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A cytosolic glucosyltransferase is required for conversion of starch to sucrose in *Arabidopsis* leaves at night

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Summary

Maltose is exported from the *Arabidopsis* chloroplast as the main product of starch degradation at night. To investigate its fate in the cytosol, we characterised plants with mutations in a gene encoding a putative glucanotransferase (disproportionating enzyme; DPE2), a protein similar to the maltase Q (MalQ) gene product involved in maltose metabolism in bacteria. Use of a DPE2 antiserum revealed that the DPE2 protein is cytosolic. Four independent mutant lines lacked this protein and displayed a decreased capacity for both starch synthesis and starch degradation in leaves. They contained exceptionally high levels of maltose, and elevated levels of glucose, fructose and other malto-oligosaccharides. Sucrose levels were lower than those in wild-type plants, especially at the start of the dark period. A glucosyltransferase activity, capable of transferring one of the glucosyl units of maltose to glycogen or amylopectin and releasing the other, was identified in leaves of wild-type plants. Its activity was sufficient to account for the rate of starch degradation. This activity was absent from *dpe2* mutant plants. Based on these results, we suggest that DPE2 is an essential component of the pathway from starch to sucrose and cellular metabolism in leaves at night. Its role is probably to metabolise maltose exported from the chloroplast. We propose a pathway for the conversion of starch to sucrose in an *Arabidopsis* leaf.

Keywords: *Arabidopsis*, glucosyltransferase, maltose metabolism, maltose transporter, starch degradation, starch mutants.

Introduction

The aim of this work was to discover how products of starch degradation exported from chloroplasts in the dark are metabolised in the cytosol of leaf cells. In spite of its central importance in assimilatory carbohydrate metabolism, little is known about the pathway of conversion of starch to hexose phosphate for sucrose synthesis and cellular metabolism in leaves in the dark (Smith *et al.*, 2003). In particular, the identity of the metabolite(s) exported from the chloroplast to the cytosol *in vivo* remained unknown until very recently. Isolated chloroplasts have been shown to export triose phosphates, glucose and maltose (Heldt *et al.*, 1977; Kruger and ap Rees, 1983; Servaites and Geiger, 2002; Stitt and ap Rees, 1980; Stitt and Heldt, 1981), and NMR studies with tomato and spinach leaves indicated that chloroplasts in these species export hexoses or malto-oligosaccharides rather than phosphorylated intermediates (Schleucher *et al.*, 1998). New work with mutant *Arabidopsis* plants has now provided good evidence that maltose is the major exported metabolite *in vivo*. We showed that an *Arabidopsis* mutant with reduced rates of starch degradation and extremely high levels of maltose in leaves has a mutation in a gene encoding a maltose transporter (maltase excess 1; MEX1) located in the chloroplast envelope (Niittylä *et al.*, 2004). This transporter appears to be highly conserved throughout the plant kingdom.

Maltose entering the cytosol from the chloroplast is likely to be converted to hexose phosphates, as substrates for sucrose synthesis and for metabolism to meet the growth and energy requirements of the cell in the dark. Three alternative initial steps in maltose metabolism can be envisaged:

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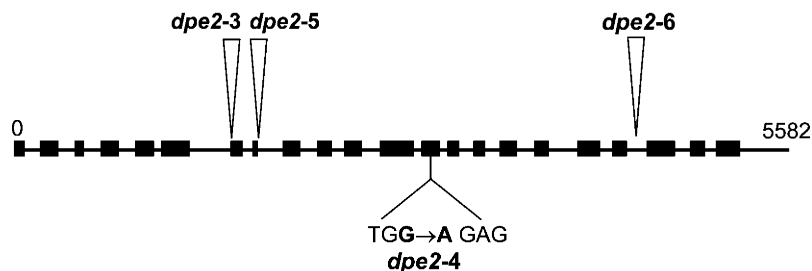

Figure 1. Structure of the *dpe2* locus.
The *dpe2* locus is displayed from 0 to 5914 bp (translation start to polyadenylation site) including 22 exons (black boxes), introns and non-coding sequences (horizontal lines). The triangles show the location of T-DNA in lines *dpe2-3* (exon 7), *dpe2-5* (intron 8) and *dpe2-6* (intron 19). The vertical line shows the guanine to adenine transition at 60 bp into exon 13 for *dpe2-4*.

(i) hydrolysis by a maltase (α-glucosidase, EC 3.2.1.20) to produce glucose for phosphorylation via hexokinase; (ii) phosphorolysis by a maltose phosphorylase (EC 2.4.1.8) to produce glucose 1-phosphate and glucose; and (iii) a glucosyl- or glucanotransferase reaction (EC 2.4.1.24 and 2.4.1.25), in which glucosyl or glucanosyl units are transferred between maltose and another glucan with the release of a free glucose. The *Arabidopsis* genome encodes at least five putative α-glucosidases and two 4-α-glucanotransferase-like proteins. There is no evidence for a maltose phosphorylase gene, and plant glucan (starch) phosphorylases do not act on maltose (Steup, 1988). We have shown that one of the putative glucanotransferases is disproportionating or D-enzyme (DPE1; At5g64860) – a chloroplastic enzyme responsible for the metabolism of the minor, maltotriose product of starch degradation at night, presumably generated by chloroplastic beta-amylolysis (Critchley *et al.*, 2001). The other (DPE2; At2g40840) lacks obvious targeting signals and is more similar in sequence to the 4-α-glucanotransferases of bacteria than it is to DPE1. In many bacteria, a 4-α-glucanotransferase is responsible for the metabolism of maltose entering the cell (for example, the maltase Q (MalQ) gene product of *Escherichia coli*; Boos and Shuman, 1998).

The likely location of DPE2 in the cytosol and its similarity to known maltose-metabolising glucanotransferases encouraged us to investigate whether it plays a role in the cytosolic metabolism of maltose derived from leaf starch in the dark. Here, we report the characterisation of mutants lacking DPE2 and provide evidence that the enzyme is a cytosolic maltose: 4-α-glucan transferase. Our results indicate that the enzyme is an essential component of pathway of utilisation of starch for sucrose synthesis and cellular metabolism in leaf cells.

Results

Isolation of the dpe2-3 *mutant*

To discover whether DPE2 has a function in the metabolism of *Arabidopsis* leaf starch, four mutants were isolated from different sources (Figure 1, and see below). These mutants were named *dpe2-3*, *dpe2-4*, *dpe2-5* and *dpe2-6*; *dpe2-1* and *dpe2-2* are described in Lu and Sharkey (2003). Here, we describe the detailed characterisation of the phenotype of a homozygous T-DNA knockout mutant *dpe2-3*, which was isolated from the Feldmann (1991) collection. *dpe2-3* contains a T-DNA insertion in exon 7 (Figure 1).

The DPE2 protein is missing in the dpe2-3 *knockout line*

A region of DPE2 unique to this enzyme (amino acids 366–379) was selected for antigen synthesis. A predicted 3D structure was obtained by modelling DPE2 against the resolved crystal structure of amylomaltase from *Thermus aquaticus* (Przylas *et al.*, 2000), which further established the location of this 14-amino-acid region (LDKNDVDYEATMET) on the surface of the DPE2 protein. The peptide was synthesised and used to raise an antiserum. The antiserum strongly recognised a band of about 100 kDa on immunoblots of crude extracts of wild-type leaves (Figure 2). This mass corresponds well to the predicted molecular mass of 110 kDa for DPE2, based on its amino acid composition. Extracts of *dpe2-3* lacked this specific

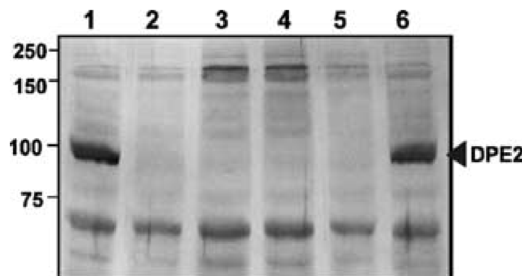

Figure 2. Immunoblot analysis for the DPE2 protein.
Soluble proteins from leaves of wild-type (lane 1, Ws, and lane 6, Col-0) and four homozygous *dpe2* mutant plants (lane 2, *dpe2-3*; lane 3, *dpe2-4*; lane 4, *dpe2-5* and lane 5, *dpe2-6*) were separated by SDS–PAGE, blotted onto a membrane and probed with a DPE2 synthetic-peptide antiserum. The mobility of molecular mass markers is indicated on the left-hand side in kDa, and the DPE2 protein is indicated as a 100-kDa band.

band; thus, the DPE2 protein is absent from the *dpe2-3* knockout mutant.

dpe2-3 *plants have a starch excess phenotype*

To discover whether DPE2 is necessary for starch metabolism, leaves were harvested at different times of day, decolourised, and then stained with iodine solution (Figure 3a). Leaves of wild-type and *dpe2-3* plants stained with approximately the same intensity at the end of the light period, indicating that they had similar starch contents. However, whereas wild-type leaves were unstained at the end of the dark period – indicating that, as expected, they contained no starch – *dpe2-3* leaves stained darkly. This suggests that *dpe2-3* leaves have a reduced rate of starch degradation at night. The high-starch phenotype was observed in leaves of very young (9-day-old) *dpe2-3* plants as well as mature rosettes (data not shown). Leaves of *dpe2-3* plants still contained starch after plants were placed in darkness for periods of up to 60 h (results not shown).

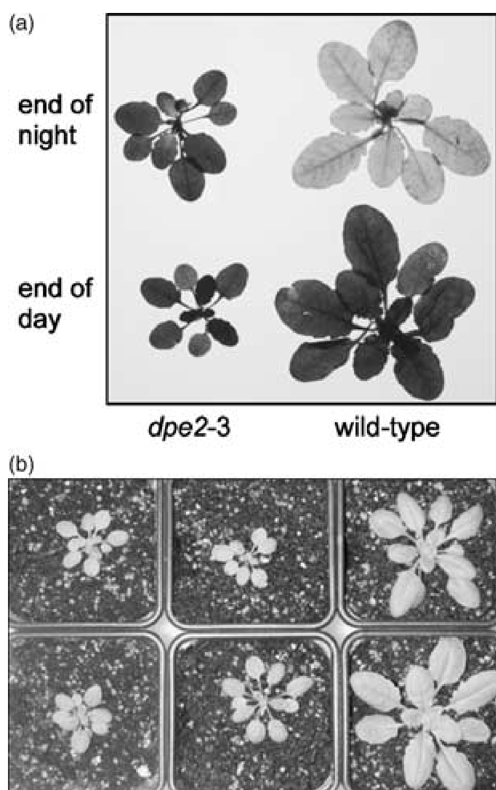

Figure 3. Presence of starch in wild-type and *dpe2-3* mutant plants.
(a) Plants harvested at the end of a 12-h dark period (end of night) and the subsequent 12-h light period (end of day) were decolourised with hot 80% (v/v) ethanol and stained with iodine solution. The presence of starch is revealed by the dark staining of the leaves.
(b) Wild-type plants and plants of the four *dpe2* mutant lines were grown together in a 12-h light/12-h dark photoperiod. The photograph was taken 21 days after vernalisation. Plants are: bottom row, left to right: *dpe2-3*, *dpe2-4* and Col-0; top row, left to right: *dpe2-5*, *dpe2-6* and Ws.

dpe2-3 *leaves have elevated levels of starch, maltose and hexoses but low levels of sucrose*

To provide more detailed information about the impact of loss of DPE2 on leaf carbohydrate metabolism, we determined the starch, malto-oligosaccharide and sugar contents of whole plants throughout a diurnal period (Figure 4). Wild-type plants showed a steady rate of starch accumulation in the light and almost complete degradation of the starch during the subsequent 12 h in the dark. *dpe2-3* plants had much higher levels of starch at the end of the night than wild-type plants, but similar levels at the end of the light period. Both the rate of accumulation during the light period and the rate of degradation in the dark were less than half of the wild-type values. This diurnal pattern of starch content was highly reproducible between independently grown batches of plants (Figure 4a,b).

Hexoses, especially glucose, were higher in *dpe2-3* than in wild-type leaves during most or all of the 24-h period (Figure 4d,e). Sucrose levels were similar in wild-type and *dpe2-3* leaves during the light period, but whereas the sucrose level in wild-type leaves showed a characteristic fall and recovery (Zeeman and ap Rees, 1999) at the start of the dark period, the level in *dpe2-3* leaves fell and remained low throughout the night (Figure 4f).

The impact of loss of DPE2 on starch degradation suggested that, like the related enzyme DPE1 (Critchley *et al.*, 2001; see Introduction), it might metabolise malto-oligosaccharides derived from starch. Accordingly, we compared amounts of individual malto-oligosaccharides in wild-type and *dpe2-3* leaves during a diurnal cycle. Malto-oligosaccharides (maltose to maltoheptaose) were barely detectable in wild-type leaves throughout the light period but increased immediately when the dark period began (Figure 4g). Maltose accounted for an average of 50% of the total malto-oligosaccharides throughout the diurnal cycle. In *dpe2-3*, the maltose level was massively elevated (average of 200-fold) throughout the 24-h period compared to the wild type. The level declined in the light and rose during the dark period. The accumulation of maltose during the dark period was comparable in magnitude with the decrease in starch content over the same period (Figure 4c). Maltose accounted for 90% of the total malto-oligosaccharides in *dpe2-3* leaves. The remaining 10% was mostly accounted for by maltotriose and maltotetraose. These malto-oligosaccharides were also massively elevated in *dpe2* relative to wild-type leaves, but behaved completely differently from maltose over a diurnal cycle. Levels were highest in the light in *dpe2* leaves, and fell at night. All malto-oligosaccharides from maltotriose to maltoheptaose showed this pattern (Figure 4h and data not shown).

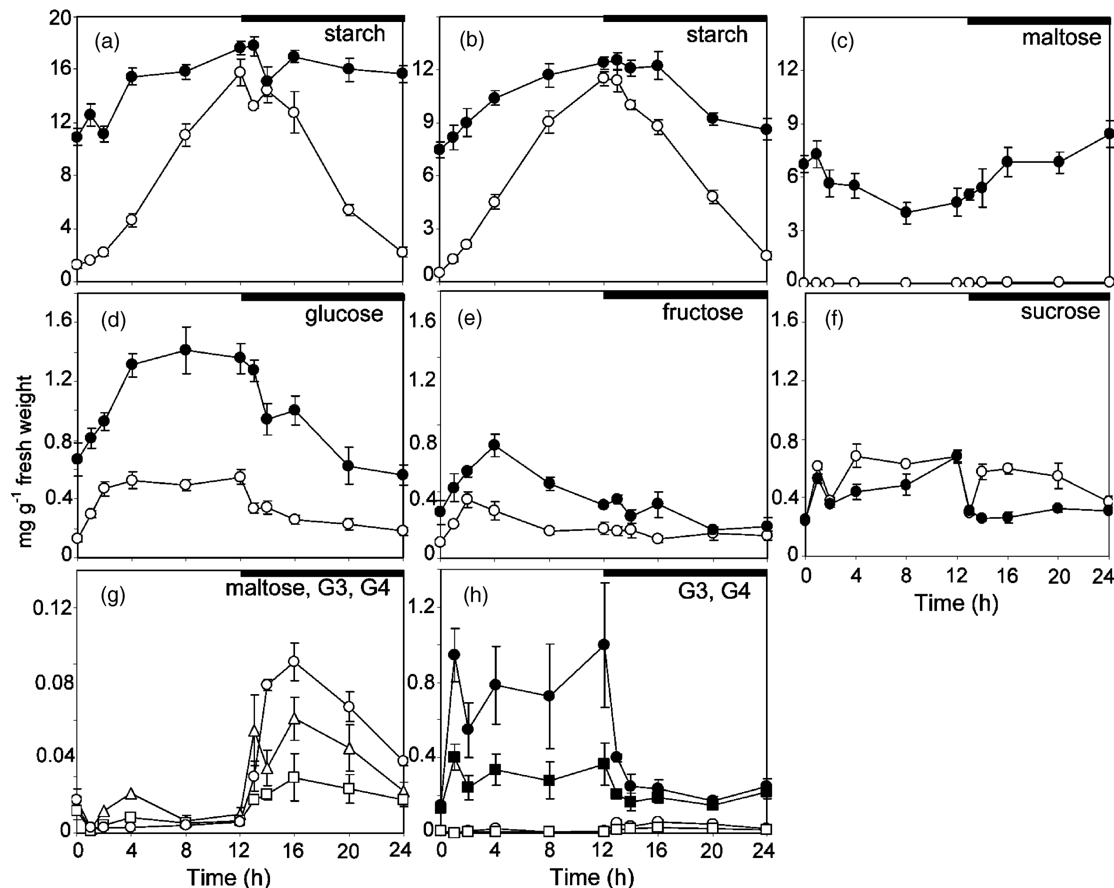

Figure 4. Starch, sugar and malto-oligosaccharide contents of wild-type and *dpe2-3* mutant leaves.
For each graph, measurements were made on plants harvested over a single 24-h period. Closed symbols are wild-type values; open symbols are *dpe2-3* values. Values are means of measurements made on the following numbers of individual plants. Starch and malto-oligosaccharides: four plants for wild-type and six for *dpe2-3*. Sugars: four plants for all measurements. Bars show SE. (a) and (b) are results for starch from two independently grown batches of plants. (g) shows an expanded version of the wild-type values for malto-oligosaccharides, also shown in (c) and (h). Note that the scale on the *y*-axis in (g) is one-tenth of that in (h), and one-hundredth of that in (c). In (g) and (h), values for maltose, maltotriose (G3) and maltotetraose (G4) are represented by circles, triangles and squares respectively.

Activities of some other starch-metabolising enzymes are affected by the loss of DPE2

The large disturbances of carbohydrate metabolism caused by the loss of DPE2 led us to investigate whether activities of other enzymes involved in starch metabolism are affected. We assayed the activities of six enzymes known to be involved, or putatively involved, in starch degradation, using optimised assays. No differences (*P*-values > 0.1) between wild-type and *dpe2-3* plants were found with respect to activities of maltase, pullulanase, α-amylase and disproportionating enzyme DPE1 (Table 1). β-amylase activity was twofold higher in *dpe2-3* plants than in wild-type plants. Other starch-excess mutants also have elevated activities of β-amylase (Caspar *et al.*, 1989). Starch phosphorylase activity was 3.5 times higher in *dpe2-3* than in wild-type leaves. We investigated whether this increase was specific for one of the two isoforms of the enzyme, using native gel analysis. The intensity of the activity band attributable to the chloroplastic form of the enzyme was the same in extracts of *dpe2-3* and wild-type leaves, but the intensity of the band attributable to the cytosolic isoform was considerably greater in extracts of *dpe2-3* leaves than in extracts of wild-type leaves. These results indicate that the activity of cytosolic, but not chloroplastic, starch phosphorylase was elevated in *dpe2-3* leaves (Figure 5a and similar, independent experiments not shown).

Three additional dpe2 *mutants have the same phenotype as* dpe2-3

To check whether the starch- and maltose-excess phenotype of *dpe2-3* plants was indeed a consequence of loss of the DPE2 protein, we searched for phenocopies of *dpe2-3* in our collection of ethyl methylsulphonate (EMS)- and X-ray-generated mutant lines pre-selected for starch-excess phenotypes (Eimert *et al.*, 1995; Zeeman *et al.*,

Table 1 Activities of enzymes of starch degradation

	Activity	
	Wild-type	*dpe2-3*
Disproportionating enzyme	273 ± 28	266 ± 27
Maltase	20 ± 2	15 ± 3
Pullulanase (limit-dextrinase)	36 ± 4	24 ± 8
α-Amylase	0.08 ± 0.004	0.08 ± 0.004
β-Amylase	140 ± 27	308 ± 38
Starch phosphorylase	40 ± 7	144 ± 15

Activities were assayed on extracts of leaves from mature rosettes. All values are means ± SE of measurements made on five extracts, from five different plants. Disproportionating enzyme was measured as the release of glucose from maltotriose. Pullulanase was assayed with pullulan as the substrate. α-amylase was assayed with starch-azure as the substrate. Starch phosphorylase was assayed with a coupled, spectrophotometric assay. For all enzymes except α-amylase, the activity is presented as nanomole glucose equivalents released from the substrate per minute per gram FW. Values for α-amylase are arbitrary units.

1998). This search yielded an EMS mutant that was slow growing and had an elevated starch content at the end of the night. Initial mapping data indicated that the mutation giving rise to the phenotype was in chromosome 2, and sequencing of the *dpe2* gene in this line revealed a G to A transition in the third position of a codon encoding tryptophan, at 60 bp into exon 13. This point mutation results in a stop codon. The allele was named *dpe2-4* (Figures 1 and 3b). We crossed the *dpe2-4* mutant to the wild type and found that all the F_1 progeny exhibited a wild-type phenotype, demonstrating that *dpe2-4* carries a homozygous recessive mutation. To further confirm that the mutations in *dpe2-3* and *dpe2-4* are in the same gene, reciprocal crosses were performed between *dpe2-3* and *dpe2-4*. A total of 40 F_1 progeny plants were derived from the two crosses, and all had the mutant phenotype. Like *dpe2-3*, *dpe2-4* plants lacked the 100-kDa DPE2 protein, were deficient in starch degradation at night, and had very high levels of maltose in their leaves (Figure 2, Table 2).

Two additional, independent T-DNA knockout lines, named *dpe2-5* and *dpe2-6*, were obtained from the Salk collection (Figure 1). These were shown to lack DPE2 protein (Figure 2). Like *dpe2-3*, plants carrying mutant alleles *dpe2-5* and *dpe2-6* were slower growing than wild-type plants of the same genetic background (Figure 3b), had high starch contents at the end of the night, and had very high levels of maltose (Table 2). The same phenotype was also recently reported for two further *dpe2* knockout mutants, *dpe2-1* and *dpe2-2* (Lu and Sharkey, 2003). These results confirm that the starch- and maltose-excess phenotype is a consequence of loss of the DPE2 protein.

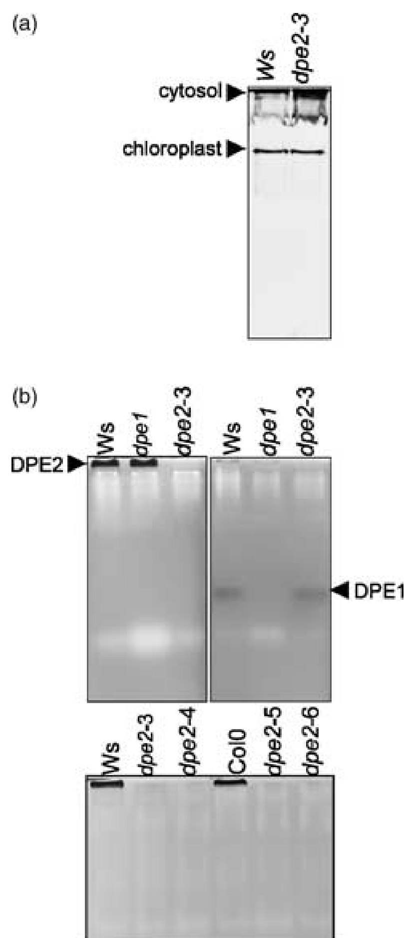

Figure 5. Native PAGE of enzyme activities in extracts of wild-type and *dpe2* mutant plants.
(a) Gel stained for starch phosphorylase activity. Soluble proteins were separated on a polyacrylamide gel containing glycogen. The gel was incubated with 20 mM glucose 1-phosphate for 16 h at room temperature and stained with iodine solution. The two isoforms of starch phosphorylase (chloroplastic and cytosolic) are distinguished by their different mobilities on the gel.
(b) Gel stained for DPE1 and DPE2 activities. Soluble proteins were separated on a polyacrylamide gel containing glycogen. Gels were incubated with either maltose (to reveal DPE2 activity) or maltoheptaose (to reveal disproportionating enzyme (DPE1) activity), and stained with iodine solution. The upper panel compares wild-type, *dpe1* and *dpe2* extracts on a DPE2 gel (left) and a DPE1 gel (right). The lower panel shows two wild-type and four *dpe2* mutant lines on a DPE2 gel.

DPE2 encodes an enzyme that transfers glucosyl units from maltose to glycogen

The very high levels of maltose in the *dpe2* mutants suggested that the glucanotransferase-like enzyme encoded by *DPE2* uses maltose as one of its substrates: as either the donor or the acceptor for a transferase reaction. If this is the case, the enzyme is expected to catalyse the release of glucose in the presence of maltose and a second appropriate glucan substrate. Accordingly, we looked for glucose production in desalted extracts of wild-type, *dpe1* and

Table 2 Starch and maltose contents of *dpe2* mutant plants

Lines	Starch content (mg g^{-1} FW)	Maltose content (mg g^{-1} FW)
Ws	0.75 ± 0.08	0.012 ± 0.001
Col-0	0.37 ± 0.05	0.008 ± 0.001
dpe2-3	8.7 ± 0.3	6.3 ± 0.5
dpe2-4	11.2 ± 0.7	5.5 ± 0.4
dpe2-5	9.1 ± 0.5	5.6 ± 0.3
dpe2-6	8.3 ± 0.8	4.7 ± 0.1

Starch and maltose were assayed as for Figure 4. Plants were harvested immediately prior to the start of the light period. Values are means ± SE of measurements made on five separate plants. Ws and Col-0 are wild-type lines. The *dpe2-3* mutation is in the Ws background, and the other mutations are in the Col-0 background.

dpe2-3 leaves in the presence of both maltose and another glucan. Experiments were carried out with *dpe1* as well as wild-type extracts because the presence of disproportionating enzyme – which catalyses glucanotransferase reactions with malto-oligosaccharides from G3 upwards – would render results with G3, G5 and G6 in wild-type extracts uninterpretable. Apparent transferase activity was detected with both amylopectin and glycogen in extracts of wild-type and *dpe1* leaves, but not in extracts of *dpe2-3* leaves. No transferase activity was detected in incubations of *dpe1* extracts containing maltose together with G3, G5 or G6.

To check whether the glucose produced in the presence of both maltose and glycogen by extracts of wild-type and *dpe1* leaves (putative DPE2 activity) was accompanied by transfer of glucosyl units between these substrates – rather than the result of stimulation by one substrate of a hydrolase producing glucose from the other substrate – incubations were carried out with [U-^{14}C]maltose and the incorporation of ^{14}C into glycogen was measured by methanol-KCl precipitation of glycogen. In experiments with five independent extracts of wild-type leaves, the amounts of glucose transferred from maltose to glycogen were between 83 and 95% of the amounts of free glucose produced in the putative transferase reaction by the same extract. In experiments with two independent extracts of *dpe1*, these values were 90 and 97%. This experiment confirms that the putative DPE2 activity is indeed a transferase, and provides good evidence that the enzyme uses maltose as a donor rather than an acceptor – in other words, it releases one of the glucosyl units of maltose as free glucose and transfers the other to glycogen.

We optimised the assay for the transferase with respect to maltose and glycogen concentrations and pH, and used these conditions to measure activity in extracts of wild-type (Wasserilewskija (Ws)), *dpe1* and *dpe2-3* leaves. Activity in wild-type and *dpe1* leaves was 0.146 ± 0.003 and 0.156 ± 0.008 μmol min^{-1} g^{-1} FW, respectively (expressed as glucose released, mean ± SE of values from five independent extracts of each sort of plant). For both sorts of leaf, activity with glycogen was about three times greater than that with amylopectin (not shown). However, activity was not detectable in extracts of *dpe2* leaves. This was true whether the activity was measured as glucose produced, or as glucosyl units transferred to glycogen, and whether assays contained glycogen or amylopectin. The detection limit of the assay was approximately 5% of the activity obtained for wild-type leaves. We checked whether failure to detect activity in *dpe2* leaves was because of the presence of an inhibitor in leaf extracts. When *dpe1* and *dpe2-3* leaves were co-extracted, activity in the mixed extract was 96% of that predicted from separate extracts of replicate samples of the two sorts of leaf, indicating that inhibition cannot account for the lack of activity in *dpe2* leaves.

As a further check on the occurrence of the maltose:4-α-glucan transferase activity in wild-type, *dpe1* and *dpe2* leaves, we devised a native gel assay for this enzyme. When extracts of wild-type leaves are subjected to electrophoresis on native, glycogen-containing gels, activity of disproportionating enzyme (DPE1) can be detected as a dark band after incubation of the gels with maltoheptaose (Critchley *et al.*, 2001). This band was also present in extracts of *dpe2* leaves (Figure 5b). Incubation of similar gels of wild-type extracts with maltose rather than maltoheptaose gave a single dark band of very low mobility on the gel. This band was missing in extracts of all four *dpe2* mutants but not in extracts of *dpe1* mutant plants (Figure 5b). We conclude that it represents the activity of DPE2.

DPE2 is located in the cytosol of Arabidopsis *leaves*

To discover the subcellular location of the DPE2 protein, protoplasts from wild-type leaves were lysed to yield a total homogenate, from which a supernatant fraction enriched in cytosol and a pellet fraction enriched in chloroplasts were prepared. The distribution of DPE2 between the chloroplasts and the cytosol was examined by immunoblotting, using the DPE2 antiserum described above. When homogenate, supernatant and pellet fractions were loaded so that each lane contained the same activity of PEP carboxylase, an enzyme known to be confined to the cytosol, the intensity of the DPE2 band in all three fractions was approximately the same (Figure 6). When fractions were loaded so that each lane contained the same activity of an enzyme known to be confined to the chloroplast (NADP glyceraldehyde 3-phosphate dehydrogenase, not shown), or the same amount of chlorophyll (Figure 6), there was a strong DPE2 band in the cytosol-enriched fraction, a weaker band in the homogenate and no visible band in the chloroplast fraction. These results indicate that the DPE2 protein is extraplastidial, most likely in the cytosol.

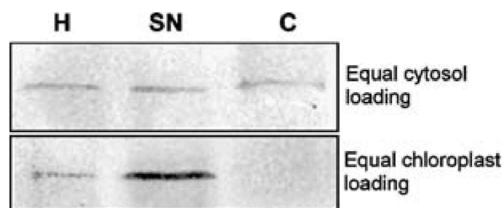

Figure 6. Subcellular localisation of DPE2 protein.
Protoplasts were prepared from wild-type leaves, and a total homogenate (H) fraction; a supernatant (SN) fraction (enriched in cytosolic components) and a pellet (C) fraction (enriched in chloroplastic components) were isolated. Lanes on an SDS-polyacrylamide gel were loaded either on the basis of equal activities of a cytosolic marker enzyme (PEP carboxylase: equal cytosol loading) or on the basis of equal amounts of chlorophyll (equal chloroplast loading). Proteins were blotted onto a membrane and probed with a DPE2 synthetic-peptide antiserum.

Discussion

The phenotypes of *dpe2* mutants indicate that this enzyme is a critical component of the pathway of conversion of starch to sucrose in leaf cells in the dark. First, the large reduction in sucrose content at night in *dpe2* relative to wild-type leaves is consistent with a lesion in the pathway of its synthesis. In wild-type leaves, sucrose content initially falls at the onset of darkness, and then rises again as its synthesis from the products of starch degradation commences (Zeeman and ap Rees, 1999). There is no rise in sucrose level after the initial fall in *dpe2* leaves. Second, the reduction in the rate of starch degradation suggests a lesion in the pathway of starch degradation. Although DPE2 does not directly attack the granule, it is possible that enzyme(s) directly attacking the granule are inhibited by the high levels of malto-oligosaccharides in *dpe2* leaves. We observed similar inhibitions of starch degradation in other mutants lacking components of the starch degradation pathway downstream of the attack on the starch granule. *dpe1* and *mex1* mutants lack the chloroplastic disproportionating enzyme (the *dpe1* mutant; Critchley *et al.*, 2001) and the chloroplastic maltose transporter (*mex1* mutants; Niittylä *et al.*, 2004), respectively. Neither of these proteins directly attacks starch granules, but in both cases, malto-oligosaccharides accumulate in mutant leaves and degradation of the starch granule is inhibited. Third, the reduction in growth rate of *dpe2* plants is consistent with a reduced supply of photoassimilate to sink organs of the plant. Reductions in growth rate are also observed in other types of mutant in which starch degradation is reduced or prevented (Caspar *et al.*, 1991; Critchley *et al.*, 2001; Niittylä *et al.*, 2004; Zeeman *et al.*, 1998).

The specific function of DPE2 in the conversion of starch to sucrose appears to be in the metabolism of maltose. We have established previously that maltose is the main form in which the products of starch degradation are exported from the chloroplast, with a more minor contribution from glucose. This conclusion comes from study of mutants that lack either a chloroplastic maltose transporter, MEX1, or the chloroplastic disproportionating enzyme, DPE1, or both of these proteins (Critchley *et al.*, 2001; Niittylä *et al.*, 2004). The very high levels of maltose in the leaves of *mex1* mutants reveal that starch degradation in the chloroplast proceeds largely via β-amylase, the main product of which is maltose. As β-amylase cannot use malto-oligosaccharides of fewer than four glucosyl units, a small amount of maltotriose is also produced during β-amylolysis of linear glucans. *dpe1* mutants accumulate maltotriose in the dark, suggesting that maltotriose is normally metabolised via a disproportionation reaction in which a maltosyl unit from maltotriose is transferred onto another oligosaccharide, producing glucose and a longer oligosaccharide. Thus the combined actions of β-amylase and disproportionating enzyme on linear glucans in the chloroplast yield both maltose and a small amount of glucose. The phenotype of *mex1*/*dpe1* double mutants – in which both maltose export and production of glucose from maltotriose in the chloroplast are prevented – is consistent with the idea that these products together account for essentially all of the carbohydrate exported to the cytosol at night. The double mutants are extremely slow growing and much smaller than the *mex1* and *dpe1* parents (Niittylä *et al.*, 2004). Figure 7 illustrates the pathway defined by these mutants.

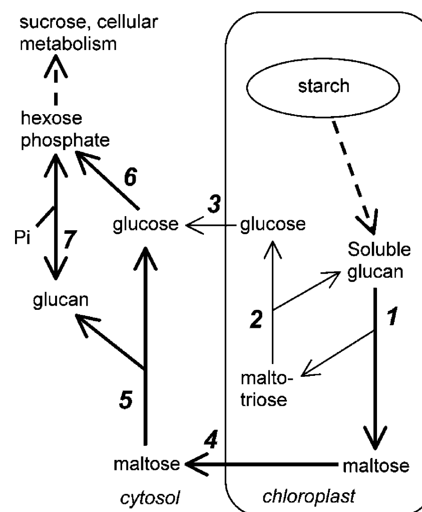

Figure 7. Proposed scheme for the conversion of starch to hexose phosphates in an *Arabidopsis* leaf cell at night.
Glucans derived from starch in the chloroplast are hydrolysed by β-amylase (*1*) to maltose, with a small amount of maltotriose. A glucosyl unit is released from maltotriose and the remaining two glucosyl units are transferred to longer glucan chains, via disproportionating enzyme DPE1 (*2*). The glucose is exported to the cytosol via the glucose transporter (*3*). Maltose is exported from the plastid via the maltose transporter MEX1 (*4*). In the cytosol, the action of DPE2 (*5*) releases one glucosyl unit as free glucose and transfers the other to a glucan. Cytosolic glucose – derived via disproportionating enzyme and the glucose transporter, and from DPE2 – is converted to hexose phosphate via hexokinase (*6*). The cytosolic glucan (starch) phosphorylase (*7*) releases glucose 1-phosphate from the glucan. Note that the cytosolic glucan and the means by which it is degraded are at this stage purely speculative.

The accumulation of maltose in *dpe2* leaves, together with the cytosolic location of the DPE2 protein, indicates that DPE2 activity is involved in the further metabolism of the maltose exported via the MEX1 transporter. DPE2 appears to be by far the major or the sole route by which this maltose is metabolised. First, the accumulation of maltose in *dpe2* leaves at night is almost sufficient to account for the degradation of starch in these leaves. Second, maltose levels in *dpe2* leaves are actually two- to threefold higher than those in *mex1* leaves when plants are grown under identical conditions (see Niittylä *et al.*, 2004; data not shown).

Levels of hexoses, and of malto-oligosaccharides longer than maltose, were also considerably elevated in leaves of *dpe2* mutant plants, and were higher during the day than at night. This diurnal trend is the opposite of that of maltose, and indicates that these compounds are at least in part derived from maltose. It is possible to speculate, for example, that during the day maltose in the chloroplast is elongated by starch synthase to produce malto-oligosaccharides that are then acted on by disproportionating enzyme (D-enzyme, DPE1), resulting in a decline in maltose and an increase in malto-oligosaccharides of various lengths and in glucose. Full understanding of these changes will require more detailed knowledge of the specificity of the DPE2 enzyme (see below), and of the partitioning of maltose between the cytosol and the chloroplast in mutant leaves.

Measurements of maltose-metabolising activities in extracts of wild-type and *dpe2* mutant leaves indicate that DPE2 is a transferase that transfers one glucosyl unit from maltose to a branched glucan, and releases the other as free glucose. When measured with glycogen as a substrate, this activity is present in leaves of wild-type plants at a level sufficient to account for observed rates of starch degradation. The rate of starch degradation is nearly 1 mg h^{-1} g^{-1} FW (Figure 4), or about 0.1 μmol glucose min^{-1} g^{-1} FW, and the activity of DPE2, expressed as total glucosyl units from starch, is about three times this value (0.3 μmol glucose min^{-1} g^{-1} FW: double the value in Results above, in which activity is expressed as release of one of the two glucosyl units from maltose). This activity was absent from leaves of *dpe2* mutants; hence, it is highly likely that it is a function of the DPE2 protein. In contrast, activity of maltase in leaves is much lower than is required to account for the rate of starch degradation, and is the same in wild-type and *dpe2* mutant leaves. We have also been unable to detect significant activity of maltose phosphorylase in wild-type leaves (data not shown).

Under the conditions we used, the DPE2 activity transferred a glucosyl unit from maltose to glycogen or amylopectin, but not from maltose to longer, linear malto-oligosaccharides. It also appears to be unable to act on single species of malto-oligosaccharides to produce longer chains. This latter type of activity is a function of disproportionating enzyme (DPE1), and is absent from the *dpe1* mutant which lacks disproportionating enzyme but contains DPE2 (Critchley *et al.*, 2001). The glucosyltransferase-like activity of DPE2 is not seen in many bacterial 4-α-glucanotransferases. The MalQ gene product from *E. coli* can use maltose as an acceptor of chains transferred from other glucans, but does not cleave the α-1,4 linkage of maltose (Boos and Shuman, 1998). The 4-α-glucanotransferase of *Thermococcus litoralis* cleaves this linkage, but can use maltose as both an acceptor and a donor in the synthesis of longer malto-oligosaccharides (Xavier *et al.*, 1999). DPE2 appears not to use maltose as an acceptor. However, caution must be exercised in drawing further conclusions about the reaction catalysed by DPE2 from our measurements: the presence in crude extracts of other enzymes that metabolise maltose, glucans and other potential substrates of DPE2 precludes further analysis, and the nature of endogenous glucan substrates is not known (see below). The production of pure enzyme for further analysis is under way. It may be appropriate to re-name this enzyme and gene when more information is available.

Glucosyltransferases with the same specificity as that of DPE2 have not previously been reported from plants. However, a gene encoding a protein 75% identical to DPE2, and of approximately the same size, is present in the rice genome (TIGR 8355.t04493). This class of enzyme may thus be widespread among higher plants.

From our present understanding of the role and nature of DPE2, we tentatively propose the following pathway from maltose to sucrose in the cytosol. DPE2 transfers one of the glucosyl units of maltose to a hypothetical glucan and releases the other for phosphorylation and conversion to sucrose. The glucosyl unit transferred to a glucan is subsequently released, and can also be used for sucrose synthesis (Figure 7). This hypothetical glucan could thus be regarded as a cytosolic carbon 'buffer' between starch degradation and sucrose synthesis. Plant cells contain soluble glycans that might fulfil this role (Steup *et al.*, 1991; Yang and Steup, 1990), but little is known of their precise location and turnover. Enzymes that might synthesise and degrade cytosolic glycans are probably present in leaf cells. A cytosolic glucan synthase has been reported from spinach leaves (Tacke *et al.*, 1991), and a putative glucan synthase gene with no apparent organellar targeting information is present in *Arabidopsis* (At5g65685). The *Arabidopsis* genome also encodes several putative glucan-degrading enzymes without obvious organellar or secretory targeting sequences. These include a putative α-glucosidase (At3g23640), α-amylase AMY2 (At1g76130) and four putative β-amylases (At4g15210, At2g45880, At5g45300 and At5g18670). It is striking in this context that the activity of starch phosphorylase was 3.5-fold higher in *dpe2* mutant than in wild-type leaves, and that most or all of

the increased activity was attributable to the cytosolic isoform of the enzyme. A cytosolic isoform of glucan (starch) phosphorylase, with a high affinity for glycogen, is of widespread occurrence in higher plants (Duwenig *et al*., 1997; Steup *et al*., 1987). The enzyme from pea seeds has a high affinity for soluble heteroglycans isolated from pea and spinach leaves (Yang and Steup, 1990). Attempts to discover the role of the cytosolic isoform, through reduction of its activity in antisense potato plants, revealed no major impact on carbohydrate metabolism (Duwenig *et al*., 1997). However, the possibility that it releases glucose 1-phosphate from a cytosolic glycan, as a step in the conversion of maltose to sucrose (Figure 7), now merits further investigation. The potential reversibility of the phosphorylase reaction under physiological conditions (Steup, 1988) would render the rate of release of glucosyl units sensitive to changes in the rate of utilisation of hexose phosphates for sucrose synthesis and cellular metabolism, consistent with our speculation that this system acts as a buffer between starch degradation and cytosolic metabolism at night.

Experimental procedures

Plant materials

Wild-type *Arabidopsis thaliana* of the ecotypes Ws and Columbia (Col-0) and mutant lines in these backgrounds were sown in a peat-based compost. Sown seeds were incubated at 4°C for 3 days, and then grown at 20°C, 75% relative humidity, in a 12-h light/12-h dark photoperiod with an irradiance of 180–200 μmol m^{-2} sec^{-1}.

Isolation of lines dpe2-3*,* dpe2-4*,* dpe2-5 *and* dpe2-6

dpe2-3 was identified by screening *Arabidopsis* plants containing random T-DNA insertions (from the collection of Feldmann, 1991) using PCR with multiple primer combinations, for insertions in the *DPE2* gene (Krysan *et al*., 1996). The mutant was found in pool N6486 from the Nottingham Arabidopsis Stock Centre. PCR primers close to the T-DNA left border with *DPE2*-specific primers from the 5′ end of the gene gave a product, and sequencing confirmed the location of the insertion. The point mutation in *dpe2-4* was discovered by amplification and sequencing of the complete DPE2 coding region in the mutant line. Independent PCR reactions using replicate DNA preparations were used to verify the point mutation and discount the possibility of a PCR error. To identify plants homozygous for the *dpe2-5* and *dpe2-6* alleles, the F_3 populations of T-DNA insertion lines from the Salk collection were screened for DPE2 protein using the DPE2 antiserum.

Production and use of a DPE2 antiserum

A polyclonal antibody was raised in a rabbit by immunisation with a peptide predicted from the sequence of the DPE2 protein (performed by Sigma-Genosys, http://www.genosys.co.uk). This synthetic peptide (LDKNDVDYEATMET) was designed by first, a pairwise comparison of the protein sequences of DPE1 and DPE2 using CLUSTALW (http://www.ebi.ac.uk/clustalw/index.html), and second, a structural prediction for hydrophilic regions on DPE2 based on a 3D modelling program INSIGHT II (http://www.accelrys.com) against amylomaltase from *T. aquaticus*, found in the FUGUE database (http://www-cryst.bioc.cam.ac.uk/~fugue/).

Leaves from 3-week-old wild-type and mutant plants were extracted at 4°C in 50 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 0.05% (v/v) Triton X-100, 5 mM DTT. After centrifugation (14 000 ***g***, 10 min), the supernatant fraction was separated on a 7.5% SDS-polyacrylamide gel (Laemmli, 1970) and then immunoblotted (Denyer *et al*., 1993) with the DPE2 antiserum at a dilution of 1 : 3000.

Extraction and measurements of metabolites

To quantify hexose, sucrose, starch and malto-oligosaccharide contents, leaves were rapidly frozen in liquid nitrogen and extracted by a modification of the method of Critchley *et al*. (2001). Samples were homogenised in 0.7 M perchloric acid and then centrifuged (3000 ***g***, 10 min, 4°C). One-half of the supernatant was adjusted to pH 7 (for sugar measurements) and the other half to pH 5 (for malto-oligosaccharide measurements). The fraction for malto-oligosaccharide measurement was further subjected to anion- and cation-exchange chromatography (Critchley *et al*., 2001) before use. The pellet was used for starch assays after washing to remove free sugars.

Starch, hexoses and sucrose were assayed enzymatically according to Hargreaves and ap Rees (1988) and Zeeman *et al*. (1998). Malto-oligosaccharides were assayed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Carbopac PA-100 column as described previously by Critchley *et al*. (2001).

Enzyme assays

Pullulanase (limit-dextrinase), α-amylase, β-amylase, maltase, starch phosphorylase and disproportionating enzyme (using maltotriose as substrate) activities were assayed as described by Zeeman *et al*. (1998). Disproportionating enzyme activity was also assayed by incubating extracts with individual malto-oligosaccharides (maltotriose, maltotetraose, maltopentaose, maltohexaose or maltoheptaose) and measuring products by HPAEC-PAD. This method revealed no differences between wild-type and *dpe2-3* plants (results not shown).

Measurement of DPE2 activity

Freshly harvested whole rosettes (0.16–0.3 g FW) were extracted in a mortar in a total of 1.2 ml of 100 mM 3-(*N*-morpholino)propanesulphonic acid (Mops, pH 7), 10% (v/v) glycerol and 1 mM DTT, with 50 mg polyvinylpolypyrrolidone. After centrifugation at 15 000 ***g*** for 10 min, the supernatant was desalted on a NAP5 (Amersham Biosciences Ltd., Bucks, UK) column and used immediately for assays. All extraction procedures were at 0–4°C. The activity of a putative transferase was measured as the difference between the amount of glucose produced in incubations that contained both maltose and the second substrate, and the sum of the amounts of glucose produced in separate incubations that contained either maltose or the second substrate alone.

Incubations were in triplicate and were at 25°C for 2 h. For assay of glucose production, they contained, in 0.4 ml, 100 mM Mops (pH 7), 10% (v/v) glycerol, 30 mM maltose, 1.5% (w/v) oyster

glycogen and 0.05 ml of extract. Control incubations lacked either maltose or glycogen. Incubations were stopped by heating to 90°C for 2 min, and glucose was assayed enzymatically (Hargreaves and ap Rees, 1988). For assay of incorporation of glucosyl units into glycogen, incubations were as above with 80 MBq mol^{-1} [U-^{14}C]maltose. Control incubations contained boiled extract. After heating as above, incubations were processed by precipitation of glycogen with 75% (v/v) methanol, 1% (w/v) KCl, as for starch synthase assays described by Jenner *et al.* (1994), followed by liquid scintillation counting.

Native PAGE

Constituents and procedure for the preparation of native PAGE were as described by Critchley *et al.* (2001). For detection of DPE1 and DPE2 activities, the incubating buffer contained either 5 mM maltose or 5 mM maltoheptaose, or both.

Preparation of protoplasts and chloroplasts

Arabidopsis leaf protoplasts were isolated from 4-week-old Ws plants based on the method described by Fitzpatrick and Keegstra (2001). Approximately 15 g of leaf material was harvested at 30 min into the light period. Leaves immersed in plasmolysing medium (400 mM sorbitol, 20 mM Mes-KOH (pH 5.2), 0.5 mM $CaCl_2$) were sliced with a sharp razor, incubated for 30 min with illumination, and then incubated for a further 3 h in plasmolysing medium containing 1% (w/v) Cellulase R-10 and 0.25% (w/v) Macerozyme R-10 (Yakult, Tokyo, Japan). Enzymes were desalted before use. Protoplasts were released by gentle agitation with a glass rod. All subsequent manipulations were carried out at 4°C. The suspension was passed through a pre-wetted 200-μm nylon mesh and centrifuged at 150 ***g*** for 5 min. The pellet was washed two times in 20 ml of plasmolysing medium (adjusted to pH 6) and finally re-suspended in 20 ml of 300 mM sorbitol, 20 mM Tricine-KOH (pH 8.4), 5 mM EGTA, 5 mM EDTA, 10 mM $NaHCO_3$, 0.1% (w/v) BSA. Chloroplasts were released by passing the protoplast suspension through a 25-μm followed by 10-μm nylon mesh. Intact chloroplasts were isolated from the resulting total homogenate by centrifugation at 480 ***g*** for 5 min. Triton X-100 (0.05% v/v) was added to samples of total homogenate and supernatant. The chloroplast pellet was re-suspended in 50 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 0.05% (v/v) Triton X-100, 5 mM DTT. Homogenate, supernatant and pellet factions were repeatedly agitated by expulsion from a syringe needle, and then used directly for chlorophyll assay or cleared by centrifugation prior enzyme assays. Fractions were assayed for chlorophyll and for activity of phosphoenolpyruvate carboxylase (Wong and Davies, 1973).

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