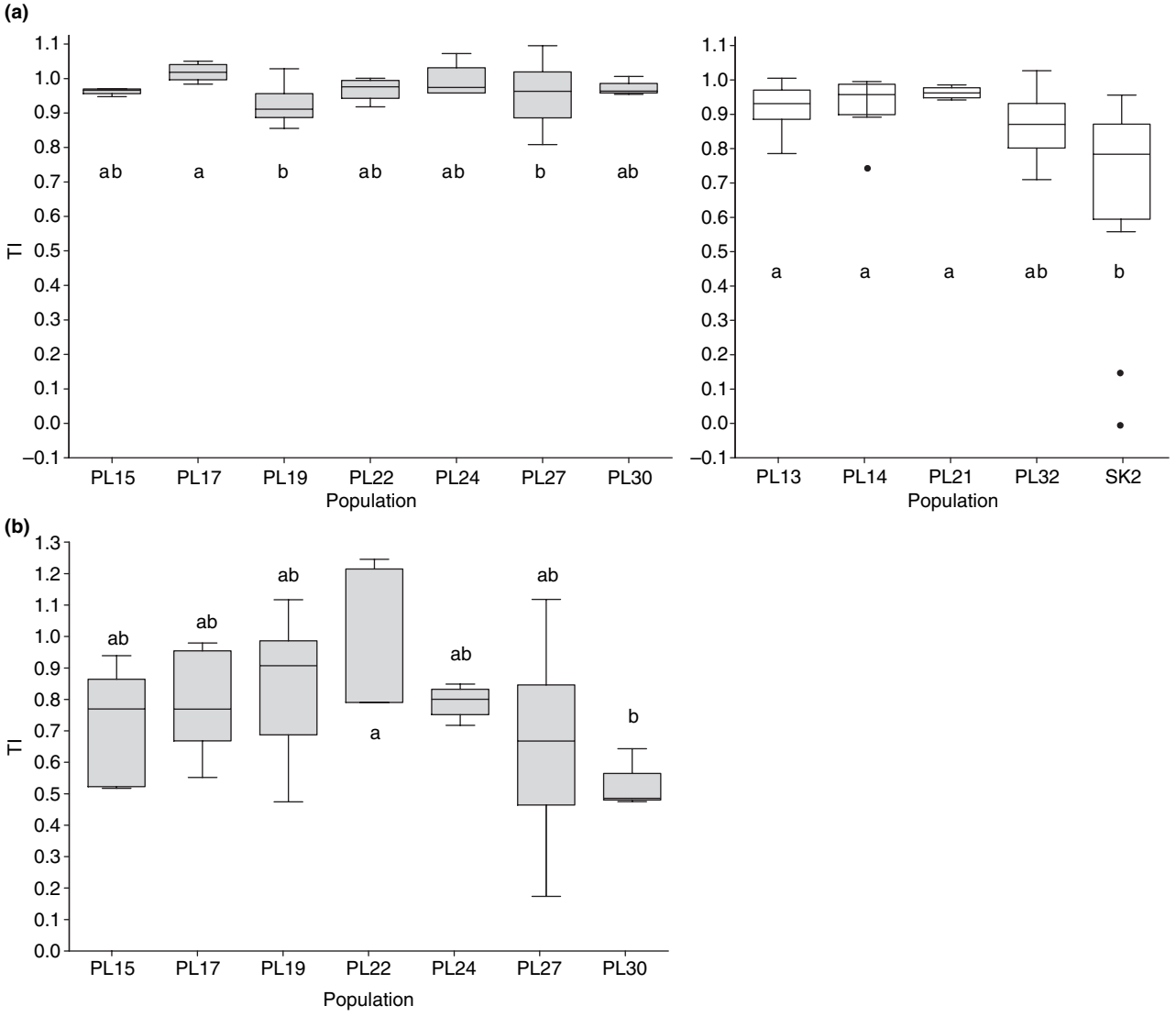

Fig. 4 Zinc (Zn) tolerance indices showing significant differences among metallicolous (M, tinted bars) or nonmetallicolous (NM, open bars) populations. (a) Index based on photosystem II yield; (b) index based on relative chlorophyll content. The box represents the 25th and 75th percentiles of the data and the mean is indicated by the horizontal line. Vertical lines and bullets show the highest and lowest data and the outlier values, respectively. Different letters indicate significant differences at the 5% level.

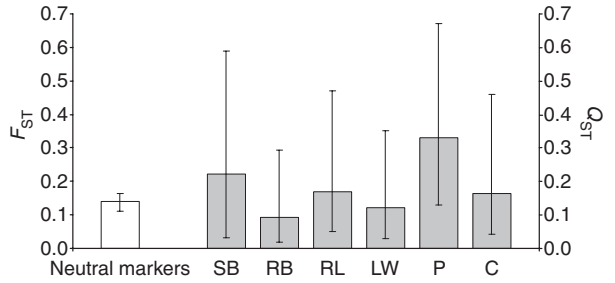

Fig. 5 Comparison of genetic differentiation for molecular markers (F_{ST}) and quantitative traits (Q_{ST}). Tinted bars correspond to estimates for different characters: SB, shoot biomass; RB, root biomass; RL, root length; LW, leaf width; P, photosystem II yield; C, relative content of chlorophyll. The 95% confidence interval for genetic differentiation is represented by the vertical lines.

all smaller than the molecular differentiation, suggesting neutral evolution or stabilizing selection.

Discussion

Evaluating variability of Zn tolerance with root and shoot measurements

Overall, our results demonstrated and quantified variability of Zn tolerance at a local scale in the pseudometallophyte species *A. halleri*. In the 12 populations sampled, the morphological (shoot and root dry biomass, root length and leaf width) and physiological (leaf photosystem II yield and relative chlorophyll content) traits used to estimate Zn tolerance showed congruent trends towards higher mean values for M populations. Nevertheless, it is the response of shoot, mainly through photosystem II yield, that seemed to capture most variability of tolerance, in particular among populations. These results are congruent with those reported by Assunção *et al.* (2003) on *Thlaspi caerulescens* and support the hypothesis that the shoot is more sensitive than the root in hyperaccumulator species because of a preferential accumulation of metal in the leaves (Assunção *et al.*, 2001, 2003). Among all the measures we performed, the most informative trait to evaluate tolerance in our study species seems to be the photosystem II yield of leaves. This trait showed high heritability, weaker variability within M than NM populations and clear differences among populations. The photosystem II yield, an indicator of functional photosynthesis and vitality of the photosynthetic tissue, has been used to investigate the mechanisms of heavy metal toxicity in previous studies (Baryla *et al.*, 2001; Küpper *et al.*, 2007), and to estimate the difference of metal tolerance between hyperaccumulator and nonhyperaccumulator species using incubation of leaf slices (Cho *et al.*, 2003). We showed that this physiological measure was also reliable to estimate polymorphism of Zn tolerance in a pseudometallophyte hyperaccumulator species. Macnair (1983) and Schat & Ten Bookum (1992) suggested that integration of possible innate variation may bias tolerance indices. However, in our study on quantitative polymorphism of Zn tolerance, such bias seems to be limited because M and NM plants showed very similar trait values under NC control conditions. This similar response of M and NM plants is interesting because it may suggest that enhanced tolerance does not imply a cost in terms of elevated Zn requirements. These results contrast with the classical hypothesis that the most tolerant and hyperaccumulator plants requires more metal for normal growth (van de Mortel *et al.*, 2006). Nevertheless, this possible absence of cost concerns only the Zn concentration tested in our survey (i.e. 10 μM) and cannot be inferred to apply to lower concentrations. Overall, in order to rigorously demonstrate this hypothesis it would be essential to measure survival and reproductive parameters as was achieved for *T. caerulescens* (Dechamps *et al.*, 2006).

Variability of Zn tolerance at a local scale and evolutionary inference

Arabidopsis halleri is a species constitutively tolerant to Zn (Bert *et al.*, 2000) that shows at a broad-scale continuous variation from NM to M populations (Pauwels *et al.*, 2006). Our results revealed that this pattern of variation is also present at a local scale, among populations that are likely to exchange genes. Hence, these results suggest that gene flow, in the network of populations considered in our survey, is probably not intense enough to prevent increasing tolerance in response to the high concentrations of metals at metalliferous sites. We also found important variability of tolerance within edaphic types, which was mostly explained by variability among individuals within populations. Such a high level of variability could be interpreted in two different ways. First, it is consistent with gene flow between NM and M populations, whereby NM individuals with tolerance close to that of M individuals would have acquired enhanced tolerance from M populations through gene flow of metal tolerance genes. This observation and the fact that the less tolerant populations (PL32 and SK2) were the most distant from the M populations may indicate that gene flow plays an important role in the distribution of this quantitative trait. Second, the large variation of Zn tolerance observed within NM populations could suggest that these *A. halleri* populations have the genetic potential to evolve towards a higher tolerance. Thus, the enhanced tolerance in recently founded M populations may result from selection on standing variation existing in NM populations rather than on newly arisen mutations. Indeed, recurrent mutations, genetic drift and, particularly in this case, gene flow could preserve a relatively high amount of neutral or deleterious variation upon which selection may proceed (Barrett & Schluter, 2007). The fact that polluted sites, which are

dramatically altered environments, become rapidly colonized is suggestive of pre-existent suitable genetic variants from standing variation in the surrounding NM populations. In addition, alleles with small effects, proposed to be involved in the variability of heavy metal tolerance among populations (Macnair *et al.*, 2000), are expected to largely contribute to adaptation from standing variation.

It is interesting to note that populations PL19 and PL30, which are geographically very close to one another (< 1 km) and are both at less Zn-contaminated metalliferous sites, clearly differed from other M populations and from NM populations in the canonical analysis of the TI. These results suggest possible different mechanisms of adaptation among M populations in our system study. Convergent evolution has often been assumed to concern distantly related species rather than closely related species or populations. Nevertheless, empirical studies of the genetics of adaptation have shown that different populations within a species may use different genetic solutions to solve similar ecological problems (for review see Arendt & Reznick, 2007). This was observed by Hoekstra *et al.* (2006) in Atlantic and Gulf coast populations of mice, for an extreme pigmentation phenotype. They showed that the mutation *Mc1r* implicated in the light coloration of the Gulf coast mice was not present in the pale Atlantic coast mice. In the context of heavy metal tolerance, Smith & Macnair (1998), using crosses between two lines that differed in copper tolerance and a single nontolerant plant, showed that several modifier genes explain different levels of tolerance in the metallophyte plant *Mimulus guttatus.* The genetic architecture of heavy metal tolerance in *A. halleri* was investigated in a cross between one M individual from *A. halleri* and one from the nontolerant species *A. lyrata* (Courbot *et al.*, 2007; Willems *et al.*, 2007). They identified several QTLs for Zn and Cd tolerance, probably implicated in different pathways (Roosens *et al.*, 2008b), which means that genetic change at either of them can, in itself, enhance metal tolerance. Evolution of Zn tolerance by population specific mechanisms is thus a possibility in *A. halleri.* Moreover, based on a genome scan approach, Meyer *et al.* (2009) showed that different loci may be involved in adaptation in two M populations present in our survey (PL22 and PL27), consistent with a scenario of convergent evolution towards increased tolerance in these two populations.

Our results for the NMp population are congruent with those obtain by Pauwels *et al.* (2006). Indeed, they showed that at the European scale, Zn tolerance in these populations was intermediate between M and NM populations. They proposed two hypotheses to explain the features of the NMp populations. These populations could be founded from neighboring M populations, followed by selection for lower tolerance owing to a possible cost for excessive tolerance. Conversely, the phenotype of NMp populations could also be explained by intense gene flow of metal tolerance genes. In these two hypotheses the NMp populations could be in a situation of maladaptation. Therefore, to gain a better knowledge of the role of gene flow, selection and drift in shaping Zn tolerance in *A halleri*, it would be interesting to investigate the genetic structure and the variability of tolerance on larger samples of NMp populations.

Adaptive value of Zn tolerance variability

To assess the role of natural selection in shaping the variability of Zn tolerance among *A. halleri* populations, we compared differentiation of this quantitative trait to differentiation of neutral molecular markers. The higher value of Q_{ST} for tolerance indices compared with F_{ST}, particularly for leaf photosystem II yield, lends support to the hypothesis that divergent selection at metalliferous and nonmetalliferous sites has played a role in phenotypic differentiation. Another observation supporting this hypothesis, and congruent with the results of Pauwels *et al.* (2006), is the higher variance observed within NM populations compared with M populations. Indeed, reduction of genetic diversity is typically considered as one of the most striking signatures of directional selection (Nielsen, 2005). Hence, our survey supports the view that tolerance to Zn, the usual trait used to explore adaptation to metal-polluted sites and the genetic basis underlying such adaptation, has probably been shaped by divergent selection within the species. However, the conclusion of our Q_{ST}–F_{ST} approach has to be confirmed because of first, the large confidence interval of Q_{ST}, and second, the possible genetic × environment effect. In *Thlaspi caerulescens*, a pseudometallophyte that tolerates and hyperaccumulates Zn and Cd, Jiménez-Ambriz *et al.* (2006) and Dechamps *et al.* (2006) have shown that several life history traits differed between edaphic types probably through divergent selection. Similarly, in *A. halleri*, life-history traits, particularly reproductive ones, could be involved in adaptation to metalliferous sites and thus will have to be investigated in parallel with Zn tolerance.

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References

Al-Hiyaly SA, McNeilly T, Bradshaw AD. 1988. The effect of zinc contamination from electricity pylons – evolution in a replicated situation. *New Phytologist* **110**: 571–580.

Al-Shehbaz IA, O'Kane SLJ. 2002. Taxonomy and phylogeny of *Arabidopsis* (Brassicaceae). In: Somerville CR, Meyerowitz EM, eds. *The Arabidopsis book*. Rockville, MD, USA: American Society of Plant Biologists. doi: 10.1199/tab.0001, http://www.aspb.org/publications/arabidopsis/

Antonovics J, Bradshaw AD, Turner RG. 1971. Heavy metal tolerance in plants. *Advances in Ecological Research* **7**: 1–85.

Arendt J, Reznick D. 2007. Convergence and parallelism reconsidered: what have we learned about the genetics of adaptation? *Science* **23**: 26–32.

Assunção AGL, Da Costa Martins P, De Folter S, Vooijs R, Schat H, Aarts MGM. 2001. Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell & Environment* **24**: 217–226.

Assunção AGL, Ten Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO. 2003. Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* **159**: 411–419.

Barrett RDH, Schluter D. 2007. Adaptation from standing variation. *Trends in Ecology and Evolution* **23**: 38–44.

Baryla A, Carrier P, Franck F, Coulomb C, Sahut C, Havaux M. 2001. Leaf chlorosis in oilseed rape plants (*Brassica napus*) grown on cadmium-polluted soil: causes and consequences for photosynthesis growth. *Planta* **212**: 696–709.

Bert V, Macnair MR, De Laguerie P, Saumitou-Lapdrade P, Petit D. 2000. Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri*. *New Phytologist* **146**: 225–233.

Bert V, Bonin I, Saumitou-Laprade P, de Laguerie P, Petit D. 2002. Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **155**: 47–57.

Bradshaw AD. 1952. Populations of *Agrostis tenuis* resistant to lead and zinc poisoning. *Nature* **169**: 1098.

Broadley MR, White PJ, Hammond JP, Zelko I, Lux A. 2007. Zinc in plants. *New Phytologist* **173**: 677–702.

Cho M, Chardonnens AN, Dietz K-J. 2003. Differential heavy metal tolerance of *Arabidopsis halleri* and *Arabidopsis thaliana*: a leaf slice test. *New Phytologist* **158**: 287–293.

Courbot M, Willems G, Motte P, Arvidsson S, Roosens N, Saumitou-Laprade P, Verbruggen N. 2007. A major quantitative trait locus for cadmium tolerance in *Arabidopsis halleri* colocalizes with HMA4, a gene encoding a heavy metal ATPases. *Plant Physiology* **144**: 1052–1065.

Dechamps C, Lefèbvre C, Noret N, Meerts P. 2006. Reaction norms of life history traits in response to zinc in *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites. *New Phytologist* **173**: 191–198.

Galardi F, Corrales I, Mengoni A, Pucci S, Barletti L, Barzanti R, Arnetoli M, Gabbrielli R, Gonnelli C. 2007. Intra-specific differences in nickel tolerance and accumulation in the Ni-hyperaccumulator *Alyssum bertolonii*. *Environmental and Experimental Botany* **60**: 377–384.

Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron-transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* **990**: 87–92.

Goudet J. 1995. Fstat version 1.2: a computer program to calculate *F* statistics. *Journal of Heredity* **86**: 485–486.

Hoekstra HE, Hirschmann RJ, Bundey RA, Insel PA, Crossland JP. 2006. A single amino acid mutation contributes to adaptive beach mouse color pattern. *Science* **313**: 101–104.

Humphreys MO, Nicholls MK. 1984. Relationships between tolerance to heavy metals in *Agrostis capillaris* L. (A. tenuis Sibth.). *New Phytologist* **98**: 177–190.

Jiménez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri S, Ronce O. 2006. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* **173**: 199–215.

Koch M, Mummenhoff K, Hurka H. 1998. Systematics and evolution history of heavy metal tolerant *Thlaspi caerulescens* in Western Europe: evidence from genetic studies based on isozyme analysis. *Biochemical Systematics and Ecology* **26**: 823–838.

Küpper H, Lombi E, Zhao F-J, McGrath SP. 2000. Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta* **212**: 75–84.

Küpper H, Parameswaran A, Leitenmaier B, Trtílek M, Šetlík I. 2007. Cadmium-induced inhibition of photosynthesis and long-term acclimation to cadmium stress in the hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **175**: 655–674.

Llaurens V, Castric V, Austerlitz F, Vekemans X. 2008. High paternal diversity in the self-incompatible herb *Arabidopsis halleri* despite clonal reproduction and spatially restricted pollen dispersal. *Molecular Ecology* **17**: 1577–1588.

Macnair MR. 1983. The genetic control of copper tolerance in the yellow monkey flower, *Mimulus guttatus*. *Heredity* **50**: 283–293.

Macnair MR, Baker JM. 1994. Metal-tolerant plant: an evolutionary perspective. In: Farago ME ed. *Plants and the chemical elements, biochemistry, uptake, tolerance and toxicity*. New York, NY, USA: VCH, 68–83.

Macnair MR, Bert V, Huitson SB, Saumitou-Laprade P, Petit D. 1999. Zinc tolerance and hyperaccumulation are genetically independent characters. *Proceedings of the Royal Society of London* **266**: 2175–2179.

Macnair MR, Tilstone GH, Smith SE. 2000. The genetics of metal tolerance and accumulation in higher plants. In: Terry N, Banuelos G, Vangronsveld J, eds. *Phytoremediation of contaminated soil and water*. Boca Raton, FL, USA: CRC Press, 235–250.

Meerts P, Van Isaker I. 1997. Heavy metal tolerance and hyperaccumulation in metallicolous and nonmetallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* **133**: 221–231.

Mengoni A, Barabesi C, Gonelli C, Galardi F, Gabbrielli R, Bazzicalupo M. 2001. Genetic diversity of heavy metal-tolerant populations in *Silene paradoxa* L. (Caryophyllaceae): a chloroplast microsatellite analysis. *Molecular Ecology* **10**: 1909–1916.

Meyer CL, Vitalis R, Saumitou-Laprade P, Castric V. 2009. Genomic pattern of adaptive divergence in *Arabidopsis halleri*, a model species for tolerance to heavy metal. *Molecular Ecology* **18**: 2050–2062.

van de Mortel JE, Villanueva LA, Schat H, Kwekkeboom J, Coughlan S, Moerland PD, Ver Loren van Themaat E, Koornneef M, Aarts MGM. 2006. Large expression differences in genes for iron and Zinc homeostasis, stress response, zand lignin biosynthesis distinguish roots of Arabidopsis thaliana and the related metal hyperaccumulator Thlaspi caerulescens. *Plant Physiology* **142**: 1127–1147.

Mundry R, Sommer C. 2007. Discriminant function analysis with nonindependent data: consequence and an alternative. *Animal Behaviour* **74**: 965–976.

Nielsen R. 2005. Molecular signatures of natural selection. *Annual Review of Genetics* **39**: 197–218.

O'Kane SL, Al-Shehbaz IA. 2003. Phylogenetic position and generic limits of Arabidopsis (Brassicaceae) based on sequences of nuclear ribosomal DNA. *Annals of the Missouri Botanical Garden* **90**: 603–612.

Pauwels M, Saumitou-Laprade P, Holl C, Petit D, Bonin I. 2005. Multiple origins of metallicolous populations of the pseudometallophyte *Arabidopsis halleri* (Brassicaceae) in central Europe: the cpDNA testimony. *Molecular Ecology* **14**: 4403–4414.

Pauwels M, Frérot H, Bonin I, Saumitou-Laprade P. 2006. A broad-scale analysis of population differentiation for Zn tolerance in an emerging model species for tolerance study: *Arabidopsis halleri* (Brassicaceae). *Journal of Evolutionary Biology* **19**: 1838–1850.

Reznick DN, Ghalambor CK. 2001. The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. *Genetica* **112–113**: 183–198.

Roosens NCJ, Willems G, Saumitou-Laprade P. 2008a. Using *Arabidopsis* to explore zinc tolerance and hyperaccumulation. *Trends in Plant Science* **13**: 208–215.

Roosens NCJ, Willems G, Godé C, Courseaux A, Saumitou-Laprade P. 2008b. The use of comparative genome analysis and syntenic relationships allows extrapolating the position of Zn tolerance QTL regions from *Arabidopsis halleri* into *Arabidopsis thaliana*. *Plant and Soil* **306**: 105–116.

Schat H, Ten Bookum WM. 1992. Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68**: 219–229.

Schat H, Vooijs R. 1997. Multiple tolerance and co-tolerance to heavy metals in *Silene vulgaris*: a co-segregation analysis. *New Phytologist* **136**: 489–496.

Schat H, Kuiper E, Ten Bookum WM, Vooijs R. 1993. A general model for the genetic control of copper tolerance in *Silene vulgaris*: evidence from crosses between plants from different tolerant populations. *Heredity* **70**: 142–147.

Schat H, Vooijs R, Kuiper E. 1996. Identical major gene loci for heavy metal tolerances that have independently evolved in different local populations and subspecies of *Silene vulgaris*. *Evolution* **50**: 1888–1895.

Siegel S, Castellan NJ. 1988. *Non parametric statistics for the behavioural sciences*. New York, NY, USA: MacGraw-Hill.

Smith SE, Macnair MR. 1998. Hypostatic modifiers cause variation in degree of copper tolerance in *Mimulus guttatus*. *Heredity* **80**: 760–768.

Van Rossum F, Bonnin I, Fénart S, Pauwels M, Petit D, Saumitou-Laprade P. 2004. Spatial genetic structure within a metallicolous population of *Arabidopsis halleri*, a clonal, self-incompatible, and heavy metal tolerant species. *Molecular Ecology* **13**: 2959–2967.

Vekemans X, Lefèbvre C. 1997. On the evolution of heavy-metal tolerant populations in *Armeria maritima*: evidence from allozyme variation and reproductive barriers. *Journal of Evolutionary Biology* **10**: 175–191.

Von Frenckell-Insam BAK, Hutchinson TC. 1993. Occurrence of heavy metal tolerance and co-tolerance in *Deschampsia cespitosa* (L.) Beauv. From European and Canadian populations. *New Phytologist* **125**: 555–564.

Waldmann P, Garcia-Gil MR, Sillanpaa MJ. 2005. Comparing Bayesian estimates of genetic differentiation of molecular markers and quantitative traits: an application to *Pinus sylvestris*. *Heredity* **94**: 623–629.

Weir BS, Cockerham CC. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.

Whitlock MC. 2008. Evolutionary inference from Q_{ST}. *Molecular Ecology* **17**: 1885–1896.

Wilkins DA. 1978. The measurement of tolerance to edaphic factors by means of root growth. *New Phytologist* **80**: 623–633.

Willems G, Dräger DB, Courbot M, Godé C, Verbruggen N, Saumitou-Laprade P. 2007. The genetic basis of zinc tolerance in the metallophyte *Arabidopsis halleri* ssp. *halleri* (Brassicaceae): an analysis of quantitative trait loci. *Genetics* **176**: 659–674.

Wu L, Bradshaw AD, Thurman DA. 1975. The potential for evolution of heavy metal tolerance in plants. III. The rapid evolution of copper tolerance in *Agrostis stolonifera*. *Heredity* **34**: 165–187.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Summary statistics of the 10 microsatellites

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Résumé

Les activités humaines génèrent souvent des milieux extrêmes dans lesquels certains organismes parviennent localement à survivre et à se reproduire grâce à leurs capacités d'adaptation. Les sols calaminaires, hautement pollués par des métaux tels que le zinc, le plomb et le cadmium, sont par exemple le résultat des activités minières ou industrielles. *Arabidopsis halleri* et *Noccaea caerulescens* (Brassicacées) sont deux espèces pseudométallophytes, c'est-à-dire présentant à la fois des populations dites métallicoles, présentes sur les sols calaminaires, et des populations non métallicoles localisées à proximité. Ces deux espèces sont tolérantes aux métaux, mais aussi hyperaccumulatrices de zinc et de cadmium dans leurs feuilles. Ces deux traits adaptatifs sont quantitatifs et le plus souvent bien structurés entre populations métallicoles et non métallicoles.

Dans ce contexte d'adaptation locale et rapide, j'aborde deux grandes questions. Première question : quelle est l'architecture génétique de la tolérance au zinc et de l'hyperaccumulation du zinc chez *A. halleri* et *N. caerulescens* ? Pour répondre à cette question, je produis de nombreuses descendances de croisements que j'analyse par QTL Mapping. En parallèle, j'explore la possibilité de modifications épigénétiques responsables des différences entre populations métallicoles et non métallicoles. Deuxième question : comment prendre en compte dans les études génétiques et écologiques le fait que la tolérance au zinc et l'hyperaccumulation du zinc sont des traits complexes ayant évolué dans un environnement complexe ? Je tente de répondre à cette question en utilisant des phénotypes décrivant plusieurs aspects de la tolérance au zinc et de l'hyperaccumulation du zinc. Je cherche également quels paramètres environnementaux abiotiques, différenciant les habitats calaminaires des habitats non pollués, pourraient représenter de potentielles pressions de sélection agissant en particulier sur l'hyperaccumulation du zinc chez *A. halleri*. Une démarche plus directe d'évolution expérimentale menée chez *N. caerulescens* est destinée à démontrer que le zinc constitue bien une pression de sélection agissant à la fois sur la tolérance au zinc et l'hyperaccumulation du zinc.

Mots-clés : anthropisation, adaptation locale, architecture génétique, *Arabidopsis halleri*, épigénétique, évolution expérimentale, hyperaccumulation, *Noccaea caerulescens*, QTL Mapping, sols calaminaires, tolérance, trait complexe, zinc

Abstract

Human activities often generate extreme environments in which only some organisms are able to survive and to reproduce thanks to their adaptive capacities. As an example, calamine soils resulting from mining and industrial activities are highly enriched with zinc, lead and cadmium. *Arabidopsis halleri* and *Noccaea caerulescens* (Brassicaceae) are two pseudometallophytes species that develop on both metalliferous (*i.e.* calamine) and non-metalliferous soils, leading to metallicolous and non-metallicolous populations geographically close to each other. These two species are metal tolerant and hyperaccumulate zinc and cadmium in their leaves. These adaptive traits are quantitative and quite structured between metallicolous and non-metallicolous populations.

In such context of local and rapid adaptation, I address two main questions. First, what is the genetic architecture of zinc tolerance and hyperaccumulation in *A. halleri* and *N. caerulescens*? To tackle this question, I produce many offspring and analyze them by QTL Mapping. In parallel, I explore the epigenetic modifications which could differentiate metallicolous and non-metallicolous populations. Secondly, how to integrate, either in genetic or ecological studies, that zinc tolerance and hyperaccumulation are complex traits that evolved in complex environments? I tempt to answer this question by seeking phenotypes describing several aspects of zinc tolerance and hyperaccumulation. I also examine which environmental abiotic parameters distinguishing calamine and non-polluted habitats could represent potential selective pressures acting on zinc hyperaccumulation in *A. halleri*. Then, an approach of experimental evolution in *N. caerulescens* has been designed to investigate more directly the role of zinc as a potential selective pressure.

Key-words: anthropization, *Arabidopsis halleri*, calamine soils, complex trait, epigenetics, experimental evolution, genetic architecture, hyperaccumulation, local adaptation, *Noccaea caerulescens*, QTL Mapping, tolerance, zinc