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The role of amyloamylase in maltose metabolism in the cytosol of photosynthetic cells

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Abstract Transitory starch is stored during the day inside chloroplasts and then broken down at night for export. Recent data indicate that maltose is the major form of carbon exported from the chloroplast at night but its fate in the cytosol is unknown. An amyloamylase gene (*malQ*) cloned from *Escherichia coli* is necessary for maltose metabolism in *E. coli*. We investigated whether there is an amyloamylase in the cytosol of plant leaves and the role of this enzyme in plants. Two mutants of *Arabidopsis thaliana* (L) Heynh. were identified in which the gene encoding a putative amyloamylase enzyme [disproportionating enzyme 2, DPE2 (DPE1 refers to the plastid version of this enzyme)] was disrupted by a T-DNA insertion. Both *dpe2-1* and *dpe2-2* plants exhibited a dwarf phenotype and accumulated a large amount of maltose. In addition, *dpe2* mutants accumulated starch and a water-soluble, ethanol/KCl-insoluble maltodextrin in their chloroplasts. At night, the amount of sucrose in *dpe2* plants was lower than that in wild-type plants. These results show that *Arabidopsis* has an amyloamylase that is involved in the conversion of maltose to sucrose in the cytosol. We hypothesize that knocking out amyloamylase blocks the conversion from maltose to sucrose, and that the higher amount of maltose feeds back to limit starch degradation reactions in chloroplasts. As a result, *dpe2* plants have higher maltose, higher starch, and higher maltodextrin but lower nighttime sucrose than wild-type plants. Finally, we propose that maltose metabolism in the cytosol of *Arabidopsis* leaves is similar to that in the cytoplasm of *E. coli*.

Keywords Amyloamylase · *Arabidopsis* · Ethanol/KCl-insoluble polysaccharide · 4- α -Glucanotransferase · Maltose metabolism · Starch

Abbreviations *F6P*: fructose 6-phosphate · *G1P*: glucose 1-phosphate · *G6P*: glucose 6-phosphate · *GTase*: glucanotransferase

Introduction

In leaves, transitory starch is stored during the day inside chloroplasts and then broken down at night for export. Studies have been done by several groups to investigate starch breakdown in isolated intact chloroplasts from spinach. Earlier studies showed that the principal products of starch breakdown in the presence of phosphate were triose phosphate (mainly dihydroxyacetone phosphate) and 3-phosphoglycerate (Heldt et al. 1977; Peavey et al. 1977; Stitt and ap Rees 1980). Starch was also converted to maltose and glucose in the absence of phosphate but the rates of phosphate-dependent phosphorolytic starch degradation were very much higher than those of phosphate-independent hydrolytic starch degradation (Heldt et al. 1977). Stitt and Heldt (1981) reported that the products of starch breakdown included triose phosphate, 3-phosphoglycerate (PGA), CO₂, glucose, and maltose. These studies suggest that phosphorolytic starch degradation is the major pathway of starch breakdown.

However, studies done by other groups showed that both phosphorolytic and hydrolytic starch degradation were important. Levi and Gibbs (1976) reported that the major products of starch breakdown from isolated spinach chloroplasts were PGA and maltose, and, to a lesser extent, glucose phosphate — a mixture of glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P). Servaites and Geiger (2002) found that glucose, maltose, and isomaltose were the principal products of starch mobilization exported from chloroplasts at night while Ritte and Raschke (2003) showed that starch breakdown in the light in guard cell chloroplasts results in substantial maltose export. Weise et al. (2003) found that maltose and glucose were the major products exported between 1 and 3 h after the lights were turned off, and

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no isomaltose was found in the exported products. The synthesis of sucrose is by far the major fate for the carbon released by degradation of transitory starch (Trethewey and Smith 2000). The export of maltose from chloroplasts in a significant amount indicates that there must be enzymes in the cytosol that metabolize maltose to precursors for sucrose synthesis (Servaites and Geiger 2002).

Cytosolic starch phosphorylase was found in spinach and pea leaves (Steup and Latzko 1979; Preiss et al. 1980; Steup et al. 1980) and potato plants (Mori et al. 1991; Duwenig et al. 1997). This enzyme was considered to be a starch metabolism enzyme and its presence in the cytosol has been puzzling since there is no starch in the cytosol. The chloroplastic isozyme possesses a high affinity for maltodextrin, whereas the affinity of the cytosolic isozyme for maltodextrin is much lower (Yang and Steup 1990). In contrast, the cytosolic isozyme has an extremely high affinity for a highly branched, ethanol/KCl-insoluble polysaccharide, which is a poor substrate for the plastidic counterpart (Yang and Steup 1990). The ethanol/KCl-insoluble polysaccharide isolated from spinach by Yang and Steup (1990) is a high-molecular-weight heteroglycan, with an approximate chain length range from 180 to 30,000. The heteroglycan is made up of galactose, arabinose, glucose, ribose, and, to a lesser extent, xylose and fructose. The glucose only accounts for approximately 5% of the total monosaccharide content and the non-glucose monosaccharides act as the priming groups for glucan (Yang and Steup 1990). It is generally believed that this ethanol/KCl-insoluble polysaccharide is in the cytosol due to its high affinity for cytosolic starch phosphorylase. These findings raise a question — what is the enzyme that converts maltose to the glucan part of the heteroglycan upon which the cytosolic maltodextrin phosphorylase can act? We hypothesize that amyloamylase is the enzyme that converts maltose to the glucan part of the heteroglycan in the cytosol.

Amylomaltase is a type-II 4- α -glucanotransferase (GTase; EC 2.4.1.25) that catalyzes an inter-molecular glucan transfer reaction from one 1,4- α -glucan molecule to another, or to glucose. Amylomaltase was first found in *Escherichia coli* as a maltose-inducible enzyme, and the amyloamylase gene (*malQ*) was cloned from *Streptococcus pneumoniae* (Lacks et al. 1982), *E. coli* (Pugsley and Dubrevil 1988), *Clostridium butyricum* (Goda et al. 1997), and *Thermus aquaticus* (Terada et al. 1999). According to Boos and Shuman (1998), incoming maltose and maltodextrin are metabolized to glucose and G1P by the action of three cytoplasmic enzymes: amyloamylase (MalQ), maltodextrin phosphorylase (MalP), and maltodextrin glucosidase (MalZ). Amyloamylase is essential for maltose degradation in *E. coli*, and *malQ* mutants are unable to grow on maltose; their growth is inhibited by maltose (Hofnung et al. 1971) and they accumulate large amounts of free maltose inside the cell (Szmelcman et al. 1976).

A similar type-II 4- α -GTase, disproportionating enzyme (D-enzyme), is present in plants. D-enzyme was

first found in potato tubers (Peat et al. 1956; Takaha et al. 1998). Since then it has been found in many other plant organs such as carrot roots (Manners and Rowe 1969), tomato fruits (Manners and Rowe 1969), spinach leaves (Okita et al. 1979), pea leaves (Kakefuda et al. 1986), and *Arabidopsis* leaves (Lin and Preiss 1988). By knocking out plastidic D-enzyme, it was found that the majority of the GTase activity was in the chloroplast (Critchley et al. 2001). The plastidic D-enzyme is involved in transitory-starch breakdown, by converting short oligosaccharides into longer chains providing substrates for the plastidic starch phosphorylase and β -amylase (Kakefuda and Duke 1989). *Arabidopsis* plants lacking the plastidic D-enzyme (*dpe1-1*) accumulate maltooligosaccharides (maltotriose-maltoheptaose), but not maltose (Critchley et al. 2001).

With the completion of *Arabidopsis* genome sequence in the year 2000, another gene predicted to encode 4- α -GTase was found on chromosome 2 (At2g40840). This gene appears not to code for any targeting sequence as judged by Target-P (Emanuelsson et al. 2000) and the enzyme is assumed to be in the cytosol. To investigate the function of this enzyme, we identified two T-DNA knockout mutants of *Arabidopsis thaliana* (L.) Heynh. that completely lack transcripts for cytosolic amyloamylase (*DPE2*). We propose that maltose metabolism in the cytosol of *Arabidopsis* leaves is similar to maltose metabolism in the cytoplasm of *E. coli*.

Materials and methods

Plants and growth conditions

Plants of *Arabidopsis thaliana* (L.) Heynh. ecotype Wassilewskija (WS) were grown under a 16-h photoperiod, 200 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. The temperature was 22 °C during the light period and 20 °C during the dark period. Humidity was maintained at 65%. Plants used in experiments were between 3 and 5 weeks old. Wild-type and mutant plants analyzed were at the same age.

Isolation of cytosolic amyloamylase mutants

The *dpe2-1*, *dpe2-2* mutants were identified by PCR screening of 72,960 Basta-resistant lines in ecotype WS transformed with an activation-tag vector, pSK1015 (Weigel et al. 2000), using primers specific for the *DPE2* gene (5' GCA GTT CCC ATG TTC TCT GTA AGG TCA GA 3' and 5' CCA AAC AGG AAA TCA GAA GGC TTA TTG CT 3') and the T-DNA (5' CAT TTT ATA ATA ACG CTG CGG ACA TCT AC 3'). The presence of T-DNA in the *DPE2* gene was confirmed by Southern blots. The left borders of T-DNA insertions were sequenced using Big Dye Terminator technology at the UW-Madison Biotechnology Center. Candidate heterozygous *dpe2* plants were backcrossed to WT three times to clean up the background and then self-crossed to produce homozygous *dpe2* plants.

Preparation of RNA and northern blots

Total RNA was extracted from 2–3 g of mature *Arabidopsis* plants as described by Takaha et al. (1993), and electrophoresed on a formaldehyde-agarose gel (Maniatis et al. 1982) and blotted onto a

Biodyne B transfer membrane (Pall Corp., East Hills, NY, USA). The membrane was pre-hybridized in Church buffer (7% SDS, 0.5 M KH_2PO_4 , 2 mM EDTA, pH 7.0) at 65 °C for 2 h and hybridized with ^{32}P -labeled DNA probes in Church buffer overnight at 62 °C. The membrane was washed with buffers containing 2–0.1% SSC and 2–0.1% SDS at 62 °C. Washed membrane was placed on a phosphor screen for 3 days. The exposed phosphor screen was scanned by a Cyclone storage phosphor system (Packard Instrument Corp., Meridian, CN). ^{32}P -labeled DNA probes (derived from *DPE2* cDNA and elongation factor 1 α cDNA) were made by labeling DNA fragments using the Klenow fragment of *E. coli* DNA polymerase I primed with random hexanucleotide primers (Feinberg and Vogelstein 1983).

Extraction and measurements of carbohydrates

Leaf samples were taken and added to microfuge tubes containing killing solution (80% ethanol, 5% formic acid) at 80 °C for 20 min. Following centrifugation, 80% ethanol was added to the pellet at 80 °C for another 20 min. The solvents in the killing solution and 80% ethanol were evaporated in a SpeedVac SC110 concentrator (Savant Instruments). The pellets were resuspended with H_2O and centrifuged, and the supernatants were assayed for soluble carbohydrates. The bleached leaves were dried in the concentrator, ground with pestles and incubated in 0.2 M KOH at 95 °C for 20 min. The gelatinized starch solutions were brought to pH 5.0 by adding 1 M acetic acid. The starch solutions were then hydrolyzed by amyloglucosidase (Sigma) and α -amylase (Sigma) at room temperature overnight. The hydrolysates were centrifuged and the supernatants were assayed for glucose.

To extract the water-soluble, ethanol/KCl-insoluble polysaccharides, a method modified from Yang and Steup (1990) was used for small-scale preparation. Leaf samples taken at different time points were ground in liquid N_2 , suspended in chilled H_2O , and centrifuged. The supernatants were deproteinized with an equal volume of chilled water-saturated phenol twice, an equal volume of chloroform once, and de-ionized with an anion- and cation-exchange resin Sordolit MB-1 (Serva). The polysaccharides were precipitated with 1% KCl and 70% ethanol, the pellets were washed twice with 75% ethanol to remove residual ethanol-soluble carbohydrates. The pellets were resuspended and hydrolyzed in 2 N HCl for 90 min at 95 °C. The hydrolysates were neutralized with 2 N NaOH and the glucose contents were determined.

Carbohydrate determination was made using NADP(H)-linked assays (Lowry and Passonneau 1972) in a Sigma ZFP 22 dual-wavelength filterphotometer (Sigma Instruments, Berlin, Germany). Phosphate buffer (pH 7.4, 50 mM KH_2PO_4 , 20 mM KCl, 10 mM MgCl_2 , 2 mM EDTA) was used. Phosphate buffer (800 μl) containing 0.8 mM NADP and 0.8 mM ATP, 1.25 U (Unit = 1 $\mu\text{mol mg}^{-1}$ protein min^{-1}) of G6P dehydrogenase and 20 U of maltose 1-epimerase was added into the cuvette before carbohydrate samples were added. Reactions were started by the addition of 1.25 U of hexokinase, and the glucose content was determined. Maltose content was determined by the addition of 10 U of maltose phosphorylase. Phosphoglucosomerase (5 U) was added to measure fructose 6-phosphate (F6P) plus fructose. F6P without fructose was measured in a subsequent reaction so that the amount of fructose could be calculated. Sucrose in the carbohydrate samples was hydrolyzed by invertase in 50 mM citrate buffer (pH 4.5) and glucose constituent in the sucrose was determined by the same enzymatic assay. Maltose epimerase and maltose phosphorylase were purchased from the Kikkoman Corp (Tokyo). These enzymes allow accurate, precise and specific assay of maltose even in the presence of millimolar levels of sucrose (Shirokane et al. 2000). All the other enzymes used were purchased from Sigma-Aldrich (St. Louis MO, USA).

HPLC analysis

Monosaccharides in HCl-hydrolyzed polysaccharides were measured using an HPLC system consisting of a Waters 626 pump

(Waters Corp., Milford, MA, USA), a Waters 717plus autosampler, a CarboPac PA100 4x50 mm guard column (Dionex Corp., Sunnyvale, CA, USA), a CarboPac PA-100 4x250 mm column, and a Waters 464 pulsed electrochemical detector with a gold electrode (Weise et al. 2003). To separate the monosaccharides, 20 mM NaOH was used isocratically. The flow rate was 1 ml min^{-1} . The settings of the pulse amperometric detector were the following: $E_1 = 50$ mV, $T_1 = 0.4$ s, $E_2 = 750$ mV, $T_2 = 0.2$ s, $E_3 = -150$ mV, $T_3 = 0.4$ s. The range was set to 0.2 μA .

Chloroplast isolation experiments

To isolate chloroplasts, a method modified from Weise et al. (2003) was used for *Arabidopsis* leaves. Rosette leaves of six wild-type *Arabidopsis* plants (nine *dpe2* plants) were taken in the middle of the light period. Leaves were macerated in a blender using four bursts of 1 s each. Maceration buffer used was 50 ml ice-cold 50 mM Hepes buffer (pH 7.6), 330 mM mannitol, 1 mM EDTA, 5 mM MgCl_2 , 1 mM MnCl_2 , 5 mM ascorbic acid and 0.25% BSA. After maceration, the leaf slurry was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 4 °C in a Beckman JS-13.1 swinging-bucket rotor (Beckman Instruments) at 1,200 g for 10 min. The pellet was gently resuspended using a soft #3 paint brush in 1 ml 50 mM Hepes buffer (pH 7.6) containing the same ingredients as maceration buffer except ascorbic acid. Resuspended solution was then placed on top of two Percoll gradients. The Percoll gradients were then centrifuged at 4 °C at 1,200 g for 7 min. The bottom band between the 60% and 80% Percoll solutions containing intact chloroplasts was removed. An equal volume of resuspension buffer was added to the chloroplast solution and centrifuged for 2 min. The chloroplast pellet was then resuspended with 0.35 ml resuspension buffer and 0.05 ml of chloroplast solution was used for chlorophyll assay, 0.25 ml was used for maltodextrin extraction and the rest was used for chloroplast intactness assays. NADP(H)-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity before and after osmotic shock was measured to determine the percentage of intact chloroplasts.

Chlorophyll assays

To convert maltodextrin content from $\mu\text{mol mg}^{-1}$ chlorophyll to $\mu\text{mol g}^{-1}$ fresh weight, total chlorophyll from fully expanded *Arabidopsis* leaves was assayed according to Wintermans and De Mots (1965). Leaves were taken in the middle of the light period and ground into fine power in liquid N_2 . Chlorophyll was extracted with 2 ml of 96% ethanol, and the leaf slurry was further homogenized with sonication, and centrifuged for 2 min. The supernatant was used for chlorophyll assay.

Results

Isolation of cytosolic amylomaltase mutants

Arabidopsis plants containing random T-DNA insertions were screened using PCR with multiple primer combinations, for insertions in the *DPE2* gene. Two insertion lines were found. DNA sequence analysis confirmed the presence of the T-DNA left border sequences. One insertion was at the end of the sixth exon of the *DPE2* gene. There was a deletion of 47 bases at the 5' end of the T-DNA. The other insertion was in the sixth intron of the *DPE2* gene. There was a deletion of 17 bases at the 5' end of the T-DNA and a rearrangement of 6 bases at the junction of the T-DNA and the *DPE2* gene (Fig. 1).

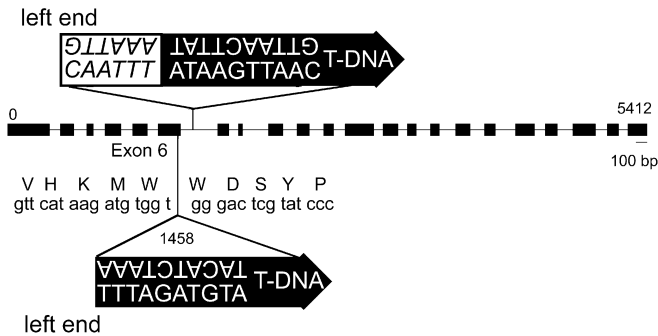


Fig. 1 Structure of the *dpe2-1* and *dpe2-2* loci. *DPE2* gene structure is depicted from 0 (translation start) to 5412 (translation stop) base pairs. *Black boxes* represent exons; *lines* represent introns. *Two vertical lines* show the sites of T-DNA insertion, and nucleotide and amino acid sequences on both sides show the sequences of the insertion site. One insertion is in the sixth exon (*dpe2-1*); the other insertion is in the sixth intron (*dpe2-2*). The left end of T-DNA ends with partial left border nucleotide sequences (*in capitals*), and with nucleotides of unidentified origin at junction of T-DNA and *DPE2* gene (*italicized capitals*, only in *dpe2-2*). The gene designation is At2g40840



Fig. 2 13-day-old wild-type, *dpe2-1* and *dpe2-2* *Arabidopsis thaliana* plants

Phenotype of *dpe2-1* and *dpe2-2* mutants

Arabidopsis dpe2-1 and *dpe2-2* mutants exhibited a dwarf phenotype. They were smaller than wild-type plants but their development was not affected (Fig. 2). Both *dpe2-1* and *dpe2-2* mutants germinated, budded, and flowered at the same time as wild-type plants, indicating that wild-type and *dpe2* plants at comparable ages are at comparable developmental stages. The dwarf phenotype of *dpe2* plants was found to co-segregate with T-DNA insertion and Basta resistance, indicating that the dwarf phenotype was caused by the T-DNA insertion in the *DPE2* gene. The leaves of *dpe2* plants were yellowish compared with leaves of wild-type plants, suggesting that *dpe2* plants might accumulate carbohydrates. However, there was no measurable difference in chlorophyll content, indicating the yellow color could result from a higher carotenoid content.

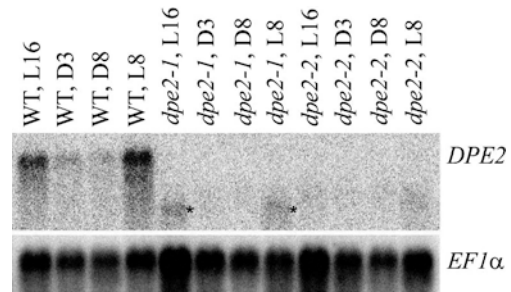


Fig. 3 Northern blot for the expression of *DPE2* in wild-type and *dpe2 A. thaliana* plants. The transcript level of *DPE2* was higher during the light period and lower during the dark period. Full-length *DPE2* mRNA was not detected in either *dpe2-1* or *dpe2-2* plants. A truncated *DPE2* mRNA, which had a T-DNA insertion in the sixth exon, was detected in *dpe2-1* plants during the light period (indicated by *asterisks*). As a loading control, the amount of *EF1α* mRNA is shown. *L16* indicates hours in the light period; *D3* and *D8* indicate hours in the dark period; *L8* indicates hours in the next light period

Northern blot and diurnal transcription of *DPE2*

The transcript level of *DPE2* varied between day and night. There were higher transcript levels during the light period and lower transcript levels during the dark period, suggesting that the transcription of *DPE2* is light inducible (Fig. 3). The full-length *DPE2* mRNA was missing in both *dpe2-1* and *dpe2-2* plants. A truncated *DPE2* mRNA, which had a T-DNA insertion in the sixth exon, was detected during the light period in *dpe2-1* plants.

dpe2-1 and *dpe2-2* mutants accumulate a large amount of maltose and starch

The amount of maltose was 20–90 times higher in *dpe2* mutants than in wild-type plants at the same age (Fig. 4). Consistent with the maltose content in bean, *Phaseolus vulgaris* L. (Weise et al. 2003), the maltose content in wild-type *Arabidopsis* was higher at night than during the day, especially early in the night. There was a peak in maltose content in the *dpe2* plants 4 h after the lights came on. The starch level in both wild-type and *dpe2* plants increased throughout the light period and dropped throughout the dark period. The overall change in amount was similar in wild-type and *dpe2* plants. However, the *dpe2* plants had a large amount of starch that was never broken down (Fig. 4).

dpe2-1 and *dpe2-2* mutants have reduced levels of G1P, G6P, and sucrose at night

The metabolites presumed to be downstream of amylo-maltase were lower at night in the *dpe2* plants than in wild-type plants. Specifically, levels of sucrose, G1P, and G6P in the *dpe2* plants were about half those of the wild type during the dark period but during the light period

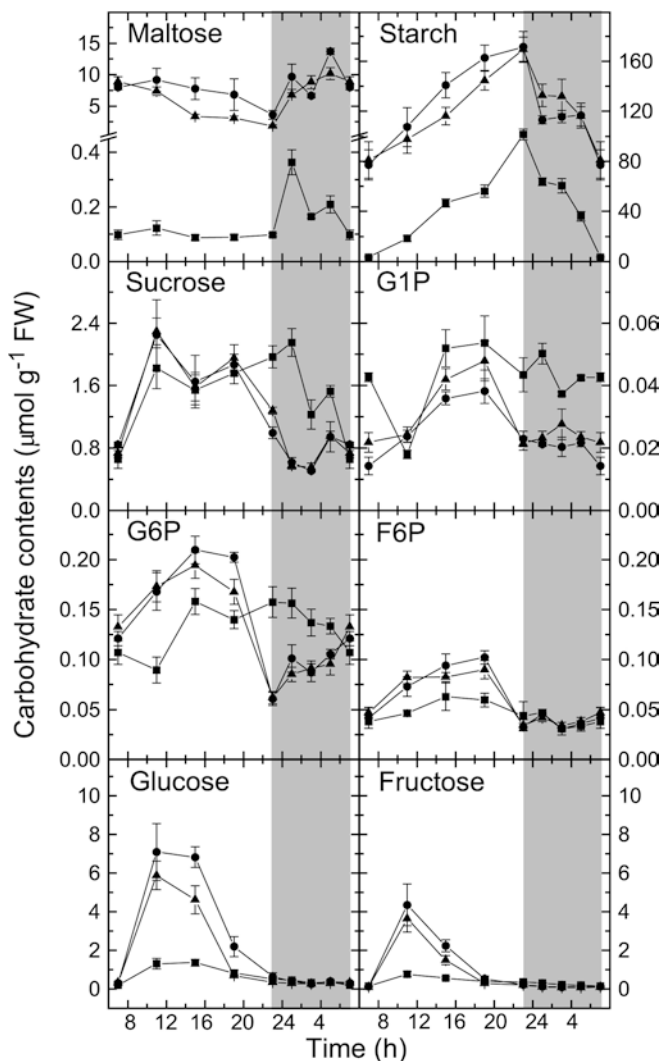


Fig. 4 Time course of maltose, starch, sucrose, G1P, G6P, F6P, glucose, and fructose contents of *Arabidopsis* leaves. The last data point is a repeat of the first data point and is shown for clarity. Data from homozygous *dpe2-1* (●) and *dpe2-2* (△) plus wild-type (■) plants are shown. Values are mean \pm SE ($n=5$). The gray area in the figure indicates the time of darkness

they were similar. Glucose and fructose contents were high in the *dpe2* plants during the day but were low in all plants at night (Fig. 4).

dpe2 mutants accumulate ethanol/KCl-insoluble maltodextrin in chloroplasts

There are two ethanol/KCl-insoluble polysaccharides in *Arabidopsis*, high-molecular-weight maltodextrin in chloroplasts, and a high-molecular-weight heteroglycan in the cytosol. HPLC analysis showed that the polysaccharide fraction from *Arabidopsis* leaves was made up of arabinose, galactose, glucose, xylose and ribose. The glucose content in the total ethanol/KCl-insoluble polysaccharides from *dpe2* leaves was three times as much as that in wild-type leaves (Fig. 5). But the con-

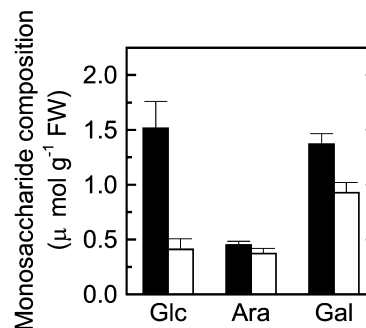


Fig. 5 Glucose (Glc), arabinose (Ara) and galactose (Gal) contents in the ethanol/KCl-insoluble polysaccharides from *dpe2* (black columns), and wild-type (white columns) *A. thaliana* leaves. Values are mean \pm SE ($n=5$)

tents of arabinose and galactose (Fig. 5) and other monosaccharides — xylose and ribose (data not shown) — did not change as much. This is consistent with the fact that in *Arabidopsis sex1* (*starch excess*) mutants only the portion of glucose in non-starch polysaccharides was increased (Schneider et al. 2002).

To investigate whether the increased amount of glucose is from chloroplastic maltodextrin or the cytosolic heteroglycan, we extracted the high-molecular-weight maltodextrin from isolated chloroplasts. HPLC analysis showed that the chloroplast maltodextrin was made up of glucose, with little or no other monosaccharide. The amount of glucose in the ethanol/KCl-insoluble polysaccharides extracted from intact leaves (Fig. 5) was not statistically different from the amount of glucose in the maltodextrin extracted from isolated chloroplasts (Fig. 6). This indicates that most, if not all, of the increased amount of glucose observed in the *dpe2* polysaccharides was in the maltodextrin in the chloroplast.

Discussion

The *DPE2* (cytosolic disproportionating enzyme, another name for the GTase) gene, At2g40840, is necessary for maltose metabolism in *Arabidopsis*. Knocking out the cytosolic GTase gene resulted in mutants having a large amount of maltose and starch and chloroplast maltodextrin compared to wild type. (T. Chia, D. Thorneycraft, S.M. Smith, and A.M. Smith reported similar findings at the 14th International Conference on *Arabidopsis* Research in Madison, WI.) Metabolites that would be downstream from amyloamylase (e.g. G1P, G6P, and sucrose) were lower at night but not during the day. We hypothesize that knocking out the *DPE2* enzyme blocks the conversion of maltose to the cytosolic polysaccharide. This results in a large amount of maltose in *dpe2* mutants. This is consistent with the fact that *malQ* mutants of *E. coli* accumulate large amounts of free maltose inside the cell (Szmecman et al. 1976). On the other hand, the large amount of maltose may feed back to limit starch degradation reactions in chloroplasts. This would explain why *dpe2* mutants have higher starch

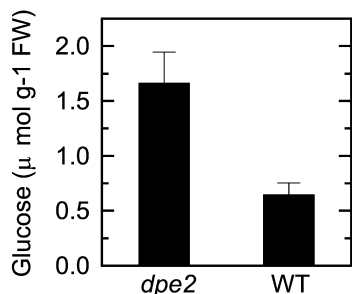


Fig. 6 Glucose content in the maltodextrin from chloroplasts isolated from wild-type (*WT*) and *dpe2* *A. thaliana*. Values are mean + SE ($n=5$)

and maltodextrin contents. By extracting maltodextrin from isolated chloroplasts, we could tell that most, if not all, of the increased amount of glucose in the non-starch polysaccharides is from chloroplast maltodextrin, not from the cytosolic polysaccharide.

The *dpe2* plants showed high levels of glucose and G6P 4 h after the lights came on. The reason for this is not clear to us. It could represent clearing of metabolite pools that built-up during the night but which could not be metabolized until the lights were turned on. This may explain why the *dpe2* plants can break down as much starch as wild-type plants, albeit with a higher background level of starch. In *dpe2* plants, starch accumulates during the first 4 h of light at least as fast as in the wild-type plants. Therefore, we believe the peak in glucose and fructose pools 4 h after the light came on represents preexisting carbon, not new photosynthate.

Type-II 4 α GTase is a member of the α -amylase superfamily; these enzymes have four distinct conserved regions containing proposed catalytic and binding sites (Matsuura et al. 1984; Takaha and Smith 1999). Examination of the amino acids in these regions suggested that the gene product we studied is more like the *E. coli* protein than other higher plant D-enzymes. To further investigate the relationships we aligned the protein sequences with MegAlign (Clustal method, gap penalty = 10, gap length penalty = 10), and generated phylogenetic trees by 1,000 bootstrap replicates under distance settings with PAUP (Fig. 7). Whether including or excluding the putative cTP from the protein sequences of 4 α GTases in *Arabidopsis*, potato, and rice, the same tree topology and bootstrap values were obtained. The phylogenetic trees from the conserved regions (not shown) and the protein sequences both show that the cytosolic GTase in *Arabidopsis* is more closely related to amyloamylase in *E. coli* than to plastidic D-enzymes in plants (Fig. 7).

The smallest substrate that the plastidic D-enzyme recognizes is maltotriose (Palmer et al. 1976) but recent data show that maltose can act as an acceptor for amyloamylase from *E. coli* (Kitahata et al. 1989; Takaha and Smith 1999). We propose that the cytosolic amyloamylase in *Arabidopsis* leaves can act on maltose. There was no detectable difference in GTase activity towards maltotriose in *dpe2* and wild-type plants (data not

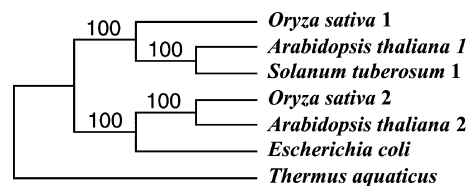


Fig. 7 Phylogenetic relationships of the protein sequences of type-II 4 α GTases from *Arabidopsis thaliana*, rice (*Oryza sativa*), potato (*Solanum tuberosum*), and *Escherichia coli*. 4 α GTase from *Thermus aquaticus* was used to root the tree as convention. The protein sequences were aligned by MegAlign (DNASTar) and analyzed by PAUP. Numbers on the branches are bootstrap values. Whether including or excluding the putative cTP from the protein sequences of 4 α GTases in *Arabidopsis*, potato, and rice, the same tree topology and bootstrap values were obtained. The accession numbers for the genes used are *A. thaliana* 1, NP_201291; *S. tuberosum* 1, CAA48630; *O. sativa* 1, BAC07076; *A. thaliana* 2 (*DPE2*), AAL91204; *O. sativa* 2, BAC22431; *E. coli*, NP_756057; *T. aquaticus* BAA33728. For plant enzymes, 1 refers to the plastidial form while 2 refers to the form that lacks a transit peptide

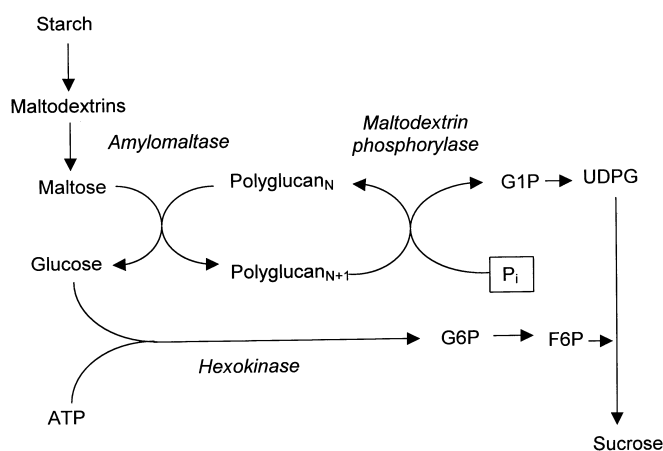


Fig. 8 Hypothetical pathway for transitory-starch breakdown and conversion to sucrose. We hypothesize that the glucan part of the cytosolic polysaccharide is the direct intermediate between maltose and sucrose. Therefore, this cytosolic polysaccharide is described as polyglucan in this diagram

shown). However, preliminary assay of GTase activity with maltose showed that there was a significant decrease of maltose-dependent GTase activity in *dpe2* mutants. This is consistent with the proposed cytosolic amyloamylase activity towards maltose. We are going to express the amyloamylase cDNA in *E. coli* and study the substrate specificity of this enzyme in greater detail.

We propose that maltose metabolism in the cytosol of *Arabidopsis* leaves (Fig 8) is similar to maltose metabolism in the cytoplasm of *E. coli* (Boos and Shuman 1998). At night, starch in leaves is broken down and the carbon is exported to the cytosol in the form of maltose and glucose. After maltose is exported into the cytosol, amyloamylase transfers a polyglucan (the glucan part of the heteroglycan) onto the maltose and releases glucose, just as amyloamylase (MalQ) does in *E. coli* (Boos and Shuman 1998). Preliminary data using heterozygous

knockout plants for a cytosolic maltodextrin phosphorylase indicate that lack of this enzyme also results in starch and maltose accumulation and low sucrose levels at night. Cytosolic maltodextrin phosphorylase recognizes maltopentaose and longer maltodextrins to form G1P and a maltodextrin that is one glucosyl unit smaller, as maltodextrin phosphorylase does in *E. coli* (Boos and Shuman 1998). By this cycle, maltose is converted to glucose and G1P. In the presence of hexokinase, which is attached to the outer membrane of chloroplasts (Wiese et al. 1999), glucose is converted to G6P. Subsequent steps are those well-known in sucrose synthesis: G6P is converted to fructose 6-phosphate (F6P) by the action of phosphoglucose isomerase (PGI) while G1P is converted to UDP-glucose (UDPG) by the action by UDPG pyrophosphorylase in the presence of UTP; F6P and UDPG are converted to sucrose 6-phosphate by sucrose-phosphate synthase (SPS); sucrose 6-phosphate is then converted to sucrose by the action of sucrose-phosphate phosphatase (SPP). Finally, sucrose is transported to other plant organs through phloem, or stored locally in the vacuoles.

The nature of the cytosolic polysaccharide involved in this metabolism in *Arabidopsis* is not clearly understood yet, but it seems significant that the heteroglycan possesses priming capacity for the phosphorylase reaction (Yang and Steup 1990). The advantages of having a cytosolic polysaccharide pool in the cytosol may include: (1) Acting as an overflow for carbohydrates (such as maltose and glucose) formed from starch degradation at night when the production of hexoses exceeds the demand for and transport of sucrose. It acts like a soluble starch pool in the cytosol just as transitory starch is a carbon overflow in chloroplasts (Stitt and Quick 1989). (2) By forming polysaccharide, the molarity of total sugar in the cytosol is kept low; as a result, the cell keeps an intracellular osmotic pressure that is good for its overall metabolic reactions in the cytosol.

In summary, the *DPE2* gene is necessary for maltose metabolism in *Arabidopsis*. We propose that maltose metabolism in the cytosol of *Arabidopsis* leaves is similar to maltose metabolism in the cytoplasm of *E. coli*. If the maltose/maltodextrin system for maltose metabolism is the primary mechanism by which carbon in starch is converted to sucrose, then the regulation of starch metabolism may be easier to understand as the regulation of the enzymes involved are discovered.

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