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David DAUVILLEE

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Implication des enzymes de débranchement dans la biosynthèse de l'amylopectine

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Président :	M. J-C. Michalski
Rapporteurs :	M. A. Buléon M. A.M. Myers
Examinateur :	Me A. Smith
Directeur de thèse :	M. S.G. Ball

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NADP GAP DH	NADP glycéraldéhyde phosphate déshydrogénase
nm	Nanomètre
PEP	Phosphoénol pyruvate
PAB	Acide paraminobenzoïque
PGM	Phosphoglucomutase
Pi	Phosphate inorganique
ppm	parts per million
SDS	Sodium dodécyl sulfate
STP	Amidon phosphorylase (Starch Phosphorylase)
Tris	Tris-(Hydroxyméthyl)-aminométhane
UGPase	UDP-glucose pyrophosphorylase
WSP	Polysaccharide soluble dans l'eau

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GENERALITES

I. L'amidon

I.1 Généralités

L'amidon représente une des plus importantes sources polysaccharidiques de l'industrie agroalimentaire. Extrait des plantes de grande culture, il est le point de départ de la fabrication de plus de 700 produits commerciaux de basse ou moyenne valeur ajoutée. Il définit aussi la forme de réserve glucidique par excellence du règne végétal. On le trouve également chez certains parasites tels qu'*Eimeria brunetti* ou *Eimeria tenella* (Ryley et coll., 1969). Les bactéries ou les cellules animales synthétisent quant à elles du glycogène. Ces deux molécules, bien que composées de la même unité de base (le glucose) et les mêmes types de liaisons (α-1,4 et α-1,6), possèdent des structures et des propriétés radicalement différentes (Gunja-Smith et coll., 1970). La structure organisée du grain d'amidon permet aux végétaux de stocker une quantité bien supérieure de glucose relativement à celle des cellules animales ou bactériennes accumulant du glycogène. Le caractère insoluble et semi-cristallin du grain d'amidon constitue en fait la différence majeure entre ces deux formes de réserve polysaccharidique. La solubilité du glycogène ne permet pas aux cellules animales ou aux bactéries d'en accumuler de grandes quantités sans risquer des perturbations de l'osmolarité. De plus, sa structure intrinsèque l'empêche de croître à l'échelle du grain d'amidon (Gunja-Smith et coll., 1970). L'intérêt majeur de l'amidon repose sur les grandes quantités disponibles et les facilités d'extraction et de transformation. Mais son utilisation à des fins industrielles nécessite une meilleure compréhension de sa structure. Bien que formé essentiellement de glucoses reliés uniquement par deux types de liaisons chimiques (α-1,6 et α-1,4), la structure organisée totalement conservée à travers tout le règne végétal pose le problème de sa biogenèse. Par ailleurs, l'utilisation contrôlée de l'amidon de structure optimisée pour des transformations passe par une élucidation des mécanismes enzymatiques aboutissant à son architecture.

I.2 Localisation des grains d'amidon

L'amidon se retrouve dans la plupart des plastes des cellules végétales. On peut distinguer deux types de polysaccharides en fonction de leur localisation dans l'organisme. L'amidon transitoire, formé à partir du dioxyde de carbone fixé lors de la photosynthèse, est observé dans les chloroplastes des tissus photosynthétiques (feuilles, tiges, etc.). Il sera dégradé à l'obscurité afin de fournir du carbone nécessaire aux autres tissus de la plante. Les amyloplastes des tissus non chlorophylliens tels que les racines, les tubercules ou les graines contiennent de l'amidon dit de réserve (pour revues, Shannon et Garwood, 1984 ; Preiss J, 1991 ; Müller-Röber et Kossman, 1994). Bien que de structure comparable, les grains d'amidon possèdent des tailles et des formes différentes qui permettent d'identifier leur source biologique par un examen microscopique (Gallant et coll., 1992). Dans tous ces tissus et organismes, ce matériel glucidique est constitué en fait de deux homopolymères de composition chimique similaire : l'amylose et l'amylopectine.

I.3 L'amylose (Am) et l'amylopectine (Ap)

L'amylose et l'amylopectine sont constitués de chaînes de glucoses liés en α-1,4 et ramifiés en α-1,6 par des liaisons O-glycosidiques. Ils constituent environ 99% du poids du grain d'amidon. Les autres composants du grain d'amidon sont des lipides, des protéines ou des minéraux.

Les proportions relatives d'amylose et d'amylopectine varient en fonction de l'origine botanique de l'amidon. En moyenne, la fraction amylosique représente 18 à 35% de la quantité totale du polysaccharide. Le mutant *waxy* (amidon de maïs cireux) ne contient plus que de l'amylopectine. A l'opposé, on mesure jusque 70% d'amylose dans l'amidon du pois ridé (*rugosus* : *r*) ou l'amylomaïs (*amylose extender* : *ae*). Un matériel de structure intermédiaire entre l'amylose et l'amylopectine s'observe parfois dans quelques génotypes mutants enrichis en

amylose (*amylose extender*). Bien que de composition identique, l'amylose et l'amylopectine possèdent des propriétés bien distinctes.

I.3.1 L'amylose

De longues chaînes de résidus de glucose liés en α-1,4 avec d'occasionnels points de branchement en α-1,6 forment l'amylose (Manners DJ, 1989 ; figure 1). Son taux de ramification augmente avec la masse de la molécule. La fraction amylosique se définit également par l'hétérogénéité de masse des molécules qui la compose. Si le degré moyen de polymérisation s'élève à 500 unités de glucose, on y retrouve en général des molécules dont la taille oscille entre quelques dizaines et quelques milliers de ces résidus.

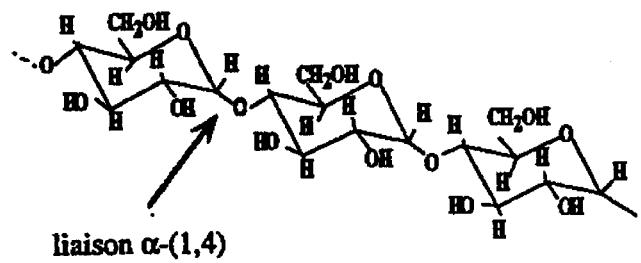

Figure 1 : **Une chaîne d'amylose.** Cette molécule est essentiellement composée de résidus de glucoses liés par des liaisons α-1,4.

La β-amylase convertit totalement en maltose un polysaccharide composé de liaisons α-1,4 mais ne peut aboutir à ce résultat sur un polymère contenant des liaisons α-1,6. Or, cette enzyme dégrade 70% de l'amylose démontrant d'une part la nature quasi linéaire de la molécule mais également la présence d'une quantité non négligeable de points de ramification.

Les longues chaînes linéaires de l'amylose sont susceptibles de complexer des petites molécules à caractère hydrophobe. On explique ainsi la présence des lipides au sein même de l'amidon. L'amylose forme également un complexe avec l'iode caractérisé par une longueur d'onde au maximum d'absorption (λmax) de l'ordre de 620nm (Banks et coll.,1971).

I.3.2 L'amylopectine

L'amylopectine définit la fraction majeure du grain ; elle représente 65 à 82% du poids sec d'un grain d'amidon. Le taux de ramification en α-1,6 de cette molécule varie de 4 à 6%. Aucun mutant sans amylopectine et de ce fait constitué uniquement d'amylose n'a été isolé à ce jour. La structure de la molécule a été élucidée en partie grâce à l'utilisation successive d'enzymes hydrolysant spécifiquement les liaisons α-1,6 et α-1,4 (Manners DJ, 1989 ; figure 2).

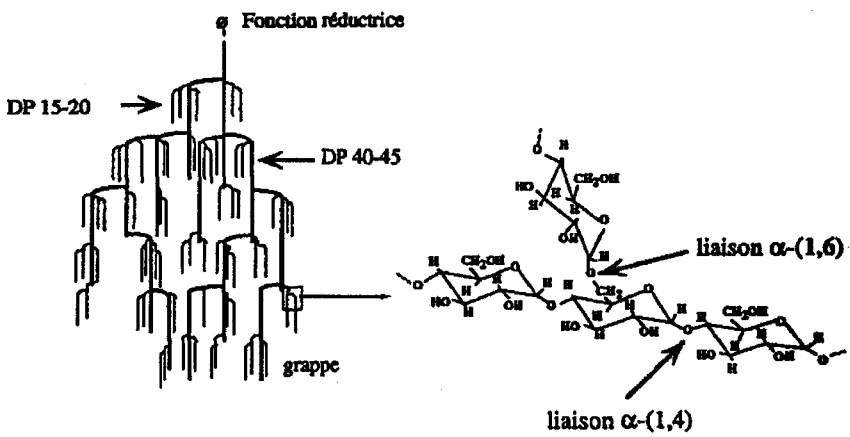

Figure 2 : Une molécule d'amylopectine. Le détail d'une chaîne montre les résidus de glucose liés en α-1,4 et ramifiés en α-1,6.

La structure est intimement associée à l'assemblement d'unités formatrices appelées grappes (figure 3). Différents types de chaînes composent la structure d'une grappe : les chaînes externes de type A qui ne participent aux

liaisons α-1,6 que par le biais de leur extrémité réductrice (elles sont donc non branchées) ; les chaînes de type B quant à elles sont branchées puisqu'elles sont liées aux autres chaînes via le carbone en position 6 d'un de leurs résidus de glucose. On distingue plusieurs chaînes de type B (B1, B2 et B3) en fonction de leur degré de polymérisation et de leur position dans la molécule (Hizukuri S, 1986). Les chaînes A et B1 sont regroupées dans la catégorie des chaînes S (short), les B2 et B3 appartiennent aux chaînes L (long). L'organisation de ces différentes chaînes est résumée dans la figure 3. Les grappes résultent de l'agencement particulier des chaînes courtes entre-elles (French, 1972 ; Robin et coll., 1974).

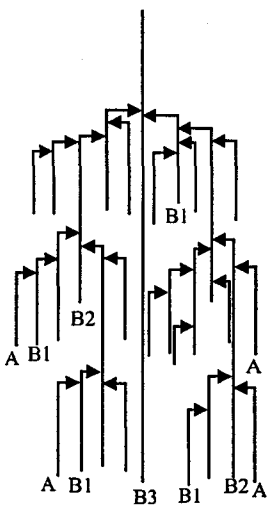

Figure 3 : Modèle d'organisation de l'amylopectine selon Hizukuri (1986).

Les chaînes les plus courtes (A) sont les plus externes de l'amylopectine. Elles sont reliées aux chaînes de type B par une liaison α-1,6. Les chaînes B1 d'un DP moyen de 19 se situent à l'intérieur d'une seule grappe élémentaire. Les chaînes B2 (DP moyen 41) réunissent deux grappes élémentaires. L'unique chaîne B3 quant à elle porte l'unique extrémité réductrice de la grappe d'amylopectine.

Selon Hizukuri, les chaînes de DP 6-12 conditionnent le type d'agencement cristallin qui sera rencontré dans la structure de l'amidon étudié. En effet, il suggère que la présence de polymorphes de cristaux (de type A cristallin ou de type B faiblement cristallin) dans l'amidon dépend de l'abondance de ces très courtes chaînes (1997). La structure plus ou moins cristalline permet de discriminer des amidons entre eux. Pfannemüller, en 1987, obtient des diagrammes de diffraction de type A pour des polyosides de glucose de DP 10-12 alors que l'utilisation de chaînes plus longues conduisent à un diffractogramme de type B. Le rapport des chaînes courtes sur celui des chaînes longues représente un autre facteur déterminant la caractéristique cristalline du polysaccharide. Celui-ci varie en fonction de l'origine botanique, de 5 pour l'amylopectine de pomme de

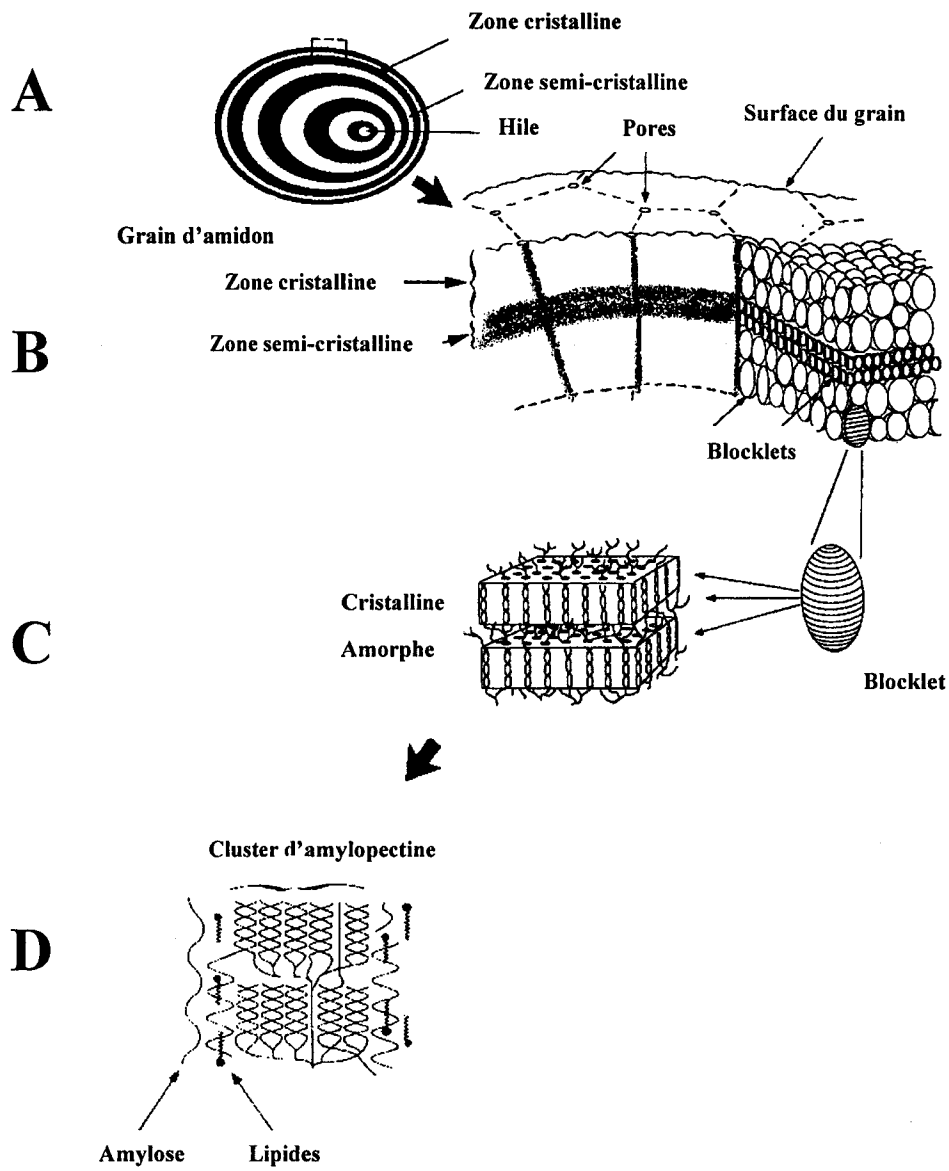

Figure 4 : Une vue d'ensemble du grain d'amidon. A) L'alternance des régions cristallines et semi-cristallines (en noir et blanc respectivement). B) La structure en "blocklets" possède différentes tailles en fonction de sa position dans le grain. C) Détail d'un "blocklet" contenant les lamelles amorphes et cristallines. D) La dernière représentation nous rappelle que l'amylose interagit avec la molécule d'amylopectine (D'après Blanshard JMV, 1987 et Gallant et coll., 1997).

terre à 8 ou 10 pour les amylopectines de céréales. Ces derniers se caractérisent par une amylopectine de type A, l'amylopectine de pomme de terre quant à elle exhibe le type B. La longueur des chaînes de l'amylopectine semble donc définir un facteur déterminant de la structure cristalline de l'amidon (Hizukuri S, 1985).

A l'échelle supérieure, on décrit la structure du grain d'amidon par une succession de zones semi-cristallines et cristallines (Yamaguchi et coll., 1979 ; French D, 1984). De nombreuses évidences expérimentales prouvent que la zone cristalline contient une lamelle amorphe et une lamelle cristalline de 9 à 10 nm d'épaisseur quelque soit l'origine botanique de l'amidon étudié (Kassenbeck, P, 1975, 1978 ; Jenkins et coll., 1993). Ces lamelles représenteraient la région cristalline des grappes et la région amorphe (où se groupent les points de branchement) des modèles de l'amylopectine proposés par Robin et coll. (1974) et French D, (1984). Manners (1989) estime que 80 à 90% du nombre total de chaînes composant l'amylopectine participent à la formation des clusters et que les 10% à 20% restant forment les connexions entre les grappes. L'utilisation de l'imagerie en microscopie révèle la présence de "blocklets" au sein du grain d'amidon (Gallant et coll., 1997). Ce terme dérive de l'allemand "blöckchen Strüktur" utilisé la première fois en 1934 par Hanson et Katz pour désigner une fraction de l'amidon résistante à la dégradation chimique. La taille de ces blocklets varient (de 20 à 500 nm) non seulement en fonction de l'organisme étudié mais aussi selon la position dans le grain d'amidon (Duprat et coll., 1980 ; Gallant et coll., 1992).

Enfin à l'échelle du microscope optique, le grain d'amidon contient des anneaux de croissance. Nous savons aujourd'hui, que l'apparition de ces anneaux résulte d'une alternance de zones cristallines et semi-cristallines dont la taille et le nombre peuvent varier en fonction des conditions de culture (Buttrose MS, 1962).

Les différents niveaux d'organisation du grain d'amidon sont représentés dans la figure 4 (Gallant et coll., 1997).

L'amylopectine possède une résistance supérieure à la digestion par la β-amylase puisqu'elle contient un plus grand nombre de points de branchement.

Sa nature branchée la rend moins susceptible d'interagir avec l'iode. Le complexe formé avec ce composé se caractérise par une coloration rouge de λmax 550 nm.

I.4 L'amidon et la cristallinité

Plusieurs niveaux d'organisation conduisent à la structure cristalline de l'amidon. L'amylopectine semble conférer à elle seule la cristallinité au grain d'amidon. L'étude de mutants ne contenant plus d'amylose ne révèle que peu de modifications de cristallinité du grain. On peut citer en exemple le cas de la souche *sta2* de *Chlamydomonas reinhardtii* ou du mutant waxy du maïs (Delrue et coll., 1992 ; Tsai CY, 1974). Cette souche déficiente pour l'enzyme responsable de la synthèse de l'amylose (la GBSSI pour Granule Bound Starch Synthase I ou amidon synthétase liée au grain) produit un amidon uniquement composé d'amylopectine sans effet sur la quantité de polysaccharide accumulée. L'étude de la cristallinité de ce polysaccharide ne montre pas de différence convaincante avec un amidon sauvage (Buléon et coll., 1997). Toutefois, il est important de noter qu'aucun résultat expérimental n'exclut la présence de l'amylose dans les cristallites : les unités de base du cristal.

La molécule d'amylopectine arbore des niveaux d'organisation qui peuvent expliquer le caractère cristallin de l'amidon. A l'échelle du nanomètre, on peut décrire cette molécule en fonction de la longueur des chaînes qui la composent et de la position des points de branchement. L'analyse et la quantification des chaînes composant l'amylopectine indique une bonne conservation de sa structure à travers le règne végétal (Morell et coll., 1998). A une échelle supérieure, on caractérise la molécule d'amylopectine par la présence des grappes provenant de l'organisation particulière des courtes chaînes de l'amylopectine (French D, 1972 ; Robin et coll., 1974). Ce caractère cristallin représente une spécificité de l'amidon vis à vis des autres polysaccharides de réserve. Les bactéries, les mammifères ou les champignons accumulent quant à eux d'autres composés glucidiques de nature proche de celle de l'amidon mais qui

pour la plupart restent hydrosolubles et amorphes. Parmi ceux-ci, le glycogène reste de loin le plus répandu.

I.5 Le pullulane et le glycogène

Le pullulane produit par certains hyphomycétes se caractérise par une structure primaire comparable à celle de l'amylopectine (Simon et coll., 1998). Il se compose d'une succession de résidus de maltotriose (trois glucoses liés en α-1,4) reliés les uns aux autres par des liaisons α-1,6 (Bender et coll., 1959 ; Catley et Whelan, 1971 ; Whelan WJ, 1971 ; figure 5).

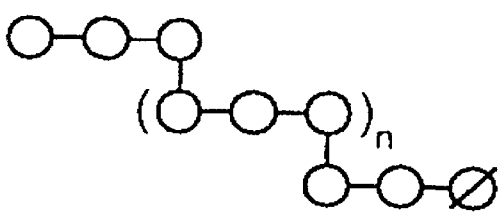

Figure 5 : Structure du pullulane. La molécule est composée d'une répétition monotone de chaînes de trois résidus de glucose liées par des liaisons α-1,6. Le résidu barré représente le glucose en position réductrice (Whelan WJ, 1971)

Le glycogène définit le polysaccharide de réserve par excellence des eubactéries et des mammifères. Après extraction de ce composé de tissus animaux, on observe de très grandes particules (d'un poids moléculaire d'environ 10^9 Da). La microscope électronique révèle qu'elles s'organisent en rosettes que l'on dénomme "particules α" d'un diamètre compris entre 60 et 200 nm (pour

revue, Manners DJ, 1991). Les particules α se composent d'un assemblement de plus petites particules dénommées β (Hata et coll., 1984 ; figure 6). Ces dernières ne constituent pas une population homogène mais un mélange d'au moins deux types de particules (diamètres 48 et 38 nm).

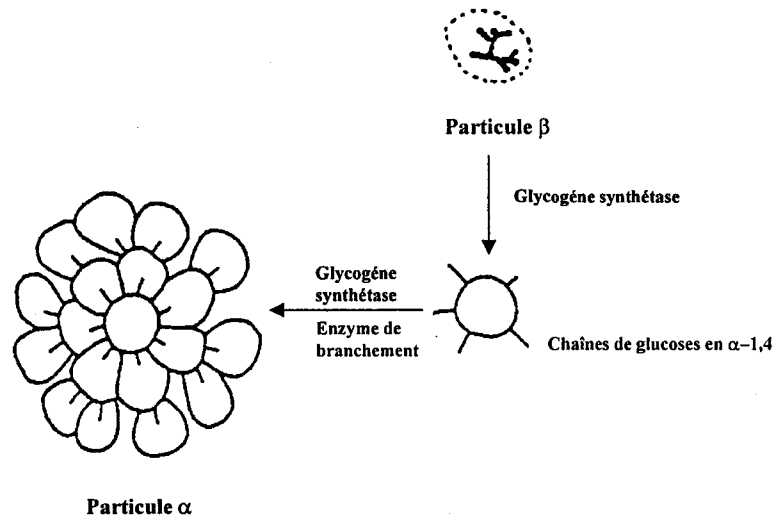

Figure 6 : Une molécule de glycogène. L'assemblement des particules β grâce à des chaînes de résidus de glucose permet la formation de la particule α en rosette.

On observe dans la molécule de glycogène des chaînes de type A et de type B selon leur position dans la molécule (figure 7).

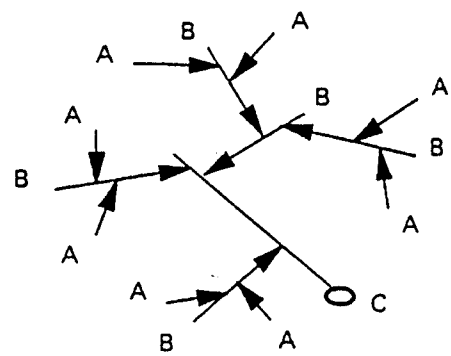

Figure 7 : Représentation schématique d'une molécule de glycogène (pour revue, Manners DJ, 1985).

Le taux de branchement de la molécule varie entre 8 et 10 %. La distribution unimodale de la longueur des chaînes constitutives du polysaccharide se distingue de la distribution polymodale des chaînes de l'amylopectine (Manners DJ, 1989). Les premiers modèles représentatifs du glycogène lui attribuaient une répartition homogène des points de branchement. Des résultats plus récents prouvent que ce n'est pas le cas (Gidley et Bulpin, 1987). On retrouve dans des génotypes particuliers de végétaux (les mutants *sugary*), un polymère soluble de constitution et de structure similaires au glycogène bactérien que l'on dénomme phytoglycogène pour souligner son origine végétale (Putaux et coll., 1999).

Le glycogène peut interagir avec l'iode. Le complexe ainsi formé possède une longueur d'onde au maximum d'absorption (λmax) d'environ 450nm.

Le tableau 1 résume les principales caractéristiques de l'amylose, de l'amylopectine et du glycogène.

	AMYLOSE	AMYLOPECTINE	GLYCOGENE
Proportions dans le grain d'amidon	18 à 35 %	65 à 82 %	-
Taux de branchement	0,2 à 1 %	4 à 6 %	8 à 10%
Poids moléculaire	10^5à 10^6	10^7 à 10^8	10^7
Nombre de résidus de glucose par chaîne	100 à 550	18 à 25	12 à 15
Digestibilité par la β-amylase	70%	55%	45%
Solubilité dans l'eau	Insoluble	Peu soluble	Soluble

Tableau 1 : Quelques propriétés physico-chimiques de l'amylose, de l'amylopectine et du glycogène.

II. La biosynthèse de l'amidon

Si on considère l'amidon comme une simple molécule composée de résidus de glucose reliés entre eux par deux types de liaison (α-1,4 et α-1,6), seules trois étapes enzymatiques apparaissent primordiales. L'ADP-glucose pyrophosphorylase (AGPase) aboutit à la biosynthèse d'un précurseur qui fournit le résidu de glucose à une seconde enzyme (amidon-synthétase, AS) qui forme les liaisons α-1,4. Il suffit alors d'une enzyme de branchement (BE) pour assurer la formation des liaisons α-1,6 ou points de branchement.

II.1 La biosynthèse du nucléotide-sucre précurseur : l'ADP-Glucose (ADPG)

L'adénosine diphosphate glucose pyrophosphorylase (AGPase, EC 2.7.7.27) catalyse la réaction de formation de l'ADP-Glucose selon la réaction suivante.

$$\text{Glucose-1-phosphate} + \text{ATP} \leftrightarrow \text{ADPG} + \text{PPi}$$

Cette enzyme existe chez les végétaux sous la forme d'un hétérotétramère composé de deux sous-unités catalytiques et deux sous-unités régulatrices (Copeland et Preiss, 1981). On retrouve cette organisation chez *Chlamydomonas reinhardtii* (Iglesias et coll., 1994). L'AGPase utilise de l'adénosine triphosphate (ATP) et du glucose-1-phosphate (G1P) provenant de l'action de la phosphoglucomutase (PGM) sur le glucose-6-phosphate (G6P) formé lors de la néoglucogenèse dans les organes photosynthétiques. L'approvisionnement en ADPG dans les organes de réserve se ferait soit par une forme plastidiale (Neuhaus et coll., 1993), soit par une forme cytoplasmique (Denyer et coll., 1996c) de l'enzyme. Une régulation allostérique exercée par le rapport 3-phosphoglycérate/phosphate inorganique (3PGA/Pi), jouant respectivement les rôles d'activateur et d'inhibiteur, contrôle surtout l'activité de l'enzyme plastidiale (Ghosh et Preiss, 1966). Cette réaction enzymatique représente une étape

primordiale de la synthèse de l'amidon puisqu'elle apporte le précurseur indispensable à la biosynthèse de l'amylose et de l'amylopectine. Chez *Chlamydomonas reinhardtii* l'étude d'une souche mutante possédant une enzyme insensible à son activateur, le 3PGA, accumule moins de 10% de la quantité d'amidon normalement présente (Ball et coll., 1991). Un autre mutant de l'algue touché au locus codant pour la sous unité catalytique ne contient plus du tout d'amidon (Zabawinski C, 1999). Des études structurales ont même montré qu'en limitant l'apport en ADPG, la longueur moyenne des chaînes d'amylopectine pouvait être modifiée. La modification de la concentration du précurseur peut donc entraîner non seulement une diminution de la quantité de polysaccharide mais aussi des changements structuraux majeurs (Van Den Koornhuyse et coll., 1996). Celles-ci sont vraisemblablement dues aux affinités distinctes pour le même substrat des enzymes d'élongation responsables de l'édification de structures différentes.

II.2 Les enzymes d'élongation ou amidon synthétases (AS)

ADPG + α-1,4-D-glucane → ADP + α-1,4-D-glucosyl- α-1,4-D-glucane

Les amidon synthétases (adénosine diphosphate glucose α-1,4-glucosyltransférases, EC 2.4.1.21) transfèrent des résidus de glucose du précurseur vers un glucane en cours d'élongation (Recondo et Leloir, 1961). Il existe de multiples isoformes de ce type d'enzyme chez les végétaux. Certaines formes sont solubles (amidon synthétase soluble ou ASS), d'autres sont retrouvées associées au grain (amidon synthétase liée au grain ou GBSS). Chacun de ces types, eux mêmes composés de plusieurs isoformes, jouent des rôles bien distincts lors de la synthèse du polysaccharide.

II.2.1 L'amidon synthétase liée au grain (GBSS)

La GBSSI est retrouvée intimement associée au grain d'amidon lui-même. Elle représente à elle seule plus de 90% des protéines associées à la matrice polysaccharidique. L'étude des mutants *waxy* du maïs (Tsaï CY, 1974), *amf* de la pomme de terre (Hovenkamp-Hermelink et coll., 1987), *lam* du pois (Denyer et coll., 1996a) ou *sta2* de *Chlamydomonas reinhardtii* (Delrue et coll., 1992) ont montré sans ambiguïté son rôle dans la synthèse de la fraction amylosique. La GBSSI n'est pas la seule amidon synthétase liée au grain, d'autres isoformes sont retrouvées aussi bien chez le pois, la pomme de terre, le maïs ou encore le blé (Smith A, 1990 ; Dry et coll., 1992 ; Mu et coll., 1994 ; Denyer et coll., 1996a). Ces enzymes, piégées au sein de la matrice polysaccharidique, possèdent une activité mineure lorsqu'on la compare à celle de la GBSSI et se retrouvent par ailleurs pour une plus large part dans la fraction soluble du stroma (Denyer et Smith, 1992 ; Edwards et coll., 1995 ; Edwards et coll., 1996).

II.2.2 Les amidon synthétases solubles (ASS)

Il existe au moins trois isoformes solubles d'amidon synthétases chez les plantes (Cao et coll., 1999 ; Li et coll., 1999). L'étude de mutants a là encore été d'un grand secours pour comprendre leur fonction *in vivo*. Que ce soit chez le mutant *sta3* de *Chlamydomonas reinhardtii* (Fontaine et coll., 1993), *rug5* du pois (Craig et coll., 1998) ou encore *dull* du maïs (Gao et coll., 1998), les amidon synthétases allongent certaines chaînes de l'amylopectine en fonction de leurs spécificités. Selon l'organisme étudié, l'activité d'une des trois formes prédomine. Ainsi, l'amidon synthétase soluble I (ASSI) représente l'activité majoritaire chez le maïs, l'ASSII tient ce rôle dans les embryons de pois, et l'ASSIII chez la pomme de terre. Les trois amidon synthétases sont pourtant retrouvées chez tous ces

végétaux démontrant l'importance de chaque isoforme. Les altérations structurales observées pour les amylopectines produites par des souches mutantes pour l'une des isoformes montrent que les activités non affectées sont, au moins en partie, incapables de pallier l'activité manquante (Edwards et coll., 1999 ; Fontaine et coll., 1993 ; Lloyd et coll., 1999).

II.3 Les enzymes de ramification ou enzymes de branchement (BE)

α-1,4-D-glucane (chaîne linéaire) → α-1,4/ α-1,6-D-glucane (glucane ramifié)

Les enzymes de branchement (α-1,4-glucane α-1,4-glucane-6-glycosyltransférases, EC 2.4.1.18) assurent la formation des liaisons α-1,6 dans le grain d'amidon. L'enzyme clive un fragment d'un α-1,4 glucane et greffe celui-ci en α-1,6. Il existe là encore plusieurs isoformes avec des affinités spécifiques variables selon le substrat. D'autre part, on différencie les BE selon la longueur des fragments transférés en α-1,6 (Takeda et coll, 1993 ; Guan et Preiss, 1993). Elles sont classées en enzymes de branchement de type I et de type II (BEI et BEII). La classe II contient deux isoformes appelées BEIIa et BEIIb. En 1995, l'expression de deux isoformes d'enzymes de branchement de l'albumen de maïs (BEI et BEIIb) dans une souche d'*Escherichia coli* mutante pour l'enzyme de branchement du glycogène a aboutit à une structure dont la distribution polymodale de la longueur des chaînes rappelle celle de l'amylopectine mais dont les propriétés physico-chimiques sont radicalement différentes (Guan et coll.).

Il faut noter que les enzymes de branchement à elles seules ne peuvent aboutir à la formation d'un polymère cristallin. La figure 8 représente une vue simplifiée du métabolisme de l'amidon. La production de l'ADP-glucose, la formation des liaisons α-1,4 et l'ajout de points de branchement constituent donc un ensemble de fonctions permettant en théorie la formation du grain d'amidon. La multiplicité des formes présentes (2 AGPase, 4 AS et 3 BE) distingue le

métabolisme de l'amidon de celui de son ancêtre présumé : le glycogène des cyanobactéries sans que l'on sache à l'heure actuelle ce qui a pu motiver une telle diversification des formes enzymatiques.

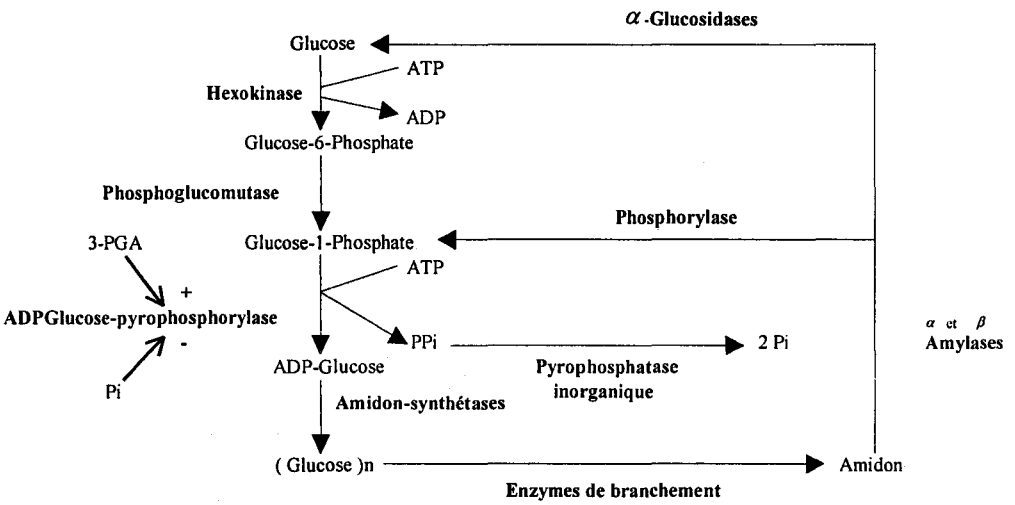

Figure 8 : Une vue simplifiée du métabolisme de l'amidon. Le nucléotide-sucre précurseur (ADP-glucose) est synthétisé par l'ADP-glucose pyrophosphorylase. Cette enzyme est régulée de manière allostérique par l'acide 3-phosphoglycérique (3-PGA) et le phosphate inorganique (Pi). L'ADP-glucose formé est pris en charge par les amidon synthètases afin d'allonger les α-1,4 glucanes en formation. Les enzymes de branchement, quant à elles, clivent et transfèrent une chaîne de glucose préexistante en position α-1,6 (point de branchement). L'amidon est dégradé par les enzymes hydrolytiques telles que les amylases ou les enzymes phosphorolytiques (phosphorylases).

Cette relative simplicité du métabolisme de l'amidon n'explique pas la variabilité qui existe entre la composition des amidons des différentes espèces ou organes étudiés. Elle ne reflète pas non plus la complexité de la structure du polysaccharide. Il est clair depuis quelques années que d'autres fonctions enzymatiques sont indispensables à la synthèse d'une structure aussi élaborée. Les trois enzymes décrites ci-dessus ne suffisent pas en effet à aboutir à la formation de la structure cristalline du grain. C'est ainsi que l'analyse de mutants de

synthèse de l'amidon a permis de mettre à jour le rôle des enzymes de débranchement (Pan et Nelson, 1984) ou de l'enzyme disproportionnante (Colleoni et coll., 1999a et 1999b).

II.4 Les enzymes de débranchement ou α-1,6-glucanohydrolases (DBE)

Les enzymes de débranchement appartiennent à trois sous familles : les enzymes de débranchement du glycogène des animaux ou de la levure, les pullulanases appelées R-enzyme ou encore limite dextrinases (EC 3.2.1.41) et enfin les isoamylases (EC 3.2.1.68). On retrouve les deux dernières chez les eubactéries et les végétaux. Ces deux types d'enzyme hydrolysent les liaisons α-1,6 mais n'agissent pas sur les mêmes substrats (Manners DJ, 1997). La pullulanase clive préférentiellement les liaisons α-1,6 du pullulane. Elle agit également sur l'amylopectine mais avec une plus faible efficacité et s'avère incapable de cliver les points de branchement du glycogène.

L'isoamylase quant à elle se révèle très active sur l'amylopectine et le glycogène mais incapable de digérer le pullulane. Ces deux enzymes coexistent dans la plupart des cellules végétales (Lee et coll., 1971 ; Doehlert et Knutson, 1991 ; Manners DJ, 1997). Avant l'étude de mutants défectueux pour leurs activités, ces enzymes étaient décrites comme intervenant uniquement dans la dégradation du polysaccharide de réserve. Nous reviendrons ultérieurement plus en détail sur les connaissances acquises à ce jour concernant le rôle de ces enzymes dans le métabolisme de l'amidon.

II.5 L'enzyme disproportionnante (EC.2.4.1.25)

L'enzyme disproportionnante (Enzyme D) clive une liaison α-1,4 d'une chaîne de glucoses et transfère l'oligosaccharide sur une autre chaîne en reformant

le même type de liaison. Cette enzyme caractérisée pour la première fois chez la pomme de terre (Peat S, 1956) possède un mode de fonctionnement particulier régit par trois règles :

-Le glucose ne sert que d'accepteur

-Le maltose ne peut pas être donneur et constitue un très mauvais accepteur

-La première liaison suivant l'extrémité non-réductrice et l'avant dernière liaison du côté réducteur ne peuvent pas être hydrolysées par l'enzyme D. Ces liaisons dites "interdites" ont été mises en évidence par Jones et Whelan en 1969. Ces règles sont illustrées dans la figure 9.

A) $\mathbf{DP_n + DP_m \leftrightarrow DP_{(n-i)} + DP_{(m+i)}}$

$\mathbf{DP_n}$: maltodextrine avec n résidus de glucose liés en α-1,4
$\mathbf{DP_m}$: maltodextrine avec m résidus glucose liés en α-1,4
i : groupe maltodextrinyle transféré avec i ≥2.

B) $\mathbf{G\sim G\text{-}(G)_n\text{-}G\sim G\text{-}G_{OH}}$
G : résidu de glucose
~ : liaison interdite.
- : liaison α-1,4
G_{OH}: résidu de glucose en position réductrice.

Figure 9 : Le mode d'action de l'enzyme disproportionnante.

A) L'enzyme clive une liaison α-1,4 d'un glucane et transfère le fragment ainsi libéré sur un autre oligosaccharide.
B) L'étude des spécificités de clivages de cette enzyme a révélé son incapacité d'agir sur des liaisons baptisées "interdites".

On confinait le rôle de cette enzyme à la dégradation des oligosaccharides libérés lors de l'action des activités hydrolytiques mais certaines observations révèlent son importance dans l'anabolisme des polysaccharides.

Chez *Escherichia coli,* l'amylomaltase joue un rôle proche de l'enzyme disproportionnante des végétaux. Le gène *malQ* appartenant à l'opéron maltose

code cette protéine. Les souches mutantes à ce locus sont incapables de croître en présence de maltose, de maltotriose ou de maltotétraose comme unique source de carbone (Hofnung et coll., 1971). Une souche ayant de nouveau la capacité de croître dans ces conditions a été isolée, celle-ci possède une maltodextrine glucosidase modifiée ayant acquis une activité transférasique (Peist et coll., 1996). Ces observations dévoilent le caractère essentiel de la fonction transférasique de l'amylomaltase pour le catabolisme des malto-oligosaccharides.

Dans les végétaux, l'utilisation d'ARN antisens dirigé contre les transcrits de l'enzyme de la pomme de terre n'a révélé aucune modification phénotypique concernant la biosynthèse de l'amidon (Takaha et coll., 1998). Il est important de noter que les auteurs dosent une activité résiduelle de l'enzyme dans les cultivars transformés et ne peuvent donc conclure sur l'absence d'effet observé alors que cette enzyme ne semble être limitante.

Récemment, l'étude d'un nouveau mutant de l'algue unicellulaire *Chlamydomonas reinhardtii* montre l'implication de l'enzyme D dans la biosynthèse de l'amidon. Cette souche portant la mutation *sta11-1* ne contient plus que 10% de la quantité d'amidon accumulée par une souche sauvage. La grande particularité de ce mutant consiste en la présence d'une fraction polysaccharidique soluble composée uniquement d'oligosaccharides non branchés (Colleoni et coll., 1999a et 1999b). Cette étude met à jour l'importance de cette enzyme dans la biosynthèse de l'amylopectine tout en n'excluant pas un rôle de celle-ci dans le catabolisme des polysaccharides de réserve. Les auteurs proposent que l'un des rôles majeurs de cette enzyme soit de recycler les oligosaccharides libérés lors de l'épissage de la préamylopectine par les enzymes de débranchement. Le chapitre suivant consacré aux activités de débranchement revient en détail sur ce mécanisme d'épissage.

III. Les enzymes de débranchement (DBE)

III.1 Les deux modes d'action des enzymes de débranchement

Les enzymes de débranchement décrites aussi bien chez les animaux, les bactéries, la levure ou les plantes possèdent des modes d'action différents en

fonction de leur origine (Lee et Whelan, 1971). On distingue le mode de débranchement direct caractéristique des enzymes de débranchement végétales du mode indirect décrit pour les enzymes des mammifères.

III.1.1 L'enzyme de débranchement indirecte

Dans les tissus des mammifères ou chez la levure, le glycogène est débranché par un système enzymatique à deux composantes (Manners et Rowe, 1969). La phosphorylase agit de façon récurrente à partir d'une extrémité non-réductrice jusqu'à une distance de quatre résidus de glucose d'un point de branchement. Cette digestion partielle du glycogène aboutit à la formation d'une dextrine limite, substrat de l'enzyme de débranchement. Cette dernière transfère un glucane de trois résidus de glucose sur un glucane à proximité et clive le point de branchement, libérant un glucose (figure 10). Ces deux enzymes assurent ainsi une dégradation complète du glycogène dans les cellules animales et fongiques.

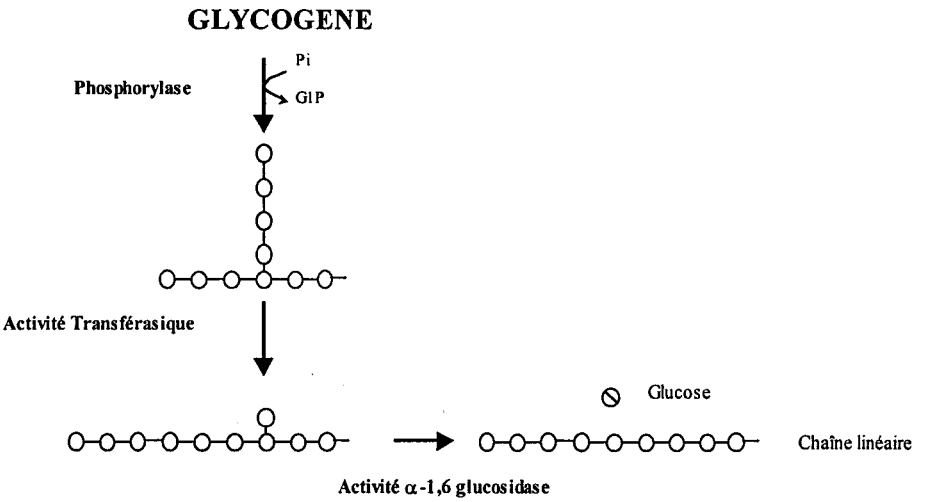

Figure 10 : Le mode d'action des activités de débranchement indirectes (Lee et Whelan, 1971).

III.1.2 Les enzymes de débranchement directes

Les enzymes de débranchement caractéristiques du monde végétal ne possèdent pas l'activité 4-α-glucanotransférasique de leurs homologues animaux ou fongiques. L'enzyme hydrolyse les liaisons α-1,6 auxquelles elle peut accéder

Enzymes	Sources	I	II	III	IV	Références
DBE	Albumen du riz (P)	DVVYNH	GFRFDLMGH	EGWD	YVSAHD	Nakamura et coll., 1996a
	Feuille d'épinard (P)	DVVYNH	GFRFDLMGH	EGWD	YVSAHD	Renz et coll., 1995
	K. aerogenes (P)	DVVYNH	GFRFDLMGY	EGWD	YVSKHD	Katsugari et coll., 1987
	Muscle de Lapin (I)	DVVYNH	GVRLDNCHS	ELFT	MDITHD	Liu et coll., 1993
	Levure (I)	DIVFNH	GFRIDNCHS	ELSR	MDCTHD	Waterston R, 1995
	E. coli (GlgX)	DIVLNH	GFRFDLAAV	EPWD	LVTAHD	Romeo et coll., 1988
	Albumen du maïs (I)	DVVFNH	GFRFDLASI	EAWD	FVCAHD	James et coll., 1995
	Albumen du riz (I)	DVVFNH	GFRFDLASI	EAWD	FVCAHD	Fujita et coll., 1999
	P. amyloderamosa (I)	DVVYNH	GFRFDLASV	EFTV	FIDVHD	Amemura et coll., 1988
BE (A)	Albumen de riz	DVVHSH	GFRFDGVTS	EDVS	YAESHD	Nakamura et Yamanouchi, 1992
BE (B)	Albumen de maïs	DVVHSH	GFRFDGVTS	EDVS	YAESHD	Baba et coll., 1991
	Levure	DVVHSH	GFRFDGVTS	EDVS	YCESHD	Thon et coll., 1992
	E. coli	DWVPGH	ALRVDAVAS	EEST	LPLSHD	Baecker et col., 1986

Tableau 2 : Alignement des séquences en acides aminés des sites actifs putatifs d'enzymes de la super famille des α-amylases (d'après Nakamura Y, 1996 ; Fujita et coll., 1999). BE, enzyme de branchement : (A) de type A, (B) de type B (d'après la classification de Burton et coll., 1995). DBE, enzyme de débranchement : (I) de type isoamylasique, (P) de type pullulanasique.

mais ne peut cliver un point de branchement reliant un seul résidu de glucose à une chaîne glucanique. La taille minimale des oligosaccharides libérés lors de son action est donc de deux résidus glucose (Abdullah et French, 1966 ; pour revue : Manners DJ, 1985 ; Lee et Whelan, 1971). Ce mode d'action regroupe en fait deux sous-classes enzymatiques définies par des spécificités de substrat : les pullulanases et les isoamylases. La purification de nombreuses enzymes de débranchement ou le clonage des gènes qui les codent qu'elles soient de source végétale, animale ou bactérienne, ont permis de les regrouper dans une super famille d'enzymes, celle des α-amylases.

III.2 La superfamille des α-amylases

La superfamille des α-amylases contient de nombreuses enzymes amylolytiques (pour revue, Nakamura Y, 1996). On y retrouve bien entendu des enzymes de débranchement (pullulanase et isoamylase) mais également des α-amylases (enzymes clivant les liaisons α-1,4) ou des enzymes de branchement. Toutes ces enzymes contiennent des régions consensus correspondant aux sites de fixation du substrat ou aux sites catalytiques (Jespersen et coll., 1993 ; Svensson B, 1994 ; Takata et coll., 1992). Ces enzymes effectuent quatre types de réactions enzymatiques. Ce sont les réactions d'hydrolyse de la liaison α-1,4, de la liaison α-1,6, la transglycosylation aboutissant à la formation d'une liaison α-1,4 et finalement la transglycosylation formant une liaison α-1,6. L'α-amylase (EC 3.2.1.1), la pullulanase (EC 3.2.1.41), la cyclodextrine glucanotransférase (EC 2.4.1.19) et l'enzyme de branchement (EC 2.4.1.18) sont respectivement des représentants des enzymes effectuant ces réactions (Takata et coll., 1992). Le tableau 2 compare les séquences consensus putatives de quelques enzymes de la superfamille des α–amylases.

La comparaison des séquences en acides aminés réalisée cette fois-ci non pas sur ces régions conservées mais sur l'entièreté de la séquence protéique révèle de grandes similitudes entre des enzymes de même spécificité provenant d'organismes phylogénétiquement éloignés. On trouve cette forte identité par exemple entre la pullulanase de l'albumen du riz et celle de *Klebsiella aerogenes*

ou le produit du gène *su-1* du maïs et l'isoamylase de *Pseudomonas amyloderamosa* (Nakamura Y, 1996). Le produit du gène *glgX* d'*Escherichia coli* présente des similitudes avec l'isoamylase de Pseudomonas mais pas avec les pullulanases bactériennes ; on peut donc penser que ce gène produit une protéine de spécificité isoamylasique. On observe également de grandes similitudes entre les séquences protéiques des enzymes de branchement de l'amidon des plantes (Burton et coll., 1995 ; Mizuno et coll., 1991 ; Cangiano et coll., 1993) et celles des enzymes de branchement du glycogène des animaux (Thon et coll., 1993), de la levure (Thon et coll., 1992) ou des bactéries (Baecker et coll., 1986 ; Kiel et coll., 1994). Toutes ces enzymes possèdent donc des régions hautement conservées bien qu'elles aient des activités différentes. L'analyse détaillée des séquences complètes des protéines de cette famille a permis d'identifier des régions qui rendent compte des spécificités enzymatiques.

III.3 Les enzymes en tonneau $(\beta/\alpha)_8$

Plusieurs groupes ont récemment publié des informations détaillées sur la structure de différentes hydrolases et quelques données cristallographiques concernant certaines α-amylases ont été obtenues (Matsuura et coll., 1984 ; Buisson et coll., 1987 ; . Boel et coll., 1990).

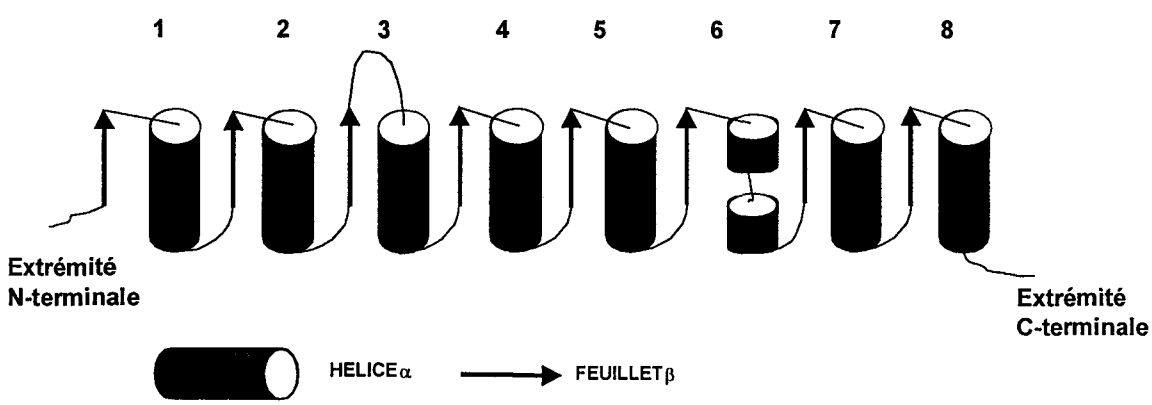

Figure 11 : Représentation schématique de l'organisation structurale d'un domaine $(\beta/\alpha)_8$. Les boucles protéiques 1 à 8 font la liaison entre les extrémités C-terminales des feuillets β et les extrémités N-terminales des hélices α et portent les acides aminés formant les sous-sites du site actif de l'enzyme (D'après Mac Gregor EA, 1993).

Toutes ces enzymes contiennent un motif en tonneau $(\beta/\alpha)_8$ composé de 8 feuillets β parallèles (E1 à E8) et de 8 hélices α (H1 à H8) elles aussi en orientation parallèle (figure 11 ; Mac Gregor EA, 1993). Ce repliement particulier se retrouve non seulement chez des enzymes hydrolysant les liaisons α-1,6 (isoamylase, pullulanase) ou qui catalysent la formation de ce type de liaison (enzymes de branchement), mais aussi dans celles qui scindent les liaisons α-1,4 (amylases). Des enzymes actives sur les deux types de liaison telles que les néopullulanases (EC 3.2.1.-), l'α-amylase-pullulanase (EC 3.2.1.1/41) ou l'enzyme de branchement du glycogène (mode de débranchement indirect ; EC 2.4.1.18), contiennent également ce repliement spécifique (Jespersen et coll., 1991 : Watanabe et coll., 1990 ; Watanabe et coll., 1991). Les boucles joignant les feuillets β et les hélices α forment le site catalytique de l'enzyme (Jespersen et coll., 1993). On retrouve les sites de fixation du substrat et de catalyse à proximité du feuillet β proche de l'extrémité carboxy-terminale ou dans la boucle β→α correspondante (Matsurra et coll., 1984 ; Buisson et coll., 1987 ; Klein et coll., 1992). L'étude des séquences en acides aminés de plus de 100 protéines de la superfamille des α-amylases prouve qu'il est possible d'identifier des motifs de conformation différente malgré la forte conservations des structures primaires. Ces motifs se retrouvent principalement situés au niveau des boucles (Svensson et coll., 1989 ; Jespersen et coll., 1991). Ainsi une nouvelle classification permet de classer les enzymes agissant sur l'amidon dans une seule et même sous-famille permettant de les distinguer de cette manière des autres protéines de la superfamille non actives sur l'amidon (Henrissat B, 1991).

L'analyse des séquences montre également, sans le prouver de façon indéniable, que la cinquième boucle contient deux larges régions hydrophobes. Or, dans toutes les enzymes de la famille agissant sur la liaison α-1,6 (pullulanase, isoamylase, α-amylase-pullulanase, enzyme de branchement), il s'avère qu'au moins l'une de ces deux régions est remplacée par un groupement polaire (Jespersen et coll., 1993). On observe également une différence de taille entre certaines boucles des deux types d'enzymes de débranchement que sont les isoamylases et les pullulanases. Ces boucles seraient donc responsables de la spécificité enzymatique des membres de la super famille des enzymes à tonneau

$(\beta/\alpha)_8$. D'autres études de ce type révèlent l'importance de certains acides aminés de ces boucles sans pour autant définir un véritable consensus quant à la relation entre la structure de la protéine et sa spécificité enzymatique. Le manque de données cristallographiques ne permet pas à ce jour de conclure définitivement sur ce point.

En 1959, Vallee et coll., ont observé la présence d'un ion Ca^{2+} fortement lié à l'enzyme. Cet ion Ca^{2+} joue un rôle essentiel dans le repliement de l'enzyme et la catalyse de la réaction. Dans la Taka-amylase d'*Aspergillus oryzae*, les données cristallographiques indiquent la présence d'un seul site de fixation du Ca^{2+} (Matsuura et coll., 1984) ; par contre, plusieurs sites sont proposés pour l'amylase pancréatique porcine (Payan et coll., 1980). L'étude de cristaux de l'amylase d'*Aspergillus niger* montre la présence de deux sites de fixation du calcium (Boel et coll., 1990). Le premier site de fixation de l'ion intervient dans la conformation de l'enzyme. Il interagit avec de nombreux résidus dont l'Asp175, l'Asn121, la Glu162 et la Glu210. Le second interagit quant à lui avec deux acides aminés présumés responsables de la catalyse. Des études d'inhibition à l'aide de fortes concentrations de calcium confirment la participation de ces acides aminés (Asp206 et Glu230) dans la réaction d'hydrolyse de la liaison α-1,4 (Boel et coll., 1990). La néopullulanase, l'α–amylase-pullulanase, les enzymes de branchement, les isoamylases et l'enzyme de débranchement du glycogène ne contiennent pas l'acide aspartique 175 interagissant avec le calcium (Baecker et coll., 1986 ; Kiel et coll., 1990, 1991 ; Baba et coll., 1991 ; Rumbak et coll., 1991). Hélas, le peu d'informations disponibles concernant les enzymes de la famille et la présence de calcium ne permettent pas de confirmer le rôle primordial de cet ion pour l'activité de ces enzymes. L'absence de l'ion Ca^{2+} dans la structure de l'enzyme offrirait une plus grande flexibilité de la chaîne peptidique permettant une accommodation du site catalytique des enzymes dépourvues de cet ion pour des substrats branchés en α-1,6 (Jespersen et coll., 1993).

On retrouve chez les plantes de nombreux membres de cette famille d'enzymes. Il s'agit en particulier de l'α-amylase, de la pullulanase ou encore de l'isoamylase. Nous allons précisément nous intéresser dans les chapitres suivants

aux deux membres de cette famille ayant fait l'objet de ce travail : l'isoamylase et la pullulanase.

III.4 La pullulanase

III.4.1 Découverte de l'activité dans les cellules végétales

La R-enzyme , pour la première fois observée dans la pomme de terre et la fève en 1951 (Hobson et coll., 1951), n'a pris le nom de pullulanase qu'après la découverte du pullulane et de la pullulanase bactérienne. L'enzyme préparée à partir de ces végétaux possède les mêmes caractéristiques que son homologue bactérien purifié d'*Aerobacter aerogenes*. Elle digère les liaisons α-1,6 contenues dans le pullulane ou l'amylopectine mais ne peut pas agir sur le glycogène (Peat et coll., 1954). De nombreuses confusions scientifiques dans l'élaboration de la nomenclature intervenues lors de la purification de cette enzyme depuis différentes sources végétales, ont conduit à l'appellation de cette enzyme sous plusieurs noms tels que pullulanase, R-enzyme mais également dextrinase limite (Pour revue : Lee et Whelan, 1971).

III.4.2 Le rôle des pullulanases végétales

La pullulanase dégrade le pullulane produit par des hypomycètes tels qu'*Aureobasidium pullulans* (Simon et coll., 1998). Malgré l'absence de ce polysaccharide chez les végétaux, cette enzyme est active sur l'amylopectine et existe dans de nombreuses plantes (Manners DJ, 1997).

La présence du phytoglycogène dans le mutant *sugary* du maïs observée pour la première fois par Sumner et Somers en 1944 a été expliquée dans un premier temps par un défaut d'activité pullulanasique (Erlander S, 1958). En 1995, James et coll. prouvent que la mutation entraîne une défectuosité de l'activité

isoamylasique et que la baisse d'activité de la pullulanase n'est pas la conséquence directe de la mutation. Les études réalisées sur les activités du riz (Nakamura et coll., 1996a) et du maïs (Beatty et coll., 1999) confortent l'hypothèse que les mutants *sugary* ne sont pas affectés au niveau d'un gène codant une pullulanase. La baisse certaine mais inexpliquée de l'activité pullulanasique qui accompagne la déficience de l'isoamylase a amené un regain d'intérêt pour l'étude de cette enzyme. Cette activité est induite par une synthèse *de novo* lors de la germination (Manners DJ, 1985). En 1980, Yamada montre l'activation de l'enzyme par des agents réducteurs et des peptidases dans diverses céréales. Toguri (1990) a même proposé que la synthèse *de novo* de réductases et de protéases lors de la germination du grain de riz augmente l'activité permettant la mobilisation de l'amidon. Une étude plus poussée de l'enzyme de la betterave sucrière montre que le dithiotréitol (DTT) rend celle-ci plus affine pour son substrat et augmente la vitesse d'hydrolyse (Li et coll., 1992). L'enzyme subirait ainsi une réduction lui permettant de prendre une conformation nécessaire à l'activité enzymatique. D'autres études révèlent une possible isomérisation de l'enzyme d'épinard en présence de son substrat (Henker et coll., 1998) et un contrôle de type redox lors de la germination du grain d'orge (Cho et coll., 1999). L'ensemble de ces observations suggère que la pullulanase tiendrait un rôle prépondérant dans la dégradation des polysaccharides.

La caractérisation de l'enzyme des embryons de pois suggère une bi fonctionnalité de la pullulanase aussi bien dans la dégradation lors de la germination mais aussi au cours de la biosynthèse de l'amidon de l'embryon en formation (Zhu et coll., 1998). Cette étude n'apporte cependant aucune preuve de l'implication de l'enzyme dans l'anabolisme du polysaccharide puisqu'elle se borne à la détection de l'activité à différentes périodes du développement de la future plante. A l'opposé, une étude similaire réalisée dans l'orge (Burton et coll., 1999) conclut simplement à l'importance de l'activité lors de la dégradation. L'absence de véritables mutants déficients pour cette activité ne permet pas d'attribuer un rôle précis à cette enzyme même si elle semble, au regard des études réalisées à ce jour, participer principalement au catabolisme des polysaccharides.

III.5 L'isoamylase

III.5.1 Découverte de l'activité dans les cellules végétales

En 1984, Pan et Nelson associent la mutation *sugary-1* du maïs avec une défectuosité en enzyme de débranchement de type pullulanasique. Ces résultats suggèrent pour la première fois qu'une enzyme de débranchement participe à la biosynthèse de l'amidon. Ils étaient alors en forte opposition à l'idée solidement installée qui place la fonction des enzymes de débranchement dans le catabolisme du polysaccharide. Une cartographie de mutations par transposon révèle plus tard que la mutation *sugary-1* touche un gène codant une activité de type isoamylasique (James et coll., 1995). Ce dernier travail prouve sans équivoque que le produit du gène possède une activité de type isoamylasique mais que les souches mutantes affichent des défectuosités pour les deux types d'activité de débranchement pour une raison encore inconnue (Rahman et coll., 1998 ; Beatty et coll., 1999).

III.5.2 Les mutants *sugary* du maïs

En 1984, Pan et Nelson prouvent grâce au mutant *sugary-1* du maïs qu'une défectuosité en enzyme de débranchement provoque le remplacement de l'amidon par un polysaccharide soluble de type glycogène appelé phytoglycogène. Ce composé contient un nombre plus important de points de branchement que l'amylopectine et se compose de chaînes en moyenne plus courtes. La longueur moyenne de ces chaînes est de 10 résidus de glucose alors qu'elle atteint 20 pour l'amylopectine. La distribution de la taille des chaînes des phytoglycogènes est d'ailleurs radicalement différente de celle de l'amylopectine (Yun et Matheson, 1993). Les derniers résultats obtenus sur le mutant *su-1* établissent de façon irréfutable que le gène *SU-1* encode une enzyme de 79 kD de spécificité isoamylasique qui s'accumule dans l'albumen lors de la biosynthèse de l'amidon (Rahman et coll., 1998). La mutation du gène de structure de l'isoamylase s'accompagne d'une défectuosité de l'expression de la pullulanase par un

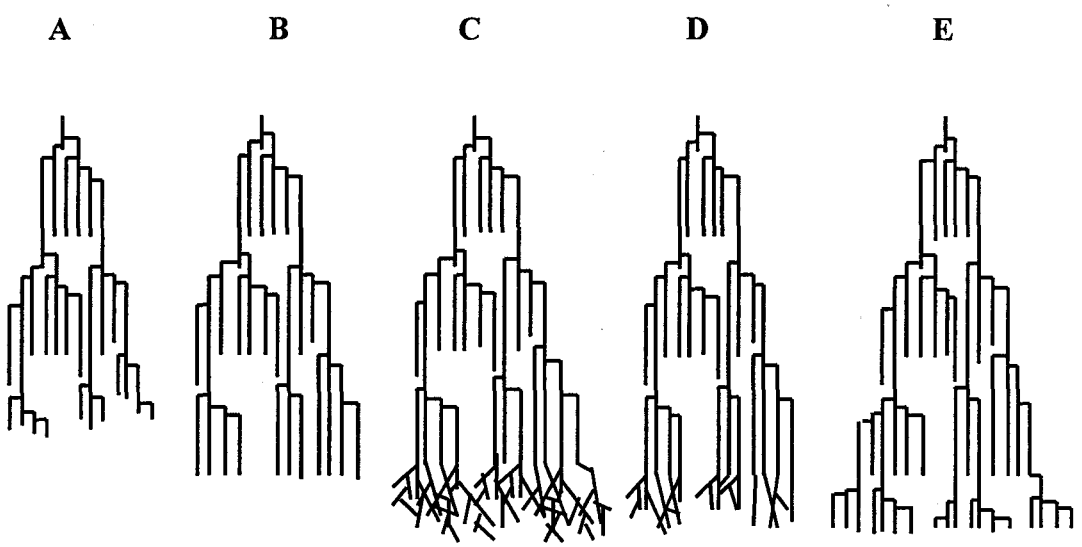

Figure 12 : Le modèle discontinu de biosynthèse de l'amylopectine : Les amidon-synthétases allongent les glucanes présents au niveau d'une lamelle amorphe (B). Lorsqu'une taille suffisante est atteinte, les enzymes de branchement créent de nombreuses liaisons α-1,6 (C) qui sont en partie clivées par les enzymes de débranchement (D). Seuls les points de ramification proches qui ne peuvent être clivés subsistent et sont pris en charge par les enzymes d'élongation afin de débuter un nouveau cycle. Le composé intermédiaire hyperbranché est appelé pré-amylopectine.

mécanisme post-transcriptionnel dont la nature n'est pas encore élucidée à ce jour (Beatty et coll., 1999).

III.5.3 Les mutants *sugary* du riz

Un mutant similaire à celui du maïs est également décrit dans le riz. Là encore, la mutation affecte le gène de structure d'une isoamylase d'une masse apparente de 83 kD (déterminée par la séquence du gène). La purification jusqu'à homogénéité de cette enzyme permet de lui attribuer une masse à l'état natif comprise entre 340 et 490kD, suggérant la présence d'un complexe d'activité isoamylasique (Fujita et coll., 1999). Une étude plus approfondie de ce complexe par électro-isofocalisation et digestion trypsique des sous-unités laisse penser à une composition de type homopolymérique de l'enzyme (Fujita et coll., 1999). Dans ce mutant aussi, l'activité pullulanasique est diminuée et les deux enzymes de débranchement apparaissent indispensables à l'édification de l'amidon (Kubo et coll., 1999). Cette fois encore, la mutation entraîne l'apparition de phytoglycogène.

III.5.4 Les mutants de *Chlamydomonas reinhardtii*

Chez *Chlamydomonas reinhardtii*, le phénotype du mutant *sta7* qui ségrège avec l'absence d'une activité de débranchement est bien plus marqué que chez ses homologues des végétaux supérieurs. Dans cette souche, l'amidon résiduel ne représente plus qu'une quantité inférieure à 0,1% de la normale et est remplacé par une petite quantité de phytoglycogène (Mouille et coll., 1996). Une telle sévérité du phénotype observé peut s'expliquer par l'absence d'activité redondante pouvant masquer ou compliquer l'interprétation des phénotypes. L'étude détaillée de ce mutant a permis de proposer un nouveau modèle de biosynthèse dit discontinu de la molécule d'amylopectine (Ball et coll.; 1996). Ce modèle est illustré dans la figure 12.

III.5.5 **Les critiques du modèle de biosynthèse discontinue**

La première critique opposée à ce modèle est l'absence de preuve irréfutable quant à la spécificité de l'activité de débranchement absente dans le mutant *sta7*.

De plus, la maturation de la molécule d'amylopectine entraînant la cristallisation représente une étape coûteuse du point de vue énergétique pour la cellule. En effet, les oligosaccharides libérés lors de l'épissage semblent n'être voués qu'à la dégradation. Il paraît peu probable qu'un mécanisme de mise en réserve passe par une étape aussi dispendieuse pour la cellule. L'obtention d'un nouveau mutant de *C. reinhardtii* a permis de répondre à cette critique. La mutation *sta11-1* (Colleoni et coll., 1999a) provoque l'absence d'activité de l'enzyme disproportionnante connue sous le nom d'enzyme D et provoque un effondrement de la synthèse d'amidon. L'étude détaillée de cette enzyme indique qu'elle peut recycler les oligosaccharides libérés lors du processus d'épissage évitant ainsi une trop grande perte énergétique (Colleoni et coll., 1999b).

Une autre critique du modèle trouve sa source dans l'étude d'un mutant déficient pour une forme chloroplastique d'isoamylase chez *Arabidopsis thaliana* (Zeeman et coll., 1998). Les auteurs proposent un autre modèle pour lequel des oligosaccharides présents dans la cellule seraient en compétition avec la molécule d'amylopectine en formation. Les enzymes de branchement et les amidon-synthétases en agissant sur ces oligosaccharides forment le phytoglycogène. L'isoamylase digère cette molécule pour éviter son accumulation dans la cellule. Ce mutant dénommé *dbe1* contient en effet un amidon de structure sauvage (10% de la quantité normale) et du phytoglycogène (Zeeman et coll., 1998). Ces deux modèles opposés demandent des études complémentaires afin de mieux appréhender le mécanisme qui prend place dans la cellule végétale et qui aboutit à la formation d'amidon cristallin.

III. *Chlamydomonas reinhardtii* : un modèle d'étude de la biosynthèse de l'amidon

L'étude du métabolisme de l'amidon chez les végétaux supérieurs demande des moyens logistiques importants. De plus, l'étude des mutants des plantes supérieures nécessite beaucoup de temps du fait d'une part du temps de croissance généralement plus long mais aussi de la polyploïdie souvent rencontrée. Un autre point en la défaveur de ces modèles provient de la possibilité de redondances fonctionnelles dues au caractère multicellulaire de la plante. Tous ces points font qu'un modèle unicellulaire représente un avantage important pour l'étude d'un métabolisme.

IV.1 Chlamydomonas : un modèle microbien

Chlamydomonas est une algue verte photosynthétique appartenant à l'ordre des volvocales. Elle se cultive aussi bien en milieu gélosé que liquide. Son temps de génération très court (8 heures dans les conditions idéales) permet d'obtenir très rapidement du matériel biologique en quantité suffisante. Toutes les techniques de microbiologie fondamentale sont applicables à cet organisme (réplique, isolement de clones, sélection par les auxotrophies etc..). Ce modèle porte même le nom de "levure verte" du fait de sa facilité de manipulation et des nombreux avantages qu'il arbore lorsqu'on le compare aux végétaux supérieurs. Chlamydomonas colonise la plupart des biotopes. Retrouvée sous une forme haploïde dans la nature, l'algue possède également un cycle sexué aboutissant à l'apparition de formes diploïdes susceptibles de redonner des ségrégeants haploïdes après méiose. Les diploïdes sont toujours sexués du fait de la dominance de la polarité sexuelle mt^- sur la polarité mt^+. Il est donc possible d'engendrer des cellules de ploïdie supérieure en répétant les croisements. Le cycle de reproduction sexuée s'amorce lors d'une carence pour un élément nutritif. Il est donc facilement induit au laboratoire en plaçant les cellules dans un milieu carencé en azote. Au cours du cycle sexué, environ 5% des zygotes formés lors de la fusion des deux cellules haploïdes aboutissent à la formation d'un diploïde végétatif stable ce qui autorise une étude des mutations par complémentation ou

par dosage de gènes. Dans la majorité des cas, le zygote évolue en zygospore qui donne 4 à 16 cellules haploïdes recombinantes après méiose. Ces cellules appelées ségrégeants représentent un matériel de qualité pour l'étude détaillée d'une mutation. Le cycle de reproduction sexuée est résumé dans la figure 13.

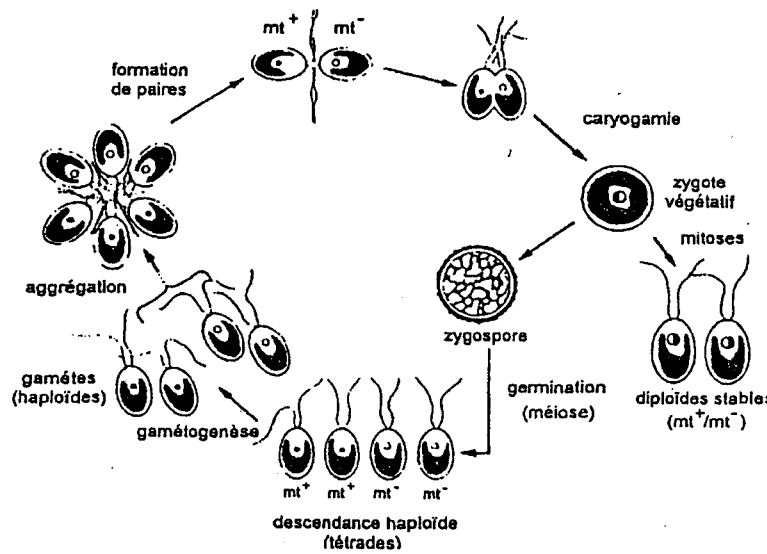

Figure 13 : Le cycle de reproduction sexuée de *Chlamydomonas reinhardtii* (d'après Harris, 1989a).

IV.2 Chlamydomonas et la biosynthèse de l'amidon

L'algue permet d'obtenir après 5 jours de culture des quantités importantes de polysaccharide (jusqu'à 60 mg d'amidon par litre de culture en milieu carencé en azote). En fonction des conditions de culture, l'algue produit un amidon dont la structure présente une forte similitude avec l'amidon transitoire des végétaux supérieurs (culture en milieu non carencé et en lumière continue ; Ball et coll., 1990), ou l'amidon de réserve des albumens de céréales (culture en carence azotée et en lumière continue ; Ball et coll., 1990 ; Fontaine et coll., 1993 ; Maddelein et coll., 1994 ; Libessart et coll., 1995). Des études physico-chimiques de cristallographie et de calorimétrie ont récemment entériné cette ressemblance (Buléon et coll., 1997). Il est très facile d'induire la dégradation des polysaccharides accumulés par l'algue en plaçant simplement les cultures à

l'obscurité. Toutes les enzymes de la biosynthèse de l'amidon sont retrouvées dans l'unique chloroplaste de cet organisme mais sont codées par le génome nucléaire.

IV.3 Les atouts majeurs de l'algue

L'atout majeur de Chlamydomonas repose sur un crible phénotypique facile à mettre en œuvre basé sur la capacité des polysaccharides à se complexer avec l'iode. Celui-ci permet l'isolement de mutants touchés dans la biosynthèse de l'amidon. Thorn et Mohazzeb ont démontré que la longueur d'onde au maximum d'absorbance (λmax) du complexe iode-polysaccharide dépend de la longueur des chaînes externes du polysaccharide (1990 ; figure 14).

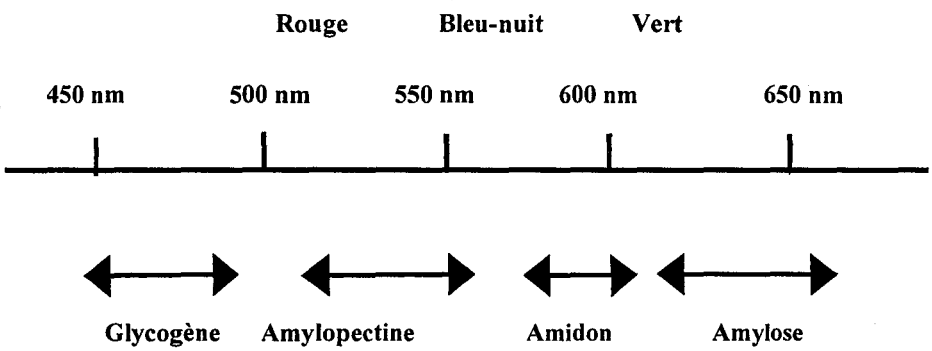

Figure 14 : λmax et couleurs des complexes iode/polysaccharides (Thorn et Mohazzeb, 1990). La couleur que revêt le complexe est caractéristique du polysaccharide.

Une suspension cellulaire est déposée sur une boite de Pétri contenant un milieu gélosé carencé en azote. Dans ces conditions (lumière vive, carence azotée), les cellules ne se divisent plus, accumulent massivement de l'amidon et la plupart des pigments finissent par être dégradés. Après 5 jours de culture, les boites sont colorées par des vapeurs d'iode et des teintes spécifiques de la nature et de la quantité de polysaccharide accumulés apparaissent. Une souche sauvage affiche une teinte bleue nuit après vaporisation. Tout changement de coloration indique la possible présence d'une mutation affectant la biosynthèse de l'amidon. Une coloration jaune apparaît pour des mutants qui ne contiennent plus ou très peu d'amidon. Des souches contenant un amidon enrichi en amylose présentent

une teinte verdâtre, alors que des mutants dont le polysaccharide de réserve est constitué uniquement d'amylopectine sont rouges après coloration.

Une autre caractéristique de l'algue est sa capacité à survivre en absence totale d'amidon. Cette caractéristique a permis à notre laboratoire d'isoler des mutants qui ne contenaient plus du tout d'amidon, alors que de tels mutants n'ont jamais été observés chez les végétaux supérieurs.

Objectif du travail de thèse

Comme nous l'avons vu, deux types d'activité de débranchement, isoamylase et pullulanase, coexistent dans la cellule végétale. La présence de mutants déficients pour ces activités dans plusieurs végétaux possédant des phénotypes dissemblables montre qu'il pourrait exister des redondances fonctionnelles entre ces enzymes. Les phénotypes observés chez le maïs et le riz sont dus à une mutation pour un gène d'isoamylase mais cette mutation s'accompagne d'une diminution de l'activité pullulanasique. De plus, la purification des enzymes chez le riz (Fujita et coll., 1999) et la pomme de terre (Ishizaki et coll., 1983) indique l'existence d'un complexe de haute masse à l'état natif. Certains auteurs émettent l'idée d'une interaction protéine-protéine entre l'isoamylase et la pullulanase (Myers A, communication personnelle) bien que celle-ci n'ait pu être démontrée par la technique du double hybride chez la levure. Certains auteurs ont même associé la sévérité du phénotype *sugary* du riz de différentes lignées à des différences d'activité pullulanasique (Kubo et coll., 1999). Chez *Arabidopsis thaliana*, la mutation du gène de l'isoamylase chloroplastique *dbe1* ne s'accompagne pourtant pas d'une baisse de l'activité pullulanasique mais au contraire d'une légère augmentation (Zeeman et coll., 1998). Le phénotype observé (amidon résiduel et phytoglycogène) s'approche pourtant de ceux des mutants *sugary*. La compréhension du mode d'action des enzymes de débranchement passe donc par une analyse détaillée de celles-ci mais aussi par l'étude de leurs interactions éventuelles. C'est dans cette optique que j'ai abordé ce travail de thèse.

RESULTATS

CHAPITRE I :

Novel, Starch-like polysaccharides are synthesized by an unbound form of granule starch synthase in glycogen accumulating mutants of *Chlamydomonas reinhardtii.*

Dauvillée D, Colleoni C, Shaw E, Mouille G, D'Hulst C, Morell M, Samuel MS, Bouchet B, Gallant DJ, Sinskey A, Ball S.

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Avant-propos

Les mutants de l'enzyme de débranchement (DBE) des végétaux supérieurs (mutants *sugary*) et de l'algue unicellulaire *Chlamydomonas reinhardtii* (mutants *sta7*) substituent la biosynthèse d'une grande partie voire de la quasi-totalité de leur amidon granulaire par la production d'un polysaccharide soluble de type glycogène : le phytoglycogène. Ces mutants offrent un matériel de choix pour la compréhension des étapes communes aux métabolismes du glycogène et de l'amidon. Le passage d'une forme insoluble cristalline (le grain d'amidon) à celle d'un polysaccharide soluble de structure peu organisée (le phytoglycogène) chez les mutants d'enzyme de débranchement illustre le rôle majeur de cette activité dans l'élaboration du grain. Les loci *STA2* et *STA3*, parfaitement décrits chez *C. reinhardtii*, conditionnent les présences respectives de l'amidon-synthétase liée au grain d'amidon (la GBSSI) et de l'amidon-synthétase soluble II (la SSII). Nous avons voulu déterminer les rôles respectifs des enzymes d'élongation (les amidon-synthétases) lors de la production du phytoglycogène chez les mutants au locus *STA7*. Dans ce but, nous avons construit par croisement, des souches contenant une mutation affectant l'activité de débranchement (*sta7*) et une voire deux mutations concernant les loci *STA2* et *STA3*.

Les polysaccharides produits par le simple mutant *sta7* ont été étudiés et comparés à ceux synthétisés par les souches doubles ou triples mutantes (*sta2 sta7* ; *sta3 sta7* ; *sta2 sta3 sta7*) . L'analyse structurale des polysaccharides accumulés par les souches *sta2 sta7* a permis d'échafauder un rôle pour la GBSSI dans la synthèse du polysaccharide insoluble de structure anormale observé et isolé dans la souche mutante *sta7*. Cette amidon-synthétase retrouvée intimement liée au grain dans un fond génétique sauvage (*STA7+*) agit sous une forme libre dans le mutant *sta7* et conditionne la formation de très faibles quantités de matériel insoluble proche de l'amylose par sa structure. La GBSSI n'est en rien impliquée dans la production du phytoglycogène. La présence d'une mutation affectant la SSII (*sta3*) dans un fond génétique mutant *sta7* engendre à l'opposé un défaut de synthèse du polysaccharide soluble.

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Novel, Starch-Like Polysaccharides Are Synthesized by an Unbound Form of Granule-Bound Starch Synthase in Glycogen-Accumulating Mutants of *Chlamydomonas reinhardtii*

David Dauvillée, Christophe Colleoni, Eudean Shaw, Gregory Mouille, Christophe D'Hulst, Matthew Morell, Michael S. Samuel, Brigitte Bouchet, Daniel J. Gallant, Anthony Sinskey, and Steven Ball*

Laboratoire de Chimie Biologique, Unité Mixte de Recherche du Centre National de la Recherche Scientifiquen no. 8576, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq cedex, France (D.D., C.C., G.M., C.D.H., S.B.); Massachusetts Institute of Technology, Department of Biology, 77 Massachussetts Avenue, Cambridge, Massachusetts 02139 (E.S., A.S.); Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, G.P.O. Box 1600, Canberra, ACT 2601, Australia (G.M., M.M., M.S.S.); and Institut National de la Recherche Agronomique, Unité de Recherches sur les Polysaccharides, leurs Organizations et Interactions, B.P. 71627, 44316 Nantes cedex 03, France (B.B., D.J.G.)

In vascular plants, mutations leading to a defect in debranching enzyme lead to the simultaneous synthesis of glycogen-like material and normal starch. In *Chlamydomonas reinhardtii* comparable defects lead to the replacement of starch by phytoglycogen. Therefore, debranching was proposed to define a mandatory step for starch biosynthesis. We now report the characterization of small amounts of an insoluble, amylose-like material found in the mutant algae. This novel, starch-like material was shown to be entirely dependent on the presence of granule-bound starch synthase (GBSSI), the enzyme responsible for amylose synthesis in plants. However, enzyme activity assays, solubilization of proteins from the granule, and western blots all failed to detect GBSSI within the insoluble polysaccharide matrix. The glycogen-like polysaccharides produced in the absence of GBSSI were proved to be qualitatively and quantitatively identical to those produced in its presence. Therefore, we propose that GBSSI requires the presence of crystalline amylopectin for granule binding and that the synthesis of amylose-like material can proceed at low levels without the binding of GBSSI to the polysaccharide matrix. Our results confirm that amylopectin synthesis is completely blocked in debranching-enzyme-defective mutants of *C. reinhardtii*.

Mutants of maize, rice, and *Chlamydomonas reinhardtii* and, more recently, Arabidopsis have been reported to accumulate in place of or in addition to starch a novel type of WSP known as phytoglycogen (Sumner and Somers, 1944; Mouille et al., 1996; Nakamura et al., 1996; Zeeman et al., 1998). In corn, Arabidopsis, and algae, the mutants were shown to lack a specific form of DBE with isoamylase-like specificity (James et al., 1995; Rahman et al., 1998; Zeeman et al., 1998). Both rice and maize were further reported to be missing a specific pullulanase-type of DBE (Pan and Nelson, 1984; Nakamura et al., 1996; Rahman et al., 1998). The major difference between algae and vascular plants consists of the severity of the phenotype recorded. Whereas maize, rice, and Arabidopsis substitute only part of their starch production by phytoglycogen, *C. reinhardtii* was reported to replace all of the starch by a low (5% of the wild type) amount of WSPs. The severity of this defect led us to suggest that debranching is mandatory to obtain significant amylopectin and, therefore, starch synthesis in plants.

The results obtained in maize, rice, and *C. reinhardtii* led us, together with a number of other authors, to propose a novel pathway for storage polysaccharide synthesis in plants (Ball et al., 1996). The latter consisted of trimming loosely spaced α-1,6 branches from a precursor of amylopectin to generate the asymmetrical distribution of branches required for amylopectin crystallization. However, this was recently questioned because in Arabidopsis leaves the loss of isoamylase activity is not accompanied by a disappearance of starch but rather by its selective decrease and partial replacement by phytoglycogen (Zeeman et al., 1998). To check for the presence of normal starch in the *C. reinhardtii* mutants, we scaled up our algal cultures to detect and characterize tiny amounts of insoluble granular polysaccharide (less than 1% of the wild-type starch amount). We further introduced mutations leading to the absence of GBSS or SS to test the dependency of the polysaccharides accumulating in DBE-defective mutants toward the different starch synthases.

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* Corresponding author; e-mail steven.ball@univ-lille1.fr; fax 33-3-20-43-65-55.

Abbreviations: DBE, debranching enzyme; DP, degree of polymerization; GBSS, granule-bound starch synthase; λ_{max}, wavelength of the maximal absorbance of the iodine-polysaccharide complex; SS, soluble starch synthase; WSP, water-soluble polysaccharide.

We now report the presence of a novel type of insoluble, starch-like material entirely constituted of amylose-like chains in the phytoglycogen-producing mutants of *C. reinhardtii*. We show the dependence of this material on GBSSI activity. Moreover, in contrast to all of the results obtained to date, we show that GBSSI does not bind to the granular polysaccharide. We believe our results demonstrate that GBSSI requires crystalline amylopectin for binding in vivo and that phytoglycogen synthesis can proceed in the complete absence of insoluble polysaccharide synthesis. We therefore confirm that amylopectin synthesis is completely blocked in the *sta7* mutants of *C. reinhardtii*.

MATERIALS AND METHODS

Material

The starch determination kit was purchased from Boehringer Mannheim. Rabbit liver glycogen and maize amylopectin were supplied by Sigma. Percoll was from Pharmacia.

Strains, Media, Incubation, and Growth Conditions

The genotypes of all of the strains of *Chlamydomonas reinhardtii* used in this work are listed in Table I. Starch and WSP were always prepared from nitrogen-starved media. Media and culture conditions used in our starvation experiments were as described by Ball et al. (1990). Formulas for Tris-acetate-phosphate and high salt-high acetate media and genetic techniques were according to the method of Harris (1989a, 1989b). All experiments were performed under continuous light (40 μE m^{-2} s^{-1}) in the presence of acetate at 24°C in liquid cultures that were shaken vigorously without CO_2 bubbling.

Purification of the Insoluble Macrogranular Fraction

Pure native starch was prepared from nitrogen-starved cultures, inoculated at 10^6 cells mL^{-1}, and harvested after 5 d of growth under continuous light (40 μE m^{-2} s^{-1}) in Tris-acetate-phosphate without nitogen medium. Algal suspensions were passed through a French press at 10,000 p.s.i. A crude starch pellet was obtained by spinning the lysate at 10,000*g* for 20 min. The pellet was resuspended in 300 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA per 10^9 starting cells, and passed twice through a Percoll gradient (1.2 mL of Percoll for the 300 μL of crude starch pellet). The purified starch pellet was rinsed in distilled water, centrifuged twice at 10,000*g*, and kept at 4°C for immediate use or dried for subsequent analysis. Starch amounts were measured using the amyloglucosidase assay as described by Delrue et al. (1992).

Purification of the WSPs

WSPs were prepared from 20 L of 1 week-old nitrogen-starved cultures that were inoculated at 10^6 cells mL^{-1}. Algae were harvested and ruptured by passing them in a French press (10,000 p.s.i) at a density of 10^8 cells mL^{-1} in the presence of pronase (1 mg mL^{-1}). The crude extract was immediately frozen at −80°C and thawed after a minimum of 4 h of storage. Cell debris were discarded by spinning at 10,000*g* for 30 min. The supernatant was extracted three times with 2:0.1 (v/v) chloroform:methanol and centrifuged at 3000*g* for 15 min. Water was added between each extraction to keep the volume of the aqueous phase constant. Emulsions were obtained by intensive shaking. The water-methanol soluble fraction was concentrated by rotary evaporation and redissolved in 10% DMSO to be subsequently loaded onto a gel-filtration column (TSK-HW-50 [F], Merck, Darmstadt, Germany) eluted by 10% DMSO as described by Maddelein et al. (1994). The high-M_r peak was desalted by dialysis at 4°C overnight and lyophilized. The low-M_r peak was desalted by gel filtration onto a column (TSK-HW-40, Merck). The peak of dextrin was lyophilized and stored at room temperature.

Debranching Analysis

Phytoglycogens (500 μg) were suspended in 10 μL of water and debranched with 1 μL of isoamylase (200 units/mL, Megazyme International, Bray, County Wicklow, Ireland) in 40 μL of 50 mM sodium acetate buffer (pH 4.0). The reaction was incubated for 2 h at 37°C and terminated by heating in a water bath at 100°C for 5 min. Completion of the reaction was ascertained by assaying the amount of reducing ends through the standard dinitrosalicyclate procedure. The latter consists of mixing 5 μL of the sample with 45 μL of water. The diluted sample was then added to 150 μL of 1% dinitrosalicyclate solution and the A_{540} was read. Complete debranching was obtained when maximal and constant absorbencies that compared favorably with those of amylopectin, amylose, and glycogen standards were recorded. The samples were then evaporated to dryness in a centrifugal vacuum evaporator.

Separation of Labeled Oligosaccharides

The debranched and undebranched samples were labeled with 8-amino-1,3,6-pyrenetrisulfonic acid and analyzed with a DNA sequencer, as fully described by O'Shea and Morell (1996).

trans Complementation Tests

The segregants (IJS) obtained from the cross between strains IJ2 and S were subjected to *trans* complementation tests to characterize their deficiencies. Diploid clone selection was achieved by growth after 5 d of the sexual fusion products on appropriate selective media. The diploids obtained were purified three times, checked for cellular volume, and then amplified. Cell patches incubated for 7 d on solid, nitrogen-deprived medium were stained with iodine. Diploids homozygous for the *sta2* mutation appear red after staining (Delrue et al., 1992).

Transmission Electron Microscopy

Cell suspensions prepared from nitrogen-starved media were harvested first and immediately embedded at 45°C in

Table I. *Strains and genotypes*

Strain	Genotype	Defective Enzyme[a]
137C	*mt − nit1 nit2*	—
BAFR1	*mt + nit1 nit2 sta2–29::ARG7*	GBSSI
IJ2	*mt − nit1 nit2 sta2–29::ARG7 sta3–1*	GBSSI SSII
S	*mt + nit1 nit2 sta7–7::ARG7*	DBE
IJS15	*mt + nit1 nit2 sta7–7::ARG7*	DBE
IJS24	*mt − nit1 nit2 sta7–7::ARG7*	DBE
IJS2	*mt − nit1 nit2 sta3–1 sta7–7::ARG7*	DBE SSII
IJS13	*mt + nit1 nit2 sta3–1 sta7–7::ARG7*	DBE SSII
IJS21	*mt + nit1 nit2 sta3–1 sta7–7::ARG7*	DBE SSII
IJS8	*mt + nit1 nit2 sta2–29::ARG7 sta7–7::ARG7*	DBE GBSSI
IJS39	*mt + nit1 nit2 sta2–29::ARG7 sta7–7::ARG7*	DBE GBSSI
IJS50	*mt + nit1 nit2 sta2–29::ARG7 sta7–7::ARG7*	DBE GBSSI
IJS3	*mt − nit1 nit2 sta2–29::ARG7 sta3–1 sta7–7::ARG7*	DBE GBSSI SSII
IJS27	*mt + nit1 nit2 sta2–29::ARG7 sta3–1 sta7–7::ARG7*	DBE GBSSI SSII

[a] Names of the enzyme activities missing in the corresponding strains.

a 3% agar gel to prevent these highly mobile cells from adopting a heterogeneous spatial distribution. Small, solidified cubes of agar containing the samples were then cut and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.1) for 3 h at room temperature. A part of these samples was postfixed in 1% osmium tetroxide for 1 h. The fixed samples were then dehydrated in an ethanol series, infiltrated with propylene oxide, and embedded in Epon. Sections of 80 nm thickness were mounted on copper or gold grids previously coated with carbon. Two complementary treatments were applied on the grids as follows: (a) sections of samples fixed by glutaraldehyde and osmium tetroxide were mounted on copper grids and then stained for 30 min in 2.8% uranyl acetate (50% methanol) at 48°C; and (b) sections of samples fixed by glutaraldehyde alone were mounted on gold grids and then stained according to the modified PATAg procedure previously described by Simon et al. (1995). The samples were first treated for 30 min in a saturated solution of 2–4-dinitrophenylhydrazine in 15% acetic acid to block free aldehydes. After several washes in deionized water, samples were oxidized for 30 min in 1% periodic acid aqueous solution, then treated for 18 h with 0.2% thiosemicarbazide in 20% acetic acid, and finally treated for 30 min with 1% silver proteinate aqueous solution.

The sections were examined with a transmission electron microscope (model 100S, Jeol) operated at 80 keV.

Zymogram Analysis

Soluble crude extracts were prepared as previously described (Fontaine et al., 1993) and immediately stored at −70°C. The lysate was cleared by centrifuging at 10,000*g* for 10 min at 4°C. Proteins were measured using the Bio-Rad protein assay kit. In 100 μL of 25 mM Tris glycine, pH 8.3, 1% SDS, 5% β-mercaptoethanol, 100 to 500 μg of protein was denaturated by boiling in a water bath for 4 min. The denatured proteins can be stored at 4°C without subsequent loss of enzyme activity. Starch synthase and DBE zymograms were as previously described by Mouille et al. (1996) and Buléon et al. (1997).

SDS-PAGE Analysis of Granule-Bound Proteins, Western Blots, and GBSS Activity Assay

Purified starches (1–6 mg) were boiled for 5 min in 80 μL of 2% SDS and 5% β-mercaptoethanol and then centrifuged for 20 min at 10,000*g* to extract proteins from the granule. Supernatants were loaded onto SDS-PAGE and stained with Coomassie brilliant blue R250.

Western-blot analysis was performed on proteins solubilized from the granule as described by Buléon et al. (1997). The specific antiserum PA55 was directed against the synthetic peptide GTGGLRDTVENC, representing a consensus sequence derived from the C-terminal part of the vascular plant starch synthases (Buléon et al., 1997). The GBSS activity assay was as described by Delrue et al. (1992).

RESULTS

Mutants Defective for the *STA7* Locus Synthesize up to 0.5% of the Wild-Type Amount of Starch in the Form of Insoluble, High-Amylose Types of Granular Polysaccharides

Because of the low amounts of polysaccharide found in the *sta7* mutants of *C. reinhardtii*, we scaled up our culture volumes to 20 L and measured the amounts of the WSP and high-density-insoluble material that sedimented in the bottom of Percoll gradients. As previously reported, in the mutants we found 5% ± 3% (n = 5) of the amount of polysaccharide measured by the amyloglucosidase assay in wild-type reference strains in the form of WSP. Both high and low-molecular-mass WSPs could be separated through TSK-HW-50 (Fig. 1A) gel-permeation chromatography. Because of the high sensitivity of the technique, we used fluorescence labeling coupled to PAGE on a DNA-sequencing gel to analyze both the size distribution of the small oligosaccharide fraction and that of the high-molecular-mass polysaccharide subjected to selective debranching through bacterial isoamylase. Results displayed in Figure 1, B and C, together with those previously pub-

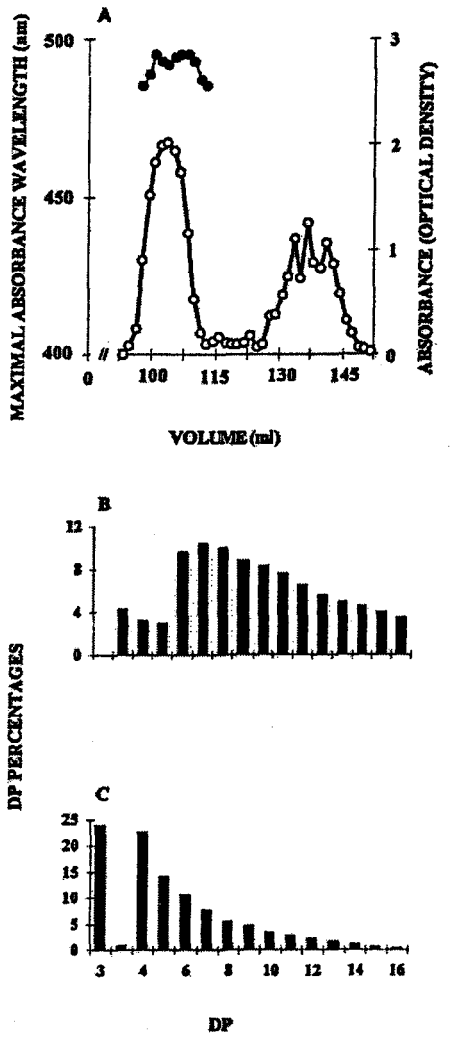

Figure 1. Determination of the chain-length distributions of both *sta7* high- and low-molecular-mass WSPs. A, TSK-HW-50 gel-permeation chromatography of *sta7* WSP. The λ_{max} (in nanometers [●]) of the undebranched fractions is scaled on the left *y* axis and the amount of Glc (in micrograms per milliliter [○]) measured for each fraction is indicated on the right *y* axis. The *x* axis shows the elution volume scale (in milliliters). B and C, Histograms of chain-length distributions obtained through gel electrophoresis of fluorescent glucans with or without isoamylase-mediated enzymatic debranching. Both chain-length distributions of debranched high-molecular-mass (B) and undebranched low-molecular-mass (C) WSPs eluted from the TSK-HW-50 column (A) are displayed as percentages of chains of DP between 2 and 16. The *x* axis displays a DP scale, and the *y* axis represents the relative frequencies of the chains expressed as percentages. The peak seen between DPs 3 and 4 is consistent with the position of a branched trisaccharide.

lished (Mouille et al., 1996), clearly confirm the high-molecular-mass fraction as glycogen-like material, whereas the oligosaccharide fraction contained both branched and linear glucans. The high-mass glucans display an iodine interaction (Fig. 1A), a branching ratio (Mouille et al., 1996), a chain-length distribution (Fig. 1B), and granule morphology (Fig. 8, C–F) similar to those reported for glycogen. The relative amounts of oligosaccharide to polysaccharide in the water-soluble fraction were between 20% and 60% (40% ± 20%). We believe that these variations reflect the presence of fluctuating amounts of α-amylase in the extracts to which the soluble polysaccharides are highly sensitive.

That these polysaccharides are made of α-1,4-linked α-1,6-branched Glc residues is beyond doubt. First, this material is 100% sensitive to amyloglucosidase. Second, it is only partly sensitive to β-amylase (not shown). Third, the ^{13}C-NMR spectra that are typical of glycogen were found for all WSPs described in this paper. These spectra were identical to those that we published previously (Mouille et al., 1996). Six major NMR signals corresponding to the six carbon atoms (numbered 1 through 6) of Glc could always be distinguished at approximately 100 ppm (C_1), 71.95 ppm (C_2), 72.44 to 73.16 ppm (C_3), 78.9 ppm (C_4), 71.64 ppm (C_5), and 60.64 ppm (C_6). These major signal chemical shifts correspond to carbons within Glc residues present in the middle of α-1,4-linked glucans. In addition and because of the abundance of the branches, we were able to pick up two additional signals at 70.25 and 61.09 ppm assigned, respectively, to C_4 and C_6 of the non-reducing-end terminal Glc residues. Other signals dispersed around the C_1 and C_4 major peaks are due to those Glc residues at or around the branch points.

The amounts of high-density-insoluble material that could be purified through isopycnic Percoll gradient centrifugation were slightly greater than what we previously reported (Mouille et al., 1996) and were estimated at 0.4% ± 0.2% of what would have otherwise been the normal starch amount in wild-type algae. Although very low, this still amounted to 10% of the total WSP fraction present in the mutants and was characterized by a λ_{max} of 590 to 600 nm, consistent with the presence of amylose-like chains within the polymer structure. This fraction was detected in all eight *sta7* mutants investigated. Because in Arabidopsis normal starch is reported to be present with phytoglycogen in isoamylase-defective mutants, we undertook a detailed, structural characterization of this material purified from 80-L algal cultures.

The Amylose-Like Polysaccharide Displays an Entirely Novel Structure

The high-amylose material consisted of both a high- (30%) and a low (70%)-molecular-mass component that could be separated through TSK-HW-75 gel-permeation chromatography (Fig. 2). The high-molecular-mass fraction displayed a λ_{max} of 590 nm, comparable to that of amylopectin components found in high-amylose starches of *C. reinhardtii* and the *su*-2 mutant of maize (Fontaine et al., 1993; Takeda and Preiss, 1993; Libessart et al., 1995). However, unlike all other wild-type or mutant amylopectins analyzed to date, we found less than 1% branches in this material by proton NMR (Fig. 3). The ^{13}C-NMR spectra were indistinguishable from those published for amylose

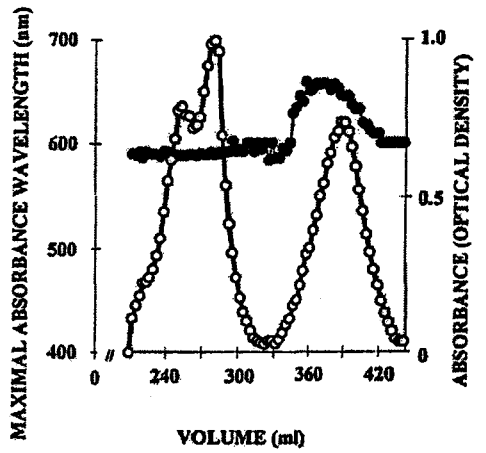

Figure 2. Separation of amylopectin-like material and amylose extracted by TSK-HW-75 gel-permeation chromatography. Absorbance ([O]) was measured for each fraction at λ_{max} (*y* axis on the right). The λ_{max} (in nanometers) is displayed as an unbroken, thin line on the left *y* axis. The *x* axis represents the elution volume scale (in milliliters).

(Fontaine et al., 1993), further establishing this material as composed of α-1,4-linked Glc residues. A low 1% ± 0.5% branching level was measured with greater precision through comparative fluorescence labeling of the debranched polysaccharide fraction. The size distribution of the debranched glucans displayed a pattern similar to that of the high-mass amylose present in wild-type starches (Fig. 4A). Because of both the high mass and the low λ_{max} of this fraction, these results could not have been produced by contamination of low amounts of amylopectin by amylose. The size distribution, branching ratio (<1%), and λ_{max} of the low-molecular-mass fraction were similar to those of amylose. However, upon debranching, this fraction yielded an unusual chain-length distribution that could distinguish it from standard amylose and amylopectin (Fig. 4B).

Because of the overall exceptional structure of this polysaccharide, we reasoned that it arose through a complete block in amylopectin synthesis and was probably due to the selective action of GBSSI, the amylose-biosynthetic enzyme. To test this hypothesis and to ascertain whether such a polymer is required to obtain phytoglycogen subsequently, we introduced mutations inactivating selectively GBSSI and SSII in a *sta7* mutant background.

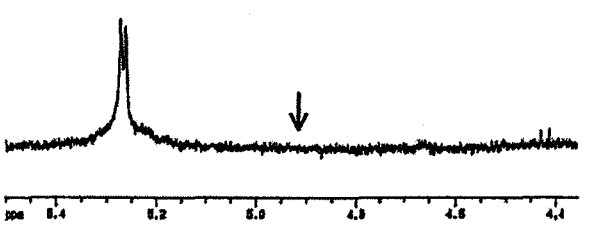

Figure 3. Proton NMR analysis of the *sta7* high-molecular-mass amylopectin-like component. The ^1H spectrum was made in 90% deuterated DMSO, 10% ^2H (Mouille et al., 1996). The position of the α-1,6-specific signal is displayed by an arrow and is shown to be below the noise level (<2%).

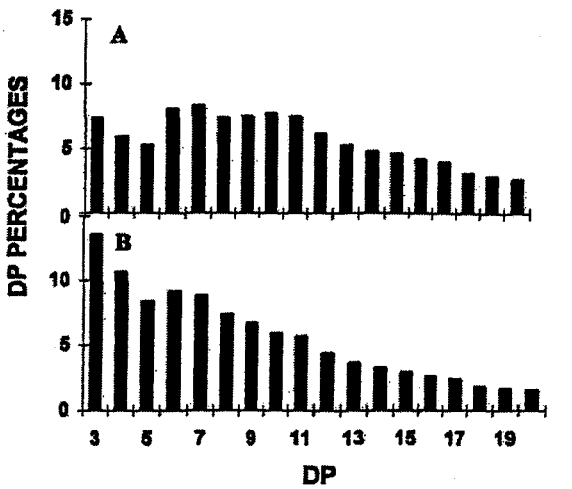

Figure 4. Histograms of the chain-length distribution of starch-like polysaccharides obtained through gel electrophoresis of fluorescent glucans after isoamylase-mediated enzymatic debranching. Both chain-length distributions of debranched high-molecular-mass (amylopectin-like; A) and debranched low-molecular-mass (amylose-like; B) fractions are displayed as percentages of chains of DP ranging from 3 to 20. Separation of both fractions was achieved through TSK-HW-75 gel-permeation chromatography of granular polysaccharides (Fig. 2) produced in the *sta7* glycogen-synthesizing mutants.

The Amylose-Like Polysaccharide Is Not Required for Phytoglycogen Synthesis

Strain S (*sta7–4::ARG7*) was crossed with IJ2 (*sta2–29::ARG7 sta3–1*). The interesting double- or triple-mutant genotypes were detected with *trans* complementation tests (see "Materials and Methods"). We selected three genotypes for each of the following classes (Table I): wild type, *sta7*; *sta7 sta2*, *sta7 sta3*, *sta7 sta2 sta3*. The genotypes were double-checked through zymogram analysis of DBE and SS. We found no differences in behavior of the SS in a mutant *sta7* background. SSII disappeared, as expected, upon introduction of the *sta3* mutation. For each genotype class, three measurements of WSP and granular starch levels were taken on three randomly selected recombinants (n = 9; Table II). For each class a complete WSP characterization was made, including a determination of the chain-length distribution of the novel types of phytoglycogens (Figs. 5 and 6). These were compared with the phytoglycogen produced by our reference *sta7* strains (Fig. 1, A and B).

It is clear that the absence of GBSSI because of the presence of the *sta2* mutation (*sta7 sta2*) is sufficient to lead to the disappearance of granular polysaccharides (Table II). Surprisingly, selective disappearance of SSII also led to an 85% quantitative decrease of the granular polysaccharide fraction but not to its disappearance. The residual material still displayed a high (600 nm) λ_{max} upon complexation with iodine. The WSP fraction was qualitatively and quan-

Table II. *Determination of the amounts of granular and soluble polysaccharides produced*

Values are the averages of three different experiments involving three distinct segregants with the exception of the *sta2Δ1 sta3 sta7Δ7* class, which involved two distinct strains (in μg per 10^6 cells).

Genotype[a]			Granular Material	WSP
+	+	*sta7Δ7*	0.12 ± 0.01	0.43 ± 0.03
sta2Δ1	+	*sta7Δ7*	0.007 ± 0.0003	0.48 ± 0.03
+	*sta3*	*sta7Δ7*	0.014 ± 0.001	0.14 ± 0.01
sta2Δ1	*sta3*	*sta7Δ7*	0.003 ± 0.0002	0.20 ± 0.01

[a] *sta2Δ1, sta2–29::ARG7; sta3, sta3–1;* and *sta7Δ7, sta7–7::ARG7.*

titatively unaffected by the absence of GBSSI and, consequently, by that of the high-amylose granular fraction (Table II; Figs. 5A and 6A). On the other hand, the phytoglycogen structure, the molecular mass distribution of the WSP fraction, and the amount of WSP were significantly affected by a defect in SSII (Table II; Figs. 5, B and C, and 6, B and C). A mutation in *STA3* leading to a defective SSII always resulted in reduction by one-half of the WSP content in the *C. reinhardtii* DBE-defective strains. Therefore, we conclude that *sta3* mutations are epistatic on *sta2* defects with respect to WSP amount, whereas the reverse proved to be true for the presence of the amylose-like material.

GBSSI Can Synthesize Amylose-Like Granular Material in the Absence of Polysaccharide Binding

In all cases in which starch synthesis was impaired in the presence of a wild-type *STA2* gene, there was a net increase of GBSSI-specific activity within the granule (Libessart et al., 1995; Van den Koornhuyse et al., 1996). A dramatic example of such an increase is provided in Figure 7A, comparing the amount of granule-bound proteins present after high or low starch synthesis occurring, respectively, under nitrogen-starvation-induced growth arrest (storage starch) or nitrogen-supplied growing conditions. These differences can be explained by the fact that an equivalent amount of GBSSI protein binds to a restricted polysaccharide amount. Because of both the low levels of granular material (0.5% of the wild-type amount) and the overrepresentation of long glucans, we expected this fraction to be filled with GBSSI protein and enzyme activity. We were surprised to find SDS-PAGE profiles generated from granule-associated proteins that could not be distinguished from those of strains completely lacking GBSSI (Fig. 7B). Moreover, the amount of GBSS activity monitored under these conditions decreased to less than 1% of the wild-type activity and was also less than those measured for strains carrying a gene-disrupted GBSSI structural gene (Fig. 7C). These results were confirmed by western blotting using an antibody directed against a C-terminal peptide sequence of higher-plant starch synthases (Fig. 7D). Western blots demonstrated the presence of a small amount of 75-kD cross-reacting polypeptide. Because SSI displays a 75-kD mass (Buléon et al., 1997), we suspect that this protein represents the residual 0.5% to 1% GBSS enzyme activity in a fashion analogous to that described for the 77-kD GBSSII in potato and pea (Edwards et al., 1996). Mutants lacking both GBSSI and SSII behaved in an identical fashion. Because GBSSI and SSI display identical masses in *C. reinhardtii*, we were unable to discriminate between these two proteins and saw no convincing differences between western blots of soluble proteins extracted from wild-type and *sta7* mutants.

Electron Microscopy

Because both the glycogen found in animal cells and the maize phytoglycogen were reported to display some level of organization, including rosette-like structures (Lavintman, 1966), we embarked on cytochemical observations made by transmission electron microscopy using the PATAg stain. This procedure enables the selective staining of starch granules and WSP in the wild-type and single or double mutants (Fig. 8). The wild-type cells prepared from nitrogen-starved media (Fig. 8, A and B) showed a signif-

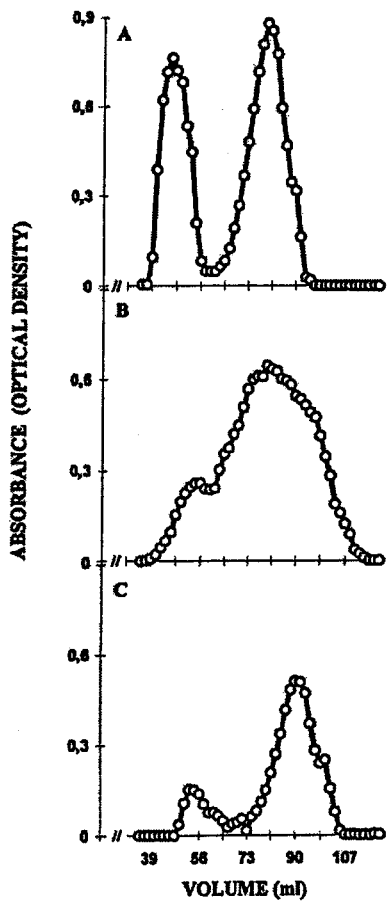

Figure 5. Separation of WSP through TSK-HW-50 gel-permeation chromatography. A, *sta2 sta7* WSP. B, *sta3 sta7* WSP. C, *sta2 sta3 sta7* WSP. The *x* axis shows the elution volume (in milliliters), and the *y* axis represents the amount of Glc ([O]; in micrograms per milliliters) measured for each fraction.

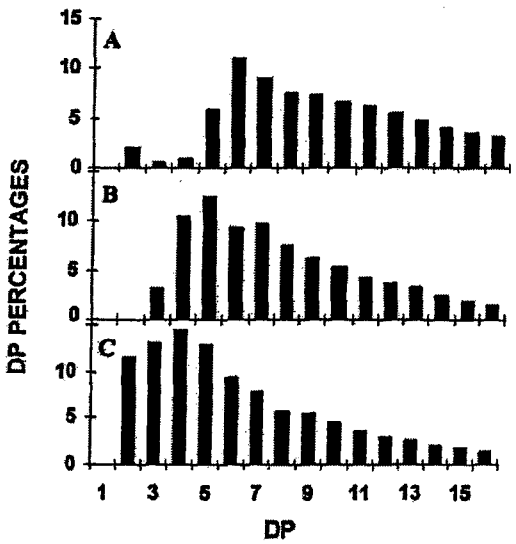

Figure 6. Histograms of chain-length distributions obtained through gel electrophoresis of fluorescent glucans after isoamylase-mediated enzymatic debranching of phytoglycogens produced by double or triple *C. reinhardtii* mutants. Chain-length distributions of debranched phytoglycogens from *sta2 sta7* (A), *sta3 sta7* (B), and *sta2 sta3 sta7* (C) mutant strains are displayed as percentages of chains of DP between 2 and 16. Phytoglycogen purification was achieved by taking the high-mass fraction after TSK-HW-50 gel-permeation chromatography, as displayed in Figure 5.

icant amount of starch granules, which were electron dense with the PATAg staining (Fig. 8B) and electron transparent (inverted contrast) with osmium-uranyl staining (Fig. 8A). The latter, which is used for contrasting unsaturated lipids and for proteins and cellular membranes, has no contrasting effect on polysaccharides. In both the single *sta7*-carrying mutant reference (Mouille et al., 1996) and in the double-mutant strains (Fig. 8, C–F), starch granules disappeared, whereas other polysaccharide structures were selectively revealed in the mutants through the PATAg staining procedure. These polysaccharides displayed no detectable morphological organization at this scale. According to both the biochemical and staining results, these structures correspond to phytoglycogen. This material appeared in the form of either small granular units, as in the single *sta7* mutant or in the double *sta2 sta7* mutant (Fig. 8, E and F), or in the form of elongated groups of units in the double *sta3 sta7* mutants (Fig. 8, C and D). Moreover, the size distribution of the WSP fit that which was expected for glycogen polymers. No analogous structures were ever detected in the wild-type algae. As usual, many lipid droplets were observed in all cells undergoing nitrogen starvation.

DISCUSSION

In this work we report the purification and characterization of an insoluble and dense polysaccharide that is synthesized in very low amounts in DBE mutants of *C. reinhardtii*. To account for the low λ_{max} of the high-mass fraction present within this polysaccharide, we propose the existence of a high-mass network of 80 to 100 Glc residue long chains hooked together through α-1,4 linkages, with occasional small branches comparable to those defined for amylose. To probe the origin of this fraction, we have introduced, in addition to the DBE defect, mutations leading to the selective inactivation of GBSSI or SSII or both of these elongation enzymes. The disappearance of the insoluble fraction correlated perfectly with that of the GBSSI activity. Because GBSSI is known to synthesize long glucans in wild-type cells, we believe that this strongly suggests that GBSSI is responsible for the synthesis of this anomalous, amylose-like material. Similar fractions have been previously observed in DBE mutants of maize. It must

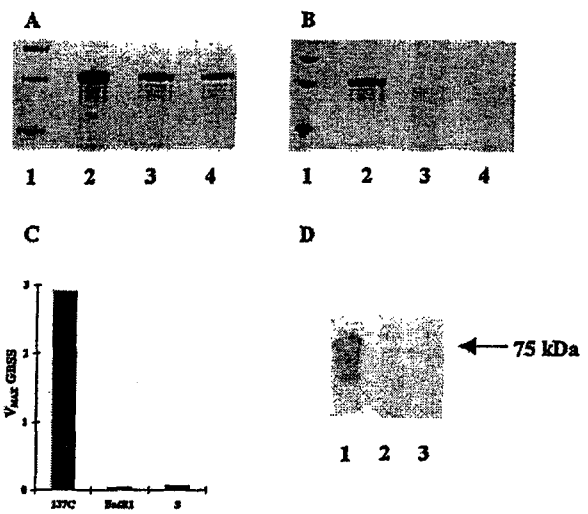

Figure 7. Detection of GBSS activity and protein from starch-like granules of phytoglycogen-producing strains. A and B, Coomassie brilliant blue R-250 stained 5% to 7.5% SDS-acrylamide gels of starch-bound proteins. In A and B, lanes 1 display molecular-mass size standards (from top to bottom 94, 67, and 43 kD). A, Lane 4 represents starch-bound proteins extracted from 6 mg of polysaccharide purified from the wild-type strain 137C after nitrogen starvation. Lanes 2 and 3 display starch-bound proteins extracted from 6 and 3 mg, respectively, of starch purified from nitrogen-supplied wild-type cultures. The major 76-kD band corresponds to the GBSS protein and displays the typical GBSS N-terminal sequence (Delrue et al., 1992). B, Lane 2 represents starch-bound proteins extracted from 2 mg of starch purified from nitrogen-supplied wild-type cultures (strain 137C). Lanes 3 and 4 display starch-bound proteins extracted from two samples of starch purified, respectively, from nitrogen-supplied *sta7–7::ARG7* (strain S) and *sta2–29::ARG7* (strain BafR1). C, Histograms of GBSSI activity are expressed as nanomoles of ADP-Glc incorporated into glucan per minute and per milligram starch. Starch was extracted from nitrogen-starved algae. Strains BaFR1 and S contain gene disruptions inactivating GBSSI and DBE, respectively. 137C is the wild-type strain. D, Western blot of starch-bound proteins extracted from equal amounts of polysaccharide. Proteins cross-reacting with antibodies directed against the C-terminal consensus peptide sequence found on vascular plant starch synthases (see "Materials and Methods") were selectively revealed. Lanes 1, 2, and 3, Strains 137C, BAFR1, and S, respectively.

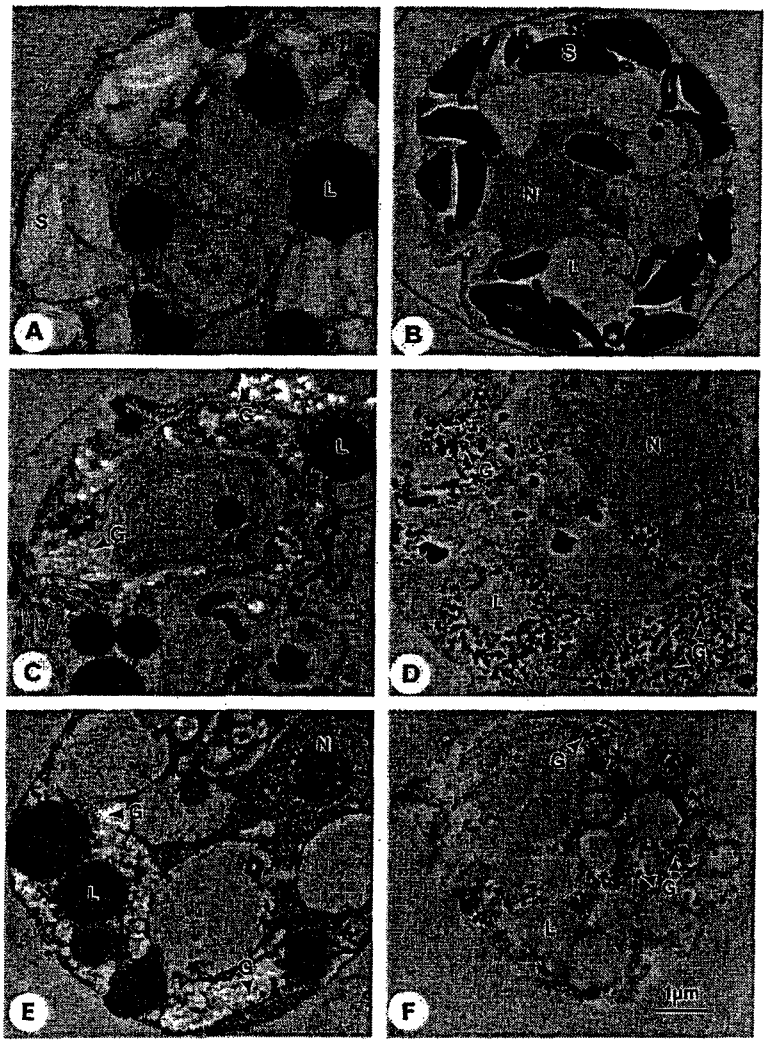

Figure 8. Electron microscopy of nitrogen-starved wild-type and double-mutant cells. A, Strain containing wild-type *STA7* showing part of the cell filled with starch granules (S) seen as electron-transparent structures of about 2 μm in length. Lipid bodies (L), approximately 1 to 2 μm in diameter, appear as structures highly contrasted after a double fixation of the cells by glutaraldehyde osmium and uranyl acetate poststaining. B, Same strain as in A. In comparison with A, contrast appears inverted because of PATAg staining of the sections used after a single fixation of the cells with glutaraldehyde. Selectively, the starch granules are deeply black and the lipid bodies are transparent. N, Nucleus. C, Nitrogen-starved double-mutant strain *sta3–1 sta7–7::ARG7*. Starch is not synthesized by this strain. Storage carbohydrate is phytoglycogen (G), which appears as very small electron-transparent inclusions (<0.2 μm in diameter) when the cells are fixed by glutaraldehyde osmium and the sections are poststained by uranyl acetate. D, Same nitrogen-starved double mutant as in C. Phytoglycogen, when contrasted with PATAg staining, appears more clearly as very dense granular particles, either unit-like particles or associated with more or less elongated strands. E, Nitrogen-starved double-mutant strain *sta2–29::ARG7 sta7–7::ARG7*. Again, starch is not synthesized by this strain and the storage carbohydrate is phytoglycogen, which appears similar to that in C when the cells are fixed by glutaraldehyde osmium and the sections are poststained by uranyl acetate. F, Same nitrogen-starved double mutant strain as in E. Phytoglycogen appears more clearly than in E when the cells are contrasted with PATAg staining. However, phytoglycogen particles are only in the form of small granular units and are smaller than in D.

be stressed, however, that the interpretation is complicated in sweet corn by the presence of the incomplete block that is recorded on the phenotype. Therefore, in this case, it is not known whether the anomalous material comes from the solubilization of preexisting granules. This could result from the presence of a progressively more severe defect in DBE activity during kernel development. Such a temporal delay in the expression of the mutant phenotype is expected to lead to the appearance of hybrid amylose-phytoglycogen structures that were reported by Boyer et al. (1981) and by Matheson (1975). In *C. reinhardtii* the situation is much simpler in that there is no starch to start with in the mutant and none to end with after subjecting the cells to polysaccharide accumulation conditions. In this case, the anomalous, amylose-like material and the phytoglycogen are discontinuous, because we find no evidence of amylose chains in the water-soluble fraction and no traces of phytoglycogen in the insoluble material.

Mutants defective for GBSSI (and consequently with no amylose) have been described in many different plant systems. These mutants build wild-type amounts of seemingly normal organized starch granules. However, amylopectin-less mutants accumulating wild-type amounts of starch granules solely made of amylose have never been reported to our knowledge. This can be interpreted simply by stating that amylose synthesis requires the presence of amylopectin, whereas synthesis of the latter is independent of amylose.

Our data imply that this requirement comes from both the need for a primer and the need for an organized crystalline amylopectin matrix to activate the normally granule-bound enzyme. In the *sta7* (DBE-defective) mutant background, the crystallization of amylopectin is prevented and GBSSI cannot bind to the polysaccharides. In the *sta7 sta3* (DBE- and SSII-defective) double-mutant background, the synthesis of the anomalous amylose-like ma-

terial is further limited by the availability of a primer substrate that is suitable for GBSSI. Recently, we demonstrated that amylose synthesis proceeds through extension and cleavage from a preexisting amylopectin primer (van de Wal et al., 1998).

In the course of searching for the determinants of biosynthesis of the amylose-like material, we constructed double- and triple-mutant genotypes that enabled us to ascertain the origin of the phytoglycogen fraction. We were not surprised to find that GBSSI had no role in the synthesis of this fraction, which was entirely under the control of SS. Thus, synthesis of phytoglycogen is completely independent of the presence of a preexisting insoluble polysaccharide.

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CHAPITRE II :

The debranching enzyme complex missing in glycogen mutants of *Chlamydomonas reinhardtii* displays an isoamylase-type specificity

Dauvillée D, Mestre V, Colleoni C, Slomianny MC, Mouille G, Delrue B, D'Hulst C, Bliard C, Nuzillard JM, Ball S.

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Avant-propos

Deux types d'enzyme de débranchement sont observés chez les végétaux : les isoamylases et les pullulanases. On distingue facilement ces deux enzymes en fonction de leurs spécificités de substrat ; alors que les isoamylases s'avèrent très largement actives sur un substrat de type glycogène, les pullulanases s'attaquent préférentiellement au pullulane, un polysaccharide dont les chaînes sont plus courtes. Les études effectuées chez le maïs ou le riz indiquent que les mutations de type « *sugary* » affectent les gènes de structure codant dans chaque cas une enzyme de débranchement de spécificité isoamylasique. Cependant, ces mutations s'accompagnent également d'une diminution de l'activité pullulanasique dans ces plantes. La situation s'inverse chez *Arabidopsis thaliana* où une mutation du gène de structure d'une isoamylase (mutation *dbe1*) provoque une augmentation de l'activité pullulanasique. Néanmoins, tous ces mutants produisent des quantités réduites d'amidon alors que des quantités non négligeables de polysaccharide soluble (phytoglycogène) s'accumulent. Le mutant *sta7* de *Chlamydomonas reinhardtii*, lui aussi déficient pour une activité de débranchement, contient également du phytoglycogène, mais contrairement aux autres végétaux, n'accumule plus d'amidon. La sévérité du phénotype de Chlamydomonas pourrait s'expliquer par l'absence de redondance fonctionnelle que laisse supposer la nature à dominante haploïde de cet organisme.

Afin de déterminer à quelle classe (isoamylase ou pullulanase) appartient l'enzyme absente chez le mutant au locus *STA7*, nous avons entrepris la purification des deux types d'activités de débranchement de l'algue et analysés leurs spécificités catalytiques. Nous avons pu mettre en évidence que le défaut de biosynthèse d'amidon observé dans le mutant *sta7* correspond à l'absence sélective de la sous-unité catalytique (dénommée CISI) d'un complexe enzymatique de haut poids moléculaire arborant une spécificité de type isoamylasique. La seconde activité de débranchement de l'algue est véhiculée par une protéine d'une masse de 95 kD (dénommé CLDI) dont l'activité enzymatique est de type pullulanasique. L'absence d'activité isoamylasique dans les mutants *sta7* de *Chlamydomonas reinhardtii* n'entraîne aucune modification de la pullulanase contrairement à ce qui se passe chez les autres végétaux. La production de phytoglycogène par le mutant *sta7* s'avère donc directement corrélée à la disparition de l'activité isoamylasique. L'article qui suit, publié dans la revue Plant Science, reprend en détails le développement de tous les arguments qui nous ont conduit à émettre ces conclusions.

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The debranching enzyme complex missing in glycogen accumulating mutants of *Chlamydomonas reinhardtii* displays an isoamylase-type specificity

David Dauvillée [a], Virginie Mestre [b], Christophe Colleoni [a], Marie-Christine Slomianny [a], Gregory Mouille [a], Brigitte Delrue [a], Christophe d'Hulst [a], Christophe Bliard [c], Jean-Marc Nuzillard [c], Steven Ball [a,*]

[a] *Laboratoire de Chimie Biologique, Unité Mixte de Recherche du C.N.R.S. no 8576, Bâtiment C9, Cité Scientifique, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France*

[b] *Roquette Frères, F62136 Lestrem, France*

[c] *Laboratoire de Pharmacognosie, ESA 6013 CNRS, CPCB Europol'Agro, bat 18, Moulin de la Housse, Université de Reims, BP 1039, 51097 Reims Cedex 2, France*

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Abstract

To investigate the functions of debranching enzymes in starch biosynthesis, we have partially purified and characterized these activities from wild type and mutant *sta7 Chlamydomonas reinhardtii*. Mutants of the *STA7* locus substitute synthesis of insoluble granular starch by that of small amounts of glycogen-like material. The mutants were previously shown to lack an 88 kDa debranching enzyme. Two distinct debranching activities were detected in wild-type strains. The 88 kDa debranching enzyme subunit missing in glycogen-producing mutants (CIS1) is shown to be part of a multimeric enzyme complex. A monomeric 95 kDa debranching enzyme (CLD1) cleaved α-1,6 linkages separated by as few as three glucose residues while the multimeric complex was unable to do so. Both enzymes were able to debranch amylopectin while the α-1,6 linkages of glycogen were completely debranched by the multimeric complex only. Therefore CLD1 and the multimeric debranching enzyme display respectively the limit-dextrinase (pullulanase) and isoamylase-type specificities. Various mutations in the *STA7* locus caused the loss of both CIS1 and of the multimeric isoamylase complex. In contrast to rice and maize mutants that accumulate phytoglycogen owing to mutation of an isoamylase-type DBE, isoamylase depletion in Chlamydomonas did not result in any qualitative or quantitative difference in pullulanase activity.

Keywords: Amylopectin; *Chlamydomonas reinhardtii*; Isoamylase; Pullulanase; Starch debranching enzyme

1. Introduction

Most archaebacteria, eubacteria and non-photosynthetic eukaryotes store glucose in the form of glycogen, an homogeneous water-soluble α-1,4 linked polysaccharide with 8–10% α-1,6 branches leading to a monomodal chain-length distribution (for review see [1]). Because of physical constraints, β glycogen granules cannot exceed 25 nm in diameter, a size-limit that was clearly overcome in the plant kingdom. The solution to the problem caused by the large amounts of glucose to be stored within the plant cell came with the substitution of glycogen by insoluble macrogranular starch synthesis [2,3].

In contrast to glycogen, starch is an heterogeneous structure composed of two distinct polysaccharide fractions [4]. Amylopectin, the major

Abbreviations: Am, Amylose; Ap, Amylopectin; DBE, Debranching enzyme; DP, Degree of polymerization.

* Corresponding author. Present address: Unité Mixte de Recherche du C.N.R.S. no 8576, Bâtiment C9, Cité Scientifique, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France. Fax: +33-320-845190.

E-mail address: steven.ball@univ-lille1.fr (S. Ball).

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fraction of starch is composed of large molecules (10^4–10^5 kDa) with 5% α-1,6 branches. Amylose is composed of smaller (10–10^2 kDa) molecules with less than 1% α-1,6 branches. Amylopectin is a complex and ordered polysaccharide displaying an asymmetrical pattern of branches and a trimodal chain-length distribution.

The reasons that plants are able to synthesize such large glucans are not yet fully understood although amylopectin crystallization was lately proposed to be generated by a glucan trimming pathway [5]. This suggestion followed the discovery in maize, rice and *Chlamydomonas reinhardtii* of debranching enzyme-deficient mutants substituting starch synthesis by that of glycogen-like polymers [6–9]. In algae, this substitution was complete. Since an 88 kDa debranching enzyme (6-glucanohydrolase) was absent in the *C. reinhardtii* mutants, it was concluded that polysaccharide debranching is mandatory to obtain significant amylopectin synthesis in plants [9].

The model proposes the existence of pre-amylopectin, a precursor whose surface is undergoing successive rounds of disorganized branching and ordered debranching [5]. Discontinuous branching and debranching cycles were first envisioned. Later refinements of this model propose that debranching enzyme merely assists the process of amylopectin crystallization by continuously cleaving off those chains which prevent proper alignment of the glucan chains. Selectivity of the debranching enzyme has been proposed to facilitate the pruning of those branched glucans that otherwise prevent polysaccharide crystallization [5].

Two types of debranching enzymes are known in plants. Limit-dextrinases (pullulanase-type) define debranching enzymes capable of cleaving pullulan, a bacterial homopolymer of maltotriosyl residues linked together by α-1,6 branches [10]. A limit dextrinase (EC 3.2.1.41) displays very slow and intermediate cleavage kinetics when acting respectively on glycogen and amylopectin [10–12].

Isoamylase-type of debranching enzymes (EC 3.2.1.68) were first reported in bacteria. These are incapable of cleaving pullulan while displaying full cleavage of both amylopectin and glycogen [12,13]. The *su1* locus of maize was shown to encode an isoamylase-type of debranching enzyme [7,14]. Despite this, the interpretation of phytoglycogen accumulation in the *su1* mutants is complicated by the observation of a simultaneous decrease of a limit-dextrinase activity not encoded by the *su1* gene [6]. Recent characterization of a rice *sugary-1* allelic series hints that in cereals the severity of the sugary phenotype is equally related to that of the decrease in limit-dextrinase activity [15]. The reason for this decrease is presently not understood.

In Arabidopsis a mutant accumulating both phytoglycogen and starch was recently shown to lack a plastidial isoamylase [16] with no concomitant decrease in limit-dextrinase. In our initial characterization of the *sta7* mutants of *C. reinhardtii* we reported the absence of an 88 kDa debranching enzyme (CIS1) in all *sta7* carrying strains. Proof of the 6-glucanohydrolase (debranching) activity of the missing hydrolase was obtained through NMR characterization of the dextrin products eluted from starch filled PAGE zymogram gels [9]. However the results described do not distinguish between isoamylase and limit-dextrinase-types of enzymes. In potato, maize and rice [13,17,18] the isoamylase was shown to be part of a large multimeric complex whose precise composition remains unknown while the pullulanase behaved as a monomer in maize and rice.

We now report a more detailed investigation on the debranching enzymes of *C. reinhardtii*. A major multimeric enzyme complex (500 ± 100 kDa) is shown to contain the CIS1 88 kDa DBE subunit. In addition one minor 95 kDa debranching enzyme (CLD1) was found in these algae. The multimeric and 95 kDa DBEs were shown to harbor respectively isoamylase and limit-dextrinase-type of activities. The limit-dextrinase activity had been previously demonstrated to be entirely located within the algal plastid [19]. We report the absence of both the multimeric and CIS1 isoamylase subunit debranching enzymes in glycogen producing mutants defective for the *STA7* gene. In contrast to rice and maize mutants that accumulate phytoglycogen owing to mutation of an isoamylase-type DBE, the minor single limit dextrinase of Chlamydomonas was unaffected by the presence of a *sta7* mutation.

2. Materials and methods

2.1. Material

Sigma Chemical Co. (St. Louis, MO) supplied the apoferritin and thyroglobulin mass standards,

rabbit liver glycogen, potato amylose, pullulan from *Aureobasidium pullulans* and maize amylopectin. Phosphorylase limit dextrin was prepared from glycogen [20]. *Pseudomonas amyloderamosa* isoamylase was from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). *Klebsiella pneumoniae* pullulanase was from Sigma Chemical Co. The catalase mass standard was from Boehringer (Mannheim,GmbH).

2.2. Strains, media, incubation and growth conditions

Our wild type (with respect to debranching enzyme activities) reference strains used in this work are IJ2 or A35 while strain S [9] was used as our reference *sta7* mutant strain. Cultures were always prepared from nitrogen supplied TAP medium supplemented with yeast extracts and harvested in late log-phase (2.10^6 cells ml^{-1}). Media and culture conditions used in our experiments were as described in [21]. Recipes for TAP and HSA media and genetic techniques can be found in [22,23]. All experiments were performed under continuous light (40 $\mu E\ m^{-2}\ s^{-1}$) in the presence of acetate at 24°C in liquid cultures that were shaken vigorously without CO_2 bubbling.

2.3. Enzyme purification

Crude extracts were prepared from 10 to 20 l of nitrogen-supplied cultures. Algae were ruptured by passing them in a French press (10 000 p.s.i) at a density of 10^8 cells ml^{-1} and immediately frozen at −80°C. Protein content was determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories GmbH, Munich). All purification steps were carried-out at 4°C. After thawing, the crude extract was subjected to a 10 000 × *g* centrifugation for 20 min. The pellet was discarded and the supernatant was cleared by protamine sulfate precipitation (50 μl of a 10% w/v protamine sulfate solution added per ml of extract). After 15-min incubation, the supernatant obtained was precipitated with 35% saturation of ammonium sulfate and centrifuged at 10 000 *g* for 20 min. The precipitate was resuspended in 2 ml of Bis-Tris propane-HCl 20 mM, DTT 20 mM pH 7 buffer (buffer A) and immediately loaded on a 1 cm diameter, 60 cm long FPLC Sephacryl S-300 HR (Pharmacia, Uppsala, Sweden) gel permeation chromatography column pre-equilibrated in the same buffer. The chromatography was performed at a flow rate of 1 ml min^{-1}. The same S-300 column was precalibrated by subjecting 5 mg of thyroglobulin (669 kDa), apoferritin (443 kDa) and catalase (240 kDa) to the same chromatographic procedure. The position of each peak fraction was used to measure the apparent size of large-size Chlamydomonas proteins. Fractions (1 ml) were immediately subjected to analysis in starch containing and pullulan azure-containing zymograms (see below). The same fractions (50 μl) were tested for production of reducing ends from amylose, glycogen and pullulan (see enzyme assays). The purified fractions could be stored frozen at −80°C for up to 2 weeks without significant activity losses. The S-300 fractions containing the 88 kDa blue-staining DBE activity [9] free of amylase activity (fractions 52–65) were pooled and loaded on a FPLC UnoQ1 (Bio-rad) column equilibrated in buffer A. Elution was obtained with a linear gradient from 0 to 50% NaCl in 50 min. Again, fractions were checked for contaminating activities by zymograms and immediately frozen at −80°C. The S-300 fractions (66–89) containing pullulanase and amylase activities were subjected to the same procedure. The fractions containing the 88-kDa debranching enzyme subunit (fractions 19 and 20 of the first UnoQ1) and the pullulanase (fractions 7 and 8 of the second UnoQ1) free of contaminating activities were immediately used for debranching analysis or kept at −80°C for subsequent analysis. The pullulanase could be stored with no loss of activity for over 6 months while significant (30%) decreases of the 88-kDa debranching enzyme subunit containing fractions was evidenced after 2 weeks at −80°C. The amount of enzyme activity harvested from 20 l cultures was sufficient to perform all characterizations reported below. However this amount was insufficient to repeat the experiments from a unique cell extract.

2.4. Enzyme assays

Isoamylase activity were assayed by incubating 50 μl of the enzyme preparation in Bis-Tris propane-HCl 20 mM, DTT 2 mM pH 7 buffer containing 5 mg ml^{-1} substrate (pullulan, amylopectin, glycogen, amylose) at 30°C in a final volume of 1 ml. Aliquots (100 μl) were taken and

the reaction was stopped by incubation at 100°C for 2 min. The activity was determined by measuring the increase in reducing power on the basis of the method of Nelson [24] and Somogyi [25] using maltotriose as a standard. The same procedure was used for pullulanase activity but incubation was at 35°C. Debranching analysis with commercial enzymes was monitored using the same procedure except that pullulanase was assayed in Sodium Acetate 55 mM pH 5 at 25°C and isoamylase in Sodium Acetate 55 mM pH 3.5 at 45°C. After stopping the reaction, samples were neutralized by NH_4OH before measuring the amount of reducing ends produced. Isoamylase and pullulanase activities were assayed in the presence of glycogen and pullulan respectively by the procedure described above but in the presence of various concentrations of Hydrogen peroxide.

2.5. Zymogram analysis

Zymograms in starch containing gels allowing the detection of most starch hydrolases and branching enzymes have been described for undenatured samples by Kakefuda and Duke [26] and for denatured enzymes by Mouille et al. [9].

To detect limit-dextrinase, 70 μl final volume (up to 400 μg of crude extract protein) of sample in purification buffer was loaded on a 29:1 (acrylamide:bisacrylamide) 10% (1.5 mm thick) polyacrylamide gel (mini-protean II cell (Bio-Rad)) containing 0.6% pullulan azure (Sigma Chem. Co.) ran at 20 V cm^{-1} for 90 min in 25 mM Tris glycine pH 8.3; 1 mM DTT. Gels were incubated for 1–12 h in the same buffer. The Chlamydomonas activity could also be monitored under denaturing conditions. 70 μl of extract in purification buffer was added to 15 μl of freshly prepared 10% SDS 50% β-mercaptoethanol and boiled in a water-bath for 5 min. The experimental conditions were as before except that 0.1% (w/v) SDS was included in the gel and the migration buffer. Electrophoresis was carried out at room temperature at 15 V cm^{-1} for 120 min. At the end of the run, the gel was washed four times with gentle shaking for 30 min in 100 ml of 40 mM Tris at room temperature to remove SDS and renaturate proteins. The gel was then incubated for 1–12 h in 25 mM Tris glycine pH 8.3 1 mM DTT. The zymogram was immediately photographed. The molecular mass of the enzyme activity detected on zymogram was measured on the same polyacrylamide gel. Some sections of the gel were stained with Coomassie Brilliant Blue while others were renaturated and incubated as described above.

2.6. NMR analysis

Nuclear magnetic resonance analysis was performed with the same set-up and conditions as those described in Fontaine et al. [27]. The level of branching was estimated by integration of the same regions of proton resonance of the monosubstituted and disubstituted glucose (δ, 5.2 and 4.9 ppm, respectively) [28]. Reducing end signals appeared in the dextrin samples. To estimate the percentage of reducing ends, we integrated the signals due to the α (5.1 ppm) and β (4.5 ppm) anomeric forms of the reducing ends with respect to same region of the monosubstituted glucose proton resonance.

3. Results

3.1. Wild-type strains of C. reinhardtii contain at least two kinds of debranching activities

Crude extracts from late log-phase algae (20 l) were subjected to protamine and ammonium sulfate precipitations, gel filtration on sepharose S-300 columns followed by UnoQ-mediated anion exchange. Since a limit-dextrinase activity had been reported previously by Levi and Gibbs [19], we followed pullulan degradation on pullulan azure containing gels. The latter always displayed a 95 kD limit dextrinase activity (shown in Fig. 8). Other zymograms performed in starch-containing gels subsequently stained with iodine allowed the detection of both an 88 kDa blue staining and a 53 kDa white staining band. These were previously shown to respectively contain debranching and α-amylase enzyme activities [9].

Results are summarized in Table 1 and Table 2 and Fig. 1. The zymogram data are not shown but are summarized by a schematic drawing at the bottom of Fig. 1. Position of size standards is highlighted (Fig. 1). It is clear that the limit-dextrinase (pullulanase) activity elutes with the bulk of the other starch hydrolases in a single wide peak (fractions 66–89) on the S-300 column precluding any significant enzymological characterization to

be made (Fig. 1). The activity containing the 88 kDa debranching enzyme (fractions 52–65) eluted as a large size complex whose apparent mass was estimated at 500 ± 100 kDa. The complex was purified 10-fold and can be considered free of other interfering activities with the exception of a small amount of trailing limit dextrinase that could be detected on pullulan azure containing gels but not on starch containing gels.

A second chromatographic step through the UnoQ column increased the purity of both activities (Table 1 and Table 2). Fig. 2A displays the level of purification of the 88 kDa debranching enzyme subunit that was achieved in these experiments. In zymograms performed without denaturation [24] we systematically observed three blue activity bands (Fig. 2B) co-eluting with the single 88 kDa band (CIS1) appearing after denaturation. It remains possible that some DBE subunits do not refold properly after denaturation and that several distinct DBE subunits are visualized under native conditions. Yields and purification factors were calculated for both the limit dextrinase and the CIS1-containing enzyme complex activities (see Section 2). A sum of 5.7% of the total limit-dextrinase activity was recovered after two-chromatographic steps. The activity was stable after freezing for up to 6 months. Yield and purification factors of 2.5% and 140-fold, respectively were measured for the 88 kDa debranching enzyme subunit-containing activity. This is probably an underestimate since the bulk of the activity measured in crude extracts is due to other hydrolases such as α-amylase. This debranching activity was lost within a few hours in buffers containing less than 1 mM DTT and could not be recovered even after prolonged incubation in up to 20 mM DTT. The enzyme proved to be very sensitive to oxidation.

The sensitivity of both enzymes towards hydrogen peroxide is compared in Fig. 3. Because of their distinctive elution patterns and behavior we could already conclude that the limit-dextrinase and the CIS1 DBE containing complex were distinct activities. The stable limit-dextrinase at best afforded for very faint bluish bands that could only be seen on starch-containing zymograms after purification and concentration but never in crude extracts.

Table 1
88 kDa Debranching enzyme purification

	Yield (%)	Pruification factor	Total activity[a]
Crude extact	100	0	322
Protamine sulfate	65	1.3	210
Ammonium sulfate	40	1.6	130
Gel permeation (fractions 52–65)	17	9.9	55
Anion exchange (fractions 19–20)	2.5	140	8.2

[a] Purification was from 4×10^{10} cells. Debranching activity corresponds to μmoles of maltotriose equivalents produced per hour from amylopectin for the whole fraction under study (see Section 2). The activity was measured without interference of other hydrolases after the gel permeation step. Debranching activity in crude extracts or after both precipitation steps is skewed by the presence of α amylase.

Table 2
95 kDa Limit dextrinase purification

	Yield (%)	Purification factor	Total activity[a]
Crude extract	100	0	14
Protamine sulfate	93	1.9	13
Ammonium sulfate	86	3.4	12
Gel permeation (fractions 66–89)	79	28	11
Anion exchange (fractions 7–8)	5.7	180	0.8

[a] Purification was from 4×10^{10} cells. Debranching activity corresponds to μmoles of maltotriose equivalents produced per hour from pullulan for the whole fraction under study (see Section 2).

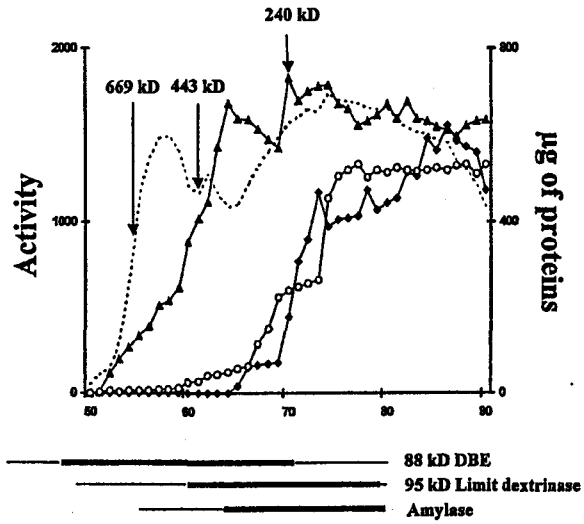

Fig. 1. Purification of Chlamydomonas debranching enzymes. A. 400 mg of crude protein was loaded on a S-300 HR (1 × 60 cm) gel filtration FPLC. Fractions of 1 ml were collected at a rate of 1 ml min^{-1}. Proteins were assayed using the Bio-Rad determination kit (broken thin line). We have drawn the chromatograph section containing the relevant DBE activities. The α-amylase activity peak tails off at fraction 110. Estimates of DBEs and α-amylase were obtained by assaying the reducing ends (nmoles maltotriose produced per hour and for the whole fraction studied) upon incubation of 20 µl of each fraction with pullulan (◆), glycogen (▲) and amylose (○) (see Section 2). Note that glycogen and amylose will generate reducing ends from both amylase and isoamylase activities. Therefore the beginning of the amylose digestion will not necessarily precisely coincide with the beginning of the α-amylase peak. Enzyme activities were localized by loading 50 µl of each fraction on both native and denaturing starch containing PAGE zymogram gels and on pullulan azure containing PAGE gels. The nature of the 88 kDa and 53 kDa activity bands activity bands had been previously ascertained by proton NMR of the eluted dextrins [9]. The nature of the pullulanase band has been ascertained by correlation to standard activity assays (such as production of reducing ends from pullulan) on the peak fractions. The position of each activity as revealed by zymograms is illustrated by lines whose thickness reflects the intensity of the zymogram bands. The 95 kDa limit dextrinase band intensity cannot be compared to the DBE, or amylase bands since it was revealed on pullulan-azure gels. The column was pre-calibrated with the 669, 443 and 240 kDa mass standards corresponding respectively to thyroglobulin, apoferritin and catalase. The arrows correspond to the peak fraction position for each of the proteins.

3.2. Characterization of the 88 kDa subunit containing debranching enzyme complex

We tested the activity of the 88 kDa subunit containing debranching enzyme complex towards four different substrates. These included pullulan, amylopectin, amylose and glycogen. The pooled peak fractions from the UnoQ1 anion exchange step were used for these studies (fractions 19 and 20 in Table 1). Results obtained with the debranching enzyme are displayed in Fig. 4A and can be compared to those obtained with the *Pseudomonas amyloderamosa* isoamylase (Fig. 4C) and the *Klebsiella pneumoniae* pullulanase (Fig. 4D). It is evident that because of both the limiting amounts of enzyme activity available from 20–40 L cultures and because of the sensitivity of the enzyme to oxidation we have obtained only 50–75% of the level of polysaccharide debranching obtained through commercial enzymes. To ensure that the Chlamydomonas enzyme was able to digest over 80% of the branches hydrolyzed by the commercial enzyme references, we have devoted a 40 l culture to the sole digestion of 5 mg of glycogen, pullulan and amylopectin in 12 h. The

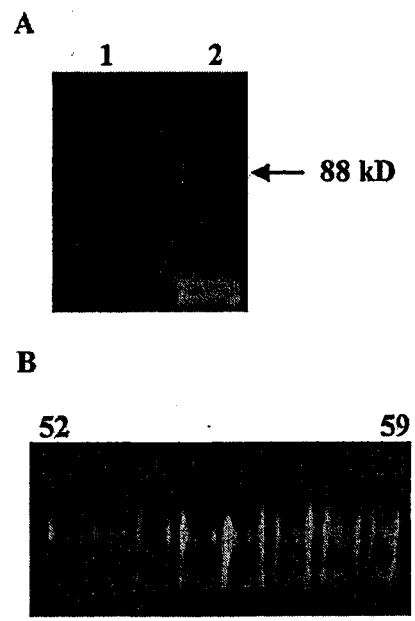

Fig. 2. Zymogram analysis of the semi-pure 88 kDa polypeptide-containing DBE complex. A. Denatured purified (fraction 19 of the anion exchange) (lane 1) and denatured crude extract (lane 2) were loaded on a starch-containing gel according to the procedure detailed in [9]. After renaturation, the gels are flooded with an iodine-containing buffer. Those enzymes that are able to modify the structure of starch within the gel will alter the iodine staining properties accordingly. B. The undenatured samples (80 µl) from fraction 52–59 of the S300 GPC separated by PAGE under native conditions were blotted onto starch-containing gels according to Kakefuda [26].

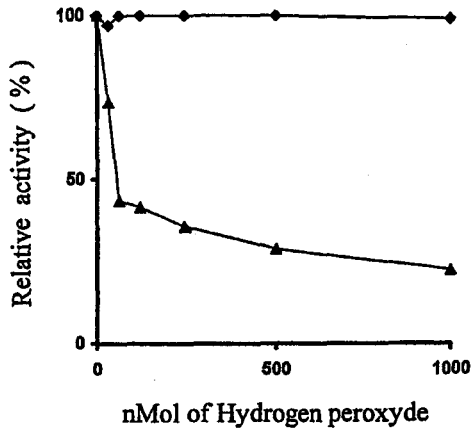

Fig. 3. Hydrogen peroxide inhibition of Chlamydomonas DBEs 5 mg of either pullulan or glycogen were digested with 50 μl (the activities increased proportionally when 10–950 μl peak fraction volumes were used) of fraction containing, respectively the 95 kDa limit dextrinase (◆, fraction 7 from the anion exchange (Table 2) and the semi-pure 88 kDa polypeptide-containing DBE complex (▲, fraction 20 from the anion-exchange (Table 1)) in a final volume of 1 ml. If the hydrogen peroxide concentration was raised to 2 mM the limit dextrinase and the semi-pure 88 kDa polypeptide-containing DBE complex displayed respectively 100 and 8% residual activity.

unincubated polysaccharides were compared to the debranched products through proton NMR analysis (Fig. 5) which directly demonstrated elimination of α-1,6 linkages. It is clear that the 88 kDa enzyme activity hydrolyses the branches of amylopectin to near completion (over 80%) whereas pullulan is not a substrate. Similar results were obtained with glycogen (data not shown). We therefore conclude that the CIS1 containing debranching enzyme complex displays an isoamylase-type of specificity. Glycogen phosphorylase limit dextrin was also tested because the *Escherichia coli* debranching enzyme purified by Jeanningros et al. [29] cleaves the α-1,6 branches of glycogen phosphorylase limit dextrin 100-fold faster than that of intact glycogen. However no significant differences were found in the initial rates of polysaccharide debranching when these substrates were compared (Fig. 6)

3.3. Characterization of the pullulanase

Fractions 7–8 of the UnoQ1 column, which were used for further characterizations, still contained 5.7% of the measurable crude extract activity and were purified 180-fold. We repeated the analysis performed on the CIS1 containing debranching enzyme complex (Fig. 4A) with fractions 7–8 containing the pullulanase activity (Fig. 4B) and compared this to the results obtained with the *P. amyloderamosa* isoamylase (Fig. 4C) and the *K. pneumoniae* pullulanase (Fig. 4D). It is clear that the Chlamydomonas enzyme can debranch pullulan (Fig. 4B). It also cleaved amylopectin (Fig. 4B), as expected for a plant limit-dextrinase. It is remarkable that both the Chlamydomonas limit-dextrinase and the Klebsiella pullulanase were able to cleave some of the branches of glycogen. However this digestion remained limited and glycogen was never debranched to completion. Cleavage of pullulan was obtained and yielded the appearance of numerous reducing ends detected by proton NMR (Fig. 7). Selective hydrolysis of the α-1,6 linkage was evidenced by a specific decrease of the α-1,6 proton NMR signals although the absolute level of pullulanase activity in the fractions used in this study proved insufficient to cleave 5 mg of pullulan, amylopectin or glycogen to completion within 12 h (Fig. 7). We further characterized the pullulanase from crude or purified extracts by performing zymograms in pullulan azure-containing gels. We found a perfect correlation during purification between the zymogram and pullulan debranching enzyme assays. We used both native or denatured extracts with subsequent PAGE respectively without or with SDS. The denatured proteins were allowed to renature before incubation. This denaturation-renaturation step induced a two to threefold reduction in enzyme activity. Under native conditions a unique major band was always observed after purification, while in crude extracts a very faint additional slower migrating smear was irregularly observed. Under denaturing conditions and upon renaturation a single sharp activity band was systematically observed. The high quality of these zymograms enables us to report a 95 ± 2 kDa mass for the *C. reinhardtii* pullulanase-type of debranching enzyme. This activity will be referred to as CLD1.

3.4. CLD1 is unaffected by the absence of a functional STA7 gene

The data shown here and our previous studies demonstrate that *STA7* is required for the pres-

ence of multisubunit isoamylase-type 6-glucanohydrolase enzyme complex. Because maize and rice mutants with such a mutation exhibit pleiotropic defects in the pullulanase-type of activity [6,8,17], we proceeded to characterize the debranching enzymes from all our *sta7* defective mutants. In addition to the seven previously described alleles that were generated by insertional mutagenesis we have selected a novel (*sta7-8*) allele through standard UV mutagenesis. The mutants displayed identical phenotypes and lacked both the CIS1 subunit detected through denaturing zymograms and the large size multimeric debranching enzyme complex as evidenced by zymograms from undenatured extracts blotted according to Kakefuda et al. [26]. Zymogram analysis performed on pullulan azure-containing PAGE gels failed to display any modification of CLD1. In addition the meiotic progeny of a cross involving strain S (containing the *sta7-4::ARG7* disruption) and a wild-type strain was subjected to zymogram analysis under native or denaturing conditions. No marked quantitative or qualitative difference could be detected between the $sta7^{-}$ and the $STA7^{+}$ segregants (Fig. 8). Moreover the faint slow-migrating smear of pullulanase activity was detected irregularly in both wild type and mutants. To ensure that no small quantitative activity modifications existed between wild type and mutant populations we confirmed the zymogram results by quantitative pullulanase assays. We measured pullulanase activities in a population of 10 wild-type (with an

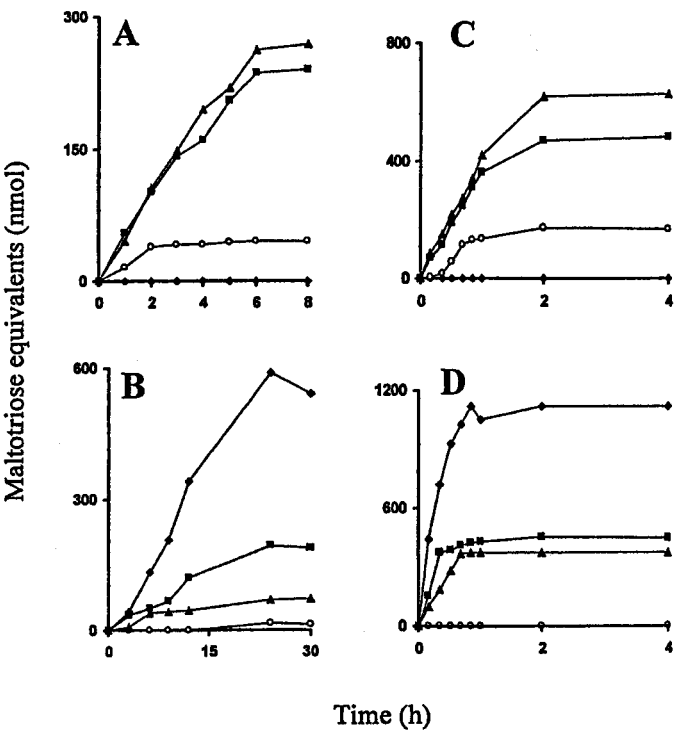

Fig. 4. Substrate specificity of debranching enzymes. A. 5 mg of substrate polysaccharide were digested with 50 μl of fraction containing the semi-pure 88 kDa polypeptide-containing DBE complex of Chlamydomonas (fraction 19–20 from the anion exchange (Table 1)) in a final volume of 1 ml. 50 μl of sample was subjected at various time to the reducing end assay as described in Section 2. Substrate polysaccharide include pullulan (◆), maize amylopectin (■), potato amylose (○) and rabbit liver glycogen (▲). B. Five miligrams of substrate polysaccharide was digested with 50 μl of fraction (the activity increased proportionally when 50–750 μl peak fraction volumes were used) containing the semi-pure 95 kDa Chlamydomonas limit-dextrinase (fractions 7–8 from the anion exchange (Table 2)) in 1 ml final volume. Fifty microlitres of sample was subjected at various times to the reducing end assay. Substrate polysaccharide symbols are as described above. C. Five miligrams of substrate polysaccharide was digested with 20 units of reference *Pseudomonas amyloderamosa* isoamylase in 1 ml final volume. Fifty microlitres of sample was subjected at various time to the reducing end assay as described in Section 2. D. Five miligrams of substrate polysaccharide was incubated with 0.3 units of reference *K. pneumoniae* pullulanase in a 1 ml final volume. Fifty microlitres of sample was subjected at various time to the reducing end assay as described in Section 2. Substrate polysaccharide symbols are as described above.

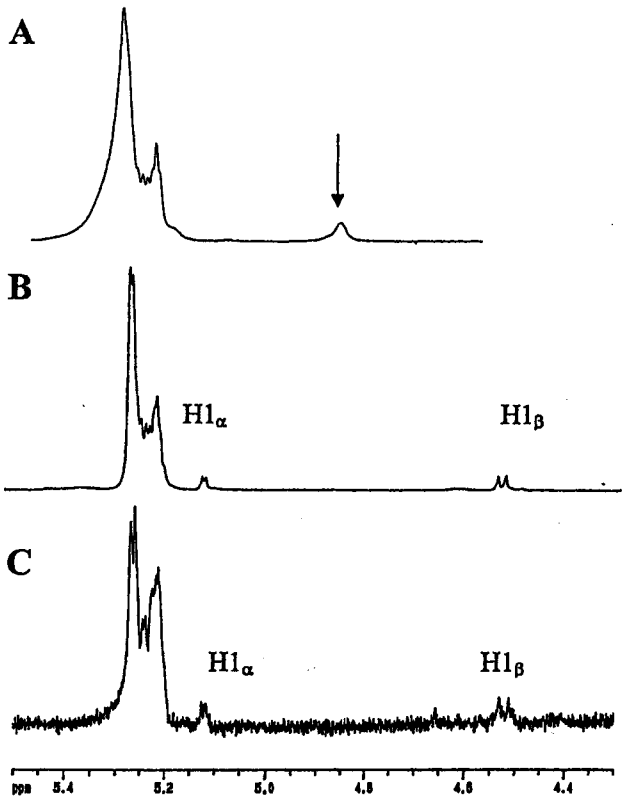

Fig. 5. Proton NMR spectra of amylopectin digested with the 88 kDa debranching enzyme. Part of the ^{1}H NMR spectra of amylopectin in dimethyl-sulfoxyde-δ6/D_2O (80:20) at 80°C is displayed. The chemical shifts for the α and β anomers of the reducing end are respectively at 5.1 and 4.5 ppm and are displayed as $H1_\alpha$ and $H1_\beta$. The arrow display the signal corresponding to the anomeric proton of carbons engaged in an α-1,6 linkage. Results obtained are analogous to those previously reported [9]. A. Undigested amylopectin; B. Amylopectin subjected to overnight digestion with 20 units of commercial Pseudomonas isoamylase; C. Amylopectin subjected to overnight digestion with 200 μl of the 88 kDa DBE from fraction 19–20 of the UnoQ as described in Section 2.

average and standard deviation respectively of 16.9 and 1.6 nmoles maltotriose equivalents produced per hour per mg protein) and 10 mutant (with an average and standard deviation, respectively of 15.9 and 1.1 nmoles maltotriose equivalents produced per hour per mg protein) segregants from the same cross in three series of experiments. No significant differences were found. To further prove the absence of subtle modifications of CLD1, we analyzed the behavior of this activity under our semi-purification conditions and found no significant modification. We therefore conclude that in *C. reinhardtii* the pullulanase activity is not significantly affected by the presence of a defect leading to phytoglycogen production.

4. Discussion

As was shown first in potato tubers [30], in the rice and maize endosperm [31,32] and more recently in pea embryos [12] and Arabidopsis leaves [16], at least two distinct types of starch debranching enzymes were found in *C. reinhardtii*. We provide evidence for the existence of a multimeric enzyme containing the CIS1 DBE subunit while the behavior of the CLD1 DBE is consistent with that of a monomeric protein. We show that the multimeric enzyme displays an isoamylase-type of cleavage specificity while the CLD1 minor activity displays the classical limit-dextrinase-type of activity. This situation is reminiscent of that initially reported for potato tubers [13] and more recently for maize and rice [17,18]. In the case of potato and rice the activity was purified to homogeneity and the protein composition of the complex analyzed in detail [13,18]. In potato two proteins of 95 and 83 kDa were separated from the complex but were not further characterized [13]. However the apparent mass of the isoamylase complex was estimated at 520 kDa. In rice one major and one minor protein was purified from the complex by

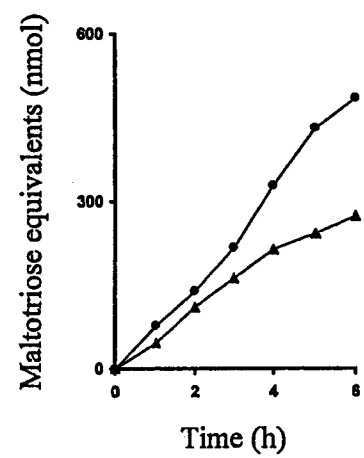

Fig. 6. Relative activities of the Chlamydomonas isoamylase with respect to rabbit-liver glycogen and to glycogen phosphorylase limit dextrin. Glycogen (5 mg ml^{-1}) (▲) and glycogen phosphorylase limit dextrin (●) were incubated with 50 μl of the 88 kDa glucanohydrolase from fraction 19–20 of the UnoQ as in Fig. 4A.

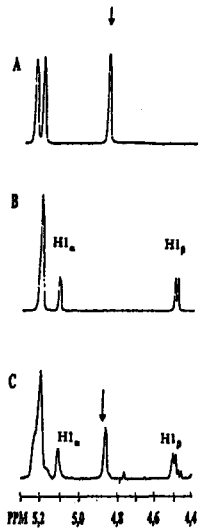

Fig. 7. Proton NMR spectra of pullulan digested partially or to completion with the Chlamydomonas 95-kDa glucanohydrolase and the *Klebsiella pneumoniae* pullulanase. Part of the ^1H NMR spectra of amylopectin in dimethyl-sulfoxyde-δ6/D_2O (80:20) at 80°C is displayed. The chemical shifts for the α and β anomers of the reducing end are respectively at 5.1 and 4.5 ppm and are displayed as $H1_\alpha$ and $H1_\beta$. The α-1,6 linkage anomeric proton is displayed by an arrow at 4.85 PPM. The integration of the α and β anomers reducing end signals matched closely the decrease recorded in the 4.85 ppm signal area. The origin of the small signals around 4.78 ppm and 4.47 ppm is not known and could reflect specific partially digested products. A. Undigested pullulan. B. Pullulan digested to completion with the *Klebsiella pneumoniae* pullulanase (0.3 units in 2 ml containing 10 mg pullulan) as described in Section 2. C. Pullulan subjected to partial (12 h) digestion with the 95-kDa Chlamydomonas glucanohydrolase as described in materials and methods.

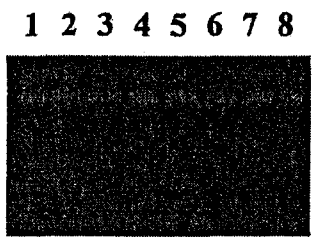

Fig. 8. Limit-dextrinase activity in wild-type and mutant *sta7* offspring. 100 μg of crude extract were loaded in a pullulan azure-containing PAGE gel and subjected to electrophoresis in native conditions as described in Section 2. The smear of activity on top of the gel results from substrate degradation during migration. Lanes 1, 3, 5 and 7 and lanes 2, 4, 6 and 8 display the activities respectively from 4 wild-type and mutant recombinants from a cross between a wild-type (strain A35) and a mutant strain (strain S containing the *sta7-4::ARG7* gene disruption) lacking the 88 kDa isoamylase.

2D-PAGE. Both proteins displayed the same N-terminal amino acid sequences and similar peptide maps upon digestion with *Staphylococcus aureus* V8 protease. The apparent mass of the rice complex was estimated between 340 to 490 kDa depending on the GPC column used. Both the values published for potato or those estimated for rice are within the size-range we now report for Chlamydomonas. The pure rice isoamylase complex systematically yielded three to four blue bands upon analysis in starch containing zymogram gels [18]. We have observed the very same behavior for the algal isoamylase complex although the enzyme was not pure enough to investigate precisely the subunit composition. Also shared by other vascular plant enzymes is the exquisite sensitivity of the isoamylases to oxidation. This sensitivity could explain the special behavior and the multiple activity bands seen in zymogram gels. On the other hand the redox state of the isoamylase might be of functional relevance. Indeed Fu et al. [33] have recently demonstrated that potato tuber ADP-glucose pyrophosphorylase can be activated by thioredoxin thereby increasing the sensitivity of the enzyme to 3-PGA activation. Such a regulation could be at work at least in the chloroplast of leaf cells and in the Chlamydomonas plastid. We have been however unable to recover activity after oxidation of the isoamylase complicating thus future investigations on this issue. Of particular significance in this respect is the recent finding that transgenic barley expressing potato thioredoxin have increased levels of limit dextrinase activity [34].

It also remains possible that in rice and other plants the multimeric enzyme is composed of distinct yet related isoamylase subunits. As was demonstrated for plant ADP-glucose pyrophosphorylases, subunits with related primary sequences can take over a predominantly catalytic or regulatory function. In maize the *su-1* gene product is able to function on its own since activity was successfully recovered upon expression in the *E. coli* cell [14].

The work reported here establishes that the 88 kDa 6-glucanohydrolase (CIS1) lacking in the *sta7* mutants of Chlamydomonas is part of a multimeric enzyme belonging to the isoamylase-type class of activity. If the *STA7* gene codes for a protein within this complex then the latter would have to be a component essential for catalysis

since all of the zymogram bands disappear in the presence of a *sta7* mutation. All *sta7* mutants have replaced starch synthesis by that of a small amount of glycogen-like material [9]. This defines debranching through isoamylase as a mandatory step of starch biosynthesis. The picture that is now emerging from the study of the glycogen-producing mutants of plants is that they all lack a particular form of isoamylase-type of debranching enzyme. However the interpretation of the phenotype recorded in cereals is further complicated by the observation of a concomitant decrease of limit-dextrinase activity. Recent evidence gathered from the analysis of *sugary-1* mutants of rice suggest that the limit-dextrinase might play an important role in the expressivity of the sugary phenotype [15]. In *C. reinhardtii* our data suggest the presence of a single pullulanase isoform with a mass similar to those reported for the vascular plant enzymes [8,17]. The insensitivity of this activity to the presence of a mutated *STA7* locus distinguishes *C. reinhardtii* from both maize and rice. Zeeman et al. [16] also failed to detect decreases of limit-dextrinase activity in the isoamylase-defective mutants of Arabidopsis. In vascular plant mutants, the appearance of phytoglycogen is not accompanied by the disappearance of starch but rather by its quantitative decrease. It is interesting to note however that from the zymograms published by Zeeman et al. [16] the levels of limit-dextrinase assayed with starch or amylopectin are comparable to those of the Arabidopsis isoamylase while in Chlamydomonas they differ nearly by two orders of magnitude. This major quantitative difference in the balance between isoamylase and limit-dextrinase in the two organisms could be related to the severity of the phenotype recorded in algae. If we assume that debranching enzymes are required to trim pre-amylopectin [5], it is very possible that in this respect some functional overlap exists between isoamylases and limit-dextrinases. Limit-dextrinase, if particularly abundant, might allow for a low rate of amylopectin crystallization in the absence of isoamylase. It is particularly striking to note that in Arabidopsis the already abundant limit-dextrinase is further enhanced by the presence of an isoamylase defect [16]. In addition such a functional overlap offers a logical explanation for the influence of the residual pullulanase activity in the expressivity of the rice sugary phenotype [15]. It would thus be of interest to increase the amount of the Chlamydomonas limit-dextrinase by, for instance, changing some of the physiological growth conditions. This might in turn lead to the limited synthesis of semi-crystalline starch. Another option would be to identify mutants lacking limit-dextrinase activity in plants such as maize or Arabidopsis where specific structural gene mutations can be identified by methods of transposon insertion screening.

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CHAPITRE III :

Two loci control phytoglycogen production in the monocellular alga *Chlamydomonas reinhardtii*

Dauvillée D, Colleoni C, Mouille G, Buléon A, Gallant D, Bouchet B, Morell M, D'Hulst C, Myers AM, Ball S.

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Avant-propos

Une nouvelle souche mutante de *Chlamydomonas reinhardtii* accumulant du phytoglycogène a été isolée suite à une mutagenèse par insertion aléatoire de plasmide dans le génome nucléaire de l'algue. Le phénotype de ce nouveau mutant dénommé « *sta8* » se rapproche fortement de celui des mutants *sugary* du maïs et du riz. Cette souche mutante accumule environ 20% de la quantité d'amidon normalement observée ainsi que du phytoglycogène. Ceci va à l'opposé des mutants au locus *STA7* dont le phénotype beaucoup plus marqué conduit à l'absence d'accumulation d'amidon granulaire et à la production de phytoglycogène, corrélées à la totale disparition de l'isoamylase (voir les chapitres précédents). La mise au point d'un dosage spécifique de l'isoamylase révèle une diminution de 65% de l'activité de cette enzyme dans l'ensemble des souches mutées au locus *STA8*. C'est la première fois que l'on observe dans un même organisme, l'impact de deux loci différents sur le contrôle de l'activité de cette enzyme. Les études génétiques et biochimiques intensives réalisées sur ces souches mutantes ne montrent pas d'autre déficience pour des enzymes du métabolisme de l'amidon.

Il nous fallait alors comprendre pourquoi les deux mutations provoquant l'accumulation de phytoglycogène n'avaient pas la même influence sur la production de l'amidon. Pourquoi n'y a t-il plus d'amidon granulaire dans les mutants *sta7* alors que les mutants *sta8* en accumulent encore 20% ? Pour tenter de répondre à cette question il nous fallait nous placer dans des conditions similaires dans les deux cas, c'est à dire en présence d'une activité isoamylasique résiduelle d'environ 35% (comme chez les mutants *sta8*). Pour ce faire, nous avons engendré par croisement une souche triploïde contenant deux allèles mutés *sta7* et un allèle sauvage *STA7*. Ce triploïde contient le même taux d'activité spécifique que la souche haploïde mutante *sta8* (c'est à dire seulement un tiers de celle du triploïde homozygote sauvage correspondant). Or, celui-ci accumule des quantités normales d'amidon sans apparition de phytoglycogène. Le facteur limitant la formation d'amidon cristallin dans les souches *sta8* ne concerne donc pas la diminution d'activité isoamylasique mais un autre élément dont la nature reste à déterminer.

Two Loci Control Phytoglycogen Production in the Monocellular Green Alga *Chlamydomonas reinhardtii*

David Dauvillée, Christophe Colleoni, Gregory Mouille, Alain Buléon, Daniel Gallant, Brigitte Bouchet, Matthew Morell, Christophe d'Hulst, Alan M. Myers and Steven G. Ball*.

Laboratoire de Chimie Biologique, Unité Mixte de Recherche du C.N.R.S. n°8576, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex France (D. D., C. C., G. M., C. d. H., S. G. B.) Institut National de la Recherche Agronomique, Centre de Recherches Agroalimentaires, Rue de la Géraudière, B.P. 71627, 44316 Nantes Cedex 03, France (A. B., D. G., B. B.) CSIRO, Division of Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia (M. M.)Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa 50011 (A. M. M.)

ABSTRACT

The *STA8* locus of *Chlamydomonas reinhardtii* was identified in a genetic screen as a factor that controls starch biosynthesis. Mutations of *STA8* cause a significant reduction in the amount of granular starch produced during nutrient limitation and accumulate phytoglycogen. The granules remaining in *sta8* mutants are misshapen, and the abundance of amylose and long chains in amylopectin is altered. Mutations of the *STA7* locus, which completely lack isoamylase activity, also cause accumulation of phytoglycogen, although *sta8* and *sta7* mutants differ in that there is a complete loss of granular starch in the latter. This is the first instance in which mutations of two different genetic elements in one plant species have been shown to cause phytoglycogen accumulation. An analytical procedure allowing assay of isoamylase in total extracts was developed and used to show that *sta8* mutations cause a 65% reduction in the level of this activity. All other enzymes known to be involved in starch biosynthesis were shown to be unaffected in *sta8* mutants. Approximately the same amount of total isoamylase activity as that present in *sta8* mutants was observed in heterozygous triploids containing two *sta7* mutant alleles and one wild type allele. This strain, however, accumulates normal levels of starch granules and lacks phytoglycogen. The total level of isoamylase activity, therefore, is not the major determinant of whether granule production is reduced and phytoglycogen accumulates. Instead, a qualitative property of the isoamylase that is affected by the *sta8* mutation is likely to be the critical factor in phytoglycogen production.

Key words : *Chlamydomonas reinhardtii* - starch debranching enzyme - isoamylase - pullulanase - amylopectin.

INTRODUCTION

Plant mutants defective in α-1,6 glucanohydrolase (starch debranching enzyme) substitute the synthesis of insoluble granular starch with that of small size hydrosoluble glycogen-like particles. Because only plant cells accumulate semi-crystalline α-1,4-linked α-1,6 branched glucans in the form of large insoluble granules, it is presumed that starch debranching enzyme may constitute one of, if not the major, biochemical step distinguishing plant starch metabolism from that of bacterial or animal glycogen. Two types of starch debranching enzymes have been detected in plants (for reviews see Manners, 1997 and Myers et al., 2000). Limit-dextrinase, also known as plant pullulanase can digest pullulan, a bacterial polysaccharide made of a regular succession of maltotriose chains linked together by α-1,6 linkages at the ends of each maltotriosyl residue. Plant isoamylases on the other hand cannot debranch such branches and thus will not hydrolyze pullulan. However, only isoamylases will rapidly debranch glycogen while both types of enzymes will readily debranch amylopectin to completion. Both families of debranching enzyme can also be clearly distinguished at the level of their protein sequences (Myers et al., 2000). Limit-dextrinase and isoamylases are present at the time of starch biosynthesis in all tissues and organs analyzed so far and could therefore both be involved in starch biosynthesis. Phytoglycogen producing mutants have been reported in maize (Correns, 1901), rice (Matuo et al., 1987), sorghum (Watson and Hirata, 1960), Arabidopsis (Zeeman et al., 1998) and the unicellular green alga *Chlamydomonas reinhardtii* (Mouille et al., 1996). In vascular plants most mutants accumulate both semi-crystalline insoluble granules and phytoglycogen while in Chlamydomonas the substitution of starch by phytoglycogen was complete. In all cases the mutants have been shown to lack an isoamylase. In addition the defective gene was shown to encode an isoamylase type of debranching enzyme (James et al. 1995, Zeeman et al., 1998, Kubo et al., 1999). In rice and maize the mutant endosperm was shown to simultaneously display a reduction in pullulanase activity while in Arabidopsis and Chlamydomonas no such decrease was ever recorded (James et al. 1995, Mouille et al., 1996, Zeeman et al., 1998, Kubo et al., 1999, Dauvillée et al., 2000). The reasons for these differences are presently unclear. In the case of cereals the resulting phenotype must thus be analyzed in the light of the absence of isoamylase and of the reduction in pullulanase. The disappearance of starch in the *sta7* phytoglycogen producing mutants of Chlamydomonas lead us to propose that polysaccharide debranching (pre-amylopectin trimming) was mandatory to obtain amylopectin synthesis in plants (Ball et al., 1996). We and others have explained this by assuming that starch debranching enzymes selectively hydrolyze those branches that prevent proper alignment and crystallisation of the polysaccharide (Ball et al., 1996; Myers et al., 2000). We also proposed that more functions may be involved in the trimming pathway and have recently shown that malto-oligosaccharide metabolism must be functional to insure proper processing of those chains released by debranching enzymes during amylopectin maturation (Mouille et al., 1996, Colleoni et al., 1999a, Colleoni et al., 1999b).

In all plants analyzed in sufficient detail it was shown that the isoamylase consists of a high mass enzyme complex (Ishizaki et al., 1983, Beatty et al., 1999, Fujita et al., 1999, Dauvillée et al., 2000). We now report a novel locus (*STA8*) which when defective leads to the simultaneous

production of both high amylose starch and phytoglycogen. We show that despite the maintenance of the 88 kD debranching enzyme subunit the total isoamylase activity has decreased by 65 % in the mutant strains. We demonstrate that it is not the quantitative change *per se* but the modification in enzyme specificity or structure which is responsible for the defect in amylopectin biosynthesis.

MATERIALS AND METHODS

Materials.

Pseudomonas amyloderamosa isoamylase was from Hayashibara Biochemical laboratories, Inc. (Okayama, Japan). Rabbit or bovine liver glycogen and maize amylopectin were from Sigma (St Louis, Missouri, USA). Boehringer (Mannheim, GmbH) provided Glc-1-P, yeast hexokinase, yeast Glc-6-P dehydrogenase and rabbit muscle phosphorylase.

***Chlamydomonas reinhardtii* strains, insertional mutagenesis, Growth conditions, Cytological Observations, and Media.**

The mutant strains BafV13 and BafO6 were obtained by transformation of the cell wall deficient arginine requiring strain 330 (*mt*$^+$ *arg7 cw15 nit1 nit2*) with 1 µg of pARG7.8 carrying the wild-type argininosuccinate lyase gene. Transformants were selected by complementation of the arginine auxotrophy and screened by spraying iodine on replica plates (Maddelein et al., 1994). All putative mutant strains were subjected to routine complementation tests.

The reference strains used in this study were 330, Baf V13 (*mt*$^+$ *cw15 nit1 nit2 sta8-1::ARG7*), BAFO6 (*mt*$^+$ *cw15 nit1 nit2 sta8-2::ARG7*) and GM7.27 (*mt*$^-$ *pab2 sta7-1::ARG7*). BGM strains from a cross performed between BafV13 and GM7.27 were used throughout this work. SJ and SN strains were used to construct the diploid and triploid strains used for gene dosage experiments and were selected from a cross between the wild-type strain A35 (*mt*$^-$ *ac14 pab2*) and the mutant strain S (*mt*$^+$ *sta7-4::ARG7 nit1 nit2*).

All experiments were carried out in continuous light (40µE m^{-2} sec^{-1}) in the presence of acetate at 24°C in liquid cultures that were shaken without air or CO_2 bubbling. Late log phase cultures were inoculated at 10^5 cells mL^{-1} and harvested at 2.10^6 cells.mL^{-1}. Nitrogen starved cultures were inoculated at 5.10^5 cells.mL^{-1} and harvested after 5 days at a final density of 1 to 2.10^6 cells mL^{-1}. Recipes for media and genetic techniques can be found in Harris (1989a and 1989b). Fixation and embedding protocols were as described in Dauvillée et al. (1999).

Structural Analysis of polysaccharides.

A full account of amyloglucosidase assays, starch purification on Percoll gradients, and, λmax, the wavelength of the maximal absorbance of the iodine-polysaccharide complex, can be found in Delrue et al. (1992). Amylopectin and amylose were separated through a CL2B gel permeation chromatography (Pharmacia) equilibrated in 10 mM sodium hydroxyde as described in Delrue et al. (1992). Phytoglycogen and oligosaccharides found in the mutant strains were separated on a TSK-HW-50 GPC column (Merck, Darmstadt, Germany) eluted in 10% DMSO as described by Maddelein et al. (1994). The water-soluble polysaccharide fraction purification can be found in Dauvillée et al. (1999).

Gel permeation chromatography purified amylopectin and phytoglycogen

were debranched by *Pseudomonas amyloderamosa* isoamylase. The APTS-tagged chains produced by isoamylase-mediated debranching and the APTS-tagged oligosaccharide fraction were separated by capillary electrophoresis carried out as previously described (O'Shea et al., 1998). Wide-angle x-ray diffraction and Transmission electron microscopy studies were as detailed in Buléon et al. (1997).

Crude extracts preparation, enzyme assays, and zymograms

Soluble crude extracts were always prepared from late-log phase cells (2.10^6 cells mL^{-1}) grown in high-salt acetate medium under continuous light (40µE m^{-2} sec^{-1}). All assays were conducted in conditions of linearity with respect to time and amount of crude extract. Phosphoglucomutase, ADP-Glc pyrophosphorylase, and phosphorylase activities were monitored by using the standard assays described in Ball et al. (1991) and Van den Koornhuyse et al. (1996). The SS and branching enzymes assays were those described by Fontaine et al. (1993) and Libessart et al. (1995). GBSSI was monitored as previously described in Delrue et al. (1992) or Van den Koornhuyse et al. (1996), from the starch purified from nitrogen-supplied cultures. The α-Glucosidase and D-enzyme activities were monitored by measuring the Glc produced from maltose or maltotriose respectively as detailed in Colleoni et al. (1999a). The analysis was completed by zymograms as detailed in Buléon et al. (1997) and Mouille et al. (1996).

Amylase and isoamylase activities were monitored by measuring the appearance of reducing ends from glycogen or amylopectin as follows. Amounts of crude extract corresponding to 20 to 200µg of total protein buffered in 2 mM DTT, 20 mM Tris, pH7, containing glycogen or amylopectin at 5 $mg.mL^{-1}$ in a one mL final volume were incubated from 10 to 120 min at 30°C in the presence of 1 mM of hydrogen peroxyde (amylase assay, see below) or 10 mM EDTA (isoamylase assay). Hydrogen peroxyde inhibition was achieved by incubating the extract for 15 min in 1mM H_2O_2 followed by extensive dialysis. Reducing ends produced during incubation were monitored by using the standard DNS assay or the Nelson (1944) and Somogyi (1952) method using maltotriose as a standard as described in Dauvillée et al. (2000).

Chlamydomonas DNA purification, Southern blot analysis.

Algal DNA was purified as described by Rochaix et al. (1991). Southern blot analysis was performed using 10 µg of PstI-digested algal DNA using an NruI/SalI 321-base pair fragment covering a small part of the bacterial tetracycline resistance gene contained in the plasmid pARG7.8 used for mutagenesis.

Gene dosage experiments

Diploid and triploid strains were constructed as follows. To obtain the homozygous mutant, we crossed SJ6 (*mt⁻ ac14 nit1 nit2 sta7-4::ARG7*) and SJ16 (*mt⁺ pab2 nit1 nit2 sta7-4::ARG7*) and selected the diploid after 4 days of growth on minimal medium supplied with ammonium. The wild-type homozygous diploid was obtained by crossing the strain SN25 (*mt⁺ pab2 nit1 nit2*) with A35 (*mt⁻ ac14 pab2*) and selecting diploids on minimum medium supplemented with paraminobenzoic acid. The heterozygous diploid were selected on the same medium after crossing the strains SJ23 (*mt⁺ pab2 nit1 nit2 sta7-4::ARG7*) and A35. Vegetative diploid strains heterozygous for mating type display an *mt⁻* mating type. After checking the phenotype, cellular volume, protein content and mating type, we crossed the heterozygous diploid with the strain S (*mt⁺ nit1 nit2*) to obtain the *sta7-4/sta7-4/+* triploid selected on

minimal medium. After selection, on the appropriate medium, the haploid, diploid, and triploid nature of the clones was confirmed by retesting the phenotypes and measuring both the average cell volume distribution and the cell protein content from unsynchronised cultures. For each construct, we selected three independent clones. Gene dosages are thus averages from three separate colonies for each construct. Phosphoglucomutase activity was assayed (Ball et al., 1991) and used as an internal standard during these experiments. Isoamylase assays with crude extracts obtained from the different genotypes were performed as described above.

RESULTS

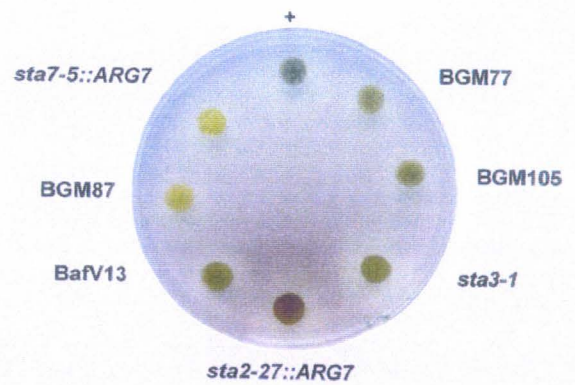


Figure 1 : Wild-type and mutant iodine-staining phenotype.

Iodine stain of cell patches incubated for 7 d on solid nitrogen-deprived medium. Genotypes with respect to starch are indicated for our reference strains. sta2-27::ARG7, sta3-1, and sta7-5::ARG7 correspond to defects respectively in GBSS, SS and debranching enzyme (Delrue et al., 1992 ; Fontaine et al., 1993 ; Mouille et al., 1996). + stands for wild-type. The original mutant strain Bafv13 (sta8-1) and two recombinants, BGM77 and BGM105, display the dark green stain of sta8-1 mutants, while the BGM87 strain shows a typical yellow stain of low-starch mutants and were proved by complementation test to carry both mutations sta7 and sta8.

STA8 defines a novel Chlamydomonas locus required for normal starch synthesis.

5.10^4 transformants were selected through complementation of the *arg7* mutation by the wild-type argininosuccinate lyase gene and screened individually through our standard iodine staining procedure (Fig. 1). Among 16 mutants defective for various aspects of starch biosynthesis and structure, two strains complemented all previously characterized mutant loci during trans complementation tests performed in vegetative diploid strains. The two mutants (BAFV13 and BAFO6) displayed similar phenotypes. They accumulated between 15 to 30% of the normal starch amounts during starch storage (Table I).

Table I : ***Phenotype of wild-type and mutant strains during storage (-N) or transitory starch (+ N) synthesis***

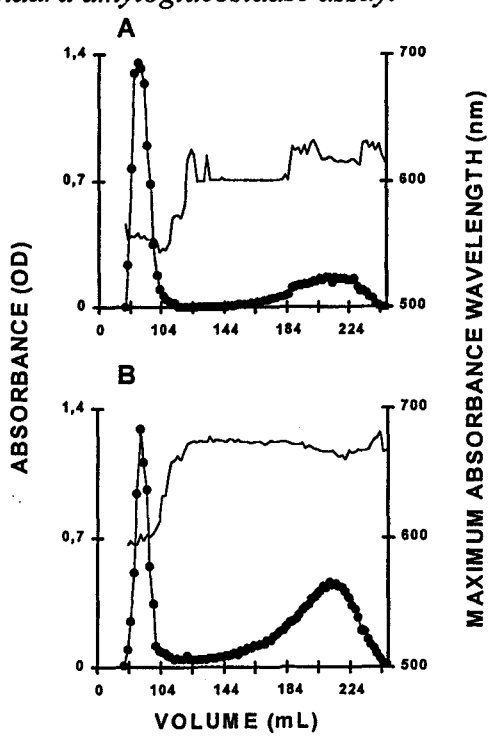
Values listed are averages of three separate measures in a single experiment

Strain	Genotype	λmax[a]		Starch[b]		WSP[c]		Am %[d]	
		+N	-N	+N	-N	+N	-N	+N	-N
BGM13	+	569	571	1.71	30	0.006	0.02	3	13
BGM34	+	565	570	1.60	26	0.003	0.07	4	16
330	+	570	565	0.80	29	0.010	0.10	1	14
BafV13	*sta8-1*	573	591	0.93	7	0.018	1.05	3	46
BafO6	*sta8-2*	571	595	1.11	9	0.025	1.20	6	53
BGM77	*sta8-1*	-	600	1.20	11	0.028	0.81	-	42

[a] : Wavelength of maximal absorbance of the iodine-polysaccharide complex of amylopectin purified by gel filtration.
[b] : Amount of insoluble polysaccharides, expressed in $\mu g.10^{-6}$ cells, purified through sedimentation as measured by the standard amyloglucosidase assay.
[c] : Amount of water-soluble polysaccharides expressed in $\mu g.10^{-6}$ cells.
[d] : The percentage of amylose in the purified starch was calculated after gel filtration of the dispersed polysaccharides.

The residual starch contained between 40 to 60 % amylose while the wild-type controls were characterized by amylose contents ranging between 12 to 30% (Table I and Fig. 2).

Figure 2 : Separation of amylopectin and amylose by CL2B-sepharose chromatography.
The optical density (•) was measured for each 2-mL fraction at λmax (unbroken thin line). The sample was loaded on the same column setup described by Delrue et al. (1992). Starches from the wild-type strain 330 (A) and the mutant strain Bafv13 (B) were extracted from nitrogen-deprived cultures. Quantification of amylose and amylopectin ratios (see Table 1) was obtained by pooling amylopectin and amylose fractions separately and measuring the amount of Glc through the standard amyloglucosidase assay.



Expressivity of the mutant phenotype was slightly reduced in nitrogen supplied cultures. Both mutations were selected during distinct transformation experiments and therefore define two different mutant alleles. Southern analysis performed with a probe representing part of the bacterial sequences used in the transformation experiments demonstrate different hybridization patterns characterizing the two *sta8* alleles.In addition the integration of pARG7 proved to involve modifications at multiple sites while only part of the Southern profile co-segregated with the mutant *sta8-1::ARG7* allele (Fig. 3). BAFV13 was subjected to extensive genetic analysis and *sta8-1::ARG7* proved to segregate as a single mendelian defect. As expected the *sta8-1::ARG7* and *sta8-2::ARG7* mutations were recessive in heterozygous diploids and did not complement with each other but complemented the isoamylase defective *sta7* mutations. We further observed that *STA8* segregated independently from *STA7* after meiosis. We have therefore defined a novel Chlamydomonas locus (*STA8*) required for normal starch biosynthesis. Strains BAFV13 and BAFO6 defined respectively the *sta8-1::ARG7* and *sta8-2::ARG7* mutant alleles.

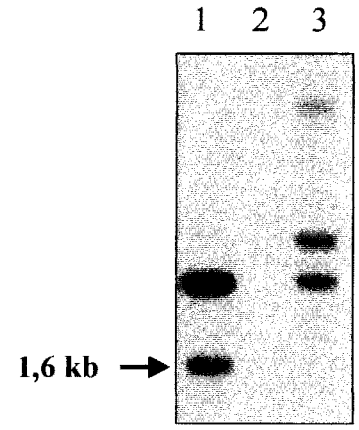


Figure 3 : Southern blot analysis
The genomic DNAs from reference strains were digested by PstI and separated by electrophoresis. The hybridization patterns were obtained with a 323 pb probe corresponding to the bacterial part of the pARG7 plasmid that was used for mutagenesis.The original sta8-1::ARG7 mutant strain (Bafv13) profile is displayed in lane 1. The wild-type strain profile 330 used for the insertional mutagenesis (lane 2) shows no signal. Strain BafO6 (sta8-2::ARG7) profile is displayed in lane 3.

Mutants of the *STA8* locus accumulate both high amylose starch and phytoglycogen.

In addition to high amylose starch we detected the accumulation of 0.5 to 2 $\mu g.10^{-6}$ cells of WSP. This material proved to contain both high and low mass glucans as evidenced by gel permeation chromatography of the purified WSP (Fig. 4).

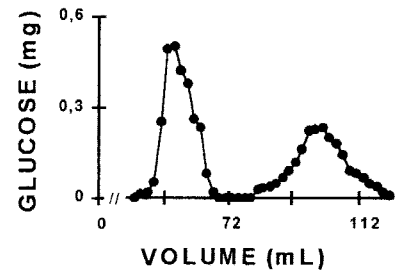


Figure 4 : TSK-HW-50 gel permeation chromatography of *sta8* WSP.
The amount of Glc (in micrograms per milliliter •) measured for each fraction by the amyloglucosidase assay is indicated on the left y axis. The x axis shows the elution volume (in milliliters).

The size distribution of the oligosaccharide fraction is displayed in Fig. 5A while the chain-length distribution of the debranched polysaccharide (Fig. 5B) is compared to those of amylopectin (Fig. 5E), glycogen (Fig. 5D) and phytoglycogen extracted from the isoamylase deficient mutants of Chlamydomonas (Fig. 5C). The degree of branching was ascertained by quantifying the amount of reducing ends generated after debranching the polysaccharide fractions. The high mass WSP contained 8.4 ± 0.3 % while branching ratios of 9 ± 0.5 %, 8.3 ± 0.2 % and 5 ± 0.3 % were measured respectively for bovine liver glycogen, phytoglycogen from the isoamylase deficient mutants of Chlamydomonas and amylopectin. In addition proton NMR was performed on the high mass WSP fractions and compared to the spectra of glycogen and amylopectin. The spectra produced were identical to those previously displayed for phytoglycogen (Mouille et al., 1996).It is clear from all these results that the *sta8* mutants accumulate glycogen-like polymers (phytoglycogen). We performed TEM analysis of wild-type and mutant cells after staining of the polysaccharides with PATAg. The glycogen granules located within the plastid were undistinguishable from those produced in the previously reported isoamylase-deficient (*sta7*) mutants of Chlamydomonas (Dauvillée et al., 1999).

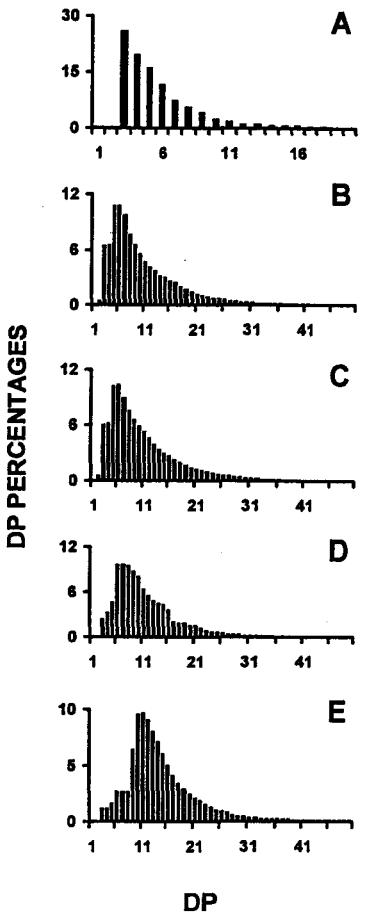


Figure 5 : Determination of the chain-length distribution of both *sta8* high and low molecular mass WSPs.

A, Histogram of chain-length distribution of low molecular mass WSP separated by TSK-HW-50 gel permeation chromatography (Fig. 4). B, chain-length distribution of debranched high molecular mass WSP from strain Bafv13 carrying sta8-1::ARG7. C, D, and E displays chain length distributions of debranched reference polysaccharides respectively high molecular mass WSP (phytoglycogen) from a sta7 mutant strain, bovine liver glycogen and maize amylopectin,. The results are displayed as percentages of chains of DP between 1 to 50. The x scale displays a DP scale, and the y axis represents the relative frequencies of the chains expressed as percentages.

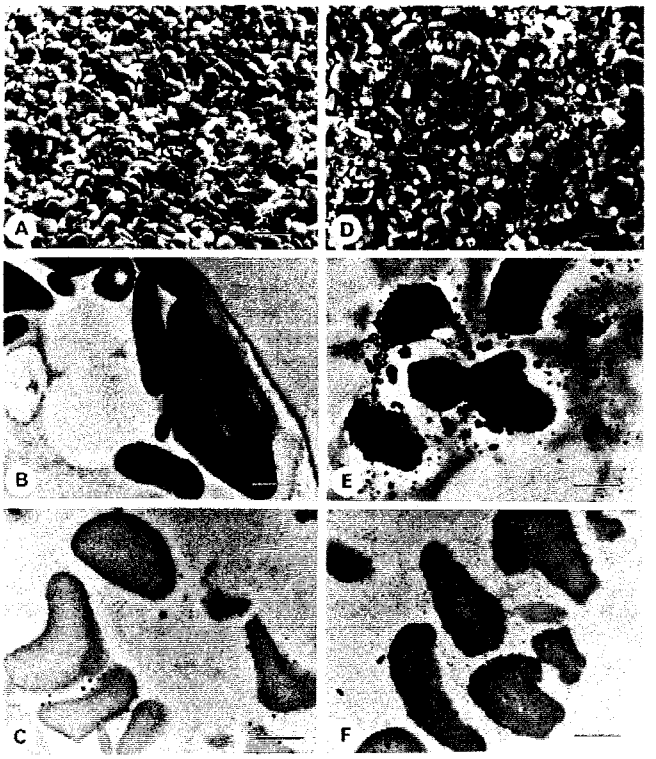


Figure 6 : SEM and TEM of starches from wild-type and mutant *sta8-1::ARG7* strains.

Electron micrographs of purified starches and in cells from nitrogen-starved wild-type (137C) and mutant sta8-1::ARG7 (Bafv13) C. reinhardtii strains. A to C, Wild-type strain 137C ; D to F, mutant sta8-1::ARG7 strain. A and D, SEM of purified starches (bar = 2 µm); B and E, TEM of starch-containing cells after PATAg staining (bar = 0.5 µm).; C and F, TEM of purified starches after PATAg staining (bar = 0.5 µm)

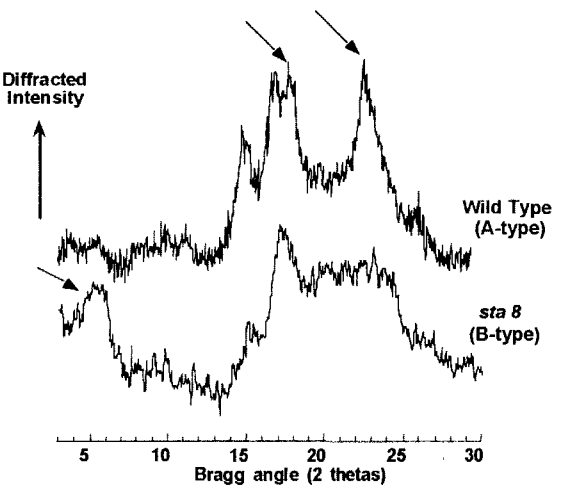


Figure 7 : X-ray diffraction of wild-type and mutant starches.

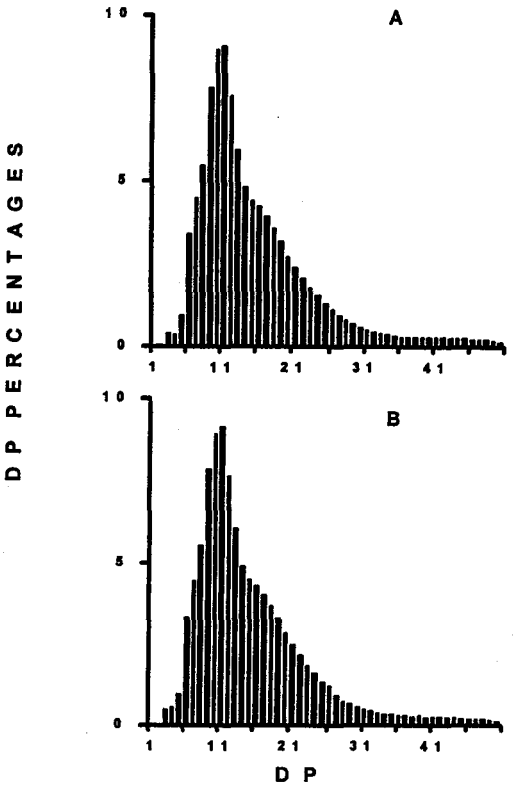
Powder X-ray diffractograms of the starches extracted from the wild-type (strain 330) and mutant (strain BafV13). Crystalline lattices of vascular plant starches fall into 3 types. The A type defines cereal endosperm starches, the B-type is found in tuber starches and some high amylose mutant starches (for review see Buléon et al., 1998). The C-type is found in pea embryos and is a mix of the A and B-type. The organization of the glucan double helical structures are completely different in A and B-type starches. The arrows display the individual reference peaks that define either the A-type or the B-type evidenced respectively in the wild-type and mutant starches.

Mutants of the *STA8* locus accumulate starch granules with a change in size, shape, X-Ray diffraction pattern and a modification of amylopectin structure.

Starch granules were purified from wild-type and mutant *sta8-1::ARG7* strains. The granules were either directly subjected to SEM or included in agar, sliced and subjected to PATAg staining. The granules of the mutant were significantly larger with distorted shapes as is often the case for high amylose starches (Fig. 6). In addition the X-Ray diffraction type of the starch has switched from the A to the B type (Fig. 7).

Figure 8 : Chain length distribution of wild-type and mutant amylopectins.

A and B, chain length distributions of wild-type and sta8-1 purified amylopectin respectively after debranching by Pseudomonas amyladeramosa isoamylase. The results are displayed as percentages of chains of DP 1 to 50. The x scale displays a DP scale, and the y axis represents the relative frequencies of the chains expressed as percentages.



The amylopectin from the wild-type and mutant starches were purified after GPC on a Sepharose CL2B column. The debranched polysaccharide was either subjected to GPC on a TSK-HW50 column to detect the long chain fraction or to capillary electrophoresis after labeling of the reducing ends with APTS to detect the chain-length distribution of chains up 50 glucose residues in length (Fig. 8A and Fig. 8B). It is clear that the mutant accumulates a significantly higher amount of long glucans (data not shown) while the small and average size chains did not differ significantly. We believe that all of these results point to a selective defect in the amylopectin biosynthetic pathway.

Devising a specific crude extract assay for isoamylase in *Chlamydomonas reinhardtii*

All phytoglycogen producing mutants presently described are specifically defective for a debranching enzyme of isoamylase specificity. Before embarking into the biochemical characterization of the *sta8* mutants we needed to establish a specific assay for this enzyme which would discriminate its activity from other starch hydrolases in crude extracts. In all plants, isoamylase and α-amylases will both generate branched or linear oligosaccharides that will be assayed without discrimination through the appearance of reducing ends from the breakdown products of glycogen or amylopectin. While branching enzymes and α-1,4 glucanotransferases will not produce soluble dextrins from polysaccharides under our experimental conditions, both limit-dextrinase (pullulanase) and α-glucosidase will readily produce oligosaccharides or glucose. However even if these enzymes are given their optimal substrates (respectively pullulan or maltose) they account for less than two percent of the reducing ends generated from both amylopectin and glycogen using the crude extract mixture of *Chlamydomonas reinhardtii* (Table II and Dauvillée et al. (2000)).

Table II. *Enzyme activities in wild-type and mutant sta8-1 progeny*

Enzyme	Wild-type strains[a]	Mutant strains[a]	Zymogram[b]
AGPase	25 ± 5 (6)	22 ± 6 (6)	No
STP	20 ± 3 (6)	19 ± 2 (6)	Yes (7,8)
PGM	5.8 ± 2.1 (6)	5.6 ± 1.7 (6)	Yes (8,8)
SS	2.7 ± 0.4 (6)	2.8 ± 0.7(6)	Yes (22,24)
BE	0.7 ± 0.1 (6)	0.9 ± 0.2 (6)	Yes (12,16)
GBSS	43 ± 4 (4)	42 ± 6 (4)	No
α-glucosidase	4.51 ± 0.37 (6)	4.68 ± 0.61 (6)	No
Limit-dextrinase	0.25 ± 0.1 (21)	0.24 ± 0.1 (23)	Yes (45,47)
D-enzyme	86 ± 7 (6)	84 ± 8 (6)	Yes (45,47)
Isoamylase	367 ± 34 (21)	122 ± 12 (23)	Yes (45,47)

[a]: The numbers between brackets correspond to the number of different strains examined.
[b]: The two numbers between brackets correspond respectively to the number of wild-type and mutant strains examined, respectively.

ADP-glucose pyrophosphorylase (AGPase) (assayed in direction of pyrophosphorolysis in the presence of 1.5 mM 3-PGA), Starch phosphorylase (STP) and phosphoglucomutase (PGM) units are expressed in nmoles Glc-1-P produced.min^{-1}.mg^{-1} protein. Soluble starch synthase (SS) and granule-bound starch synthase (GBSS) are expressed in nmoles ADP-Glc incorporated into polysaccharide.min^{-1}.mg^{-1} protein (SS) or mg starch (GBSSI). Branching enzymes (BE) is expressed as nmoles Glc-1-P incorporated into polysaccharide.min^{-1}.mg^{-1} protein (phosphorylase amplification assay). Limit dextrinase and D-enzyme are expressed in nmoles maltotriose formed from pullulan. min^{-1}.mg^{-1} protein and nmoles glucose formed from maltotriose. min^{-1}.mg^{-1} protein respectively. α-glucosidase activities are expressed in nmoles glucose formed from maltose. min^{-1}.mg^{-1} protein. The isoamylase activities were monitored by measuring the amount of reducing ends produced during incubation with glycogen in the presence of 10 mM EDTA. The activities are expressed as nmoles of maltotriose equivalents liberated from glycogen per hour and per mg of protein.

We have previously reported that the purified Chlamydomonas isoamylase is highly sensitive to oxidation by H_2O_2 (Dauvillée et al., 2000). The purified enzyme remains however insensitive to concentrations of up to 10 mM EDTA. We have recently noted that the 50-53 kD α-amylase activities of Chlamydomonas display a strong requirement for calcium. Concentrations of EDTA as low as 5 mM were sufficient to inhibit the bulk of the enzyme activities detected on zymograms (Fig. 9A) performed with both denatured-renatured extracts (Mouille et al., 1996) or with native proteins (Kakefuda and Duke, 1984). We further confirmed the sensitivity of α-amylase to EDTA by purifying the enzyme over 50-fold and obtaining fractions lacking isoamylase activity (Fig. 9B). Over 99 % inhibition was achieved through the use of 10 mM EDTA (Fig. 9C) in these purified extracts while the enzyme was insensitive to 1 mM H_2O_2 (Fig. 9D). We proceeded to establish that production of reducing-ends from glycogen breakdown in the presence of 10 mM EDTA or after treatment with 1 mM H_2O_2 defined quantitative crude extract assays respectively for isoamylase and α-amylase. We therefore compared crude extracts of wild-type and *sta7* mutants for the production of reducing-ends resulting from glycogen or amylopectin breakdown (Fig. 9E). In the presence of 10 mM EDTA reducing-end production in wild-type extracts decreased by 55 %. The *sta7* mutants lack isoamylase with no concomittant modification of amylase (Mouille et al., 1996). As predicted this resulted in the complete absence of reducing-end production (<1%) in the presence of 10 mM EDTA. Moreover the activity measured in the *sta7* mutant without EDTA precisely matched that of the amount of activity inhibited by 10 mM EDTA in wild-type extracts. In addition the wild-type reducing-end production decreased by 45 % after treatment with 1mM H_2O_2 while the *sta7* extracts remained insensitive to H_2O_2. These results prove beyond doubt that reducing-end production in the presence of either 10 mM EDTA or after treatment with 1 mM H_2O_2 do define specific crude extract assays respectively for isoamylase and α-amylases in *Chlamydomonas reinhardtii*.

Mutants of the *STA8* locus display a selective 65% quantitative reduction in isoamylase activity but still retain the 88-kD debranching enzyme subunit

We have assayed the wild-type and mutant extracts for the presence of all enzymes suspected to be involved in starch biosynthesis or degradation. Results listed in Table II demonstrate that all these enzymes are unaffected by the presence of the *sta8-1::ARG7* mutation. A 65 ± 5% reduction in isoamylase activity co-segregated with the mutant gene in 46 recombinants analyzed from a cross involving a wild-type and a mutant Chlamydomonas strain. Moreover, in contrast to the previously described *sta7* mutants (Mouille et al., 1996), the *sta8* strains still contained the 88-kD isoamylase subunit in seemingly normal amounts as far as could be evidenced by zymograms performed after denaturation-renaturation

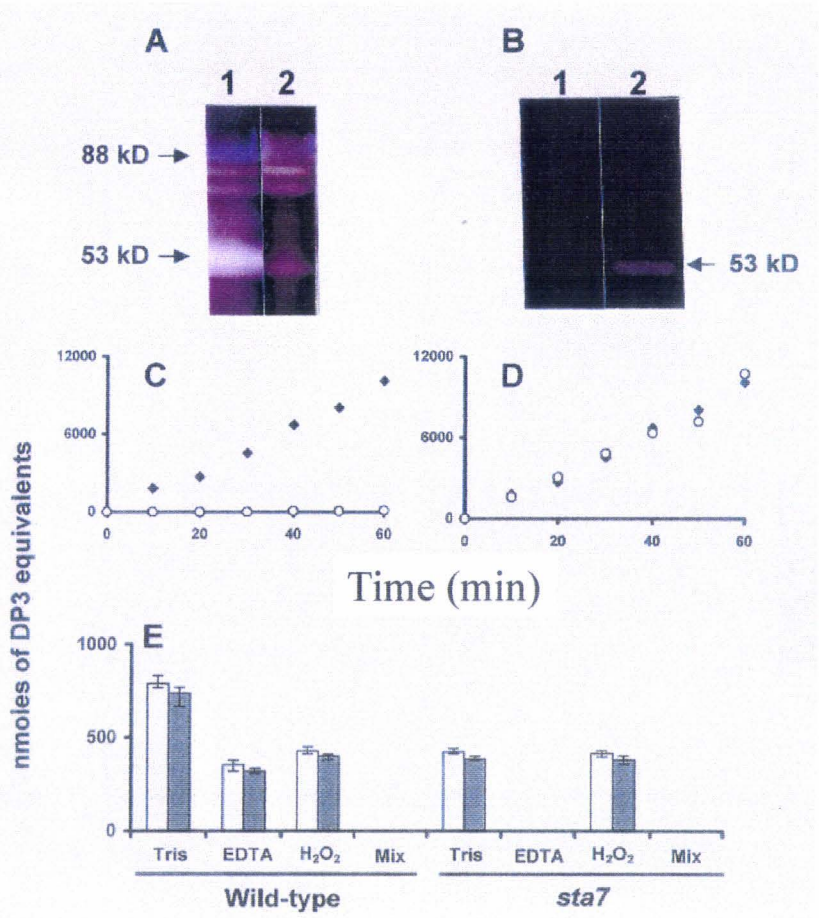


Figure 9 : Isoamylase and α-amylase inhibition experiments.

***A**, 200 µg of wild-type crude extract were denatured and loaded on a starch containing zymogram (Mouille et al., 1996). After renaturation, part of the gel was incubated with or without 5 mM EDTA. Lane 1 displays the gel segment incubated in the incubation mix without EDTA, lane 2 another gel segment containing the same sample but incubated in a mix with 5 mM EDTA.**B**, 20 µg of proteins from semi-purified α-amylase were denaturated and loaded on a starch containing zymograms according to Mouille et al. (1996). After renaturation, part of the gel was incubated with or without 5 mM EDTA. Lane 1 displays the gel segment incubated in the incubation mix containing 5 mM EDTA, lane 2 another gel segment containing the same sample but incubated in a mix without EDTA.*

***C**, 20 µL of the semi-purified α-amylase (purified 50 times) were used to measure the increase in reducing ends from glycogen (see methods) with (o) or without (υ) 10 mM EDTA.*

D, The same procedure was followed but the enzyme was pretreated with 1 mM H_2O_2 (o) (see methods).

E, Wild-type and sta7 crude extracts (100 µg of proteins) were used to assay the increase in reducing power from bovine liver glycogen (white bars) or maize amylopectin (grey bars) in the presence of different inhibitors. Tris stands for the buffer used in standard assay (20 mM Tris pH7 containing 5 mg.mL^{-1} of polysaccharide). EDTA stands for the presence of 10 mM EDTA in the buffer described above and H_2O_2 for 1 mM hydrogen peroxide pretreatment.

In panels C and D, the x axis represents the incubation time expressed in minutes. In C, D and E, the y axis scale displays maltotriose nmoles equivalents liberated during incubation for the whole semi purified α-amylase fraction (C and D) or for 100 µg of crude extract (E).

Mutants of the *STA8* locus display a selective 65% quantitative reduction in isoamylase activity but still retain the 88-kD debranching enzyme subunit

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Gene dosage experiments

Establishing a specific crude extract assay for isoamylase enabled us to perform gene dosage experiments in diploid and triploid strains homozygous or heterozygous for either *STA7* or *STA8*. In addition to the three haploid standards containing *sta7*, *sta8* and the wild-type reference (+), we have prepared (see materials and methods) the following 3 diploid and 3 triploid genotypes: *sta7/sta7*, *sta7/+*, *+/+*, *sta7/sta7/sta7*, *sta7/sta7/+*, *+/+/+*. The results displayed in Fig. 10 demonstrate a linear relationship between wild type gene dose and isoamylase enzyme activity for the *STA7* locus. The results obtained with the *STA8* locus are more complex and the relationship is not strictly linear with respect to the wild-type allele dose. In addition although zymograms do not offer precise quantifications we can clearly observe an increase of the 88 kD DBE subunit zymogram stain as a function of the wild-type *STA7* allele dose. No such relation was found with the *STA8* locus suggesting that *STA7* could encode the 88 kD DBE subunit. The experiments described above provided us with two distinct genotypes (*sta7/sta7/+*, and *sta8* haploid) containing the same amount of isoamylase specific activity. We observed in the homozygous *sta8/sta8* the same mutant phenotype as that described for the haploid *sta8-1::ARG7*. However despite the presence of a 65% decrease in isoamylase activity the *sta7/sta7/+* triploid displayed a fully wild-type phenotype. These results establish that the defect in amylopectin synthesis recorded in the *sta8* mutants is not due to either a decrease in isoamylase activity nor to a modification of the branching to debranching enzyme ratio. It is therefore the residual enzyme's quality which is chiefly responsible for the dysfunction in amylopectin synthesis.

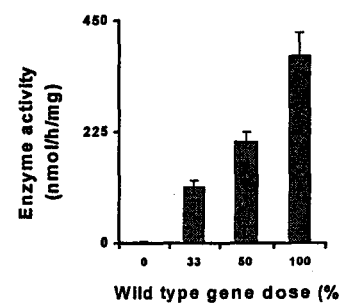

Figure 10 : Gene dosage.
Gene dosages ranging from 0% wild-type alleles (homozygous mutant) to 100% wild-type (homozygous wild-type), 50 % corresponds to the heterozygous diploids, while 33 % corresponds to a sta7/sta7/+ triploid. Histograms representing means and standard deviations (n = 3) were calculated for each gene dose. 100 µg of proteins from the different genotypes were used to assay isoamylase in the presence of 10 mM EDTA. Results are displayed as nmoles of maltotriose equivalents produced from glycogen per hour and per mg protein. Total phosphoglucomutase specific activities were monitored as internal controls and proved similar in all constructs (2.3 ± 0.5 nmol Glc-6-P formed from Glc-1-P min^{-1} mg^{-1} protein).

DISCUSSION

A fundamental aspect of plant physiology is the storage of glucose units produced initially during photosynthesis in large, insoluble starch granules. Because this carbon source storage mechanism is in stark contrast to the soluble glycogen molecules found in animals, fungi, and procaryotes, it has been of interest to seek mutant plants in which glycogen-like polymers accumulate at the expense of starch grains. Indeed, mutants of *Chlamydomonas* (Mouille et al., 1996), maize (James et al., 1995), rice (Kubo et al., 1999), and *Arabidopsis* (Zeeman et al., 1995) have been identified that display this phenotype. A striking observation is that in all these instances phytoglycogen accumulation results from a mutation that controls production of an isoamylase, and in at least three of the species the mutation directly alters the gene that codes for the enzyme.

The relation between the isoamylase and the form of the storage glucan, i.e., soluble or granular, appears to be very specific. Phytoglycogen accumulating mutants in maize are easy to detect owing to the resultant kernel phenotype, and in the past century dozens of such mutations have been isolated in the course of the extensive genetic analysis of this species. All of these are allelic mutations of the *su1* locus, indicating the high level of saturation of genetic screens for phytoglycogen accumulating mutants. Mutation of an isoamylase coding sequence, therefore, may be the sole means of altering starch biosynthesis such that glycogen-like polymers are formed in plants.

The genetic analysis reported here indicates that at least two different genes in a plant species can determine the ability to produce storage glucans in the form of insoluble granules. This is the first description of two genes controlling phytoglycogen production, so further characterization of the *STA8* gene is likely to be of interest in understanding how granular starch is produced. The *sta8* mutations cause significant reductions in the activity of isoamylase in total extracts. The genetic analysis, therefore, has not identified a completely different biochemical activity involved in phytoglycogen production at the expense of starch, and the specific relationship between phytoglycogen and isoamylase activity remains. Identification of *STA8*, however, is likely to provide novel insights into the nature of the isoamylase. Only one isoamylase in *Chlamydomonas* cell extracts is detected in activity gels. This activity migrates as an 88 kDa polypeptide, and it is completely eliminated by the *sta7* mutations (Mouille et al., 1996; Dauvillée et al., 1999; Dauvillée et al., 2000). The *sta8* mutations, in contrast, reduce this activity but do not eliminate it completely. Several possibilities may be considered to explain how *STA7* and *STA8* can both affect the same enzyme. One of the two genes may directly code for a polypeptide that possesses isoamylase activity, whereas the second gene may control expression of the enzyme. Alternatively, both *STA7* and *STA8* could code for polypeptides within the isoamylase, with the *STA7* product as an essential component and the *STA8* product as a contributing factor that is not absolutely necessary for activity. The gene dosage experiments reported in this paper strongly suggest that *STA7* encodes a catalytic subunit within the enzyme complex.

Plant isoamylases are known to be multisubunit enzymes of high molecular weight, so the possibility of heteromultimeric compounds must be

considered. This suggestion seemingly is contrary to nature of the rice endosperm isoamylase, which was defined as homomultimeric complex (Fujita et al., 1999). In potato however, two polypeptides copurified with the isoamylase activitly (Ishizaki et al., 1983). Furthermore, at least two genes coding for polypeptides highly homologous to known isoamylases are present in *Arabidopsis* and potato. Further characterization of the precise molecular nature of the isoamylase in various plant species is necessary to understand the role of this enzyme in amylopectin biosynthesis, and we expect that the genetic characterization of two loci controlling the enzyme in *Chlamydomonas* will be useful in this regard.

Mutations that affect isoamylase in various species can have different effects on amylopectin biosynthesis. This study revealed that in *sta8* mutants the chain length distribution of amylopectin in the residual starch is similar to that of wild type amylopectin, and the same result was obtained in *Arabidopsis dbe1* mutants (Zeeman et al., 1998). In contrast, mutations of isoamylase polypeptides in maize and rice cause alterations in the chain length distribution in the remaining amylopectin (C. C. and A. M., unpublished results; Nakamura et al., 1997). A third phenotype is observed in the *Chlamydomonas sta7* mutants as well as in rice plants bearing specific alleles of the *su1* locus. In these plants granular starch is completely lacking. A possible explanation for these varying results is that there are various means of altering isoamylase activity and that different types of mutation have different effects on amylopectin biosynthesis. These considerations raise the question of whether the total activity of isoamylase determines if storage glucans are converted into soluble or insoluble polymers or, alternatively, whether specific qualitative aspects such as substrate specificity also are involved in the process.

The different phenotypes caused by *sta7* and *sta8* mutations offered an opportunity to address this question. Comparison of a *sta8/sta8* strain to *a sta7/sta7/STA7* heterozygous triploid revealed two distinct phenotypes. In the latter instance starch production is normal, whereas in the former phytoglycogen is present and granular starch is strongly reduced. Yet the two strains possess the same total amount of isoamylase activity measured in total extracts. To explain these results we suggest that a qualitative aspect of isoamylase activity is critical in amylopectin biosynthesis. One possibility is that the substrate specificity of the isoamylase is different in the enzyme found in homozygous *sta8* strains compared to that present in the *sta7* heterozygotes. A second possibility is that the interactions of the multimeric isoamylase complex with other polypeptides are altered in the *sta8* mutant.

A technical advance described here is the ability to measure total isoamylase activity *in vitro* in crude cell extracts. The presence of several different amylolytic enzymes that can produce new reducing ends from polysaccharide substrates is a significant complication in measurement of debranching enzyme activity. The fact that *sta7* mutant extracts assayed in the presence of EDTA contain undetectable amounts of amylolytic activity indicates that the assay used here is effective in eliminating all extraneous enzyme activity above the isoamylase that is dependent on *STA7*. EDTA treatment, therefore, is an effective means of measuring isoamylase in *Chlamydomonas reinhardtii*. Whether this method is applicable to other species has however to be thoroughly checked and may vary according to the tissue and species. Three requirements that were met in the Chlamydomonas have to be ascertained in each case. First the contributions of the many EDTA insensitive starch hydrolases have to account for less than 1% of the crude

extract mixture activity to be considered negligible. Second mutants lacking isoamylase must be available to confirm that in the presence of EDTA no activity can be detected in the assay. Third the amount of activity assayed in the absence of EDTA in the mutant should match precisely the decrease in activity measured in the presence of EDTA in the wild-type controls.

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CHAPITRE IV :

Biochemical characterization of wild-type and mutant isoamylases of *Chlamydomonas reinhardtii* supports a direct function of the multimeric enzyme organization in amylopectin maturation

Dauvillée D, Colleoni C, Mouille G, Morell M, D'Hulst C, Myers AM, Ball S.

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Avant-propos

L'activité isoamylasique de *Chlamydomonas reinhardtii* est supportée par un complexe enzymatique de haute masse moléculaire. Ce dernier se présente sous la forme de trois bandes d'activité sur des zymogrammes réalisés en conditions non dénaturantes. Après dénaturation, les trois bandes n'en forment plus qu'une dont on associe l'activité à la sous-unité catalytique du complexe. L'ensemble des trois activités observées en conditions natives disparaît dans un contexte mutant *sta7*.

Nous savons que la diminution de la quantité d'amidon et l'apparition de phytoglycogène observées dans les mutants *sta8* ne sont pas des conséquences directes de la diminution de l'activité isoamylasique (voir le chapitre précédent). Afin de découvrir la nature de l'élément responsable de ce phénotype particulier, nous nous sommes engagés dans la semi-purification des complexes enzymatiques responsables de l'activité isoamylasique des souches sauvage et mutante *sta8*. Un seul complexe enzymatique est observé dans les souches mutantes *sta8* sur les zymogrammes en conditions natives. Ce complexe possède en outre une taille réduite qui correspond à la taille de la plus petite des trois formes du complexe des souches sauvages.

Sachant que la quantité d'activité isoamylasique dans les souches *sta8* n'étaient pas le facteur entraînant la réduction de l'accumulation d'amidon et la production du phytoglycogène, nous avons cherché à vérifier si le complexe mutant possédait des propriétés permettant de le distinguer du sauvage. La spécificité de l'isoamylase présente dans le mutant *sta8* n'est pas modifiée au regard de celle de la souche sauvage et s'avère correctement localisée dans le stroma. Sa sensibilité à l'oxydation est similaire à celle de son homologue non mutant et enfin elle possède la même capacité à digérer totalement le phytoglycogène *in vitro* sans le faire *in vivo*. Tous ces points réunis tendent à prouver que seule l'architecture du complexe enzymatique *sta8* permet de le différencier du sauvage et apparaît comme l'élément responsable du défaut de biosynthèse de l'amidon dans les souches mutantes *sta8*. Cette étude révèle également que l'isoamylase n'est probablement pas impliquée dans le catabolisme du phytoglycogène *in vivo* dans la souche sauvage. Nous allons détailler tous ces points dans le chapitre qui suit.

Biochemical characterization of wild-type and mutant isoamylases of *Chlamydomonas reinhardtii* supports a function of the multimeric enzyme organization in amylopectin maturation

David Dauvillée, Christophe Colleoni, Gregory Mouille, Matthew K. Morell, Christophe d'Hulst, Alan M. Myers and Steven G. Ball*.

Laboratoire de Chimie Biologique, Unité Mixte de Recherche du C.N.R.S. n°8576, Université des Sciences et Technologies de Lille, 59655 VILLENEUVE D'ASCQ Cedex France (D. D., C. C., G. M., C. d. H, S. G. B.)CSIRO, Division of Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia (M. K. M.). Department of Biochemistry, Biophysics and Molecular.

ABSTRACT

Chlamydomonas reinhardtii **mutants of the *STA8* gene produce both reduced amounts of high amylose starch and phytoglycogen. In contrast to the previously described phytoglycogen-producing mutants of Chlamydomonas which contain no residual isoamylase activity, the *sta8* mutants still contained 35% of the normal amount of enzyme activity. We have purified this residual isoamylase and compared it to the wild-type Chlamydomonas enzyme. We have found that the high mass multimeric enzyme has reduced its average mass at least by half. This coincides with the disappearance of two out of the three activity bands that can be seen on zymogram gels. Both wild-type and mutant enzymes are shown to be located within the plastid. In addition they both act by cleaving off the outer branches of polysaccharides with no consistent difference in enzyme specificity. Because the mutant enzyme was demonstrated *in vitro* to digest phytoglycogen to completion, we propose that its inability to do so *in vivo* supports a function of the enzyme complex architecture in the processing of pre-amylopectin chains**

Key words : *Chlamydomonas reinhardtii* - starch debranching enzyme- isoamylase – pullulanase - amylopectin.

INTRODUCTION

Mutants lacking isoamylase, an enzyme that catalyzes the breakdown of α-1,6 branches, are unable to synthesize normal starch in maize, rice and Chlamydomonas (for reviews see Nakamura, 1996, Myers et al., 2000) . In maize and rice the so-called "sugary" mutations that map in an isoamylase structural gene lead to the disappearance of all detectable isoamylase activity. Mutants defective in the *STA7* locus of Chlamydomonas equally lack starch but the relation to the isoamylase structural gene still needs to ascertained (Mouille et al., 1996). This has led to the contention that debranching of a precursor of amylopectin known as pre-amylopectin is an integral part of the amylopectin synthesis pathway (Ball et al., 1996, Myers et al., 2000). It was proposed that the release of improperly positioned α-1,6 branches allowed proper alignment of the α-1,4-linked glucans within the growing polysaccharide. In turn this alignment facilitated crystallisation within large-size starch granules. Recently mutations in another gene (*STA8*) that leads to some partial defect in isoamylase activity have been documented in *Chlamydomonas reinhardtii*. These mutations lead to a two-third reduction in total isoamylase activity together with a substantial decrease in amylopectin content and the concomitant appearance of glycogen-like polymers (phytoglycogen) (Dauvillée et al., submitted). In Chlamydomonas, heterozygous triploids with a *sta7/sta7/STA7* genotype contain the same wild-type isoamylase specific activity as the mutant *sta8* haploids (Dauvillée et al., submitted). Yet these heterozygous triploids accumulate wild-type amounts of normal starch and no phytoglycogen. These results suggest that it is the quality of the residual isoamylase in the *sta8* mutant rather than the reduced amount of activity that is responsible for the dysfunctions in amylopectin synthesis. Because the wild-type isoamylase activity is not present in rate controlling amounts in Chlamydomonas, explanations have to be sought that are consistent with the finding of a substantial amount of residual activity in the *sta8* mutants. The first obvious explanation is that the enzyme's substrate specificity has changed. The second is that the mutant enzyme is mislocated and that only tiny and insufficient amounts of activity are transported within the plastid. The third is that while significant activity amounts can be measured *in vitro*, the mutant enzyme is nearly completely inactive *in vivo*. The fourth is that the known multimeric architecture of plant isoamylases *per se* might be of particular relevance for amylopectin synthesis. We now report the partial purification of this residual isoamylase present in the *sta8* mutant and its comparison to the wild-type enzyme. We provide evidence that the multimeric organization of the isoamylase *per se* is of paramount importance *in vivo* during amylopectin synthesis

MATERIALS AND METHODS

Materials.

The apoferritin and thyroglobulin mass standards, the maize amylopectin, and the bovine or rabbit liver glycogen were supplied by Sigma Chemical Co. (St. Louis, MO). The catalase mass standard was from Boehringer (Mannheim, GmbH).

***Chlamydomonas reinhardtii* strains, growth conditions, WSP assays and media.**

The wild type reference strain used in this work is 330 (*mt*$^+$ *arg7 cw15 nit1 nit2*) while strains Bafv13 (*mt*$^+$ *cw15 nit1 nit2 sta8-1::ARG7*) and GM7.27 (*mt*$^-$ *pab2 sta7-1::ARG7*) were used as our reference *sta8* and *sta7* mutant strains respectively. Wild-type and mutant segregants from a cross between Bafv13 and GM7.27 were used throughout this work (strains called BGM). The genotype of the segregants were checked by standard complementation tests and starch-containing zymograms. WSP was assayed as follows. One liter TAP-N cultures inoculated at 5.10^5 cells.mL^{-1} were harvested after five days and ruptured by one passage in a French Press (10000 psi).The lysate was kept at 4°C and immediately cleared by centrifugation at 3000 g for 10 min. The supernatant was immediately frozen (-20°C) and thawed after 2 hours. The thawed extract was boiled for 5 min in a water-bath and further cleared by spinning at 10000 g for 15 min at 4°C. The WSP was immediately assayed by the amyloglucosidase assay (Delrue et al., 1992).

All experiments were carried out in continuous light (40µE m^{-2} s^{-1}) in the presence of acetate at 24°C in liquid cultures that were shaken without air or CO_2 bubbling. Late-log-phase cultures were inoculated at 10^5 cells mL^{-1} and harvested at 2.10^6 cells mL^{-1}. Genetic techniques were as described by Harris (1989a). Standard TAP (Tris acetate phosphate) medium was fully detailed in Harris (1989b). TAP-N defines TAP medium where NH_4Cl was substituted by an equivalent concentration of NaCl.

Enzyme purification and zymograms.

Crude extract preparation and isoamylase purification from *Chlamydomonas reinhardtii* crude extracts were fully detailed in Dauvillée et al. (2000). Briefly, the thawed extracts were subjected to successive protamine sulfate and ammonium sulfate precipitations. Proteins obtained in the 35% ammonium sulfate pellet were loaded on a FPLC gel permeation chromatography Sephacryl S-300 HR. The fractions containing the isoamylase activity were detected by starch containing zymogram, pooled and loaded on a FPLC UnoQ1 (Biorad) column. Gel permeation columns were precalibrated by subjecting 5 mg of thyroglobulin (669 kD), apoferritin (443 kD), and catalase (240 kD) to the same chromatographic procedure.

Zymograms in starch containing gels allowing the detection of most starch hydrolases have been described for undenaturated samples by Kakefuda and Duke (1984). To detect *Chlamydomonas reinhardtii* isoamylase isoforms, 100 µg of crude extract proteins were loaded on a 29:1 (acrylamide:bisacrylamide) 7.5% (1.5 mm thick) native polyacrylamide gel containing 0.6 % of rabbit liver glycogen (Sigma Chem. Co.), ran at 20 V.cm^{-1} for 120 min at 4°C in 25 mM Tris glycine pH 8.3, 1 mM DTT. Gels were incubated for 1-12 h in the same buffer containing 20 mM DTT and finally stained by using a freshly prepared 0.2% I_2 0.02% KI stock solution.

Chloroplast isolation and characterization

Synchronized Chlamydomonas

cultures on a 12 h light/12 h dark cycle were used to extract chloroplasts. The cells were harvested in the middle of the third light period. The method used to isolate chloroplasts has been fully detailed in Mason et al. (1991). The chloroplast fractions obtained were used to measure marker enzyme activities including UDP-glucose pyrophosphorylase and PEP carboxylase as cytosolic markers and NADP GAP deshydrogenase as plastidial markers. The corresponding enzyme assays have been previously described in Borchert et al. (1993). Activities were assayed in both the plastidial fraction and the homogenate from the same cultures. Chloroplast contamination by cytosolic enzymes in all preparations were estimated to be around 20%.

Isoamylase assays and debranching kinetics

Isoamylase was always assayed at 30°C in 20 mM Tris, 10 mM EDTA, 2 mM DTT pH 7, unless otherwise specified, using either bovine liver glygogen or amylopectin at 5 mg.mL^{-1}. Activities were measured as previously detailed (Dauvillée et al., 2000) through the increase in reducing power generated by the debranching reaction. This increase was translated into maltotriose equivalents by using the latter as standard (Dauvillée et al., 2000). After stopping the reaction by boiling 5 min, the determination of the chain-length distribution of the glucans released by the Chlamydomonas isoamylases were obtained by FACE or high performance anion exchange with pulsed amperometric detection chromatography as fully detailed in O'Shea et al. (1998) and Libessart et al. (1995).

To check the sensitivity of the wild type and mutant enzyme to DTT, we used the same protocol but with several concentrations of DTT including 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2 mM. To obtain cells in the same redox state, we used 3-day old synchronized cultures undergoing 12h light / 12h dark cycles. The cells were harvested rapidly 5 min at 3000 g. All subsequent steps were carried-out under high light and on ice to avoid oxidation within the extract. The crude extract was obtained by braking the cells with a potter on ice and used immediately to assay isoamylase with the procedure described above.

RESULTS

Mutants of the *STA8* locus lack two out the three native isoamylase zymogram bands.

In *Chlamydomonas reinhardtii* zymogram procedures have been devised that allow migration of denatured proteins and their renaturation in the presence of substrate (Mouille et al., 1996). This technique enabled us to visualize an 88-kD debranching enzyme with isoamylase specificity. The presence of this polypeptide is clearly under control of the *STA7* locus and gene dosage experiments are consistent with *STA7* encoding this catalytic isoamylase subunit. *STA8* has no detectable influence on the quality or quantity of 88-kD debranching enzyme as detected in these zymograms. Because we know the isoamylase to be substantially reduced in *sta8* mutants, we embarked in a more detailed zymogram investigation by looking at native proteins. We used both the set-up detailed in Kakefuda and Duke, (1984) and a novel procedure devised by us for glycogen containing gels (see methods). In this system, three clear white-staining isoamylase bands of similar intensity were detected in all wild-type strains (Fig. 1). All three bands disappeared in *sta7* mutants. Interestingly the *sta8* mutant clearly lacked the two slow migrating

bands (Fig. 1). To ensure that it was specifically due to the presence of *sta8-1::ARG7*, we examined cosegregation of the zymogram defect with the *sta8* mutation on 26 wild-type and 27 mutant recombinants from a cross involving the BAFV13 mutant and the A35 wild-type strain. Cosegregation was observed on all 53 meiotic recombinants. In addition we observed full epistasis of *sta7* on *sta8* and no activity bands could be scored in the double mutants.

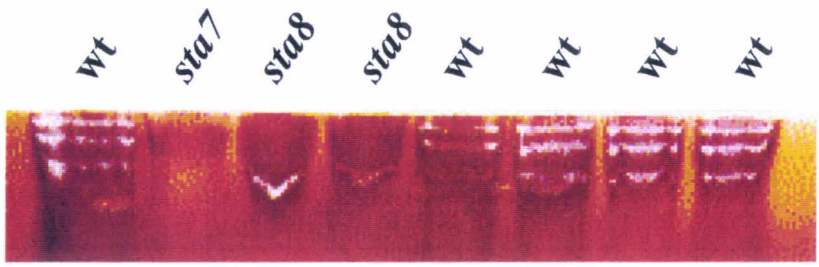

Figure 1 : Detection of isoamylase complexes on glycogen containing zymograms.

Native crude extracts (100 µg of protein) were loaded on a rabbit liver glycogen containing zymogram (see methods). Chlamydomonas isoamylase complexes appears as white bands after incubation at the top of the gel. The isoamylase defective mutants carrying the sta7-1::ARG7 mutation show no band on this zymogram. Wild-type strains always display three distinct bands of similar intensity while sta8-1::ARG7 strains display only one lower white band on over 50 meiotic segregants (27 mutant strains and 26 wild-type).

Partial purification and comparison of the wild-type and mutant isoamylase architecture.

In order to understand better the qualitative modification of isoamylase activity present in the *sta8* mutants, we partly purified the enzyme activity according to a pre-established procedure (Dauvillée et al., 2000) and compared the purification behavior with that of wild-type strains. Purification of the wild-type enzyme relied on a S-300 FPLC gel filtration followed by anion exchange on a UNOQ FPLC column. Yields and purification factors are summarized in Table I. Figure 2 exemplifies results obtained with the S-300 FPLC. We used the zymogram procedure developed by Kakefuda and Duke (1984) to visualize the isoamylase activities within the protein mixture. We also sized the proteins by using commercially available mass standards (thyroglobulin, apoferritin, and catalase respectively of 669, 443, and 240 kD). Fig. 2 displays the gel filtration step performed on the same amount of protein extract with the relevant molecular mass standard (thyroglobulin) position for wild-type (Fig. 2A) and mutant (Fig. 2B) strains of isogenic background. Three blue activity bands can again be seen in zymograms from the wild-type strain while only one such band is witnessed in the *sta8* mutant.

Table I. *Isoamylase purification*

	Yield (%)		Purification factor		Total activity[a]	
	sta8	+	*sta8*	+	sta8	+
Crude extract	100	100	0	0	225	322
Protamine sulfate	74	65	1,7	1,3	167	210
Ammonium sulfate	46	40	2,4	1,6	105	130
Gel permeation (52-65)	-	17	-	9,9	-	55
Gel permeation (58-66)	19	-	9,9	-	42	-
Anion exchange (19-20)	-	2,5	-	140	-	8,2
Anion exchange (15-16)	4,5	-	77	-	10,2	-

[a] *Purification was from 4 x 10^{10} cells. Debranching activity corresponds to µmoles of maltotriose equivalents produced per hour from amylopectin for the whole fraction in study. After the gel permeation step, wild-type isoamylase was free of interfering starch hydrolytic activity, while trace amount of α-amylase can be detected by zymograms in the sta8 semi-purified fractions.*

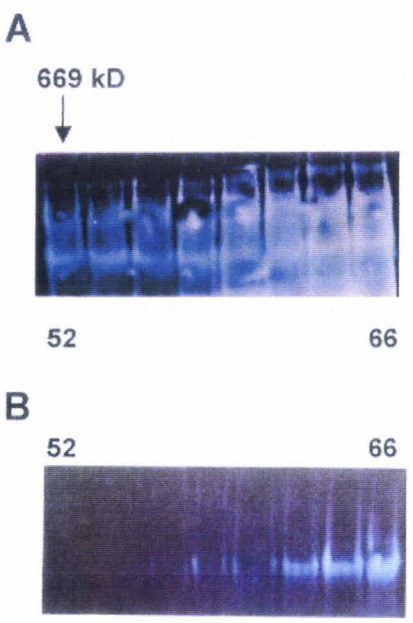

Figure 2 : Semi purification of wild-type and *sta8* isoamylases.

Crude extracts from 20 L cultures of wild-type strain A35 or the sta8-1 Bafv13 strain were subjected to the purification procedure described previously by Dauvillée et al. (2000). The undenatured samples (80µL from the fractions 52, 54, 56, 58, 60, 62, 64, and 66 of the S300 GPC) separated by PAGE under native conditions were blotted onto starch-containing gels according to Kakefuda (1984). The color of the bands witnessed after staining the gel with iodine will change according to the enzyme specificities. Branching enzymes will stain white or light red, amylases and glucosidases will stain white and debranching enzymes (pullulanases or isoamylases) will stain blue. A, zymogram obtained from wild-type isoamylase semi-purification. B, the same fractions analysed from the reference sta8-1 mutant strain.

As a control, we checked that all three bands disappeared in *sta7* mutants. In addition, we calculated the average mass of the DBE activities from three distinct purification experiments (see methods). We find values of 500 ± 100 kD for the wild-type and 150 ± 50 kD for the mutant activities. Interestingly the presence of the 88 kD DBE subunit visualized after denaturation on zymogram gels displays an equivalent shift, proving that this catalytic subunit is present within a protein complex of much larger size in the wild-type than in the mutant. We found no clear correlation in the wild-type strain chromatogram between molecular mass and the presence of any of the 3 distinct zymogram bands suggesting as was recently observed in rice that the three separate bands are probably generated by the experimental procedures from an otherwise intact multimeric complex (Fujita et al., 1999). To confirm that molecular mass of the complex and presence of a distinct subset of zymogram bands were direct consequences of the *sta8* mutation we partly purified the DBEs from 5 wild-type (strains BGM13, BGM15, BGM80, BGM200, and BGM202) and 5 mutant (strains BGM6, BGM11, BGM12, BGM97, and BGM201) meiotic recombinants and found identical results.

Substrate specificity and mode of action of wild-type and mutant enzymes

The trimming function that we envision for the plant isoamylases in amylopectin synthesis implies that the DBEs splice out only those branches that would otherwise prevent amylopectin crystallization (Mouille et al., 1996, Myers et al., 2000). This suggests that any modification of the substrate specificity of the isoamylases will interfere with polysaccharide crystallization and therefore with amylopectin synthesis. Modification of the isoamylase complex through the *sta8* mutation is thus expected to generate some change in substrate specificity in the DBE complex that would conveniently explain the mutant phenotype. We therefore embarked in experiments designed to probe the specificity of the wild-type and mutant enzyme not only in semi-pure fractions but also in crude extracts. This was done to avoid selecting any subset of the activities displayed in Fig. 1 that might differ with the bulk of the isoamylase activities and therefore lead us to biased conclusions. We used two different analytical procedures (FACE and HPAED-PAD) to characterize the chain-length distributions of the glucans liberated through enzymatic debranching from both bovine liver glycogen (Fig. 3) and maize amylopectin (Fig. 4). In addition we quantified the amount of branches present in the malto-oligosaccharides produced upon incubation with the enzyme extracts. We also double-checked the chain-length distribution within the residual polysaccharide after purification and treatment with the *Pseudomonas amyloderamosa* isoamylase. We never released branched malto-oligosaccharides during these experiments and the branching percentage always fell below our level of detection (<1%). This proves that the plant isoamylases can be defined as exoenzymes and are only able to digest the outer chains of the substrate polysaccharides. Fig. 3 and Fig. 4 exemplifies the type of results we got through all these experiments. While in some cases, we did witness some small differences, the latter vanished upon reproducing the experiment and displayed no constant pattern distinguishing the wild-type and mutant enzyme. We conclude that both enzymes display apparently the same or a very similar specificities during the debranching of amylopectin and glycogen. These conclusions hold within the limits afforded by the presently available analytical tools.

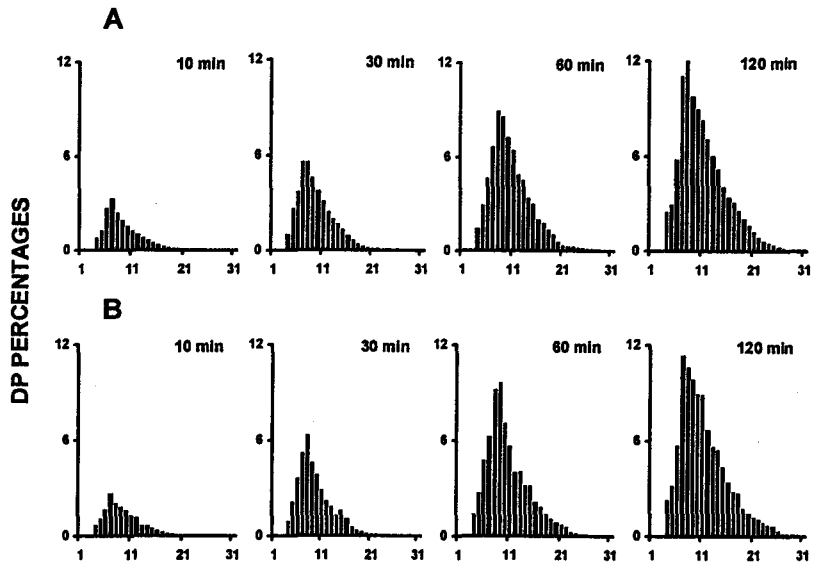

Figure 3 : Debranching kinetics of bovine liver glycogen by wild-type and mutant isoamylases. *Semi-purified isoamylases were incubated in the presence of 5 mg.mL^{-1} of bovine liver glycogen (see methods). Aliquots were boiled after 10, 30, 60, and 120 minutes and chain-length distribution of the glucans liberated were analyzed by HPAED-PAD. A, chain length distribution of glucans liberated by wild-type semi-purified isoamylase (fraction to from the gel filtration FPLC) . B, chain length distribution of glucans produced by semi-purified isoamylase from sta8 defective strains (fraction to from the gel filtration FPLC). The results are displayed as percentages of chains of DP 1 to 32. The x scale displays a DP scale, and the y axis represents the relative frequencies of the chains expressed as percentages.*

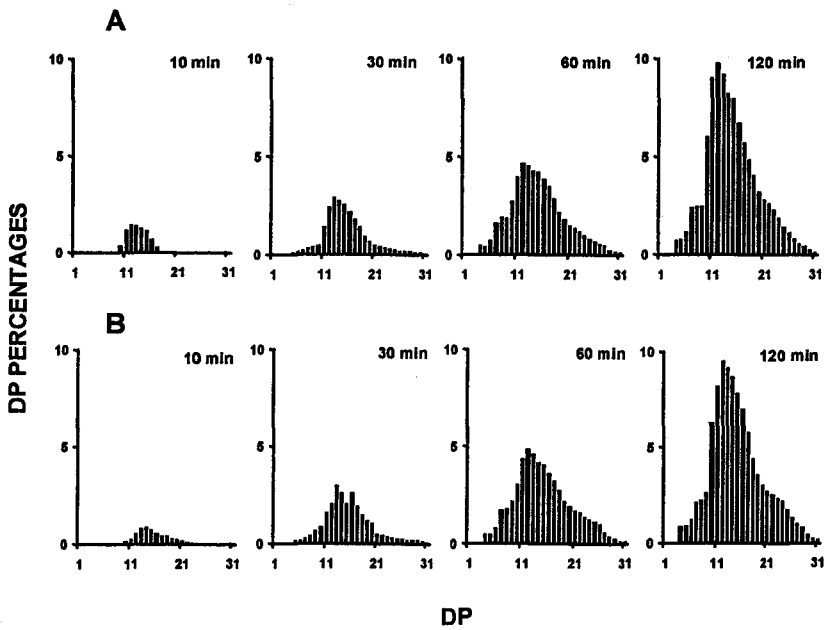

Figure 4 : Debranching kinetics of maize amylopectin by wild-type and mutant enzymes. *Semi-purified isoamylases were incubated in the presence of 5 mg.mL^{-1} of maize amylopectin (see methods). Aliquots were boiled after 10, 30, 60, and 120 minutes and chain-length distributions were analysed through HPAED-PAD. A, chain length distribution of glucans liberated by wild-type semi-purified isoamylase. B, chain length distribution of glucans liberated by semi-purified isoamylase from the sta8 defective strain. The results are displayed as percentages of chains of DP between 1 to 32. The x scale displays a DP scale, and the y axis represents the relative frequencies of the chains expressed as percentages.*

Because neither glycogen nor amylopectin define the true *in vivo* substrates for the Chlamydomonas enzyme, we reasoned that the phytoglycogen synthesized in the *sta8* mutants should be specifically enriched in chains selectively resistant to the mutant enzyme thereby explaining accumulation of the latter in the mutant plastid. We were thus surprised to find that the wild-type and mutant isoamylase are equally effective for debranching phytoglycogen *in vitro* (initial rates measured for phytoglycogen debranching were respectively of 370 ± 12 and , 359 ± 21 nmoles equivalents maltotriose protein mg^{-1} h^{-1}) moreover both enzymes were equally effective in debranching phytoglycogen to completion.

Finally we measured the Km for glycogen and amylopectin, the optimum pH and temperature values for both mutant and wild-type and were unable to find any convincing difference.

Localization of the wild-type and mutant isoamylase, *in vivo* activity

Another possible explanation for the presence of an altered phenotype within the *sta8* mutants can be easily found if the mutant isoamylase was not efficiently transported within the plastid compartment. An older study by Levi and Gibbs (1984) establish α-amylase as a plastidial enzyme. However the enzyme assays used by Levi and Gibbs would not distinguish amylases in general from isoamylases and glucosidases. We therefore embarked in cell fractionation experiments to revisit this issue with the aim to probe the localization of the wild-type and mutant isoamylase. The results listed in table II establish both wild-type and mutant isoamylases as entirely (>95%) plastidial while the amylase assay attributes only part of this family of enzymes to the plastid compartment.

Table II. *Enzyme activities of cell homogenates and chloroplasts from Chlamydomonas reinhardtii wild-type and sta8 strains*

Enzymes	wild-type		*sta8*	
	Homogenate	Chloroplasts	Homogenate	Chloroplasts
UGPase	609 ± 36	116 ± 15	567 ± 38	126 ± 14
PEP carboxylase	12 ± 0.7	2.2 ± 0.3	10 ± 1	1.6 ± 0.2
NADP GAP DH	138 ± 6	184 ± 11	120 ± 6	159 ± 4
Isoamylase	9.4 ± 0.3	10.2 ± 0.8	2.6 ± 0.1	3.7 ± 0.1

Activities are expressed in $\mu mol.mg^{-1}Chl.h^{-1}$ and are the average of three separate measures.

Finally all our measures of enzyme activity are performed in the presence of 2 mM DTT to obviate enzyme oxidation and inactivation. The wild-type Chlamydomonas enzyme is exquisitely sensitive to oxidation and no enzyme activity can be measured in its absence. This property is shared by many other plant isoamylases. In addition, once oxidized we are unable to recover any significant enzyme activity if we reintroduce DTT in the assays. It thus remains possible that *in*

vivo the residual activity present in the *sta8* mutants remains insufficiently reduced. Addition of 2 mM DTT might in this specific case restore some activity to the complex and therefore lead to an overestimation of the biologically relevant activity left within the mutant strains. To tackle this difficult question, we designed an experimental procedure aimed at maximizing our chances to observe some activity in the natural reduced state, occurring within the concentrated crude extract, without the addition of 2 mM DTT (see methods). We reasoned that if the ratio of mutant to wild-type activities remained constant within a full range of DTT concentrations, it would suggest that the residual isoamylase present within the mutants is not less active *in vivo* because of its decreased ability to be reduced. Results displayed in Fig. 5 show that both wild-type and mutant enzyme are equally sensitive to reduction by DTT suggesting that the 1/3 ratio observed by *in vitro* measures between mutant and wild-type activities reflects the *in vivo* situation within the plastid at the site of starch biosynthesis.

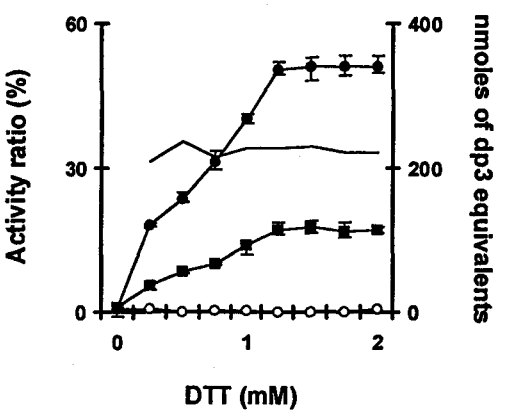

Figure 5 : DTT sensitivity of Chlamydomonas isoamylases.

The fresh crude extracts from synchronized cultures were used to assay isoamylase activities in wild-type and sta8 strains using bovine liver glycogen as substrate (see methods) at various DTT concentrations. Results are displayed as nmoles of maltotriose formed by hour and per mg of protein. The x axis represents the DTT concentrations used in the incubation buffer. Means (●, ■, O, for respectively wild-type (330), sta8-1::ARG7 (Bafv13), sta7-4::ARG7 (Bafj6) strains) and SD from three distinct measures were calculated for each DTT concentration.

Mutants of the *STA7* and *STA8* locus display identical rates of phytoglycogen degradation *in vivo*

Starch and water-soluble polysaccharide degradation can be induced by simply switching Chlamydomonas cultures to darkness. In the experiments depicted in Fig. 6, we induced accumulation of phytoglycogen from 3 different genotypes carrying respectively the *sta7*, *sta7 sta8* and *sta8* mutations. Despite differences in the initial phytoglycogen content of the strains used in these experiment the rates of polysaccharide degradation were similar in all three mutants. It is remarkable that strains and which contain respectively 35% and 0% of residual isoamylase activity display similar amounts of phytoglycogen and identical polysaccharide degradation kinetics *in vivo*. We believe these results prove that isoamylase does not play a rate

controlling role in the breakdown of phytoglycogen *in vivo*.

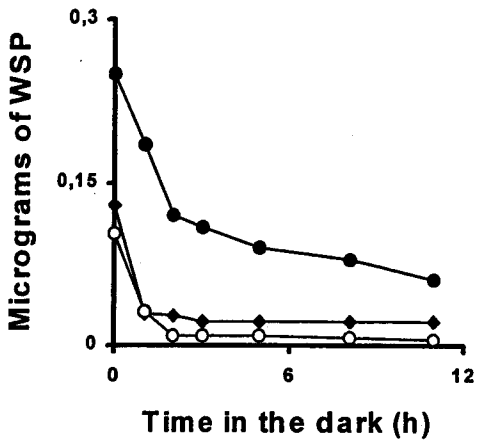

Figure 6 : Kinetics of WSP degradation
One week TAP-N cultures (6 L) inoculated at 5.105 cells.mL^{-1} in the light were switched to darkness. 500 mL samples were harvested and the WSP was assayed by the amyloglucosidase assay (see methods). ●, ◆, ○, represent strains harboring respectively the sta7-1::ARG7 (strain GM7.27), the sta8-1::ARG7 (strain Bafv13) and both mutations (strain BGM87)

DISCUSSION

The rate of semi-crystalline amylopectin formation decreases in strains harboring a defect in the assembly of the isoamylase multimer complex

Mutants of the *STA8* locus in *Chlamydomonas reinhardtii* accumulate both a restricted amounts of high amylose starch and phytoglycogen. This coincides with a drop of 65 % in isoamylase activity while no other enzyme known to interfere with starch biosynthesis seems modified (Dauvillée et al., submitted). In this work we prove that this residual activity is exported normally to the plastid compartment and that the substrate specificity has not been significantly altered. Moreover we provide suggestive evidence
that this enzyme remains functional *in vivo*. Because we know that triploids displaying one third of the wild-type isoamylase activity display a fully wild-type phenotype the mutant enzyme must differ by some other property essential *in vivo* for normal amylopectin synthesis. This work establishes that the high mass multimeric complex has collapsed from a minimum of half to one fourth of its normal size in the mutants. This coincides with a simplification of the zymogram profile from 3 to one fast migrating activity band although only one form might in fact exist *in vivo* in both wild-type and mutant strains. The modification of the isoamylase multimeric architecture is presently the only convincing qualitative difference that we can reasonably offer to explain the mutant phenotype. We therefore propose that the multimeric assembly of isoamylase subunits into a large size complex *per se* is essential to proper enzyme function.

If we do not take into account the increase in amylose content, the residual starch synthesized in the *sta8* mutants displays a structure quite similar to that of wild-type polysaccharide. The increase in amylose and in the long chain content of amylopectin can be attributed either to a relative decrease of the rate of amylopectin synthesis or to an increase in malto-oligosaccharide and (or) ADP-glucose levels which define mechanisms documented to control amylose content and synthesis by GBSSI (Leloir et al., 1961, Denyer et al., 1996, Van den Koornhuyse et al., 1996, van de Wal et al., 1998). In line with the absence of modification that we now report for the mutant enzyme's substrate specificity is the observation that the distribution in size of the small and intermediate chains of amylopectin has not significantly changed. This result is at variance with that reported for maize and rice where mutations in a gene encoding an isoamylase subunit essential for catalytic activity are reported to significantly modify the residual amylopectin. It is also at variance with the deeply modified structure of the granular polysaccharide found in *sta7* mutants of Chlamydomonas that equally lack the major isoamylase catalytic subunit (Dauvillée et al., 1999)

We believe that the presence of a significantly reduced amount of otherwise normal amylopectin points to a reduction in the rate of normal amylopectin synthesis due to a severe dysfunction of the isoamylase complex. We do not believe that the normal function of isoamylase is impeded completely in the mutants but rather that the reaction simply slows down to the point where it becomes limiting for amylopectin synthesis while simultaneously allowing the formation of phytoglycogen.

Isoamylase does not directly control the steady-state levels and structure of phytoglycogen

We have proven that the steady-state levels and phytoglycogen structure are identical both in *sta7* mutants lacking all isoamylase activities and in *sta8* mutants that still contain a significant amount of biologically active enzyme. In addition we have proven that the isoamylase activity remaining in the *sta8* mutant debranches phytoglycogen *in vitro* with an efficiency that is similar to that of the wild-type enzyme. Taken together these results make a very strong case against isoamylase being a rate controlling factor responsible for down regulating phytoglycogen production. This conclusion is also supported by the measures of identical rates of phytoglycogen breakdown *in vivo* in strains lacking or displaying reduced amounts of isoamylase.

The nature of the *sta7* and *sta8* gene products

Chlamydomonas reinhardtii is presently the only plant system where two loci have been described to control phytoglycogen production. The exact molecular nature of both loci still needs to be established. Gene dosage experiments performed with the *STA7* locus together with the absence the 88 kD isoamylase subunit documented in such mutants clearly point to *STA7* as a structural gene that would encode the 88 kD catalytic subunit of the enzyme. This hypothesis is further supported by the discovery of the molecular nature of analogous mutations in rice and maize (James et al., 1995, Kubo et al., 1999). The nature of the *STA8* gene products remains however elusive. Two equally viable assumptions can be made with respect to this locus. First the phenotype of the Chlamydomonas *sta8* mutants matches precisely that which was reported for a mutant of Arabidopsis defective in a gene known to encode an isoamylase-like subunit (Zeeman et al., 1998). One might thus be tempted to believe *STA8* encodes another isoamylase-like subunit in a heteromultimer enzyme. This subunit would have more a regulatory than a catalytic function within the enzyme

complex in a fashion which is reminiscent of the catalytic and regulatory subunits of ADP-glucose pyrophosphorylase. The potato isoamylase complex has been previously purified to homogeneity and demonstrated to contain at least two distinct subunits (Ishizaki et al., 1983). However only one isoamylase subunit has been demonstrated to exist within the pure rice isoamylase complex (Fujita et al., 1999). It remains however equally possible that the leaf enzyme complexes contain several distinct isoamylases while the cereal endosperm complex would only contain one such subunit. Another possibility would be that *STA8* encodes a protein required for the assembly of the isoamylase complex. In line with such an hypothesis is the co-purification of the maize isoamylase sugary 1 subunit together with bacterial chaperonins upon expression in *E. coli* (Myers personal communication). It must be stressed that while investigations concerning the molecular nature of *STA7* and *STA8* clearly define our next research priorities, the conclusions that we propose in this paper do not require that *STA8* encodes a functional isoamylase subunit.

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CHAPITRE V :

Participation à l'étude du mutant pour l'enzyme disproportionnante de *Chlamydomonas reinhardtii*

Colleoni C, Dauvillee D, Mouille G, Buleon A, Gallant D, Bouchet B, Morell M, Samuel M, Delrue B, D'Hulst C, and others (1999a) Genetic and Biochemical Evidence for the Involvement of α-1,4 Glucanotransferases in Amylopectin Synthesis. *Plant Physiology* **120** : 993-1004

Colleoni C, Dauvillee D, Mouille G, Morell M, Samuel M, Slomiany MC, Lienard L, Wattebled F, D'Hulst C, Ball S (1999b) Biochemical Characterization of the *Chlamydomonas reinhardtii* α-1,4 Glucanotransferase Supports a Direct Function in Amylopectin Biosynthesis. *Plant Physiology* **120** : 1005-1014

Avant-propos

Une partie de mon travail de thèse s'est porté sur l'étude d'un nouveau locus décrit chez *Chlamydomonas reinhardtii* : le locus *STA11*. Les analyses de la souche mutante indiquent que l'enzyme disproportionnante (une α-1,4 glucanotransférase) codée par ce locus joue un rôle essentiel lors de la synthèse correcte de l'amylopectine. Ma contribution à cette étude porte sur les points suivants :

- Mise au point de la méthode de purification de l'activité enzymatique
- Analyse génétique de la mutation et en particulier, vérification de la coségrégation du phénotype mutant et l'absence de l'α-1,4 glucanotransférase
- Mise au point d'une méthode de zymogramme permettant la visualisation de l'activité sur gel en conditions dénaturantes.
- Analyses structurales des polysaccharides produits par le mutant

Ce travail n'ayant pas un lien direct avec le sujet de ce mémoire, nous n'approfondirons pas les résultats obtenus mais le lecteur pourra découvrir ci après deux articles publiés dans la revue Plant Physiology, faisant état de tous les résultats biochimiques et génétiques obtenus suite aux études portant sur les souches mutantes au locus *STA11*.

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Genetic and Biochemical Evidence for the Involvement of α-1,4 Glucanotransferases in Amylopectin Synthesis[1]

Christophe Colleoni, David Dauvillée, Gregory Mouille, Alain Buléon, Daniel Gallant, Brigitte Bouchet, Matthew Morell, Michael Samuel, Brigitte Delrue, Christophe d'Hulst, Christophe Bliard, Jean-Marc Nuzillard, and Steven Ball*

Laboratoire de Chimie Biologique, Unité Mixte de Recherche du Centre National de la Recherche Scientifique no. 8576, Université des Sciences et Technologies de Lille, 59655 Villeneuve D'Ascq cedex France (C.C., D.D., G.M., B.D., C.d.H., S.B.); Institut National de la Recherche Agronomique, Centre de Recherches Agroalimentaires, Rue de la Géraudière, B.P. 71627, 44316 Nantes cedex 03, France (A.B., D.G., B.B.); Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, G.P.O. Box 1600, Canberra, Australian Capital Territory 2601, Australia (M.M., M.S.); and Laboratoire de Pharmacognosie, Groupe de Glycotechnie, Université de Reims, Moulin de la Housse–BP 1039, 51097 Reims cedex 2, France (C.B., J.-M.N.)

We describe a novel mutation in the *Chlamydomonas reinhardtii STA11* gene, which results in significantly reduced granular starch deposition and major modifications in amylopectin structure and granule shape. This defect simultaneously leads to the accumulation of linear malto-oligosaccharides. The *sta11-1* mutation causes the absence of an α-1,4 glucanotransferase known as disproportionating enzyme (D-enzyme). D-enzyme activity was found to be correlated with the amount of wild-type allele doses in gene dosage experiments. All other enzymes involved in starch biosynthesis, including ADP-glucose pyrophosphorylase, debranching enzymes, soluble and granule-bound starch synthases, branching enzymes, phosphorylases, α-glucosidases (maltases), and amylases, were unaffected by the mutation. These data indicate that the D-enzyme is required for normal starch granule biogenesis in the monocellular alga *C. reinhardtii*.

Starch is one of the most abundant biological polymers present in the earth's biosphere and remains the major supply of calories in both human and animal diets. As is the case for animal, fungal, and bacterial glycogen, starch is made solely of Glc residues linked in α-1,4 positions and branched in α-1,6 positions. Unlike glycogen, starch granules consist of complex, semicrytalline structures of unlimited size (for review, see Buléon et al., 1998). Amylopectin, the major polysaccharide fraction of starch, is considered to be the only molecular fraction required to generate normal granules. The building of the polymer structure depends on the transfer by SS of Glc in the α-1,4 position from ADP-Glc to the nonreducing end of growing chains. Introduction of the α-1,6 branch proceeds through the cleavage (by branching enzyme) of a pre-existing α-1,4-linked glucan and the transfer of the cleaved glucan in the α-1,6 position. It was previously thought that the specific features of starch structure depended solely on the concerted action of multiple forms of SS and branching enzyme; however, other enzymes are likely to be involved in the process.

Our strategy consisted of isolating mutants defective in amylopectin synthesis, thereby defining the functions involved in starch granule biogenesis. From the analysis of mutants of the monocellular green alga *Chlamydomonas reinhardtii* (Mouille et al., 1996), maize (James et al., 1995), rice (Nakamura et al., 1996), and Arabidopsis (Zeeman et al., 1998b), it was determined that debranching enzymes were required to trim α-1,6 linkages from a precursor (pre-amylopectin) into a mature amylopectin molecule (Ball et al., 1996) or that they were required to prevent glycogen production by the starch synthesis machinery (Zeeman et al., 1998b). Continued genetic analysis is likely to identify additional enzymes needed for starch biosynthesis that would not have been foreseen from the basic biosynthetic steps (Mouille et al., 1996).

Eukaryotic algae are of particular relevance for studies dealing with starch synthesis. Starch polysaccharides are not found in bacteria or fungi. Because *C. reinhardtii* is the only starch-storing unicellular organism intensively studied by geneticists, it offers a unique opportunity to understand the basic mechanisms of starch granule biogenesis. Indeed, growth-arrested *C. reinhardtii* cells accumulate glucopolysaccharides that are very similar to cereal endosperm storage starch, so this organism serves as a highly useful model for starch synthesis in crop plants. *C. rein-*

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* Corresponding author; e-mail steven.ball@univ-lille1.fr; fax 33-3-20-43-65-55.

Abbreviations: APTS, 8-amino-1,3,6-pyrenetrisulfonic acid; D-enzyme, disproportionating enzyme; DP, degree of polymerization; GBSS, granule-bound starch synthase; SEM, scanning electron microscopy; SS, soluble starch synthase; TEM, transmission electron microscopy.

hardtii cells contain the same set of starch biosynthesis enzymes and, most importantly, respond in an analogous fashion to mutations affecting these activities (for review see Ball, 1998).

D-enzymes define α-glucanotransferases that transfer unbranched malto-oligosaccharide groups from a donor α-1,4 glucan of at least three Glc residues (maltotriose) to a recipient oligosaccharide at the final expense of Glc formation (Peat et al., 1956). It is thought that this activity disproportionates short glucans into longer oligosaccharides to facilitate their degradation through phosphorylases or hydrolases (Boos and Schuman, 1998).

This study characterized a novel *C. reinhardtii* mutation, *sta11-1*, which causes decreased levels of starch, modification of amylopectin structure, increased amylose content, modification of granule size and shape, and accumulation of unbranched oligosaccharides. All enzymes previously suspected to be involved in starch biosynthesis are unaffected by the presence of the *sta11-1* mutation; however, *sta11-1* mutants lack an enzyme required for maltotriose hydrolysis. Zymogram analysis established that the missing maltotriose-metabolizing enzyme, as described for higher plants, is a D-enzyme.

These data indicate that α-1,4 glucanotransferases are important components of the amylopectin synthesis machinery in *C. reinhardtii*. The results are generally similar to those regarding debranching enzyme function, because in both instances enzymes thought to be involved in the breakdown of glucopolysaccharides are apparently involved in starch biosynthesis.

MATERIALS AND METHODS

Materials

U-^{14}C Glc-1-P and [D-Glc-U-^{14}C]ADP-Glc were purchased from Amersham. ADP-Glc and maize amylopectin were from Sigma. *Pseudomonas amyloderamosa* isoamylase was from Megazyme (Sydney, Australia). Glc-1-P, rabbit muscle glycogen, yeast hexokinase, yeast Glc-6-P dehydrogenase, and rabbit muscle phosphorylase were obtained from Boehringer Mannheim.

Chlamydomonas reinhardtii Strains, UV Mutagenesis, Growth Conditions, Cytological Observations, and Media

The wild-type reference *Chlamydomonas reinhardtii* strains used in this study were 330 (*mt*$^+$ *arg7 cw15 nit1 nit2*) and 137C (*mt*$^-$ *nit1 nit2*). According to the genotypes of the mutants or segregants tested, diploids were selected by complementation on minimal medium after crossing either with strain A35 (*mt*$^+$ *pab2 ac14*), 37 (*mt*$^-$ *pab2 ac14*), NV314 (*mt*$^-$ *pab2 ac14 sta1-1*), strain 37E-17 (*mt*$^-$ *pab2 ac14 sta3-1*), GST– (*mt*$^-$ *nit1 nit2 sta5-1*), BAFR1 (*mt*$^+$ *nit1 nit2 cw15 arg7-7 sta2–29::ARG7*), or 18B (*mt*$^-$ *nit1 nit2 sta2-1*). The *sta11-1* strains most commonly used for complementation were CO 214 (*mt*$^+$ *nit1 nit2 sta11-1*), CO 29 (*mt*$^+$ *pab2 ac14 sta11-1*), and JV45J (*mt*$^-$ *nit1 nit2 sta11-1*).

UV mutagenesis was performed by irradiating cells at 20% survival using a 254-nm transilluminator (model TS-15, Ultra-Violet Products, San Gabriel, CA) displaying a peak intensity of 7.0 mW cm^{-2}. Irradiation was followed by overnight incubation in high-salt medium in darkness.

All experiments were carried out in continuous light (80 μE/m^2 s^{-1}) in the presence of acetate at 24°C in liquid cultures that were shaken vigorously without air or CO_2 bubbling. Late-log-phase cultures were inoculated at 10^5 cells mL^{-1} and harvested at 2×10^6 cells mL^{-1}. Nitrogen-starved cultures were inoculated at 5.10^5 cells mL^{-1} and harvested after 4 d at a final density of 1 to 2×10^6 cells mL^{-1}. Genetic techniques were as described by Harris (1989a). Standard TAP (Tris acetate phosphate) medium was fully detailed in Harris (1989b), while nitrogen-starved medium (TAP-N) and diploid clone selection were described in Ball et al. (1990, 1991) and Delrue et al. (1992). Fixation and embedding protocols were as described in Dauvillée et al. (1999).

Structural Analysis of Polysaccharides

Wide-angle x-ray diffraction and TEM and SEM analyses were as detailed in Buléon et al. (1997). Gel permeation chromatography of delipidated starch fractions dispersed in 10 mM NaOH was as described in Delrue et al. (1992) and Libessart et al. (1995), and was performed on Sepharose CL2B (Pharmacia). Gel permeation chromatography-purified amylose and amylopectin were debranched with isoamylase. ^{1}H-NMR of gel permeation chromatography-purified starch fractions were as described previously (Fontaine et al., 1993). The APTS-tagged chains produced by isoamylase-mediated debranching were separated by high-resolution slab gel electrophoresis on a DNA sequencer (O'Shea and Morell, 1996). The results obtained were confirmed by capillary electrophoresis analysis of debranched amylopectins obtained from two mutant and two wild-type meiotic offspring. Capillary electrophoresis was carried out as previously described (O'Shea et al., 1998).

Measures of Starch Levels, Starch Purification, and Spectral Properties of the Iodine-Starch Complex

A full account of amyloglucosidase assays, starch purification on Percoll gradients, and λ_{max}, the wavelength of the maximal absorbance of the iodine-polysaccharide complex, can be found in Delrue et al. (1992).

Crude Extract Preparation, Enzyme Assays, Partial Purification of Enzyme Activities, and Zymograms

Soluble crude extracts were always prepared from late-log-phase cells (2×10^6 cells mL^{-1}) grown in high-salt acetate medium under continuous light (80 μE m^{-2} s^{-1}). All assays were conducted in conditions of linearity with respect to time and amount of crude extract. Phosphoglucomutase, ADP-Glc pyrophosphorylase, and phosphorylase activities were monitored by using the standard assays described in Ball et al. (1991) and Van den Koornhuyse et al. (1996). For SS and branching enzymes, the assays used were those described by Fontaine et al. (1993) and Libessart

et al. (1995). GBSS I was monitored as previously described (Delrue et al., 1992) from the starch purified from nitrogen-supplied cultures. α-Glucosidase was monitored by measuring the Glc produced from maltose hydrolysis in sodium acetate, pH 6.5, by the standard Glc-6-P dehydrogenase assay described in Ball et al. (1991). The analysis was completed by zymograms as detailed in Buléon et al. (1997) and in Mouille et al. (1996). For partial purification, a 10% (w/v) protamine sulfate precipitation was applied to 5×10^9 cells in 4 mL of 50 mM sodium acetate, pH 6.0, and 1 mM DTT, loaded on a FPLC anion-exchange column (1.6 × 10 cm, 1 mL min^{-1}, 0–0.5 M NaCl gradient; DEAE Mono-Q, Pharmacia), and 1-mL fractions were collected. Aliquots (300 μL) corresponding to each fraction were stored at −80°C, while the different enzyme activities were detected by our standard enzyme assays or zymogram procedures.

D-enzyme activity was first monitored by measuring the production of Glc from maltotriose as follows. Amounts of crude extracts corresponding to 20 to 200 μg of total protein buffered in sodium acetate, pH 6.5, in a 1-mL final volume were incubated from 5 to 30 min at 30°C with 50 mM maltotriose. Glc was monitored by the Glc-6-P dehydrogenase assay as previously described (Ball et al., 1991).

The activity was followed on zymograms under either denaturing or native migration conditions. Denaturation of the extract was as previously described (Mouille et al., 1996). The equivalent of 50 to 200 μg of undenatured or denatured crude extract protein was loaded on a 30:1 (acry: bis), 7.5% (w/v) acrylamide, 1.5-mm-thick polyacrylamide gel (native conditions) or in a similar gel containing 0.1% (w/v) SDS (denaturing conditions). Migration at 15 V cM^{-1} was performed for 90 min at 4°C. At the end of the run, the denaturing gels were first subjected to renaturation as described in Mouille et al. (1996). The renatured and native gels were incubated overnight in the dark at room temperature in 30 mL of 3 mg mL^{-1} maltotriose; 200 mM Tris/HCl pH 8.0, 1 mM EDTA, 42 mM $MgCl_2$, 0.014% (w/v) NADP, 0.027% (w/v) NAD, 0.027% (w/v) MTT, 0.015% (w/v) PMS, 1.5 mM ATP, 1 unit mL^{-1} hexokinase, and 0.5 unit mL^{-1} Glc-6-PDH. The gel was subsequently rinsed with distilled water and photographed.

To ascertain that the 62-kD Glc-producing activity displayed D-enzyme activity, we cut off 5-mm-wide strips of gels containing the renatured activity and incubated them in 0.5 mL of buffer containing 2 mM Glc, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose. After overnight incubation, the gel strip was discarded and the buffer was lyophylized and redissolved in 50 μL of distilled water to be spotted immediately on a TLC plate (Silica Gel 60, Merck) in butanol:ethanol: water (5:5:4), and revealed by orcinol sulfuric staining.

Oligosaccharide Purification

Malto-oligosaccharides were prepared from 3 L of nitrogen-starved culture, inoculated at 10^5 cells mL^{-1}, and harvested after 7 d of growth under continuous light (80 μE m^{-2} s^{-1}) on high-salt acetate medium (Harris, 1989b) with gentle shaking. Algae were ruptured by passage in a French press cell (10,000 p.s.i.) at a density of 10^8 cells mL^{-1} in water. The crude extract was immediately frozen at −80°C. After thawing, cell debris were discarded by centrifugation at 10,000*g* for 15 min at 4°C. The supernatant was boiled for 5 min to inactivate enzymatic activities, and centrifuged at 10,000*g* for 15 min. The supernatant was then lyophilized, and the lyophilized material was resuspended in 1 mL of distilled water and loaded onto a Dowex 50:2 (1- × 6-cm) column immediately coupled to a Dowex 1:2 (1- × 6-cm) column equilibrated with water. Four microliters of each fraction (1 mL) was subjected to TLC (Silica Gel 60 column). Malto-oligosaccharides were revealed by spraying with orcinol (2 g L^{-1}) dissolved in 20% (v/v) sulfuric acid. The TLC plate was subsequently incubated at 80°C for 10 min. After pooling and neutralization with one drop of 30% ammonium hydroxide, the pool was concentrated by rotary evaporation and desalted by gel filtration chromatography on a column (TSK HW-40, Merck) equilibrated in 0.5% (w/v) acetic acid. The desalted material was concentrated by rotary evaporation and lyophilized. The sample was kept at room temperature until it was subjected to NMR analysis.

Gene Dosage Experiments

Diploid and triploid strains were constructed as follows. To obtain the homozygous mutant diploid, we crossed CO 216 (*mt⁻ ac14 nit1 nit2 sta11-1*) and CO 27 (*mt⁺ pab2 nit1 nit2 sta11-1*) and selected the diploid after 4 d of growth on minimal medium supplied with ammonium. Vegetative diploid strains heterozygous for mating type display an *mt⁻* mating type. After checking the phenotype, cellular volume, protein content, and mating type, we crossed the homozygous mutant either with CO 29 to obtain the homozygous triploid mutant or with strain 37 to obtain the *sta11-1/sta11-1/+* triploid. To obtain the homozygous wild-type diploid, we crossed CO 218 (*mt⁻ ac14 nit1 nit2*) and CO 42 (*mt⁺ pab2 ac14*). The colonies were selected on

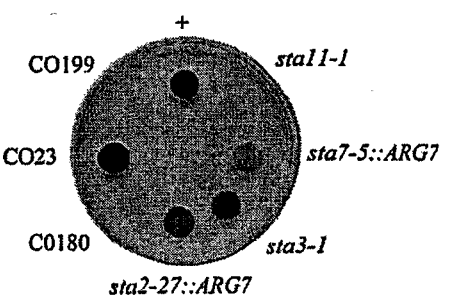

Figure 1. Wild-type and mutant iodine-staining phenotype. Iodine stain of cell patches incubated for 7 d on solid nitrogen-deprived medium. Genotypes with respect to starch are indicated for our reference strains. *sta2–27::ARG7*, *sta3–1*, and *sta7–5::ARG7* correspond to highly specific defects respectively in GBSS, SS, and debranching enzyme (Delrue et al., 1992; Fontaine et al., 1993, Mouille et al., 1996). +, Wild type. The original mutant strain JV45J (*sta11-1*) and two recombinants, CO199 and CO180, display the typical yellow stain of low-starch mutants, while the wild-type recombinant CO23 shows the typical dark-blue color of the wild-type reference strain.

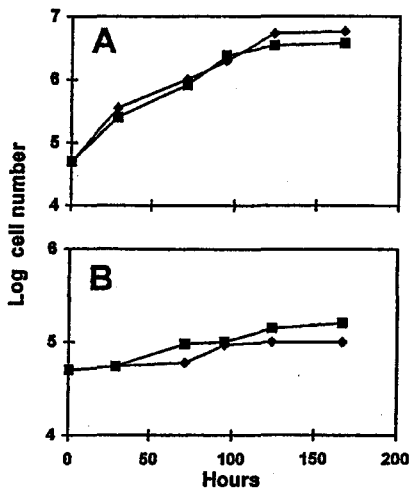

Figure 2. Growth curves of wild-type and mutant *sta11-1* strains. Unsynchronized precultures of three mutant and three wild-type progeny from a cross involving the mutant JV45J and the wild-type strain 37 were grown in TAP medium (Harris, 1989b) to late log phase at 2×10^6 cells mL^{-1}. The cultures were inoculated at 5×10^4 cells mL^{-1} and subjected to a 12-h d (80 $\mu E\ m^{-2}\ s^{-1}$)/12-h night cycle of culture at 20°C with vigorous shaking. A, TAP medium (with acetate). Average mutant cell counts (■) and wild-type (♦) cell counts are displayed as log of cell number versus time. B, TP medium (without acetate). Average mutant cell counts (■) and wild-type (♦) cell counts are displayed as log of cell number versus time. The cultures are severely CO_2 limited under these conditions and therefore grew at a very slow but comparable rate.

minimal medium supplied with acetate using nitrate as the nitrogen source (Ball et al., 1991).

The homozygous wild-type diploid was crossed with CO 35 (*mt⁺pab2 nit1 nit2*) to obtain the triploid wild-type (+/+/+) and with CO 26 (*mt⁺pab2 nit1 nit2 sta11-1*) to obtain +/+/*sta11-1*. The triploid strains were selected on minimal medium with nitrate as the nitrogen source. The heterozygous diploid strain was obtained by crossing JV45J and 37, and was selected on minimal medium with nitrate as the

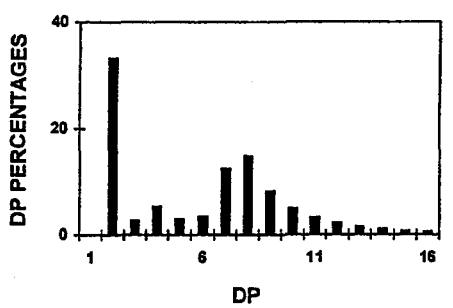

Figure 3. Relative frequency distributions of oligosaccharides. Undebranched malto-oligosaccharides accumulated by the *sta11-1* reference strain JV45J were separated according to length after APTS fluorescence labeling and separation on a DNA sequencer. Percentages of chains ranging between a DP of 1 to 16 (chains containing 1–16 Glc residues) are scaled on the *y* axis.

Table I. *Malto-oligosaccharide purification table*

Extract	Polysaccharide Quantity[a]	Yield
	mg	%
Crude extract	3.1	100
Lyophilizate	2	65
Dowex 50:2, 1:2	1.5	50
TSK HW40	1.43	46

[a] Polysaccharide amounts were measured by the standard amyloglucosidase assay. The purification reported here was from 3-L cultures of nitrogen-starved mutant strain JV45J.

nitrogen source. After selection on the appropriate medium, the haploid, diploid, or triploid nature of the clones was confirmed by retesting the phenotypes and measuring both the average cell volume distribution and the cell protein content from unsynchronized cultures. We also confirmed the mating types of the clones. For each construct we selected three independent clones. Gene dosages are thus averages from three separate colonies for each construct. Phosphoglucomutase activity was assayed (Ball et al., 1991) and used as an internal standard during these experiments.

RESULTS

Isolation and Characterization of Low-Starch Mutants

Among 5×10^4 colonies screened by the standard iodine-staining technique after UV mutagenesis of a wild-type *C. reinhardtii* strain (137C), we isolated a low-starch mutant (JV45J) that accumulated 8% of the normal amount of

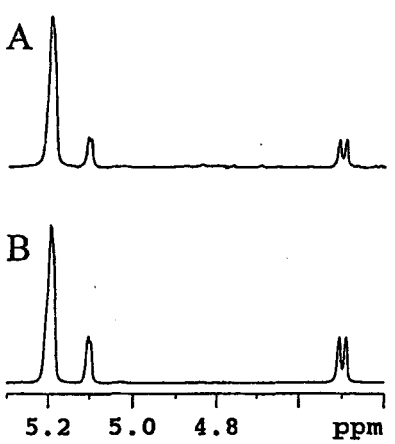

Figure 4. ^{1}H-NMR analysis of malto-oligosaccharides. Part of the ^{1}H-NMR spectra of amylopectin in dimethyl-sulfoxyde-*d6*/^2H_2O (80:20) at 80°C is displayed. The chemical shifts for the α- and β-anomers of the reducing end are at 5.1 and 4.5 ppm, respectively. If present, α-1,6-linkage anomeric protons should be found at 4.85 ppm. The integration area of the α- and β-anomers reducing end signals divided by that of the α-1,4-linkage anomeric proton at 5.2 ppm yields the average DP of the sample. NMR conditions were similar to those described in Delrue et al. (1992). A, Malto-oligosaccharides purified from strain JV45J. B, Maltotriose reference.

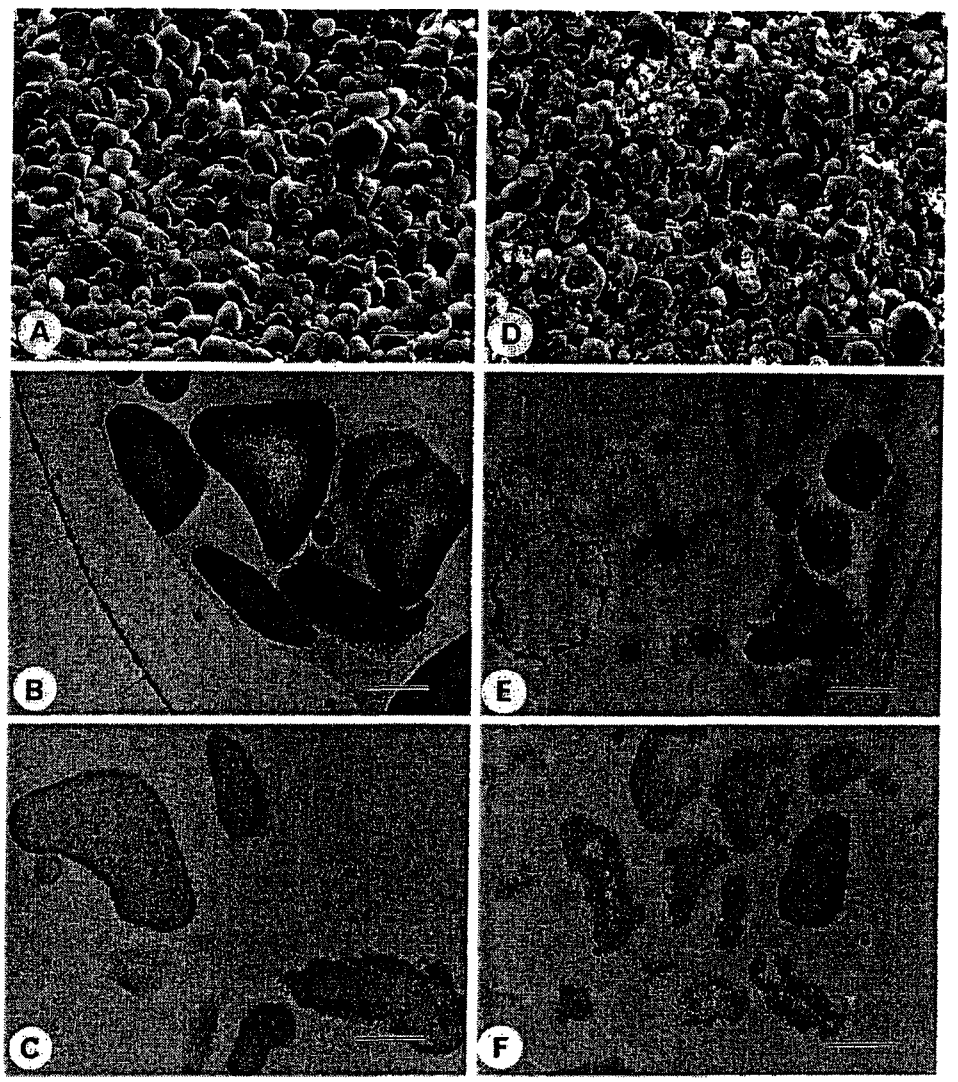

Figure 5. SEM and TEM of starches from wild-type and mutant *sta11-1* strains. Electron micrographs of purified starches and in cells from nitrogen-starved wild-type (137C) and mutant *sta11-1* (JV45J) *C. reinhardtii* strains. A to C, Wild-type strain 137C; D to F, mutant *sta11-1* (JV45J) strain. A and D, SEM of purified starches (bar = 2 μm); B and E, TEM of starch-containing cells after PATAg staining (bar = 0.5 μm); C and F, TEM of purified starches after PATAg staining (bar = 0.5 μm).

starch under conditions of maximum synthesis (Fig. 1). The defect behaved as a standard single-recessive Mendelian trait upon crossing. In addition, all diploids generated by crossing with reference mutant strains carrying a defect in the *STA1, STA2, STA3, STA4, STA5, STA6, STA7,* or *STA8* genes proved to be wild type for starch amount and structure. Therefore, we defined a novel genetic locus, which we named *STA11*. In addition to starch, the *sta11-1* mutants accumulated a significant amount (2% of the amount of starch in a wild-type strain) of water-soluble, amyloglucosidase-digestible material. No growth defects could be detected upon growing the mutants for over a week in continuous light with or without acetate or under a 12-h day/12-h night cycle with or without acetate (Fig. 2).

Characterization of the Water-Soluble, Amyloglucosidase-Digestible Material

We found no evidence for the presence of high-mass, water-soluble polysaccharides. Indeed, the amount of water-soluble amyloglucosidase-digestible material excluded from the gel permeation chromatography column was less than 1% of the total water-soluble polysaccharide found in crude extracts. The water-soluble polysaccharide

thus consisted solely of α-1,4-linked glucans, the size distribution of which is displayed in Figure 3. These glucans were further purified (Table I) and subjected to a detailed structural characterization. The size distribution before and after purification was identical. The mix of oligosaccharides generated an averaged ^{1}H-NMR spectrum very similar to that of maltotriose standards (Fig. 4). As demonstrated by ^{1}H-NMR analysis, branches, if present, fell below our detection level (1%). Co-segregation between the oligosaccharide fraction and the low-starch phenotype was found among all *sta11-1*-carrying strains tested (n = 50).

Characterization of the Residual Mutant Starch

The residual starch structure was monitored through a variety of techniques, including wide-angle x-ray diffraction analysis (not shown), TEM and SEM (Fig. 5), separation of amylose and amylopectin through gel permeation chromatography (Fig. 6), and enzymatic debranching of the purified amylose and amylopectin (Fig. 7). The starch granules showed a significant relative increase in amylose (Table II). The distributions displayed in Figure 6, A and B, suggested a decrease in the mass of the amylose fraction. This was confirmed by mixing a low amount of labeled mutant starch with a high amount of wild-type reference polysaccharide on the same column. The x-ray diffractograms switched from the wild-type A-type lattice with high crystallinity to a mix of A- and B-type lattices with very low crystallinities. The shape of the granules was particularly affected, as illustrated in Figure 5.

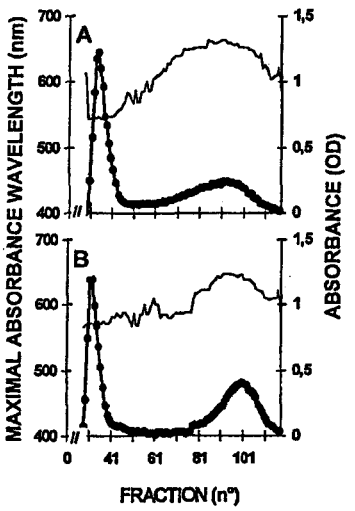

Figure 6. Separation of amylopectin and amylose by CL2B-Sepharose chromatography. The optical density (●) was measured for each 2-mL fraction at λ_{max} (unbroken thin line). All samples were loaded on the same column setup described by Delrue et al. (1992). The wild-type haploid 137C strain starch extracted from nitrogen-starved cultures (storage starch) (A) displays both amylopectin and low-M_r amylose. B, Starch from the mutant strain JV45J carrying the *sta11-1* mutation. Starch was also extracted under nitrogen starvation (storage starch). Quantification of amylose and amylopectin ratios was obtained by pooling amylopectin and amylose fractions separately and measuring the amount of Glc through the standard amyloglucosidase assay.

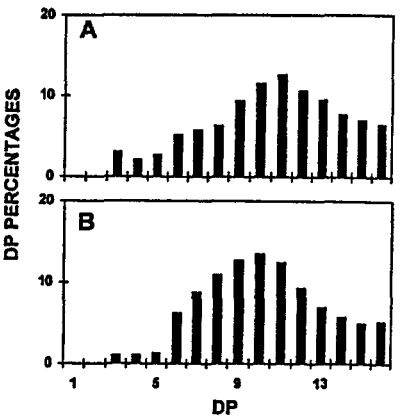

Figure 7. Chain-length distributions of wild-type and mutant amylopectin. Isoamylase-debranched chains were separated according to length after APTS fluorescence labeling and separation on a DNA sequencer. Percentages of chains ranging between DP 1 to 16 (chains containing 1–16 Glc residues) are scaled on the y axis. A, Debranched chains of gel permeation chromatography-purified amylopectin from the mutant JV45J. B, Debranched chains from gel permeation chromatography-purified *C. reinhardtii* reference amylopectin (extracted from the amylose-free BAFR1 strain).

In addition, the starch granules decreased their overall mean size and the regularly shaped smooth surface became rugged and highly irregular. The purified amylopectin and amylose were subjected to enzymatic debranching, labeling of the reducing ends by APTS, and separation of the chains according to length on DNA sequencing gels. While no changes were detected in the amylose chain-length distributions, a significant modification of the very small chain-length distribution (increase of DP 3–5 and, to a lesser extent, DP 12–16 at the expense of DP 6–11) could be detected (compare Fig. 7A with Fig. 7B). Capillary electrophoresis was performed to confirm those differences between four wild-type and four mutant strains. An example is shown for four of these strains (Fig. 8), and results are summarized by subtractive analysis in Figure 9. Figure 9 clearly confirms the enrichment found in the mutants in the extra-short glucan range (DP 3–5 at the expense of DP 6–11). The increase previously noted for DP 12 to 14, while significant, is not far from the natural variation range for each genotype class. These results were confirmed on the two other pairs of wild-type and mutant recombinants.

Finding the Enzymatic Defect

A detailed enzymological analysis was done in crude and partially purified extracts for all major enzymes reported to be involved in starch biosynthesis. This study involved quantitative and qualitative measures of enzyme activities, together with kinetic characterizations and investigations of the elution behavior during FPLC on anion-exchange columns. The enzymes tested include ADP-Glc

Table II. *Phenotype of wild-type and mutant strains during storage or transitory starch synthesis*
Values listed are average of three separate measures in a single experiment.

Strain	Genotype	λ_{max}[a]		Starch[b]		MOS[c]		Am[d]	
		+N	−N	+N	−N	+N	−N	+N	−N
								%	
CO23	+	566	560	1.2	13	0.011	0.018	1	14
CO65	+	570	542	0.4	24.7	0.007	0.001	2	15
CO35	+	564	554	0.91	22.8	0.012	0.017	5	25
CO29	*sta11-1*	576	570	1	1.7	0.45	0.45	7	38
CO137	*sta11-1*	572	564	2	0.83	0.3	0.17	10	25
CO214	*sta11-1*	575	562	0.31	0.78	0.2	0.2	12	24

[a] Wavelength of maximal absorbance of the iodine-polysaccharide complex of amylopectin purified by gel filtration. [b] Amount of insoluble polysaccharide, expressed in $\mu g\ 10^{-6}$ cells, purified through sedimentation as measured by the standard amyloglucosidase assay. [c] Amount of soluble malto-oligosaccharides expressed in $\mu g\ 10^{-6}$ cells. 0.5 $\mu g\ 10^{-6}$ cells corresponds to a plastidial concentration of 10 mM if all MOS was to be considered as maltotriose. [d] The percentage of amylose in the purified starch was calculated after gel filtration of the dispersed polysaccharides.

pyrophosphorylase, phosphoglucomutase, SS I, SS II, GBSS, both types of branching enzymes, debranching enzymes (limit-dextrinase and isoamylase), phosphorylases, maltase (α-glucosidase), and all starch hydrolases that could be detected in starch-containing zymogram gels (Table III).

No qualitative nor quantitative differences in these enzyme activities were found to co-segregate with the mutant gene. However, when we applied the standard assay used in plants to measure D-enzyme activity, we were surprised to find a 99% decrease in the rate of Glc production from maltotriose (Table III). The mutants were also clearly defective in the production of Glc from maltotetraose, maltopentaose, maltohexaose, and maltoheptaose. However, the production of Glc from maltose was comparable in mutant and wild-type strains. This production amounted to less than 5% of that measured with maltotriose in the wild-type progeny. We therefore assume that the production of Glc from maltoriose by α-glucosidase or other hydrolases is negligible in face of the amount of D-enzyme activity present in our extracts. This result is similar to that reported for higher plant leaf extracts (Zeeman et al., 1998a).

Glc production from maltotriose thus defines a quantitative and specific assay for D-enzyme activity in *C. reinhardtii* extracts. We adapted the zymogram techniques used to detect Glc production to assay the action of D-enzyme on maltotriose (Fig. 10). We were able to detect the activity after denaturation and renaturation, which allowed us to estimate the mass of the enzyme. This 62-kD band was absent in all meiotic progeny bearing the mutation ($n = 75$) and was systematically present in wild-type strains. To make sure that the 62-kD band contained oligosaccharide-disproportionating activity, we incubated gel slices in buffer containing Glc, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, or maltoheptaose (not shown). The lyophilized incubation buffers were then spotted onto TLC plates and the oligosaccharides revealed by the orcinol-sulfuric acid method (Mouille et al., 1996). The 62-kD band obeyed the rules set years ago by Peat et al. (1956) to define D-enzymes: it could not use Glc or maltose as sole substrates, but could disproportionate α-1,4-linked oligosaccharides of at least three Glc residues into longer oligosaccharides.

Gene Dosage Experiments

We were able to monitor the amount of enzyme activity present within the plastids (8.7 μmol of Glc formed from maltotriose $min^{-1}\ mL^{-1}$) or to perform gene dosage experiments in diploid and triploid zygotes. The enzyme activity correlated with the relative amount of wild-type alleles, as would be expected if *STA11* encoded D-enzyme (Fig. 11). Knowing the cell and organelle volumes of the strains used in this work, we were able to calculate the physiological enzyme concentrations (Schötz et al., 1972). We were also able to estimate the amount of malto-oligosaccharides in wild-type (50–200 $\mu g\ mL^{-1}$ of chloroplast) and mutant (0.5–1 $mg\ mL^{-1}$) strains.

The Expression of the Mutant Phenotype Is Partly Conditional

We have previously noted that expressivity of mutant phenotypes can vary when transitory starch is compared with the more classical storage form of the polysaccharide (Libessart et al., 1995). In *C. reinhardtii*, storage starch synthesis is obtained by using nutrient starvation conditions, leading to the arrest of cell division and to the accumulation of starch. On the other hand, transitory starch synthesis is obtained under conditions of active photosynthesis and cell division. Mutant phenotypes of *C. reinhardtii* fall into three classes. The first class concerns mutations whose phenotypes express themselves equally in both physiological conditions. Mutations affecting the small and large subunits of ADP-Glc pyrophosphorylase, GBSS, and debranching enzyme fall within this class (Ball et al., 1991; Delrue et al., 1992; Libessart et al., 1995; Mouille et al., 1996; Dauvillée et al., 1999). The functions thereby defined are said to be mandatory for either amylose or starch synthesis.

The second class contains mutations expressed in both conditions but with lesser expressivity upon transitory starch. These include mutations affecting the major SS

(Libessart et al., 1995). These functions are thus involved in the process of normal starch synthesis, but cannot be defined as mandatory in *C. reinhardtii*. We believe this to be the result of some level of functional redundancy due either to the presence of multiple enzyme forms belonging to same family or to the presence of alternative pathways such as those defined by hydrolysis or phosphorolysis. Finally, some loci, when defective, lead to a detectable phenotype only in storage conditions. Their expression is said to be conditional. This applies to the high-amylose *sta4* mutants, for which no enzymatic defect has yet been reported (Libessart et al., 1995).

We compared the phenotypes recorded on the wild-type and mutant *sta11-1* offspring during nitrogen starvation

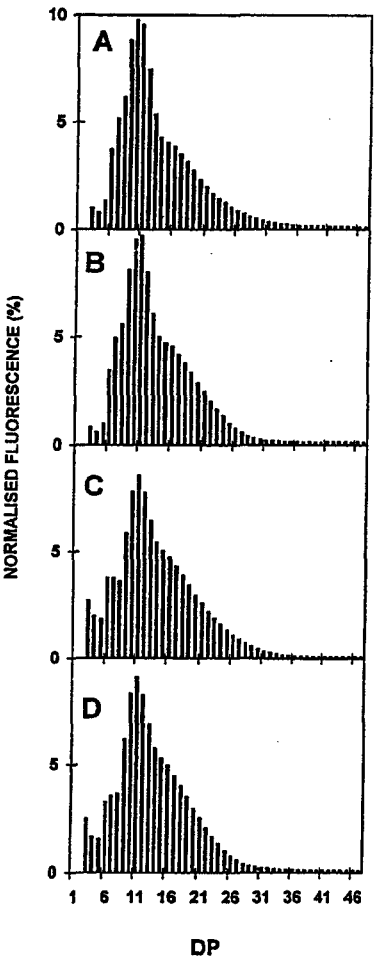

Figure 8. Chain-length distributions of amylopectin from wild-type and mutant strains. Distribution of chain lengths of wild-type and mutant amylopectin after isoamylase-mediated debranching were confirmed by capillary electrophoresis of APTS-labeled glucans following a procedure previously described (O'Shea et al., 1998). The relative amount of chains corresponding to each DP is strictly equivalent to the normalized fluorescence percentage. A and B correspond to wild-type strains 137C (A) and CO65 (B), while C and D correspond to *sta11-1*-carrying strains JV45J (C) and CO29 (D).

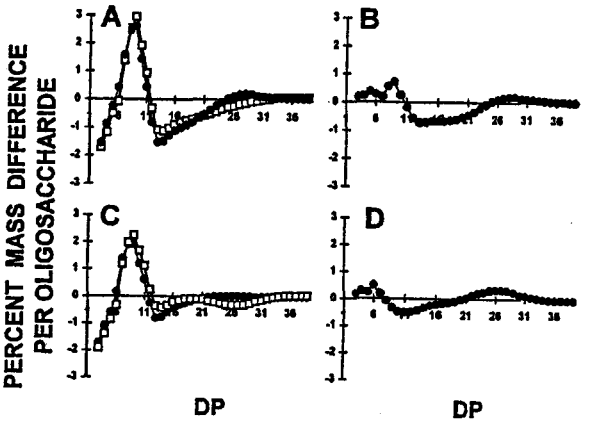

Figure 9. Comparison of the normalized masses of APTS-labeled oligosaccharides from isoamylase debranched amylopectin. The percentage difference of the total mass present in each individual oligosaccharide has been obtained by subtracting the chain-length distribution from debranched amylopectins. A, Subtractive analysis from the wild-type reference strain 137C minus that of the mutant *sta11-1* JV45J (□) and from the wild-type strain 137C minus that of the mutant *sta11-1* CO29 (●). B, Subtractive analysis from the wild-type reference strain 137C minus that of the wild-type strain CO65 (●). C, Subtractive analysis from the wild-type reference strain CO65 minus that of the mutant *sta11-1* JV45J (□) and from the wild-type strain CO65 minus that of the mutant *sta11-1* CO29 (●). D, Subtractive analysis from the mutant reference strain JV45J minus that of the mutant CO29 (●).

with those obtained during normal growth. An example of such an analysis is displayed in Table II. The results clearly define the *sta11-1* mutant defect as partly conditional, and therefore it belongs to the second class. Maximal expression of the defect is obtained under nitrogen starvation, where decreases in starch exceeding 90%, together with increases in amylose percentage and malto-oligosaccharide content, are obtained. However, only the increases in malto-oligosaccharide and amylose percentages are systematically recorded in undepleted medium. We believe this defines *STA11* as being required but not mandatory for normal starch biosynthesis.

DISCUSSION

It was recently reported that a 98% reduction in D-enzyme activity did not affect starch biosynthesis or structure in potato (Takaha et al., 1998). However, there are several known instances in which potato antisense RNA technology has yet to confirm phenotypes of mutants obtained in a wide variety of starch-synthesizing species. We believe this to be due to the presence in many cases of a sufficient amount of residual wild-type activity to carry out the steps, which could be far from rate controlling. This study establishes a correlation between the disappearance of D-enzyme and major alterations in starch structure and accumulation, and thus raises the possibility that this enzyme is a constituent of the starch biosynthetic pathway.

Table III. *Enzyme activities in wild-type and mutant sta11-1 progeny*

AGPase (assayed in direction of pyrophosphorolysis in the presence of 1.5 mM 3-PGA), starch phosphorylase, and phosphoglucomutase units are expressed in nmol Glc-1-P produced min^{-1} mg^{-1} protein. SS and GBSS are expressed in nmol ADP-Glc incorporated into polysaccharide min^{-1} mg^{-1} protein (SS) or mg starch (GBSSI). Branching enzyme is expressed as nmol Glc-1-P incorporated into polysaccharide min^{-1} mg^{-1} protein (phosphorylase amplification assay). Limit-dextrinase and D-enzyme are expressed in nmol maltotriose formed from pullulan min^{-1} mg^{-1} protein and nmoles Glc formed from maltotriose min^{-1} mg^{-1} protein, respectively. α-Glucosidase activities are expressed in nmol Glc formed from maltose min^{-1} mg^{-1} protein. N/A, Not applicable.

Enzyme	Wild-Type Strains	Mutant Strains	Zymogram
ADP-Glc pyrophosphorylase	3.2 ± 0.2 (4)[a]	4.2 ± 0.2 (6)	No
Starch phosphorylase	20 ± 4 (6)	20 ± 2 (4)	Yes (7, 15)[b]
Phosphoglucomutase	6.7 ± 2.9 (6)	6 ± 2 (4)	Yes (10, 8)
SS	2.4 ± 1 (5)	2.3 ± 0.7 (4)	Yes (9, 9)
Branching enzyme	0.6 ± 0.2 (6)	0.5 ± 0.2 (6)	Yes (6, 4)
GBSS	45 ± 10 (2)	42 ± 8 (3)	No
α-Glucosidase	5.8 ± 0.8 (9)	4.6 ± 1 (7)	No
Limit-dextrinase	0.37 ± 0.1 (6)	0.34 ± 0.1 (6)	No
α-Amylase	N/A	N/A	Yes (22, 20)
Isoamylase	N/A	N/A	Yes (22, 20)
D-enzyme	83 ± 4 (10)	1.3 ± 0.5 (10)	Yes (36, 39)

[a] The numbers in parentheses correspond to the number of different strains examined. [b] The two numbers in parentheses correspond to the number of wild-type and mutant strains examined, respectively.

In the progeny of a cross between wild type and *sta11-1* mutant strains, co-segregation was observed for the phenotypes of D-enzyme deficiency, malto-oligosaccharide accumulation, and specific starch structural defects. That unbranched malto-oligosaccharides accumulated in the mutant progeny was to be expected, because these are the normal substrates of D-enzymes. The relative abundance of maltose to that of longer oligosaccharides fluctuates because of the presence of other starch hydrolases. Maltose production probably results from the action of α-amylase on longer oligosaccharides that would otherwise accumulate in the mutants. However, the major changes in starch biosynthesis in conditions of nitrogen starvation were unexpected in light of what is currently known about the functions of D-enzyme, in particular because these enzymes are usually assumed to be utilized for catabolism of unbranched malto-oligosaccharides. The D-enzyme deficiency co-segregated not only with a reduction in total glucopolysaccharide accumulation, but also with abnormal starch granule shape and size, an altered amylose/amylopectin ratio in the mutant granules, and changes in the chain-length distribution of amylopectin in those granules. To explain these observations we propose that D-enzyme has a specific function in the process of starch biosynthesis.

If the absence of D-enzyme is not responsible for the starch biosynthesis defects, then the alternative explanation requires that *sta11-1*, through pleiotropic effects, causes a deficiency of more than one enzyme. This situation could result from, for instance, the collapse of a specific multienzyme complex or from some complex feedback-regulatory mechanism acting on enzymes that have not yet been characterized. In this view an unidenti-

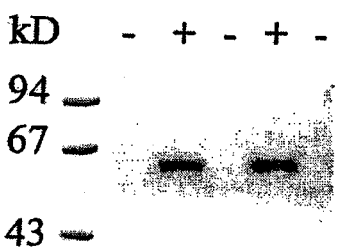

Figure 10. The enzymatic defect of *sta11-1* mutant strains. Denatured crude extracts (100 μg of protein) from two wild-type (+) and three *sta11-1* (−) were loaded on denaturing polyacrylamide gels. The proteins were renatured after electrophoresis and incubated overnight. Glc production was revealed after overnight incubation of the gel with 3 mg mL^{-1} maltotriose. The 62-kD blue-staining band can be easily distinguished in the wild-type cells. This band contained the D-enzyme activity.

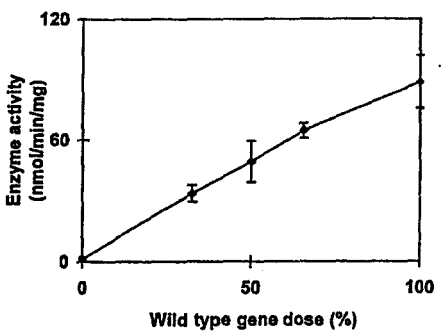

Figure 11. Gene dosages expressed from 0% (homozygous mutant) to 100% (homozygous wild-type); 50% corresponds to the heterozygous diploids, while 33% and 66% correspond to a *sta11-1/sta11-1/+* and a *sta11-1/+/+* triploid respectively. Haploid, diploid, or triploid homozygous combinations gave identical results when the activity was expressed per milligram of protein. Means (♦) and SDs of measures from three different diploid or triploid constructs were calculated for each gene dose. Total phosphoglucomutase-specific activities were monitored as internal controls and proved similar in all constructs (2.5 ± 0.4 nmol Glc-6-P formed from Glc-1-P min^{-1} mg^{-1} protein).

fied enzyme other than D-enzyme is also affected in the *sta11-1* mutant strains, and is required for normal starch biosynthesis. We consider this possibility unlikely because all known enzyme activities in the starch biosynthesis pathway have been monitored very carefully in the mutant strains.

Some minor quantitative and qualitative fluctuations were observed, however, and were found equally in the mutant and nonmutant progeny in the segregating population; therefore, they cannot be relevant to the observed starch biosynthesis phenotype. Furthermore, complementation tests established that *STA11* is distinct from any genetic element known in *C. reinhardtii* to code for or determine activity of an enzyme involved in starch biogenesis, including the genes required for GBSS I, SS II, phosphoglucomutase, the ADP-Glc pyrophosphorylase large and small subunits, and a starch-debranching enzyme. Although this alternative explanation cannot be definitively excluded, the most straightforward explanation of the phenotype caused by *sta11-1* is that D-enzyme itself is required for normal starch biosynthesis.

Gene dosage experiments in diploid and triploid genetic backgrounds established a very good correlation between the amount of D-enzyme activity and the *STA11* wild-type allele dose. These data suggest that *STA11* codes for D-enzyme, although further analysis is required to characterize definitively the nature of this gene. Even if *STA11* is proven to code for D-enzyme, however, the possibility of a pleiotropic effect causing the starch structural defects in *sta11-1* mutants cannot be ruled out definitively. Complementation of the *sta11-1* defect by transgenesis of a wild-type D-enzyme gene is entailed with similar limitations. Gene cloning, however, is inherently difficult in *C. reinhardtii* owing to technical limitations such as the absence of powerful yeast-type shuttle vectors precluding complementation cloning and the absence of complete banks of tagged mutants.

We expect, however, that application of this powerful genetic screening system to identify a previously unsuspected enzyme likely involved in starch biosynthesis enables analysis in plants such as maize or Arabidopsis. In such an application, specific structural gene mutations could be identified by methods such as transposon insertion screening. What is needed at present, before any discussion is made on the mutant phenotype and its relevance to our understanding of starch biosynthesis, is to investigate the D-enzyme wild-type activity in greater detail. In particular, we would like to determine if this enzyme could be directly involved in the building of the wild-type amylopectin structure. D-enzyme has been documented as working essentially on soluble malto-oligosaccharides, and we have thus proceeded to further investigate its activity on amylopectin structure.

ACKNOWLEDGMENTS

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Biochemical Characterization of the *Chlamydomonas reinhardtii* α-1,4 Glucanotransferase Supports a Direct Function in Amylopectin Biosynthesis[1]

Christophe Colleoni, David Dauvillée, Gregory Mouille, Matthew Morell, Michael Samuel, Marie-Christine Slomiany, Luc Liénard, Fabrice Wattebled, Christophe d'Hulst, and Steven Ball*

Laboratoire de Chimie Biologique, Unité Mixte de Recherche du Centre National de la Recherche Scientifique no. 8576, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq cedex, France (C.C., D.D., G.M., M.-C.S., L.L., F.W., C.d.H., S.B.); and Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, G.P.O. Box 1600, Canberra, Australian Capital Territory 2601, Australia (M.M., M.S.)

Plant α-1,4 glucanotransferases (disproportionating enzymes, or D-enzymes) transfer glucan chains among oligosaccharides with the concomitant release of glucose (Glc). Analysis of *Chlamydomonas reinhardtii sta11-1* mutants revealed a correlation between a D-enzyme deficiency and specific alterations in amylopectin structure and starch biosynthesis, thereby suggesting previously unknown biosynthetic functions. This study characterized the biochemical activities of the α-1,4 glucanotransferase that is deficient in *sta11-1* mutants. The enzyme exhibited the glucan transfer and Glc production activities that define D-enzymes. D-enzyme also transferred glucans among the outer chains of amylopectin (using the polysaccharide chains as both donor and acceptor) and from malto-oligosaccharides into the outer chains of either amylopectin or glycogen. In contrast to transfer among oligosaccharides, which occurs readily with maltotriose, transfer into polysaccharide required longer donor molecules. All three enzymatic activities, evolution of Glc from oligosaccharides, glucan transfer from oligosaccharides into polysaccharides, and transfer among polysaccharide outer chains, were evident in a single 62-kD band. Absence of all three activities co-segregated with the *sta11-1* mutation, which is known to cause abnormal accumulation of oligosaccharides at the expense of starch. To explain these data we propose that D-enzymes function directly in building the amylopectin structure.

In plants, the only α-1,4 glucanotransferases reported to be present at the time of starch synthesis are collectively called D-enzymes (Peat et al., 1956; Takaha et al., 1993). D-enzymes act on soluble oligosaccharides at least three Glc residues long (maltotriose) and disproportionate them into oligosaccharides of various lengths at the expense of Glc formation. In such a reaction an α-1,4 linkage is cleaved from a donor unbranched oligosaccharide of at least three Glc residues, and the resulting chain segment is transferred to another acceptor glucan, creating a novel α-1,4 linkage. Maltosyl residues are often transferred as a result of D-enzyme action, but maltose itself is not a product of the reaction. However, both Glc and maltose can be used as acceptors (Jones and Whelan, 1969). It is known that in Arabidopsis leaves, D-enzyme is the major maltotriose-metabolizing enzyme present (Lin and Preiss, 1988; Zeeman et al., 1998). The presence of this activity during potato tuber development has led investigators to suggest that D-enzyme might be required for some specific aspect of starch biosynthesis (Takaha et al., 1993). Like a few other glucanotransferases, such as branching enzyme (Takaha et al., 1996a), D-enzyme has been recently shown to lead to the formation of cyclic compounds after prolonged incubation of both amylose and amylopectin with high amounts of pure activity (Takaha et al., 1996b, 1998). It is not known if this property relates to the physiological function of this enzyme.

D-enzyme is believed to be part of the starch degradation pathway. The disproportionating of small malto-oligosaccharides into longer glucans facilitates their degradation through maltodextrin phosphorylases and glucosidases in a fashion similar to that described for amylomaltase, a similar α-1,4-glucanotransferase required for malto-oligosaccharide assimilation in *Escherichia coli* (for review, see Boos and Shuman, 1998).

We have previously shown that the absence of D-enzyme in *sta11-1* mutants correlates with an accumulation of unbranched malto-oligosaccharides. However, we also found a large decrease in starch content in conditions of maximal starch synthesis, a result that does not fit the catabolic function envisioned for these α-1,4 glucanotransferases. We were surprised to find a modification of amylopectin chain-length distribution. The latter consisted of a relative increase of very small chains that could easily be distinguished from similar distributions witnessed in other mutant amylopectin structures (Fontaine et al., 1993; Libessart et al., 1995). This surprising change correlated with an alteration in granule size morphology and crystallinity. It

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* Corresponding author; e-mail steven.ball@univ-lillel.fr; fax 33–3–20–43–65–55.

Abbreviations: APTS, 8-amino-1,3,6-pyrenetrisulfonic acid; D-enzyme, disproportionating enzyme; DP, degree of polymerization.

also resulted in significant increases in the ratio of amylose to amylopectin. Because no other known enzyme of the starch metabolic pathway was altered in the mutant, all of these phenotypes have to be explained by the disappearance of D-enzyme. The most straightforward explanation would be to assume a direct function of D-enzyme in the building of the amylopectin structure; however, the biochemical evidence supporting such a function remains to be produced.

We demonstrate that the *Chlamydomonas reinhardtii* D-enzyme is active on the outer chains of amylopectin and glycogen. We propose a novel function for the plant α-1,4 glucanotransferase that explains all phenotypic traits observed simultaneously in the *sta11-1* mutants of *C. reinhardtii*.

MATERIALS AND METHODS

Materials

[1,2-^{14}C]Acetic acid (sodium salt) was purchased from Amersham. Maltotriose-forming amylase was purchased from Unipex (Reuil-Malmaison, France). Sweet potato β-amylase and *Pseudomonas amyloderamosa* isoamylase were from Megazyme International (Sydney, Australia). Glc and glucans were assayed through the amyloglucosidase using the starch determination kit from Boehringer Mannheim. Glc, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were from Sigma. Radioactive oligosaccharides were prepared by extracting in vivo-labeled amylopectin from the *Chlamydomonas reinhardtii* mutant strain BAFR1 defective for granule-bound starch synthase I and amylose synthesis (Delrue et al., 1992). The strain was grown in the presence of 500 μCi [1,2-^{14}C] of sodium acetate at 702 μCi mg^{-1}. The culture was starved of nitrogen in TP (Tris phosphate) medium for 5 d. Fifteen percent of the total label was successfully incorporated into amylopectin. The starch was purified through Percoll gradient centrifugation as detailed previously (Delrue et al., 1992), and stored at −20°C. Radioactive oligosaccharides were prepared freshly by debranching 10 mg of in vivo-labeled amylopectin to completion, as described previously (Dauvillée et al., 1999). The specific radioactivity of the debranched oligosaccharides was 1.8 μCi mg^{-1}.

Radiolabeled maltotriose is not available commercially and was prepared as follows. Maltotriose-forming amylase (0.06 unit) from *Microbacterium* sp. was incubated with 500 μg of radioactive oligosaccharide prepared as described above in a 1-mL final volume of 10 mM $CaCl_2$, 10 mM Tris/HCl, pH 7.0. The reaction was incubated for 15 min at 55°C and stopped by heating for 5 min in a boiling-water bath. The maltotriose produced was purified by high-performance anion-exchange chromatography with pulsed amperometric detection as described previously (Fontaine et al., 1993). The yield and purity were 46% and 95%, respectively. Maltotriose was lyophilized and stored at room temperature.

C. reinhardtii Strains, Growth Conditions, and Media

The wild-type reference *C. reinhardtii* strain used in this study was 137C (*mt*$^-$ *nit1 nit2*) and JV45J (*mt*$^-$ *nit1 nit2 sta11-1*). Wild-type and mutant segregants from a cross performed between JV45J and wild-type strain 37 (*mt*$^+$ *pab2 ac14*) have been previously described and were used throughout this work. Starch from the granule-bound starch synthase I mutant BAFR1 (*mt*$^+$ *nit1 nit2 cw15 arg7-7 sta2-29::ARG7*) was used as the source of pure amylopectin.

All experiments were carried out in continuous light (40 μE m^{-2} s^{-1}) in the presence of acetate at 24°C in liquid cultures that were shaken vigorously without air or CO_2 bubbling. Late-log-phase cultures were inoculated at 10^5 cells mL^{-1} and harvested at 2×10^6 cells mL^{-1}. Standard TAP (Tris acetate phosphate) medium was used in this work and was fully detailed in Harris (1989).

Structural Analysis of Polysaccharides

^{1}H-NMR of gel permeation chromatography-purified starch fractions were as described previously (Fontaine et al., 1993). The APTS-tagged chains produced by isoamylase-mediated debranching were separated by high-resolution slab gel electrophoresis on a DNA sequencer (O'Shea and Morell, 1996). Enzymatic debranching of polysaccharides was as previously described (Dauvillée et al., 1999).

Enzyme Purification

Frozen crude extract proteins (500 mg) were cleared by centrifugation and precipitated by 10% protamine sulfate for 20 min at 4°C. The supernatant was submitted to anion-exchange chromatography (Mono-Q columns, Pharmacia) in 50 mM sodium acetate and 2 mM DTT adjusted to pH 6.0 with acetic acid. The unretained fraction was subjected to $(NH_4)_2SO_4$ precipitation at 30% saturation. The supernatant was further precipitated at 50% saturation. The pellet was ressuspended in the same buffer and subjected to gel permeation chromatography on a S100 column (Pharmacia). The pooled fractions containing D-enzyme activity were passed through a cation-exchange column (model UnoS12, Bio-Rad) in the same buffer. The enzyme activity was followed by either the previously described zymogram or by the quantitative D-enzyme assay. The latter consists of measuring the Glc produced from maltotriose by incubating up to 50 μL of sample with 75 μL of 50 mM sodium acetate adjusted to pH 6.0 with acetic acid and 25 μL of an 80 mg mL^{-1} maltotriose solution. The mixture was incubated at 30°C for 15 min and stopped by heating for 3 min in a boiling-water bath. The sample was cleared by centrifugation, and the Glc was assayed by measuring the production of NADPH during the standard hexokinase-Glc-6-P dehydrogenase reaction.

Zymogram Analyses

We used zymogram techniques adapted from the method developed by Lacks and Springhorn (1980), who used α-amylase as a model enzyme to study renaturation

and detection. An in-depth discussion on renaturation-detection techniques can be found in Gabriel and Gersten (1992). Soluble crude extracts were always prepared from late-log-phase cells (2×10^6 cells mL^{-1}) grown in high-salt acetate medium under continuous light (80 $\mu E\ m^{-2}\ s^{-1}$). Algae were ruptured by passage in a French press (10,000 p.s.i.) at a density of 10^9 cells mL^{-1} and immediately stored at −80°C. After thawing, the lysate was cleared by centrifugation at 10,000*g* for 15 min at 4°C. The amount of protein was measured using a protein assay kit (Bio-Rad). Protein (500 μg) in 100 μL of 25 mM Tris-Gly, pH 8.3, 1% (w/v) SDS, 5% (v/v) β-mercaptoethanol was denatured by heating in a boiling-water bath for 5 min.

Our zymogram procedures were established to detect starch hydrolases in starch-containing gels in Mouille et al. (1996). The oligosaccharide-incorporation zymogram technique was modified from these procedures as follows. Denatured proteins were loaded on a 30:1 (acry:bis), 7.5% (v/v) acrylamide, 0.1% (v/v) SDS, 1.5-mm-thick denaturing polyacrylamide gel containing 0.3% rabbit liver glycogen (Sigma). Electrophoresis was performed at room temperature at 15 V cm^{-1} for 90 min using the Mini-Protean II cell (Bio-Rad) in 25 mM Tris-Gly, pH 8.3, 1 mM DTT, and 0.1% (v/v) SDS buffer. At the end of the run, the gel was washed twice with gentle shaking for 1 h in 100 mL of 40 mM Tris at room temperature to remove SDS and to renature the proteins. The gel was incubated overnight in 50 mM Tris, 5 mM EDTA, 10 mM DTT, and 2 mM maltoheptaose (Sigma) at 25°C. The reaction was stopped, and the gel was stained in an aqueous solution containing 0.25% KI and 0.025% I_2.

Enzyme Treatment of Amylopectin

Five milligrams of amylopectin from the *waxy* cultivar of maize was dispersed in 100 μL of 90% DMSO. Seven-hundred microliters of 50 mM sodium acetate, adjusted to pH 6.0 with acetic acid and with an enzyme activity corresponding to 2 nmol of Glc produced from maltotriose per minute, was added subsequently and incubated overnight at 30°C; 500 μg of the sample was further subjected to a β-amylase (17 units) treatment in 50 μL of 50 mM sodium acetate adjusted to pH 4.0 with acetic acid. After 1 h of incubation, another dose of β-amylase was added and the incubation continued for an additional hour. The reaction was stopped and the enzyme inactivated by heating the sample for 5 min in a boiling-water bath. Both the β-amylase-treated and untreated samples were then debranched and analyzed.

Oligosaccharide Incorporation Assay

From 50 to 500 μg of radioactive oligosaccharides, prepared as described in "Materials and Methods," were added to 50 μg to 5 mg of either nonradioactive amylopectin (purified from the same BAFR1 strain) or rabbit liver glycogen in a 1-mL final volume of 50 mM sodium acetate adjusted to pH 6.0 with acetic acid. An activity of D-enzyme corresponding to 106 nmol of Glc produced per minute from maltotriose was added to the sample and incubated for 150 min at 30°C. The activity in the presence of 500 μg of oligosaccharides was previously proven to be strictly proportional to time (up to 4 h) and activity amount (up to 400 nmol of Glc produced per minute from maltotriose). The labeled polysaccharide was then separated from the unincorporated oligosaccharides through gel permeation chromatography on either TSK-HW50 (Merck) for glycogen or on Sepharose CL2B (Pharmacia) for amylopectin. The reverse reaction, transfer of radioactive outer chains from amylopectin to cold oligosaccharides, was monitored at or above the physiological concentration of malto-oligosaccharides (200–500 μg malto-oligosaccharide mL^{-1}) using gel permeation chromatography.

RESULTS

Enzyme Purification

D-enzyme was partially purified by a three-step chromatographic procedure involving anion exchange, selective ammonium sulfate precipitation, and gel permeation and cation exchange. The purified activity displayed a wide pH optimum over the range of 5.0 to 7.5. The activity decreased significantly above pH 9.0. It is worth noting that under our standard conditions we could not detect significant production of Glc from maltotriose in crude extracts from the *sta11-1* mutant, suggesting that D-enzyme was the only enzyme present that was able to metabolize small oligosaccharides under physiological conditions.

After these chromatographic steps, yields of approximatively 2.4% of D-enzyme activity were obtained (Table I). If all fractions containing the activity were harvested, the purification factor measured was 32-fold. However, if low-protein-containing fractions were selected, we were able to bring this factor up to 135-fold, with a 0.5% yield (Table I). The enzyme was freed from all other contaminating starch hydrolases after the second chromatographic step. These contaminating activities were monitored by assaying the fractions on starch-containing zymogram gels (Mouille et al., 1996). The activity was stable when stored for over 6 months at −20°C in purification buffer. We tested the action of the purified 62-kD enzyme separately on Glc, maltose, maltotriose, maltotetraose, maltopentaose, and maltoheptaose (Fig. 1). While the 62-kD α-1,4 glucanotransferase left Glc untouched, it successfully disproportionated all oligosaccharides above DP 3, as shown in Figure 1. The reduced but significant disproportionating activity witnessed with DP 2 was due to the presence of trace amounts

Table I. *D-enzyme purification*

D-enzyme was measured through the release of Glc from maltotriose (see "Materials and Methods").

Purification Step	Purification Factor	Yield
		%
Crude extract	1	100
Protamine sulfate	2.8	40
Mono Q	5	22
S100 + UnoS	32	2.4
Fraction 24	135	0.5

of maltotriose and maltotetraose in the commercial source of maltose. The very low amounts of maltose detected whenever oligosaccharides were successfully disproportionated further confirmed that the enzyme obeys the rules established years ago by Whelan to define D-enzymes (Jones and Whelan, 1969).

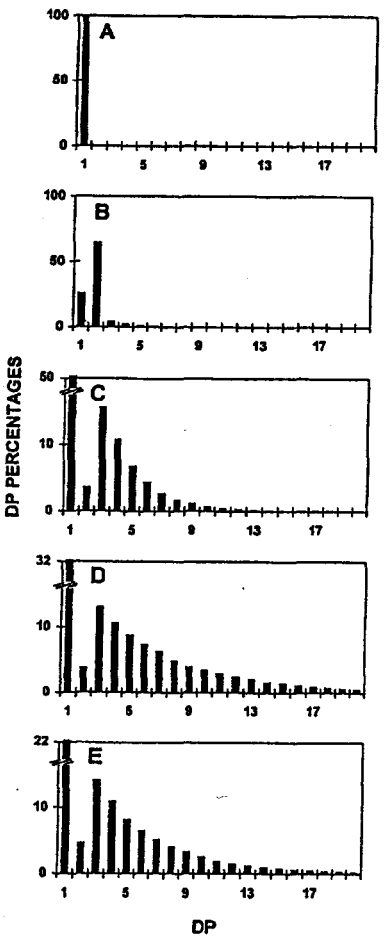

Figure 1. Chain-length distributions of oligosaccharides after incubation with the 62-kD transferase. Undebranched oligosaccharides were separated according to length after APTS fluorescence labeling and separation on a DNA sequencer. Percentages of chains ranging between DP 1 and 20 (chains containing 1–20 Glc residues) are scaled on the *y* axis. A, Undebranched malto-oligosaccharides generated through incubation of Glc with semi-pure *C. reinhardtii* D-enzyme. B, Undebranched malto-oligosaccharides generated through incubation of maltose with semi-pure *C. reinhardtii* D-enzyme. C, Undebranched malto-oligosaccharides generated through incubation of maltotriose with semi-pure *C. reinhardtii* D-enzyme. D, Undebranched malto-oligosaccharides generated through incubation of maltopentaose with semi-pure *C. reinhardtii* D-enzyme. E, Undebranched malto-oligosaccharides generated through incubation of maltoheptaose with semi-pure *C. reinhardtii* D-enzyme.

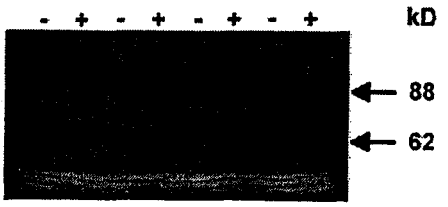

Figure 2. Zymogram analysis. Denatured crude extracts (100 μg of protein) from eight wild-type (+) and *sta11-1* (−) strains were loaded on denaturing polyacrylamide gels containing soluble potato starch (Mouille et al., 1996). The proteins were renatured after electrophoresis and incubated overnight. Starch hydrolases were revealed by iodine staining. The 88-kD blue-staining band was previously proven to be a debranching enzyme. The 62-kD dark-red-staining band can be distinguished from all other starch hydrolases by the absence of gel clearing within the staining region, which is consistent with the absence of oligosaccharide production during polysaccharide modification.

Activity of D-Enzyme toward the Outer Chains of Amylopectin

In the course of performing zymograms under denaturing conditions to detect the various starch hydrolases in *sta11-1* and wild-type strains, iodine treatment of the starch-containing gels (Fig. 2) revealed the absence in the mutant of a band staining dark red. The 62-kD mass deduced from the zymogram fit that of the *C. reinhardtii* D-enzyme (Colleoni et al., 1999). It was absent in all meiotic progeny bearing the *sta11-1* mutation ($n = 75$) and systematically present in wild-type strains. In addition, we noted co-elution throughout enzyme purification of this red-staining band and with both the quantitative D-enzyme assay and our zymogram procedure using maltotriose as a substrate (Colleoni et al., 1999). An example of this co-purification is displayed in Figure 3.

Because we have previously proven that the 62-kD zymogram band contained the D-enzyme activity, and because both D-enzyme and this activity co-purify and are clearly under the control of the same gene, we conclude that the modification of starch structure witnessed in these gels and the disproportionating activity are supported by the same 62-kD protein. Following the technique established in Mouille et al. (1996), polysaccharide was eluted from the bands present in the amylopectin-containing gels and subjected to ^{1}H-NMR analysis. A similar analysis was performed after 135-fold purification of the enzyme activity (see above) and treatment of amylopectin in purification buffer.

The ^{1}H-NMR signals of the incubated amylopectin displayed major changes. The latter consisted of the replacement of the bimodal proton signal at 5.3 to 5.2 ppm by a monomodal signal at the same 5.2 ppm position in our standard NMR conditions (Fig. 4). The relative heights of these two signals distinguishes the ^{1}H-NMR signatures of glycogen and amylopectin (Mouille et al., 1996) and are therefore suspected to reflect differences in chain-length distribution. Enzymatic debranching does not affect the relative heights of these two signals, confirming that chain-length distribution modifications are present rather than increases in the number of branches (Mouille et al., 1996).

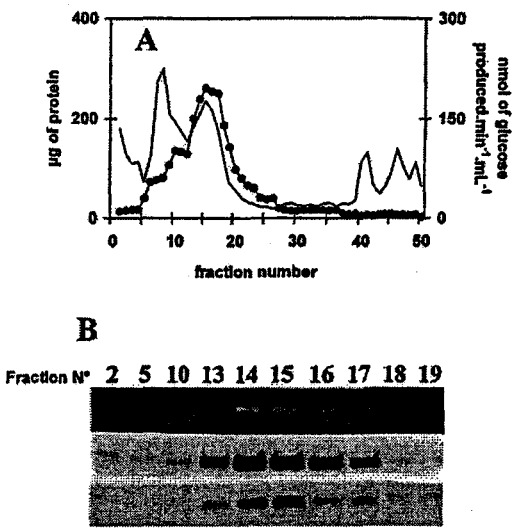

Figure 3. Co-purification of starch structure modification, Glc production from maltotriose, and incorporation of maltoheptaose into the external chains of glycogen. A, Elution profile of FPLC chromatography. In this experiment, 60 mg of crude extract protein (after a protamine sulfate precipitation step) was loaded on an anion-exchange column linked directly to a cation-exchange column, which was only eluted with 5% (w/v) NaCl. Two-milliliter fractions were collected at a 1 mL min^{-1} flow rate. Proteins (thin line) were measured in each fraction according to the Bio-Rad assay. D-enzyme activity (●) was monitored through production of Glc from maltotriose (see "Materials and Methods"). B, Fractions from all cation exchanges were assayed through three distinct zymogram procedures involving denaturation of proteins and renaturation after migration (see "Materials and Methods"). Only the active fractions are shown in B. Three distinct zymogram procedures are displayed. These include modification of starch structure (top), incorporation of maltoheptaose in the outer chains of glycogen (middle), and Glc production from maltotriose (bottom). All active bands were shown to migrate as 62-kD proteins. Note that the dark-red band that characterizes D-enzyme in starch-containing zymograms turns to a white stain upon incubation with a vast excess of enzyme in the concentrated peak fractions.

Upon incubation of amylopectin with the semi-pure D-enzyme, changes were witnessed in the chain-length distribution of the incubated amylopectin (Fig. 5, A and B). We found no evidence of oligosaccharide production by spotting the treated samples on TLC plates as described in Mouille et al. (1996). The amount of amyloglucosidase-resistant material (cyclic glucans) remained under 5% of the total polysaccharide amounts used in these experiments, suggesting that incubation times exceeding 12 h and higher enzyme activities are required to produce these structures. In addition, no release of radioactive material was witnessed when radioactive amylopectin was used as a substrate (see "Materials and Methods").

Because no oligosaccharides were liberated during amylopectin treatment with the 62-kD D-enzyme, and because the amount of α-1,6 linkages remained constant (5%), we conclude that amylopectin acts as an α-1,4 glucanotransferase, cleaving α-1,4 linkages present on donor amylopectin chains and transferring them to the nonreducing end of neighboring acceptor chains. To prove that the transfer reaction involved the outer chains of amylopectin, β-amylase digestions of the incubated polysaccharides were performed on the untreated and incubated amylopectin. The digested polysaccharides were then subjected to enzymatic debranching, yielding identical chain-length distributions for the treated and untreated samples (Fig. 5, C and D). Because β-amylase are processive enzymes that selectively digest the outer chains of the polysaccharides, this result proves that the major modifications seen in Figure 5B are confined to the polymer's external chains.

These dramatic changes are sufficient to explain a shift in color of the iodine polysaccharide complex in starch- or amylopectin-containing gels. Indeed, Banks et al. (1971) have shown that the λ_{max} (the wavelength of the maximal absorbance of the iodine-polysaccharide complex) decreases with the average DP of linear α-1,4 glucan populations. Therefore, changes in exterior chain-length distributions of D-enzyme-treated amylopectin starches are likely to affect the λ_{max} of the resulting polysaccharide. This could explain the typical dark-red stain detected in the zymograms.

D-Enzyme Favors the Incorporation of Oligosaccharides into Polysaccharide Outer Chains

We characterized further the action of D-enzyme in the presence of both amylopectin and unbranched malto-oligosaccharides. We produced ^{14}C labeled malto-oligosaccharides by debranching in vivo-labeled amylopectin. The

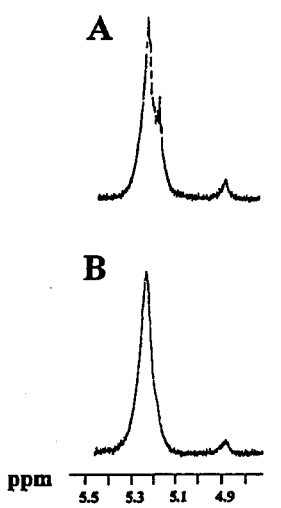

Figure 4. ^1H-NMR analysis of amylopectin incubated with or without the 62-kD transferase. Ten milligrams of maize amylopectin was incubated overnight in 1 mL of 50 mM sodium acetate/acetic acid, pH 6.0, in the absence (A) or presence (B) of 50 µL of semi-pure enzyme fraction corresponding to 2 nmol of Glc produced from maltotriose per minute. Part of the ^1H-NMR spectra revealing signals specific from the α-1,4-linked Glc residues (from 5.4–5.1 ppm) or for the α-1,6-linked Glc residues (around 4.9 ppm) is displayed. NMR analysis was as described in Mouille et al. (1996).

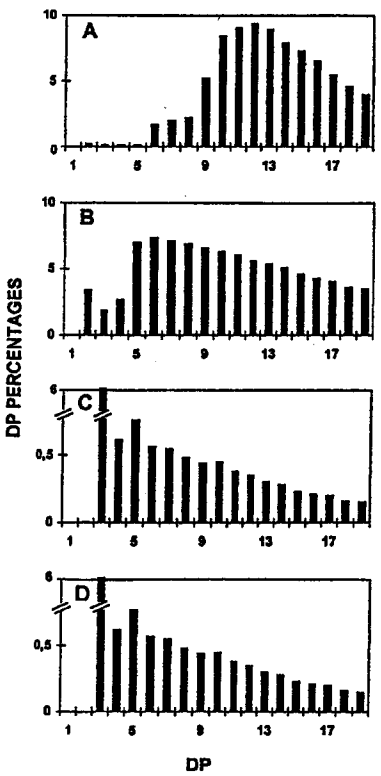

Figure 5. Chain-length distributions of amylopectin after incubation with the 62-kD transferase. Isoamylase-debranched chains were separated according to length after APTS fluorescence labeling and separation on DNA sequencer. Percentages of chains ranging between DP 1 and 19 (chains containing 1–19 Glc residues) are scaled on the *y* axis. A, Debranched chains from gel permeation chromatography-purified *C. reinhardtii* reference amylopectin (extracted from the amylose-free BAFR1 strain). B, Debranched chains from gel permeation chromatography-purified *C. reinhardtii* reference amylopectin (from strain BAFR1) after incubation with semi-pure *C. reinhardtii* D-enzyme. C, Debranched chains from gel permeation chromatography-purified *C. reinhardtii* reference amylopectin (from strain BAFR1) after incubation with semi-pure *C. reinhardtii* D-enzyme and subsequent digestion with β-amylase. D, Debranched chains from gel permeation chromatography-purified *C. reinhardtii* reference amylopectin (extracted from the amylose-free BAFR1 strain) after digestion with β-amylase. In C and D, the polysaccharide was not separated from the maltose generated by β-amylase. DP 2 is therefore not represented.

chain-length distribution of the mixture of debranched chains was the same as that displayed in Figure 5A. Incorporation of the oligosaccharides into amylopectin (Fig. 6) or rabbit liver glycogen was monitored successfully. This incorporation was linear with time for incubation times ranging between 15 min and 6 h. This radioactivity disappeared upon subsequent treatment of the polysaccharide with β-amylase, proving that D-enzyme transfers efficiently segments of malto-oligosaccharides onto the outer chains of both glycogen and amylopectin. In contrast, incubation of radioactive amylopectin (1 mg mL^{-1}) with up to 500 μg mL^{-1} of unbranched, cold malto-oligosaccharides prepared from the same source did not yield any measurable release of radioactive material into the malto-oligosaccharide fraction. The sensitivity of the assay was such that we would have detected this reverse reaction even if as little as 5% of the amount transferred in similar conditions onto the polysaccharide outer chains had occurred. The physiological concentration that we measured in *C. reinhardtii* for small malto-oligosaccharides ranged between 50 to at most 200 μg mL^{-1}. We therefore conclude that at the starch granule surface (with amylopectin at over 10 mg mL^{-1} and malto-oligosaccharide concentrations well under 200 μg mL^{-1}), incorporation of debranched oligosaccharides such as those generated through debranching of pre-amylopectin would be strongly favored.

The Specific Requirements of D-Enzyme for the Incorporation of Oligosaccharides into Polysaccharide Outer Chains

Incorporation of label from malto-oligosaccharides into glycogen gave us an additional reliable assay of D-enzyme activity. This enabled us to set-up a novel zymogram procedure for D-enzyme detection (Fig. 7) based on the incorporation of oligosaccharides into glycogen. Once again, our denaturing gels allowed us to estimate the mass of the enzyme at 62 kD. Moreover, co-elution of this activity stain was seen with those of our two other zymogram procedures and with the quantitative D-enzyme assay during

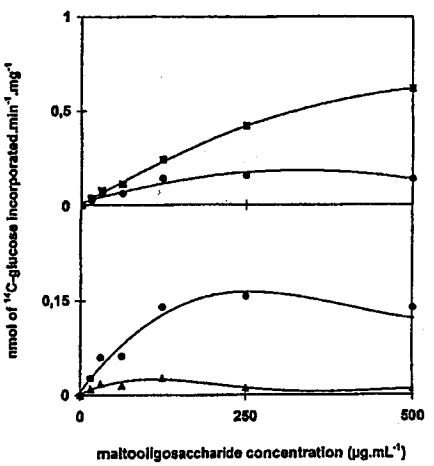

Figure 6. Malto-oligosaccharide incorporation assays. Amylopectin (2.5 mg, ■; 1 mg, ●; 0.25 mg, ▲) was incubated with increasing concentrations of radiolabeled malto-oligosaccharides produced by debranching radioactive *C. reinhardtii* amylopectin. The *y* axis represents two different scales giving net incorporation of oligosaccharides within the polysaccharide. An enzyme activity corresponding to 2 nmol of Glc produced from maltotriose per minute was used in these experiments. Incorporation was strictly proportional to enzyme activity for activities ranging between 0.5 and 20 nmol of Glc produced from maltotriose per minute. The label was confined to the outer chains of amylopectin, since all of the radioactive material was liberated upon treatment with β-amylase.

purification of the protein (Fig. 3). We found co-segregation between all three types of activities (Glc production from maltotriose, amylopectin modification, and incorporation of unbranched glucans into glycogen) and the wild-type *STA11* allele in the progeny of crosses involving wild-type and mutant *C. reinhardtii* strains. We then proceeded to monitor the incorporation of malto-oligosaccharides of controlled length into the external chains of glycogen using this novel zymogram procedure.

It is evident from our results displayed in Figure 8A that efficiency of transfer onto glycogen external chains increases with the length of the donor chain. Particularly striking in Figure 8B is the absence of stain when using maltotriose as a donor substrate even at very high substrate concentrations and after prolonged incubation. To ensure that we were not dealing with some iodine staining artifact, we prepared radioactive maltotriose (see "Materials and Methods") and compared the efficiency of transfer with that of debranched amylopectin. We found low incorporation of label from purified labeled maltotriose into the outer chains of amylopectin. Indeed, we observed 10% of the incorporation rates initially measured with the mix of debranched radioactive oligosaccharides that we displayed in Figure 6.

Surprisingly, purified and labeled maltotetraose gave incorporation rates similar to those previously achieved by the mix of debranched and labeled oligosaccharides, while in zymograms maltotetraose always gave faint stains. It must be stressed that all malto-oligosaccharides added in the label incorporation experiments were immediately disproportionnated by D-enzyme into longer oligosaccharides, while in the zymogram experiments this modification was slowed because of the vast excess of substrate oligosaccharide contained in the large volume (30 mL) of incubation buffer. Therefore, we feel that glucans longer than maltotetraose will define the actual optimal substrates of the incorporation reaction. Such longer glucans are simply not produced fast enough by disproportionating maltotriose when a large excess of oligosaccharides is used on an immobilized D-enzyme such as in the zymogram procedures. We expect that in vivo glucans of the required lengths are produced by the processing of pre-amylopectin through isoamylases.

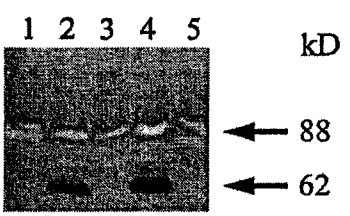

Figure 7. Malto-oligosaccharide incorporation zymograms. Two-hundred micrograms of denatured crude extract protein from wild-type (lanes 2 and 4) and mutant (lanes 1, 3, and 5) recombinants obtained after crossing JV45J with the wild-type strain 37 were loaded in glycogen-containing gels in the presence of maltoheptaose. Note that the white-staining band at 88 kD represents the debranching enzyme missing in the glycogen-producing mutants of *C. reinhardtii* (Mouille et al., 1996).

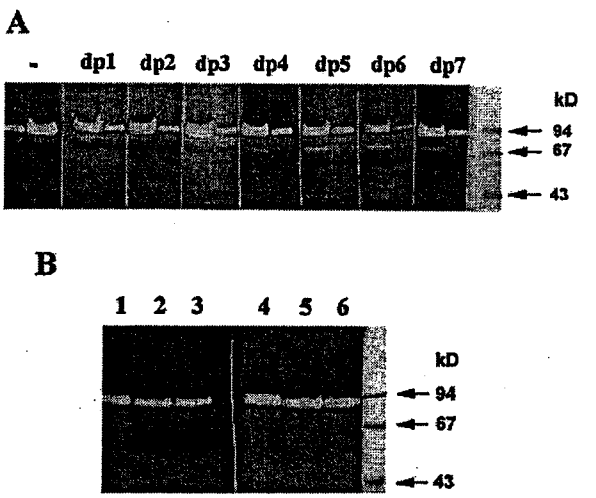

Figure 8. Comparisons of malto-oligosaccharide incorporation into glycogen. A, Crude extract protein (100 and 300 μg) was loaded eight times in pairs on two identical gel systems and run simultaneously in glycogen-containing gels. The gels were then cut into eight different fragments and incubated separately with 2 mM DP1 (Glc) to DP 7 (maltoheptaose). One gel fragment was incubated without malto-oligosaccharides (−). The gels were incubated overnight at room temperature. B, Denatured crude extract protein (200 μg) from two wild-type reference strains (lanes 2 and 3; lanes 5 and 6) and mutant strain JV45J (lanes 1 and 4) were loaded in glycogen-containing gels and incubated for 48 h with 2 mM maltoheptaose (lanes 1–3) or 20 mM maltotriose (lanes 4–6).

We would like to stress that the incorporation of labeled oligosaccharides into solubilized amylopectin, although more quantitatively reliable, is a poor reflection of the physiological situation. Both amylopectin and oligosaccharides readily diffuse in this system. However, in the zymogram procedure the oligosaccharides are allowed to diffuse in a large volume of buffer while the polysaccharide remains constrained in a small gel phase volume together with a high enzyme specific activity. This situation mimicks the synthesis of polysaccharide at the granule surface.

The Enhancement by D-Enzyme of the Phosphorylase-Mediated Malto-Oligosaccharide Degradation

In bacteria it was suggested that amylomaltase, an α-1,4 glucanotransferase similar to D-enzyme, enhanced the production of Glc-1-P by maltodextrin phosphorylase through the generation of glucans long enough (DP 5) to be used by maltodextrin phosphorylase (for review, see Boos and Shuman, 1998). We tested this suggestion by measuring Glc-1-P production from maltotriose, maltotetraose, maltopentaose, and maltoheptaose in crude extracts of *C. reinhardtii* wild-type and mutant *sta11-1* strains. As shown in Figure 9, it is evident that the presence of D-enzyme stimulates the degradation of both maltotetraose and maltotriose through phosphorylase by at least a factor of 5. Interestingly, the same treshold length (DP 5) of the glucan allowing Glc-1-P production is found both in bacteria and plants.

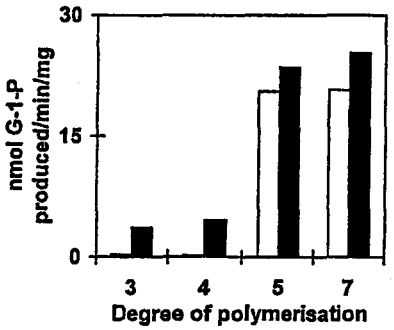

Figure 9. Stimulation of malto-oligosacharide phosphorolysis by D-enzyme. Crude extract protein (100 μg) from wild-type strain 137C (black bars) and mutant strain JV45J (white bars) were incubated for 1 h at 30°C in the presence of 2.5 mM maltotriose, maltotetraose, maltopentaose, and maltoheptaose.

DISCUSSION

D-Enzyme Readily Transfers Segments of Chains onto Polysaccharide External Chains

D-enzyme was initially described as an enzyme using and producing soluble oligosaccharides (Peat et al., 1956; Jones and Whelan, 1969). It was assumed to be an integral part of the malto-oligosaccharide assimilation pathway during starch breakdown. Recent papers using high amounts of pure potato D-enzyme activity on both amylose and amylopectin showed that the enzyme was able to produce cyclic branched or unbranched glucans of various lengths after prolonged incubation (Takaha et al., 1996b, 1998). Similar results were obtained with branching enzymes (Takaha et al., 1996a). These experiments, however, did not investigate the effect of D-enzyme on the amylopectin chain-length distribution at lower concentrations of enzyme activities and for restricted time periods. It occurred to us that the reaction that we had first observed on amylopectin alone failed to generate oligosaccharides because it uses the polysaccharide outer chains both as donor and acceptor glucans.

The reaction is driven on an unbranched soluble malto-oligosaccharide at the expense of Glc formation from the reducing end of the donor chain. However, the analogous residue from the donor amylopectin outer chain is expected to remain bound to the polymer through an α-1,6 branch. Indeed, the concentration of external glucans within starch can be estimated at 450 mM (van de Wal et al., 1998). Such high local concentrations would outcompete as a recipient any soluble oligosaccharide that might be present within the plastid. We further reasoned that if unbranched oligosaccharides and preamylopectin co-exist in the plant cell, then D-enzyme would presumably act as a polymerase, using oligosaccharide chains as donors and yielding net incorporation into insoluble mature amylopectin ultimately at the expense of Glc formation.

Because of the extremely high local concentration of potential acceptor chains in amylopectin or its precursors (pre-amylopectin), the glucan once incorporated will only very slowly be freed from the polysaccharide. The energy cost of such a polymerization reaction would be exceedingly low and would consist only of two ATP high-energy bonds required to regenerate one ADP-Glc for each glucan chain incorporated into the polysaccharide. In the present study, we have demonstrated that the D-enzyme from *C. reinhardtii* (and probably from vascular plants, too) is able to incorporate segments of unbranched malto-oligosaccharides quickly and efficiently on the outer chains of amylopectin. We were, however, unable to observe the reverse reaction (transfer from amylopectin outer chains to malto-oligosaccharides) at physiological substrate concentrations. This is not that surprising, since fructosyl transferases, enzymes of analogous properties, have been proven to function in fructan polymerization (van der Meer et al., 1998). The rates of incorporation that we measured are physiologically relevant, especially if one assumes that the reaction takes place at the growing surface of the starch granule. Indeed, we have been unable to saturate D-enzyme with amylopectin in our in vitro incorporation experiments.

The Function of D-Enzyme in Amylopectin Synthesis

D-enzyme-defective mutants accumulate large amounts of oligosaccharides with no detectable α-1,6 branches. A likely source of such oligosaccharides can be sought in the action of debranching enzymes. We and others have recently proposed that amylopectin synthesis occurs through cycles of glucan trimming by selective debranching of a precursor (pre-amylopectin) into a mature semi-crystalline amylopectin molecule (Ball et al., 1996). During this process of pruning α-1,6 branches, the plant debranching enzymes will release unbranched malto-oligosaccharides. Because such molecules accumulate in the D-enzyme-deficient mutant, we believe that the normal function of plant α-1,4 glucanotransferases is to process these chains.

There are two possible types of processing. These involve distinct mechanisms aimed at retrieving more or less of the energy that would otherwise be lost with the spliced glucans. The first involves disproportionating the small linear oligosaccharides into Glc and longer oligosaccharides, rendering the latter accessible to phosphorolytic degradation. This fits better with the postulated function of D-enzyme in the normal process of starch degradation. We did observe stimulation of degradation by phosphorylase in the presence of D-enzyme. The second would consist of direct transfer of glucans back onto the outer chains of amylopectin. Such a mechanism would be more efficient, since most of the energy contained in the α-1,4 linkages of the spliced glucans would be recovered on the maturing polysaccharide.

In this study, we provide biochemical support for the physiological relevance of this reaction. Most importantly, the second mechanism offers a straightforward explanation for all of the phenotypic traits observed in the D-enzyme-defective mutants. These consist not only of malto-oligosaccharide accumulation, but also of a structural modification of the remaining amylopectin and a downfall in starch synthesis. It is not easy to understand the basis for the modification in starch structure if the first mechanism was operating on its own. It must be stressed, however,

that changes in polysaccharide structure could also arise from competition between malto-oligosaccharides and the normal substrates of soluble starch synthases and branching enzymes. In addition, it remains possible that small modifications in structure originate from the activity of D-enzyme toward the external chains of the polysaccharide without implying that oligosaccharides are necessarily reinserted into the structure. It is also possible that both postulated mechanisms occur simultaneously, and certainly does not rule out an additional function of D-enzyme in the normal process of starch degradation.

While in *C. reinhardtii* the presence of debranching enzymes remains mandatory to obtain starch synthesis, the requirement for D-enzyme becomes acute only in conditions of maximal starch synthesis. Losses of metabolic energy generated by the action of debranching enzymes become critical only when the cell devotes its activity to carbohydrate storage and the energy supply remains limited. This is certainly the case in nitrogen-starved *C. reinhardtii* cells, but could vary according to the plant species or tissue. However, we do not expect to see differences in amylopectin structure and maltoligosaccharide accumulation vanish under any circumstances. This will probably enable the breeding of plants with modified starch structure and high yield.

We believe our data prove that D-enzyme is required for normal amylopectin synthesis. We expect not only to open new avenues for breeders to improve starch quality, but also to give new insights into our understanding of starch biosynthesis in plants. These insights stem from the functional tie that we establish between polysaccharide and malto-oligosaccharide metabolisms in building amylopectin structure. In return, the discovery of such ties in plants may encourage further investigations performed on amylomaltase, the product of the *E. coli MALQ* gene, which is the bacterial analog of D-enzyme. We have observed high rates of incorporation of maltoheptaose into the external chains of glycogen through the *MALQ* gene product, an observation that is relevant to our understanding of bacterial glycogen metabolism.

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DISCUSSION

Discussion et perspectives.

Ce travail s'est axé tout particulièrement sur l'étude de deux activités enzymatiques que l'on associait plus à la dégradation des polysaccharides qu'à leur anabolisme. La découverte des mutants aux loci *STA7* et *STA11* et les caractérisations biochimiques et génétiques réalisées sur les descendants des algues mutantes d'origine prouvent l'implication de l'enzyme disproportionnante et de l'isoamylase dans la biosynthèse de l'amylopectine (Colleoni et coll., 1999a et 1999b ; Mouille et coll., 1996). Sans remettre en cause leur participation au catabolisme des polysaccharides de réserve, ces travaux ont mis à jour de nouveaux prérequis pour une synthèse normale d'amidon chez les végétaux. Aux étapes primordiales représentées par la synthèse du nucléotide-sucre précurseur, la synthèse des liaisons α-1,4 et des liaisons α-1,6 viennent s'ajouter une étape de débranchement catalysée par l'isoamylase et une étape de recyclage conditionnée par la présence de l'enzyme D.

I. Les mutants d'enzyme de débranchement de Chlamydomonas.

Comme dans le cas des végétaux supérieurs, la nature de l'enzyme de débranchement absente dans les mutants *sta7* se révèle de spécificité isoamylasique. A l'opposé de ses homologues des plantes supérieures, la mutation du gène de l'isoamylase de l'algue n'entraîne pas de modifications de la seconde activité de débranchement: la pullulanase. En absence d'activité isoamylasique (mutants *sta7* de Chlamydomonas), la nature des polysaccharides accumulés change totalement. L'amidon est presque entièrement remplacé par un polysaccharide soluble de type glycogène. Le matériel granulaire retrouvé en très faible quantité dans les souches déficientes pour l'isoamylase possède une structure fortement altérée le rapprochant plus de l'amylose que de l'amylopectine. D'ailleurs, dans le mutant, ce matériel dépend de la présence de l'amidon synthétase liée au grain, l'enzyme responsable de la synthèse de la fraction amylosique dans un contexte sauvage. Cette enzyme, bien qu'incapable de se fixer à la matrice polysaccharidique, produit de façon limitée des polysaccharides insolubles de nature anormale dans les souches déficientes au locus *sta7*. L'ensemble de ces résultats révèle

le rôle primordial de l'activité de débranchement pour l'obtention d'une synthèse normale d'amylopectine.

II. Une caractéristique clé de l'isoamylase : son architecture.

La mise à jour d'un nouveau locus (*STA8*) conditionnant l'activité isoamylasique de l'algue ouvre de nouveaux horizons pour la compréhension exacte du rôle de l'étape de débranchement. Les mutants au locus *sta8* ne contiennent plus que 35% de l'activité isoamylasique normalement observée dans une souche sauvage. La production concomitante d'un amidon de structure peu altérée et de phytoglycogène dévoile non pas un blocage mais bien un ralentissement de la chaîne métabolique aboutissant à la synthèse d'amylopectine cristalline. Cette réduction de la quantité d'activité ne rend pas compte des conséquences phénotypiques. Notre étude ne montre pas de changement des propriétés biochimiques de l'enzyme, ni une mauvaise localisation de l'activité qui reste plastidiale chez le mutant. Seules les modifications de l'architecture du complexe enzymatique semblent provoquer le défaut de biosynthèse observé. Le complexe enzymatique portant l'activité isoamylasique dans la souche sauvage voit en effet sa taille se réduire d'un tiers dans un contexte mutant *sta8*. La structure tridimensionnelle du complexe protéique altérée par la mutation *sta8* apparaît être la cause majeure des perturbations observées.

III. La provenance du phytoglycogène

Les mutations affectant les loci *STA7* et *STA8* engendrent toutes deux le remplacement d'une partie ou de la quasi-totalité de l'amidon par du phytoglycogène. Nous ne pouvons pas à ce jour démontrer la relation qui existe entre l'absence ou la diminution de l'activité isoamylasique et la production de ce polysaccharide soluble. Nous pouvons néanmoins émettre des hypothèses quant au rôle de l'enzyme dans l'élaboration de ce matériel. Le niveau d'équilibre et la structure du phytoglycogène sont très proches dans les deux types de mutants *sta7* et *sta8*. Or, ces deux mutations n'affectent pas l'activité de la même façon. Dans un cas, les souches ne contiennent plus

du tout d'activité, alors que dans l'autre il reste toujours 35% de l'activité normale. Cette quantité apparaît suffisante pour une synthèse normale d'amylopectine comme le démontre le phénotype du triploïde hétérozygote ne contenant plus qu'une dose sauvage du gène *STA7*. De plus, l'isoamylase du mutant *sta8* débranche *in vitro* le phytoglycogène avec une efficacité comparable à celle de l'enzyme sauvage. Ces résultats, et le fait que les taux de dégradation *in vivo* du phytoglycogène sont similaires dans les souches *sta7* et *sta8*, n'arguent pas en faveur d'un rôle de l'enzyme dans la dégradation du polysaccharide soluble. Deux modèles ont été proposés, à ce jour, en fonction des résultats obtenus chez Arabidopsis (Zeeman et coll., 1998) ou chez Chlamydomonas (Ball et coll., 1996). La figure 1 résume trois hypothèses permettant de relier l'activité de l'isoamylase et la présence de phytoglycogène dans les mutants. Dans les parties 1A et 1B, l'isoamylase a pour rôle essentiel de débrancher le phytoglycogène qui représente une molécule compétitrice de la biosynthèse de l'amylopectine. L'isoamylase en participant à la dégradation de ce polysaccharide soluble permet de supprimer la compétition et favorise la synthèse d'amylopectine aux dépens de celle du phytoglycogène. Dans le modèle A, l'isoamylase représente l'enzyme essentielle à cette dégradation contrôlant le système de dégradation alors qu'en 1B, elle ne fait que participer à ce phénomène et une autre activité hydrolytique (AMYX) représente l'étape limitante de ce processus.

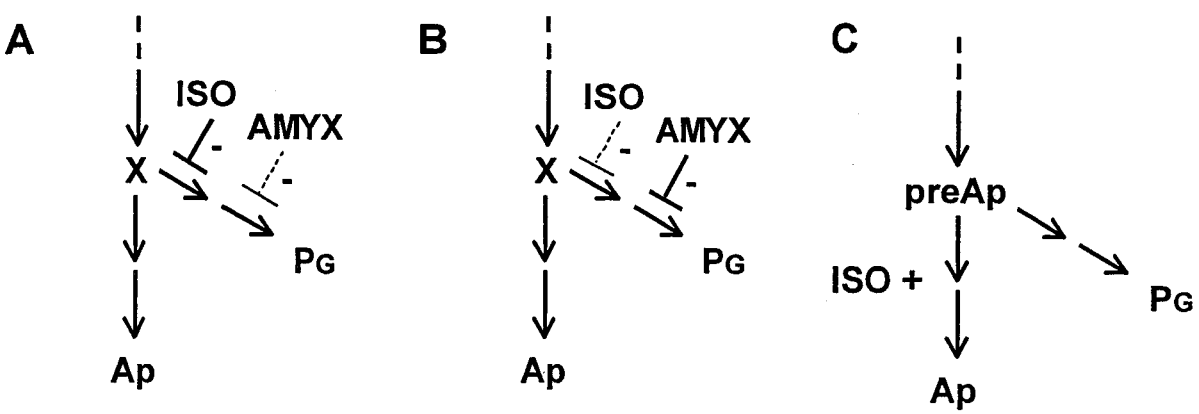

Figure 1 : Des modèles possibles expliquant l'intervention de l'isoamylase dans la production des polysaccharides végétaux. ISO : isoamylase ; AMYX : activité de type amylasique ; X : intermédiaire de biosynthèse ; PG : phytoglycogène ; Ap : Amylopectine.

Si l'isoamylase représentait l'activité contrôlant la dégradation du phytoglycogène (1A), nous n'aurions pas du observer des quantités similaires d'un polysaccharide de structure identique dans des mutants ne contenant plus cette activité (*sta7*) et des souches possédant encore une activité résiduelle non négligeable (*sta8*). Un rôle secondaire dans le débranchement du phytoglycogène de l'activité de débranchement (1B) ne peut être exclu aux regards des résultats expérimentaux mais l'absence de modifications structurales entre les phytoglycogènes retrouvés dans les mutants *sta7* et *sta8* n'argue pas en faveur d'un rôle de l'activité dans le catabolisme du polysaccharide. Dans la figure 1C, l'isoamylase possède un rôle majeur dans le contrôle du taux d'un précurseur commun aux chaînes métaboliques conduisant à la formation du phytoglycogène et de l'amylopectine. Pour cette hypothèse, nous pouvons facilement comprendre comment une absence ou une baisse de l'activité isoamylasique engendre une augmentation de ce précurseur (la pré-amylopectine) permettant la synthèse du phytoglycogène. L'isoamylase ne possède pas ici un rôle direct dans le catabolisme du phytoglycogène et une réduction de la quantité d'activité n'engendre pas de modifications de la quantité ou de la structure des polysaccharides solubles formés.

IV. Le rôle de l'isoamylase dans la biosynthèse de l'amylopectine

Le modèle d'épissage alternatif (Ball et coll., 1996 ; Myers et coll., 2000) confère un rôle essentiel à l'isoamylase lors de la biosynthèse de l'amylopectine. Dans ce modèle, l'enzyme de débranchement dégrade sélectivement certains points de branchement (liaisons α-1,6) qui empêchent la pré-amylopectine de s'engager dans un phénomène de cristallisation. Un modèle alternatif (Zeeman et coll., 1998) propose un rôle de l'enzyme dans la dégradation du phytoglycogène (figures 1A et 1B). Dans ce cas, la baisse de la quantité d'amylopectine dans le mutant déficient pour l'activité isoamylasique s'explique par un processus de compétition entre les deux types de polysaccharide pour des éléments communs (les enzymes de biosynthèse par exemple). Cette enzyme ne fait ainsi pas partie de la chaîne métabolique conduisant à la formation de l'amylopectine.

Les résultats obtenus lors de ce travail de thèse renforcent le modèle présenté en figure 1C et nous permet d'émettre des hypothèses quant aux phénomènes prenant place au sein des différents génotypes (figure 2). Dans une souche sauvage (figure 2A),

l'isoamylase dégrade les points de branchement de la pré-amylopectine empêchant la cristallisation et permet la synthèse de l'amylopectine. Le mutant *sta7* déficient pour cette activité ne produit plus d'amylopectine mais du phytoglycogène. Pour ce génotype, l'absence de l'activité enzymatique ne permet pas l'épissage et conduit à la formation de phytoglycogène (figure 2B). Pour les souches contenant une activité réduite (mutants *sta8*), l'architecture non conforme du complexe produit un ralentissement de l'épissage du précurseur entraînant la production concomitante des deux polysaccharides (figure 2C).

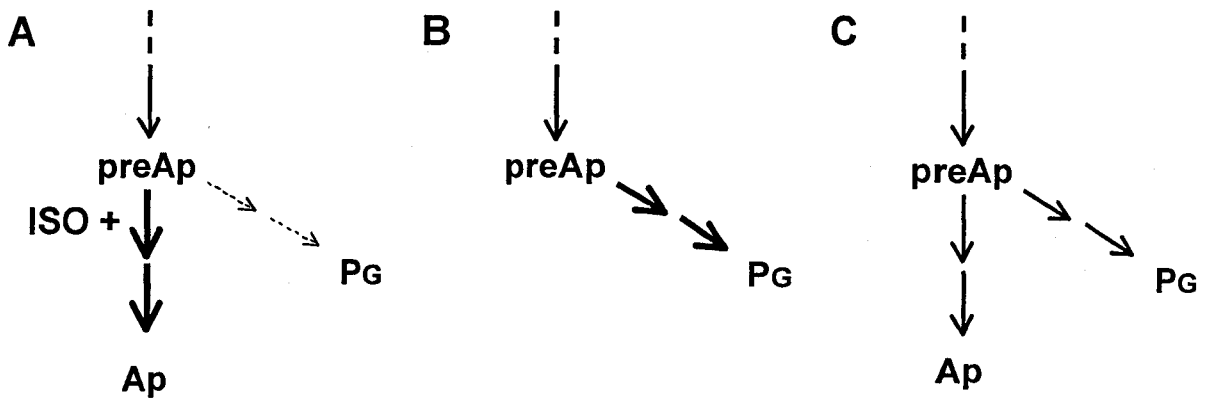

Figure 2 : Les phénomènes liés à l'activité isoamylasique dans différents génotypes. PreAp : pré-amylopectine ; ISO : isoamylase ; PG : phytoglycogène ; Ap : amylopectine. A, B et C représentent respectivement les situations prenant place dans une souche sauvage, une souche *sta7* et une souche *sta8*.

V. Perspectives.

La compréhension du rôle exact de l'isoamylase dans le métabolisme de l'amidon passe obligatoirement par l'élucidation moléculaire de la nature des protéines formant le complexe isoamylasique. Nos résultats suggèrent fortement une organisation hétéromérique de ce dernier. Nous allons donc chercher à élucider la nature des produits des loci *STA7* et *STA8* qui, au vu des résultats obtenus, font partie de ce complexe enzymatique. Afin de cloner ces gènes, nous avons récemment identifié dans une

banque d'EST de l'algue des fragments possédant une grande similitude avec des gènes codant pour des enzymes de débranchement de plantes. L'amplification de fragments par la technique de RT-PCR à partir d'une préparation d'ARNm devrait nous apporter le matériel nécessaire pour associer l'une ou l'autre des mutations avec les allèles *STA7* et *STA8*. Dans le cas d'une amplification de fragments de la taille attendue, nous utiliserons ceux-ci à des fins de clonage et le grand nombre d'allèles mutants obtenus par insertion aux loci *STA7* et *STA8* nous offriront un matériel de choix pour des expériences d'hybridations moléculaires permettant de faire le lien entre la mutation et la nature du produit du gène.

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