

STA11, a *Chlamydomonas reinhardtii* Locus Required for Normal Starch Granule Biogenesis, Encodes Disproportionating Enzyme. Further Evidence for a Function of α -1,4 Glucanotransferases during Starch Granule Biosynthesis in Green Algae¹

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In *Chlamydomonas reinhardtii*, the presence of a defective *STA11* locus results in significantly reduced granular starch deposition displaying major modifications in shape and structure. This defect simultaneously leads to the accumulation of linear malto-oligosaccharides (MOS). The mutants of *STA11* were showed to lack α -D-glucanase, a plant α -1,4 glucanotransferase analogous to the *Escherichia coli* amyloamylase. We have cloned and characterized both the cDNA and gDNA corresponding to the *C. reinhardtii* α -D-glucanase. We now report allele-specific modifications of the α -D-glucanase gene in the mutants of *STA11*. These allele-specific modifications cosegregate with the corresponding *sta11* mutations, thereby demonstrating that *STA11* encodes α -D-glucanase. MOS production and starch accumulation were investigated during day and night cycles in wild-type and mutant *C. reinhardtii* cells. We demonstrate that in the algae MOS are produced during starch biosynthesis and degraded during the phases of net polysaccharide catabolism.

Until recently plant starch was believed to be synthesized from ADP-Glc solely through a combination of starch synthases and branching enzymes. However the finding of low-starch or starchless mutants defective for a particular form of debranching enzyme in four different plant systems established that other enzymes of starch metabolism are equally important in ensuring normal starch granule biogenesis (James et al., 1995; Mouille et al., 1996; Nakamura et al., 1996; Zeeman et al., 1998). This came as a surprise because debranching enzymes were initially thought to be enzymes involved solely in starch breakdown. Mutants of the corresponding activities in yeast (Teste et al., 2000) or *Escherichia coli* (I. Kinderf, Z. Li, M.S. Samuel, B. Koshar-Hashezmi, S. Ball, L. Rampling, and M. Morell, unpublished data) are clearly glycogen over-producers, confirming the initial sus-

picion. Although the detailed interpretation of the results obtained with the plant mutants vary somewhat, there is now a general agreement that isoamylases, the particular form of debranching enzyme affected in these studies, are enzymes required during starch biosynthesis exclusively. It has been comforting to realize that all mutants affected in starch metabolism behaved in a similar fashion in plants as different as *Chlamydomonas reinhardtii*, Arabidopsis, pea (*Pisum sativum*), maize (*Zea mays*), or rice (*Oryza sativa*). Some discrepancies in expressivity of mutant phenotypes could be easily explained most of the time by subtle differences in the pathways. For instance, the presence of extraplasmidial and plastidial ADP-Glc pyrophosphorylases in cereals easily explains why mutants of cereals lacking the major enzyme form displayed reduced expressivity in their starch accumulation phenotype (for review, see Kossmann and Lloyd, 2000). The starch accumulation phenotypes of the Arabidopsis or *C. reinhardtii* mutants defective for the catalytic subunit of their sole plastidial ADP-Glc pyrophosphorylase were much more severe (Lin et al., 1988; Zabawinski et al., 2001).

At variance with animals and fungi, plants including *C. reinhardtii* have retained the bacterial pathway of malto-oligosaccharide (MOS) assimilation in the plastid stroma. Mutants of the plant α -D-glucanase (the

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equivalent of the *E. coli* *MalQ* encoded amyloamylase) display a strong phenotype on starch metabolism (Critchley et al., 2001). D-enzyme is an α -1,4 glucanotransferase that transfers mostly maltosyl residues from a donor oligosaccharide to the nonreducing end of an acceptor. The donor and acceptor molecules can be glucans of identical length. D-enzyme thus disproportionates a homogenous population into a series of oligosaccharides of increasing length (Peat et al., 1956; Jones and Whelan, 1969). The first mutants defective for D-enzyme were reported in *C. reinhardtii* and synthesized 10-fold less starch while displaying modifications in starch structure and amylose content (Colleoni et al., 1999a, 1999b). This correlated with an accumulation of unbranched soluble MOS in the plastid stroma. An equivalent mutant of *Arabidopsis* also accumulated MOS and high amylose starch but did not display any consistent decrease in starch accumulation (Critchley et al., 2001). On the contrary, the *Arabidopsis* leaf contained more starch at the end of the night than the wild-type reference, suggesting a function of D-enzyme in polysaccharide breakdown (Critchley et al., 2001). In addition, accumulation of MOS seemed confined to the dark phase. This is the first time that such conflicting behaviors between mutants of starch metabolism are reported. The results obtained in *C. reinhardtii* suggest an important function of D-enzyme in starch biosynthesis whereas those of *Arabidopsis* point to a role in starch breakdown only. The *C. reinhardtii* mutant was obtained during a classical screen for low-starch phenotypes after UV mutagenesis (Colleoni et al., 1999a), whereas the *Arabidopsis* mutants were obtained as gene disruptions after screening a T-DNA library of *Arabidopsis* for an insertion in a particular D-enzyme sequence (Critchley et al., 2001). Several issues require clarification before any further speculation is made on the functions of D-enzyme in plants. It is still unclear whether the *C. reinhardtii* *STA11* locus defines the structural gene encoding the same type of D-enzyme activity as in *Arabidopsis*. If so, it remains possible that the UV-generated *sta11-1* did not generate a true null mutant allele and that the phenotype would be different with a gene disruption at the *STA11* locus. Finally, the low-starch phenotype in *C. reinhardtii* was recorded under nitrogen starvation, which defines culture conditions that mimic the constant accumulation of starch occurring in storage organs. On the other hand, the *Arabidopsis* phenotype was investigated in leaf cells undergoing recurrent cycles of synthesis and degradation. Therefore additional documentation of the defects occurring in the *sta11* mutants of *C. reinhardtii* was needed before any further speculation could be made on the function of α -1,4 glucanotransferases in starch metabolism. We now report the molecular characterization of the *STA11* locus and mutants. We prove that *STA11* defines the structural gene of a form D-enzyme analogous to that analyzed in *Arabidopsis*. We report the

isolation of a gene disruption at the *STA11* locus and have investigated the behavior of the D-enzyme mutants following recurrent cycles of synthesis and degradation. We show that the MOS are produced mainly during the phases of active starch biosynthesis and are degraded during polysaccharide catabolism. These results confirm an important function of α -1,4 glucanotransferases during the normal process of starch biosynthesis in green algae. The possible reasons underlying the conflicting results obtained in *C. reinhardtii* and *Arabidopsis* are discussed.

RESULTS

Isolation of *sta11-2* Allele

During the first of our two large-scale campaigns of insertional mutagenesis (see "Materials and Methods"), we isolated one particular low-starch mutant that accumulated unbranched MOS (Fig. 1). Because of this phenotype, we immediately set out to assay D-enzyme activity and to perform complementation tests with the previously defined *sta11-1* UV-generated mutant (Colleoni et al., 1999a, 1999b). As expected, the mutant contained no trace of D-enzyme activity and did not complement the previously characterized *sta11-1* mutants in diploids. A set of meiotic recombinants carrying the new *sta11-2::ARG7* mutation were obtained upon crossing with a wild-type reference strain. A set of three *sta11-1* and three *sta11-2::ARG7* segregants were compared with respect to starch and soluble glucans accumulation. In addition, a more detailed investigation of the structure of both starch and glucans was performed on strain CR102 and compared with the reference JV45J *sta11-1* mutant strain. The results summarized in Ta-

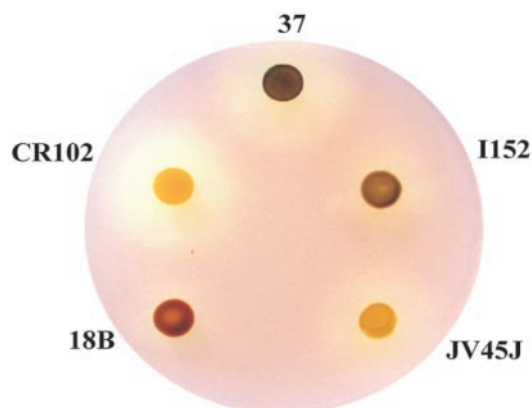


Figure 1. Wild-type and mutant iodine-staining phenotypes. Strain 37 corresponds to the wild-type reference used in this work. Strains 18B (*sta2-1*) and I152 (*sta3-1*) are defective for GBSSI and SSIII-like soluble starch synthase, respectively (Delrue et al., 1992; Fontaine et al., 1993). Both JV45J (*sta11-1*) and CR102 (*sta11-2*) are defective for D-enzyme activity. Mutations at *STA11* locus in JV45J and CR102 have been generated after UV and insertional mutagenesis, respectively. Cells patches were incubated 5 d on solid nitrogen-deprived medium and were subsequently stained by iodine vapors.

Table 1. Phenotypes of 137C (wild-type), JV45J (*sta11-1*), and CR102 (*sta11-2*) strains during storage (–N) and growth (+N) starch synthesis

Culture conditions	Starch ^a		MOS ^b		λ_{\max}^c of Amylopectin		Amylopectin ^d		Amylose ^d	
	–N	+N	–N	+N	–N	+N	–N	+N	–N	+N
	$\mu\text{g million cells}^{-1}$				nm		%			
137C (wt)	20.1	1.60	0.012	0.08	552	570	82	90	18	10
JV45J (<i>sta11hyphen1</i>)	2.3	0.15	0.46	0.33	562	577	79	78	21	22
CR102 (<i>sta11-2</i>)	0.4	0.30	1.15	0.60	568	591	72	67	28	33

^aAmounts of insoluble polysaccharide purified through sedimentation and ^b amounts of soluble MOS as measured by the standard amyloglucosidase assay. ^cWavelength at the maximal absorbance of the iodine-polysaccharide complex of amylopectin purified by gel filtration chromatography on Sepharose CL-2B column. ^dThe percentage of each fraction of starch was calculated after gel filtration chromatography on Sepharose CL-2B column of the dispersed polysaccharides.

ble I suggest that the *sta11-2::ARG7* mutation displays a phenotype very similar to that of the previously characterized *sta11-1* carrying mutants with a somewhat increased effect. During crosses ($n = 8$), the *sta11-2* mutants always accumulated less than 5% of the wild-type starch amount under nitrogen starvation, whereas the *sta11-1* mutants could accumulate up to 12% of that amount. This correlated with a significant increase in MOS production in the *sta11-2::ARG7* mutants. The chain-length distribution of this material was highly variable but always consisted in both *sta11-1* and *sta11-2::ARG7* of unbranched chains typically inferior to degree of polymerization 16 with a significant amount of maltose. In addition, the chain-length distributions of the amylopectin from both the *sta11-1* and *sta11-2::ARG7* were significantly modified. Using fluorophore-assisted capillary electrophoresis, we had previously documented a small but significant difference in the chain-length distribution of the wild-type and mutant (*sta11-1*) amylopectin (Colleoni et al., 1999a). Using high-performance anion exchange chromatography with pulsed amperometric detection (Fig. 2), we confirm these differences and demonstrate that the presence of *sta11-2::ARG7* leads to an exacerbation of the phenotype. The strains carrying the gene disruption display a more dramatic relative increase of chains of DP 12 to 16 and similar modification of the very small glucans (DP 3–6). Here again, the expressivity of the *sta11-2::ARG7* mutant phenotype is significantly increased. In nitrogen-supplied medium in the light and in the presence of acetate, the relative decrease in starch amount in the mutants compared with the wild type is far less spectacular. However, even in these conditions, the *sta11-2::ARG7* mutants displayed a higher expressivity than the *sta11-1* carrying strains because they contained higher amylose and MOS amounts.

Cloning and Characterization of a *C. reinhardtii* cDNA Homologous to Vascular Plants D-Enzymes

Alignment of known D-enzyme protein sequences revealed three blocks (Fig. 3, regions I–III) of highly conserved amino acid sequences between enzymes as

distantly related as those of *S. pneumoniae*, *C. butyricum*, potato, and *B. burgdorferi*. We used degenerated oligonucleotide primers to amplify by PCR from genomic DNA, a 1,564-bp sequence with homology to vascular plant sequences. We then used this as a probe to fish out a 2,071-bp cDNA lacking the 133 bp of 5' sequence that were subsequently obtained by RACE-PCR to yield the full cDNA sequence. The complete protein sequence is displayed in Figure 3 and was compared with the *E. coli* MalQ amyloamylase, the *Synechocystis* sp. α -1,4 glucanotransferase, the potato tuber D-enzyme, and the leaf-expressed D-enzyme sequence of Arabidopsis. A phylogenetic tree of selected α -1,4 glucanotransferases was built

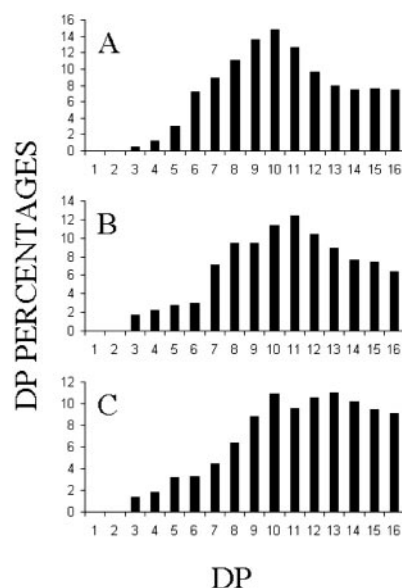


Figure 2. Chain-length distributions of the two *sta11* allelic mutants and the wild-type reference strain. Isoamylase-debranched chains were analyzed by HPAED-PAD. Percentages of chains ranging between DP 1 to 16 (chains containing 1–16 Glc residues) are scaled on the y axis. A, Debranched chains of purified amylopectin from our “waxy” reference strain BAFR1 (*sta2-29::ARG7*). B, Debranched chains of gel permeation chromatography-purified amylopectin from the mutant JV45J (*sta11-1*). C, Debranched chains of gel permeation chromatography purified amylopectin from the mutant CR102 (*sta11-2*).

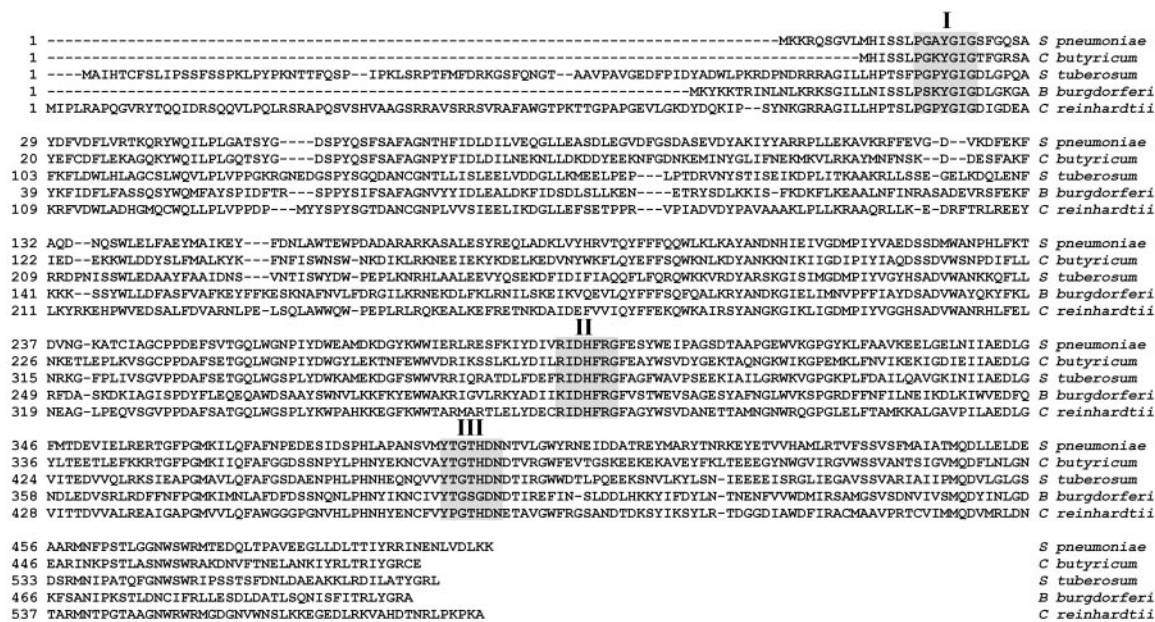


Figure 3. Sequences comparison of α -1,4 glucanotransferases from different organisms. *C. reinhardtii* D-enzyme sequence (AAG29840) was compared with those from *Streptococcus pneumoniae* (NP_346528), *Clostridium butyricum* (Q59266), potato (*Solanum tuberosum*; QO6801), and *Borrelia burgdorferi* (NP_212300). Highlighted boxes display conserved regions used for oligonucleotide primers design. Alignment was constructed using the ClustalW method with the PAM250 residue weight table. The amino acid (G) located on the right side within region I of *C. reinhardtii* D-enzyme sequence is modified to a Cys residue in the *sta11-1* mutant allele.

(Fig. 4), showing once again that the *C. reinhardtii* enzyme sequence could be placed as an intermediate between cyanobacteria and vascular plants. In addition to this, we selected a set of two distinct cosmids covering the entire gDNA corresponding to the *STA11* gene (see "Materials and Methods"). The organization of the *STA11* gene is displayed in Figure 5.

Molecular Characterization of *sta11-2::ARG7*

Because insertional mutagenesis in *C. reinhardtii* is known to lead to local rearrangements and deletions of genomic DNA that are easy to detect, we used the D-enzyme cDNA as a probe in Southern blots of wild-type and mutant recombinants of *sta11-2::ARG7*. The results shown in Figure 6A show cosegregation of a RFLP evidenced with the D-enzyme probe and the *sta11-2::ARG7* mutation. The D-enzyme transcript levels, although low in the wild-type strain, could be detected both in northern-blot analysis (data not

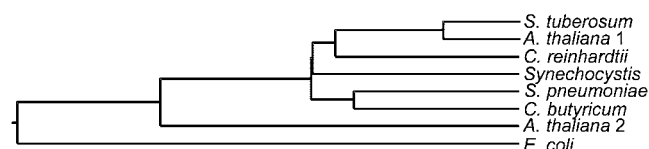


Figure 4. Phylogenetic tree of selected α -1,4 glucanotransferases. Accession numbers (from top to bottom) are as follow: Q06801, NP_201291, AAG29839, NP_440120, NP_346526, Q59266, AAL91204, and NP_417875. This phylogenetic tree was built using the ClustalW method with the PAM250 residue weight table.

shown) and in reverse transcriptase (RT-PCR) experiments (Fig. 6B). On the contrary, the *sta11-2::ARG7* mutants contained no detectable transcripts corresponding to D-enzyme, whereas mRNAs encoding D-enzyme could be routinely found in *sta11-1* mutants with levels similar to those found in wild-type strains (Fig. 6B). These results confirmed *sta11-2::ARG7* as a bona fide null mutant.

Molecular Characterization of *sta11-1*

When we set out to amplify the *sta11-1* cDNA by using the same set of degenerated primers as those that enabled us to clone our wild-type probe, we systematically met with failure. However the presence of mRNA was suggested by the use of other

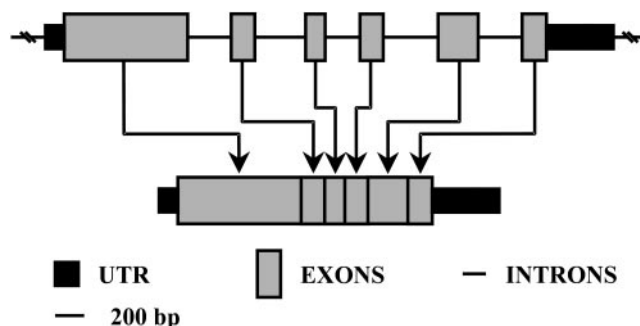


Figure 5. Intron/exon organization of *C. reinhardtii* α -1,4 glucanotransferase gene (GenBank accession no. AF307843).

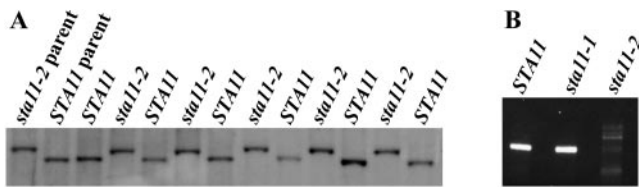


Figure 6. A, RFLP analysis from *sta11-2::ARG7* (indicated as *sta11-2*) mutant and wild-type strains. Molecular hybridization has been carried out using as a probe a 1.5 kb corresponding to an internal region of the α -1,4 glucanotransferase gene. B, A 0.8% (w/v) agarose gel loaded with RT-PCR products from a reaction performed with specific primers corresponding to the 3' end of the β -enzyme mature transcript (for more details, see "Materials and Methods").

primers corresponding to the *C. reinhardtii* sequence. We then sequenced the whole-mutant *sta11-1* cDNA and found only one consistent change in the middle of the conserved region I, thereby explaining our failure to amplify cDNAs by using oligonucleotide primers corresponding to this region of the gene. We then sequenced this region in a total of five wild-type and five *sta11-1* mutants and found the modified sequence to cosegregate with the mutant allele. This cosegregation analysis was completed by an additional round of PCR performing directly on gDNA, using the degenerated primers corresponding to region I and II on eight wild-type and six mutant progeny. The sequence modification consisted of change of a GGC codon specifying Gly to a TGC codon specifying Cys at the level of the last Gly of region I (highlighted in Fig. 3)

Analysis of Enzyme Activity and Corresponding mRNA Levels during Recurrent Starch Synthesis and Degradation

To approach the conditions used during the analysis of the Arabidopsis mutants, we subjected *C. reinhardtii* to a 12-h day/12-h night cycle of growth in nitrogen-supplied and CO₂-enriched or acetate-supplied environment. These optimal conditions ensure that *C. reinhardtii* will not be restricted for growth. The enzyme activity levels were assayed while the relative mRNA abundance was estimated by semiquantitative RT-PCR experiments (data not shown). No major oscillations of mRNA abundance were observed at the experiment's level of detection (2-fold) that could be compared with those that we previously showed for the small subunit of the *C. reinhardtii* ADP-Glc pyrophosphorylase (data not shown; Zabawinski et al., 2001). The enzyme assays afforded for more precise measurements that revealed a small but significant increase of β -enzyme activity in darkness (Fig. 7).

Analysis of Starch and MOS Contents

We followed starch and MOS accumulation in two *sta11-1* mutants and two wild-type recombinants for

36 h as detailed in "Materials and Methods." According to the preculture used and the particular strain under study, the time of minimal starch and MOS content varied somewhat (± 3 h). However, the results were all essentially the same with the minimal starch content being obtained most often in the middle of the light phase as was previously reported (Mérida et al., 1999) and the maximal starch content being always reached at the light to dark transition (Fig. 8, A and B). Most importantly, all *sta11-1* mutants MOS behaved similarly and followed closely the starch accumulation curves being degraded at the time of starch breakdown and produced at the time of polysaccharide synthesis.

DISCUSSION

α -1,4 Glucanotransferases were recently shown to define important components of starch metabolism in Arabidopsis and *C. reinhardtii* (Colleoni et al., 1999a, 1999b; Critchley et al., 2001). Both mutants accumulated MOS and high amylose starch when defective for β -enzyme activity. However, the levels of starch in the Arabidopsis mutant were either equivalent or higher than those displayed by the wild-type reference, whereas in the *C. reinhardtii* mutants, starch levels were as severely reduced as in strains defective for the large subunit of ADP-Glc pyrophosphorylase (Van den Koornhuysse et al., 1996). These vastly different phenotypes argued that in *C. reinhardtii*, β -enzyme is required in the normal process of starch biosynthesis, whereas in Arabidopsis, it appears that the same enzyme activity is required for starch degradation only.

In an attempt to resolve these contradictions, we undertook a detailed molecular characterization of the *sta11* mutants of *C. reinhardtii*. The multiplicity of enzyme forms in plants is such that it remained possible that the enzyme defective in both systems belonged to different families in a fashion reminiscent of the debranching enzymes of the isoamylase or pullulanase type. Both forms of debranching enzyme catalyze the same biochemical reaction, but isoamy-

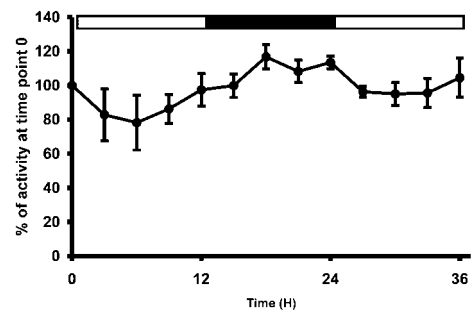
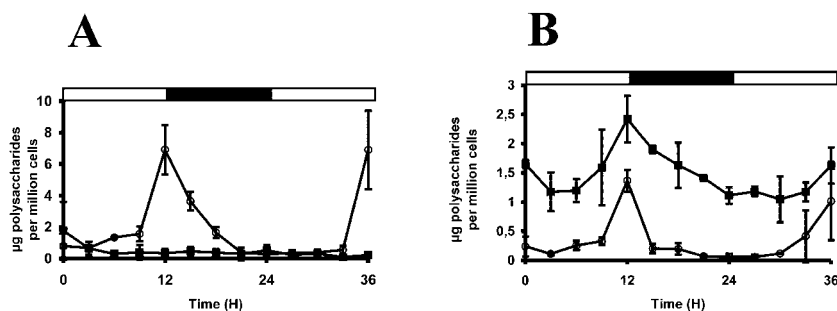


Figure 7. β -Enzyme activity (●) measured on wild-type cells grown under 12-h d/12-h night cycle with 4% (v/v) CO₂ constant bubbling. The results displayed are the mean \pm sds of four distinct and independent experiments. The results were compared by taking T = 0 measure as an arbitrary standard (assigned value of 100%).

Figure 8. Starch (○) and WSP (■) accumulations in wild-type and *sta11-1* mutant strains. Cells were grown under 12-h day (white horizontal bars)/12-h night (black horizontal bars) cycle with 4% (v/v) CO₂ constant bubbling. Results displayed in A are the mean ± SD of two distinct and independent experiments performed on wild-type strain. Results displayed in B are the mean ± SD of two distinct and independent experiments carried out on *sta11-1* mutant strain.



lase was shown to be selectively involved in amylopectin synthesis, whereas pullulanase is suspected to be active in starch degradation. Because two different kinds of α -1,4 glucanotransferases are known to occur in Arabidopsis, it was of paramount importance to better define the nature of the missing enzyme activity in *C. reinhardtii*. It is now clear that the D-enzyme of *C. reinhardtii* is more related to the Arabidopsis chromosome V form of α -1,4 glucanotransferase and to the previously characterized potato D-enzyme than to that of the Arabidopsis chromosome II. Yet it is the chromosome V form of α -1,4 glucanotransferase that is missing in the Arabidopsis mutant. Because only one kind of α -1,4 glucanotransferase is presently documented in the extensive expressed sequence tag data gathered for *C. reinhardtii* (more than 110,000 expressed sequence tags are currently available for *C. reinhardtii*), it still remains possible that α -1,4 glucanotransferases sequences have been duplicated during evolution of vascular plants and that different forms of enzymes have acquired specialized anabolic or catabolic functions.

Another possible explanation for the conflicting results obtained in Arabidopsis and *C. reinhardtii* could come from the different molecular nature of the mutations under investigation. The Arabidopsis mutant carries a T-DNA insertion essentially inactivating the gene, whereas the *C. reinhardtii* UV-generated mutant is now shown to consist of a single-amino acid change in an otherwise intact protein. It is thus possible that the very nature of these different mutations could explain the observed phenotypic contradictions. However, the report we now make of a gene disruption of the *C. reinhardtii* *STA11* gene disproves this. The absence of D-enzyme mRNA and protein in *C. reinhardtii* correlates with a further decrease in starch amount to less than 3% of the wild-type amount during nitrogen starvation, an observation that is very hard to reconcile with an absence of direct or indirect function of D-enzyme in the normal process of starch biosynthesis. The increased phenotypic expressivity of the *sta11-2::ARG7* gene disruption was also evidenced on the chain-length distribution of amylopectin. This modification can be explained through a direct function of α -1,4 glucanotransferases in amylopectin synthesis as suggested by Colleoni et al. (1999b). In that case, it was pro-

posed that the function of the α -1,4 glucanotransferase would be to transfer most of the glucans produced by isoamylases during biosynthesis back to the external pre-amylopectin chains. The modification in chain-length distribution of the mutant amylopectin can also be explained by an indirect and more likely effect consisting of the lowering of the ADP-Glc concentration. Such a substrate concentration decrease is expected if the function of D-enzyme is to harvest efficiently the energy contained in the glucans released by isoamylase by assisting the release of G1P through starch phosphorylase. The modified chain-length distribution witnessed here is very similar to that evidenced on the amylopectin of the low-starch mutants defective for either phosphoglucomutase or the large subunit of ADP-Glc pyrophosphorylase (Van den Koornhuysse et al., 1996). Future characterization of the phenotypes witnessed in both Arabidopsis and *C. reinhardtii* should therefore focus on metabolic profiling of the mutant and wild-type strains.

In a final attempt to resolve the contradictions observed between the Arabidopsis and *C. reinhardtii* mutants, we have investigated the phenotype of the algal mutants in physiological conditions that are closer to those of the Arabidopsis leaf, which defines a typical plant "source" tissue. Our previous report concerned nitrogen-starved culture where starch accumulates to very high levels and adopts a structure similar to that of storage starch, which is synthesized in the plant "sink" reserve tissues (the kernel endosperm, the tuber, the seed embryo, etc.). During nitrogen starvation, the *C. reinhardtii* cell becomes progressively non-photosynthetic. It was therefore possible that if starch turnover occurs in these conditions, the energy losses occasioned by the presence of futile cycles would not be compensated by photophosphorylation. The fact that preamylopectin processing during biosynthesis is expected to release MOS through the action of isoamylases, may explain why inefficient MOS metabolism may trigger a collapse of polysaccharide synthesis under nitrogen starvation. We therefore have investigated the behavior of the *sta11* mutants in nitrogen-supplied medium and growing in log phase in the presence of a 12-h-light and 12-h-dark cycles. The experiments detailed in this work prove that the ATP supplied through

photosynthesis does not bypass the requirement for D -enzyme activity during biosynthesis. Most importantly, the MOS accumulated at the time of starch biosynthesis and were degraded at the time of starch breakdown, a result that is in clear contradiction with the situation documented in Arabidopsis. It must be stressed that the *C. reinhardtii* system presents important differences with respect to the mature Arabidopsis leaf cell. Among these differences, the cell cycle remains active in the algae. Together with the ticking of the circadian clock, this may be responsible for the very different timing of starch biosynthesis and degradation observed in *C. reinhardtii*. This may however not be a unique property of algae because other vascular plant leaf tissues are also known to anticipate the arrival of darkness and trigger starch degradation in the light (Li et al., 1992; Mérida et al., 1999).

It is now apparent that different species of plants as well as different tissues of a same plant harbor very different levels of enzyme activities of starch and MOS metabolism. This remains true when one compares those tissues actively engaged in starch synthesis. The Arabidopsis mature leaf, the *C. reinhardtii* nitrogen-supplied cell growing in the light, the *C. reinhardtii* nitrogen-starved cell, the potato tuber, the maize endosperm, and the pea embryo all contain vastly different types and amounts of enzyme activities concerned with starch metabolism. Although the "core" synthetic pathway composed of ADP-Glc pyrophosphorylases, starch synthases, branching enzymes, and isoamylases are present in all cases (for review, see Kossmann and Lloyd, 2000; Myers et al., 2000), the systems contain very different amounts of those other enzymes whose functions in starch metabolism remain unclear. D -Enzyme activity is easy to detect in *C. reinhardtii*, in the Arabidopsis leaf, and in the potato tuber. However, the activity could not be detected at comparable levels in the maize endosperm. When maize and *C. reinhardtii* pullulanase are assayed and compared, the algae proved to contain one to two orders of magnitude less of this enzyme activity, whereas plastidial phosphorylase defines one of the most abundant enzymes of the maize endosperm amyloplast. The MOS produced by isoamylase during starch biosynthesis could be metabolized through many distinct pathways with different consequences. They can be very efficiently recovered by providing primers to the soluble or granule-bound starch synthases. They can be equally efficiently recovered by being transferred on pre-amylopectin by D -enzyme. They can be degraded with moderate energy loss by a combination of D -enzyme and phosphorylase digestion, or they can feed a futile cycle by degradation through endo or exo type of amylases or by glucosidases. The phenotypic consequences will depend on the limited or unlimited availability of substrate and ATP, which in turn will depend on the highly variable physiological conditions

prevailing in the tissue under consideration. D -Enzyme as a major enzyme of MOS metabolism is likely to be of use in both polysaccharide synthesis and degradation. The consequences of mutations affecting D -enzyme activity on starch metabolism will largely depend on the enzymatic make-up and physiological status of the cell under consideration.

MATERIALS AND METHODS

Materials

[α - ^{32}P]dCTP was purchased from Amersham Biosciences UK Ltd. (Little Chalfont, Buckinghamshire, UK). CL-2B Sepharose column and Percoll were obtained from Amersham Biosciences AB (Uppsala). Starch assay kit was obtained from Roche Diagnostics (Mannheim, Germany).

Chlamydomonas reinhardtii Strains, Growth Conditions, and Media

The reference strains of *C. reinhardtii* used in this study are 137C (*mt-nit1 nit2*), 37 (*mt+ pab2 ac14*), and BAFR1 (*mt+ nit1 nit2 sta2-29::ARG7*). The strain used for nuclear transformation is TerBD20 (*mt- sta2-1 nit1 nit2 cw15 arg7-7*). JV45J (*mt- nit1 nit2 sta11-1*) and all "CO" strains were previously described (Colleoni et al., 1999a). RC21 (*mt- sta11-2::ARG7 sta2-1 nit1 nit2 cw15 arg7-7*) was obtained by nuclear transformation of TerBD20 using plasmid pASL and strain CR102 (*sta11-2::ARG7*) represents a progeny from a cross involving both RC21 and 37 strains. Standard media are fully detailed by Harris (1989), whereas growth conditions and nitrogen-starved media are described by Ball et al. (1990, 1991), Delrue et al. (1992), and Libessart et al. (1995). For the studies concerning enzyme activity, mRNA level, starch, and MOS measurement in 12-h-day/12-h-night cycle, experiments were performed under high CO_2 level (4%) bubbling in Sueoka medium (Sueoka, 1960).

Insertional Mutagenesis

Transformations of strain TerBD20 with plasmid pASL (Adam and Lopez, 1998) were performed following method described by Kindle (1990).

D -Enzyme Assays

Protocols used for crude extracts preparations and enzyme assays were previously described by Colleoni et al. (1999a, 1999b).

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

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

Separation of Starch Polysaccharides by Gel Permeation Chromatography

Starch (1.5 mg) dissolved in 500 μL of 10 mM NaOH was applied to a column (0.5-cm i.d. \times 65 cm) of Sepharose CL-2B, which was equilibrated and eluted with 10 mM NaOH. Fractions of 300 to 320 μL were collected at a rate of one fraction per 1.5 min. Glucans in the fractions were detected by their reaction with iodine, and the levels of amylopectin and amylose were determined by amyloglucosidase assays (Roche Diagnostics).

Chain Length Distribution Analysis

Fractions from CL-2B column containing amylopectin were pooled and dialyzed 12 h against water. After lyophilization, the samples were digested by isoamylase (2 units in 55 mM sodium acetate buffer, pH 3.5; Megazyme International, Bray, County Wicklow, Ireland) before HPAED-PAD analysis. For a complete description of this technique, refer to Fontaine et al. (1993).

Cloning of the Full-Length D-Enzyme gDNA

A complete description of algal DNA extraction can be found in Rochaix et al. (1991). Degenerated primers corresponding to the two first of the three highly conserved regions of D-enzyme protein sequence (see Fig. 2) were designed as following: primer I, 5'-RTTRTCRTGNGTACCNGTRTA; and primer II, 5'-RAARCCVGCRAAATGRTCRATVCG. PCR amplification performed on wild-type genomic DNA with both primers I and II gave a specific fragment of 1,564 bp whose sequence was strongly similar to that of D-enzymes already cloned from higher plants. To isolate the full genomic copy of the structural gene of *C. reinhardtii* D-enzyme, 11,280 *Escherichia coli* clones from a cosmid library (Zhang et al., 1994) were screened using the previously obtained 1,564-bp fragment as a radiolabeled probe. This genomic library is indexed in 120 microtitration plates, and the corresponding *E. coli* clones were transferred onto nylon filters and consequently treated as described by Sambrook et al. (1989) before hybridization with the specific nucleotide probe. Out of a total of four positives clones, two were selected for further analysis because of their strong hybridizations with the probe. This prompted us to use these cosmids for complete sequencing of the D-enzyme gene subsequently submitted to GenBank (accession no. AF307843).

Cloning of the Full-Length D-Enzyme cDNA

A partial cDNA clone corresponding to algal D-enzyme was isolated as follows. Approximately 500,000 lysis plaques of a *C. reinhardtii* λZAP II cDNA library were screened with the 1,564-bp genomic probe previously described. A cDNA clone with an insert of 2,071 bp was isolated and fully sequenced on both strand. The 5' end of the D-enzyme cDNA was obtained by RACE-PCR (Invitrogen, Carlsbad, CA) following the supplier's instructions. In brief, a total fraction of RNA from the wild-type strain was reverse transcribed using the following specific primer 5'-CTCCAGCAGTCCGTCCTTG. A first PCR amplification of the subsequently produced cDNA was done using the specific primer 5'-GCTCCTCAATGCTCACCACAA while the nested PCR amplification was carried out with the next specific primer 5'-CGGGGGCACCAGCGCAGCAG. The complete cDNA obtained was submitted to GenBank (accession no. AF307842). Total RNA was extracted from the wild-type strain 330 with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the supplier's instructions.

RFLP Analysis

Standard protocols for molecular biology as described by Sambrook et al. (1989) were used for RFLP analysis, including gDNA restriction and subsequent electrophoresis on agarose gel, transfer onto nylon membranes, and hybridization with a specific probe. Approximately 10 µg of gDNA was digested with 50 units of *Sma*I. Restriction fragments were then separated on 0.8% (w/v) agarose gel and transferred onto a nylon membrane (Porablot, NY Amp, Macherey-Nagel, Germany). Hybridization was performed overnight at 65°C in the following hybridization buffer: 5× SSC, 5× Denhardt's, 0.1% (w/v) SDS, and 0.1 g mL⁻¹ denatured salmon sperm DNA, where 1× SSC is 0.15 M NaCl, 0.015 M sodium citrate and 1× Denhardt's is 0.2 g L⁻¹ Ficoll 400, 0.2 g L⁻¹ PVP40, and 0.2 g L⁻¹ bovine serum albumin. Probes were radiolabeled by random priming method as described by supplier's instruction (Amersham Biosciences). Membranes were typically washed twice in 2× SSC and 0.1% (w/v) SDS at 65°C for 10 min and twice in 0.5× SSC and 0.1% (w/v) SDS at 65°C for 10 min before exposure to x-ray film.

RT-PCR Experiments

Total RNA was extracted using the RNeasy plant mini kit as described by the manufacturer (Qiagen). Total RNA was then quantified by agarose gel

analysis and spectrophotometry. Reverse transcription was performed with SuperScript II kit (Invitrogen). The product of the reaction was then purified on mini-column (PCR purification kit, BIO 101, La Jolla, CA), and the subsequent PCR amplification was performed using the "Jump start ready mix" (Sigma-Aldrich, St Louis). Primers corresponding to the D-enzyme cDNA were the following: RTD3, 5'-TGATGCGGCTGACAACACG; and RTD4, 5'-TCCCCACAGAAACGCACCCCTACA.

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