A mutant of *Arabidopsis thaliana* lacking the ability to transport glucose across the chloroplast envelope

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At the end of a 12-h day leaves of the mutant of *Arabidopsis thaliana* L., TC265, contained 4–5 times more starch than those of the wild type. During a subsequent 12-h night the decline in the starch content of the leaves of the mutant was at least 50% of that of the wild-type leaves. Starch labelled in the light in a 30-min pulse in $^{14}CO_2$ was rapidly broken down in a subsequent 12-h chase in the dark in air in the leaves of both mutant and wild type. Chloroplasts from leaves of the wild type took up [^{32}P]P_i and [U-^{14}C]glucose at 12 and 1.6 μmol/h per mg of chlorophyll respectively; chloroplasts from the mutant showed a similar rate for [^{32}P]P_i but no uptake of [U-^{14}C]glucose. The glucose content of freshly isolated chloroplasts from the mutant was twice that of chloroplasts from the wild type; this difference was accentuated when the isolated chloroplasts were incubated in the dark. SDS/PAGE of preparations of chloroplast envelopes showed that those from the mutant were deficient in a protein band of approximate molecular mass 40 kDa. It is suggested that in mutant TC265 the primary lesion is in a hexose transporter in the chloroplast envelope, and that this transporter moves the products of starch breakdown that are destined for sucrose synthesis from the chloroplast to the cytosol.

INTRODUCTION

Much of the starch formed by leaves during the day is converted into sucrose at night (Sharkey and Pate, 1976; Fondy and Geiger, 1982). The route whereby the products of starch breakdown move from the chloroplast for conversion into sucrose in the cytosol has not been established. There is the following evidence that such movement occurs as a C-6 rather than a C-3 compound. First, measurements of the amounts of fructose 1,6-bisphosphate and fructose 2,6-bisphosphate in darkened leaves that were converting starch into sucrose strongly suggest that the concentration of the former was too low and that of the latter too high to permit significant activity of cytosolic fructose-1,6-bisphosphatase (Stitt et al., 1985; Usuda et al., 1987; Servaites et al., 1989). Secondly, there is direct evidence of the presence of a glucose carrier in the chloroplast envelope (Schäfer et al., 1977; Herold et al., 1981). Thirdly, transgenic potato plants that lack chloroplast fructose-1,6-bisphosphatase can convert sucrose into starch (Koβman et al., 1992). We wish to add to the above evidence by showing that the mutant TC265 of *Arabidopsis thaliana*, previously thought to be deficient in respect of starch breakdown (Caspar et al., 1991), actually lacks the ability to transport glucose across the chloroplast envelope.

EXPERIMENTAL

Plants

Seeds of the wild type and the mutant TC265 of *Arabidopsis thaliana* L. (Heynh.) (race Columbia) were obtained from C. R. Somerville (Michigan State University, U.S.A.). The TC265 seed was the result of five backcrosses of TC26 (Caspar et al., 1991) to wild type. For the experiments described later in Tables 1 and 2 plants were grown as described by Haughn and Somerville (1986). In all other experiments plants were grown in Novagrow, a growth medium consisting of coconut residue and animal manure, that contained the insecticide 'gamma BHC Dust' at 0.58% (w/v). Plants in the 16-h photoperiod, and in continuous light, were grown in a growth room at 18–20 °C, 50–75% relative humidity and a quantum irradiance of 100 μE/m^2 per s. For growth in the 12-h photoperiod plants were grown as just described for 3 weeks, then they were transferred to a growth cabinet at 20 °C, 75% relative humidity and a quantum irradiance of 200 μE/m^2 per s. Wild-type and mutant plants were used after 4 and 5 weeks growth respectively. Although the mutant plants had been grown for longer their slower development meant that they were at the same developmental age as the wild-type plants.

Materials

Enzymes were obtained from Boehringer–Mannheim U.K. (Lewes, East Sussex, U.K.) except Cellulase (Onozuka R10) and Macerozyme (R10) which were from Yakult Pharmaceutical Company (Unwin Ltd., Welwyn, Herts., U.K.). Radiochemicals were from Amersham International (Aylesbury, Bucks., U.K.). Protein markers were from Sigma Chemical Company (Poole, Dorset, U.K.). Silicone oil (AR200) was from Wacker-Chemie GmbH (Munich, Germany); Novagrow from Hensby Biotech Ltd. (St. Ives, Cambs., U.K.); 'gamma BHC Dust' from Fisons (Ipswich, Suffolk, U.K.) and Scintran tissue-solubilizer from BDH Chemicals Ltd. (Poole, Dorset, U.K.).

Measurement of starch

Samples (110–340 mg fresh wt.) of leaves were prepared and immediately added to 20 ml of boiling 80% (v/v) ethanol. The insoluble material was homogenized in 80% (v/v) ethanol with a pestle and mortar, and then extracted with three successive portions of boiling 80% (v/v) ethanol. Starch, in the material which was insoluble in 80% (v/v) ethanol, was measured as described by Hargreaves and ap Rees (1988) by determining the amount of glucose released when the insoluble material was treated with amyloglucosidase and α-amylase.

Metabolism of $^{14}CO_2$

Three comparable wild-type and three comparable mutant plants were selected after 4 h of the photoperiod; the complete plants were transferred to a sealed 500-ml glass vessel, which was filled with CO_2-free air. Then $^{14}CO_2$ at 0.035% (v/v) and specific

radioactivity 55.8 μCi/μmol was generated in the vessel by injecting lactic acid on to sodium [^{14}C]bicarbonate. The vessel was illuminated at 20 °C to give a quantum irradiance of 200 μE/m^2 per s at the leaf surface. After 30 min, leaves from one wild-type and one mutant plant were removed and added to boiling 80 % (v/v) ethanol. The remaining plants were put in air in the dark at 20 °C and sampled after 12 h and 72 h. Each sample of leaves was extracted as described for the measurement of starch. The ethanol extracts from a given sample were combined, reduced to 2–3 ml *in vacuo* at 25 °C and made up to 10 ml with water to give the water-soluble fraction. The insoluble substances were treated as for the assay of starch; the labelled material that was released as a result of incubation with amyloglucosidase and α-amylase was analysed by paper chromatography in ethyl acetate/pyridine/water (8:2:1, by vol.) and the ^{14}C recovered as [^{14}C]glucose is taken as a measure of the labelling of starch. Recovery of ^{14}C from the chromatograms was > 89 % of that added to the chromatogram.

Preparation of chloroplasts

To isolate envelope proteins, protoplasts were obtained by chopping mature leaves of *Arabidopsis* into 0.5 cm^2 segments and floating them, at 30 g/300 ml, on 400 mM sorbitol, 20 mM Mes/KOH, pH 5.2, 0.5 mM $CaCl_2$ (digestion buffer) that contained 2 % (w/v) Cellulase (Onozuka R10) and 0.5 % (w/v) Macerozyme (R10) for 90 min at 20 °C in light of intensity 100 μE/m^2 per s. For all other chloroplast preparations protoplasts were prepared as follows: Cellulase and Macerozyme were dissolved in digestion buffer, centrifuged at 11000 *g* for 2 min and the supernatant was loaded on a column (1 cm × 9 cm) of Sephadex PD-10 that had been equilibrated with digestion buffer. The enzymes were eluted from the column with digestion buffer. Leaf segments were prepared as described earlier and then floated on digestion buffer at 20 °C at a light intensity of 100 μE/m^2 per s. After 30 min the desalted enzymes were added to the digestion buffer to give final concentrations of 0.2 % (w/v) for Cellulase and 0.05 % (w/v) for Macerozyme. Incubation was continued at 25 °C for 15 h at 100 μE/m^2 per s. At the end of both digestion procedures described the digests were chilled to 4 °C and all subsequent steps in the preparation of chloroplasts were carried out at 4 °C. During this procedure preparations from the mutant, because of the higher starch content, were subjected to lower centrifugal force (given in parentheses) than the preparations from the wild type. The chilled digestion mixture was filtered through a 200-μm nylon mesh. The filtrate was then centrifuged, 5 min at 168 *g* (4 min at 38 *g*), and the pellet of protoplasts was resuspended in 400 mM sorbitol, 20 mM Mes/KOH, pH 6.0, 0.5 mM $CaCl_2$. The resulting suspension was centrifuged as just described and the pellet was resuspended in 300 mM sorbitol, 20 mM Tricine/KOH, pH 8.4, 5 mM EGTA, 5 mM EDTA, 10 mM $NaHCO_3$, 0.1 % (w/v) fatty-acid-free BSA. This suspension was gently expelled through a 10-μm (20-μm for the mutant) nylon mesh fitted into the end of a cut-off 25-ml syringe. The resulting lysate was centrifuged for 2 min at 256 *g* (1 min at 270 *g*) to give chloroplasts that were resuspended in 300 mM sorbitol containing 20 mM Hepes/NaOH, pH 7.6, 4 mM $MgCl_2$, 1 mM $MnCl_2$, 2.5 mM EDTA, 10 mM $NaHCO_3$ and 0.1 % (w/v) fatty-acid-free BSA (chloroplast resuspension medium). Chloroplast intactness was determined from measurements of ferricyanide reduction (Lilley et al., 1975) and chlorophyll was measured as described by Arnon (1949).

Membranes of the chloroplast envelope were isolated at 4 °C. Chloroplasts were recovered from the protoplast lysate by centrifuging at 392 *g* for 2 min and were resuspended in 0.6 M sucrose containing 10 mM Tricine/KOH, pH 8.2, 2 mM EDTA, 1 mM dithiothreitol (lysis buffer) at 1.5 mg of chlorophyll/ml. This suspension was then frozen at −20 °C and thawed twice at 20 °C, and then the sucrose concentration was increased to 1.3 M. A stepped gradient was prepared with 3 ml of chloroplast suspension being overlaid by 5 ml of 0.98 M sucrose in lysis buffer, 5 ml of 0.6 M sucrose in lysis buffer, and 1.7 ml of lysis buffer. This gradient was centrifuged for 8 h at 192000 *g* in an SW40 Ti rotor on a Beckman Ultracentrifuge L5-50. The yellow band that formed at the 0.98 M/0.6 M sucrose interface was taken as the envelope fraction (Somerville and Somerville, 1985) and was mixed with lysis buffer to give a final volume of 14.7 ml; this was then centrifuged for 2 h at 192000 *g* to give a pellet, the chloroplast envelope membranes, which was resuspended in 25 μl of 25 % (v/v) glycerol in lysis buffer at a protein concentration of 0.1–0.4 μg/μl.

The proteins in the preparation of chloroplast envelopes were separated by SDS/PAGE using the Bio-Rad Mini-Protean II electrophoresis apparatus. Samples of the envelope preparations (5 μl) were mixed with 10 μl of 80 mM Tris/HCl (pH 8.8) containing 10 % (v/v) glycerol, 10 % (w/v) SDS, 5 % (v/v) β-mercaptoethanol and 0.002 % (w/v) Bromophenol Blue, heated at 70 °C for 5 min and centrifuged at 11000 *g* for 15 min to give a supernatant that was then subjected to SDS/PAGE in a 15 % (w/v) polyacrylamide gel as described by Laemmli (1970). Proteins were revealed by silver staining (Blum et al., 1987) and the intensity of the stain was measured with a Molecular Dynamics 300S laser scanning densitometer. Protein was measured with the Bio-Rad assay kit (Bradford, 1976).

Uptake of labelled compounds by chloroplasts

P_i uptake by isolated chloroplasts was measured at 4 °C by a method based on that described by Heldt (1980). Chloroplast suspensions, 10–20 μg of chlorophyll in 100 μl of chloroplast resuspension medium, were layered above 120 μl of silicone oil (AR200) in 0.4-ml centrifuge tubes. Measurements were started by the further addition of 100 μl of [^{32}P]P_i (0.6 mM KH_2PO_4/K_2HPO_4, pH 8.0, in chloroplast resuspension medium labelled with [^{32}P]P_i to a specific radioactivity of 16.7 μCi/μmol). Incubations were terminated by centrifugation at 9650 *g* for 45 s and the samples were immediately frozen at −80 °C. The tips of the centrifuge tubes were cut off with a hot scalpel and the chloroplast pellets were eluted by shaking vigorously for 2 h in 0.25 ml of Scintran tissue-solubilizer; [^{32}P]P_i content was then determined by liquid-scintillation counting with Hionic Fluor. Parallel experiments were carried out with [U-^{14}C]sorbitol. The procedures were identical with those for P_i uptake except that the experiment was started by adding 100 μl of chloroplast resuspension buffer that contained [U-^{14}C]sorbitol so that the final concentration in the reaction mixture was 300 mM and the specific radioactivity was 0.008 μCi/μmol.

Transport of glucose was measured as described for P_i except that 150 μl of chloroplast suspension, 20–75 μg of chlorophyll, was placed above the silicone oil, the reaction was started by the addition of 50 μl of [U-^{14}C]glucose to give a final concentration of 2 mM at a specific radioactivity of 1.25 μCi/μmol, and the reaction was carried out at 20 °C in the dark. Again each measurement with [U-^{14}C]glucose was accompanied by comparable measurements made with [U-^{14}C]sorbitol, in which the reaction was started by adding 50 μl of [U-^{14}C]sorbitol to give a final concentration of 300 mM at a specific radioactivity of 0.008 μCi/μmol.

Table 1 Diurnal variation in the starch content of leaves of wild-type and mutant plants grown in a 12-h photoperiod

Wild-type and mutant plants were grown in a 12-h photoperiod for 28 and 40 days respectively. Samples of leaves, fresh weight 110–340 mg, were taken for starch analysis at the end of the night and at 6-h intervals throughout the next 24 h. Each sample was of leaves from a single plant. Each value is the mean ± S.E.M. of determinations from six different samples. Values for the mutant are compared by a standard one-tailed, unpaired, Student's *t*-test and the probability that the values are from the same numerical population is shown.

		Starch content (mg/g fresh wt.)		Statistical analysis of mutant data	
Time from start of experiment (h)	Illumination	Wild type	Mutant	Comparison	Probability (*P*)
0	Dark	1.38 ± 0.13	45.34 ± 1.32	0 h v 12 h	0.0001
6	Light	5.76 ± 0.24	49.69 ± 2.39	12 h v 24 h	0.0004
12	Light	10.82 ± 0.74	52.76 ± 1.59	0 h v 6 h	0.0450
18	Dark	5.92 ± 0.54	48.78 ± 1.17	6 h v 12 h	0.0450
24	Dark	1.30 ± 0.11	47.84 ± 1.59	12 h v 18 h	0.0016
				18 h v 24 h	0.2330

Table 2 Metabolism of ^{14}C-labelled starch by wild-type and mutant plants during darkness

Wild-type and mutant plants were grown in a 16-h photoperiod for 4 and 5 weeks respectively. Three comparable wild-type and three mutant plants were selected after 4 h of the photoperiod, placed in a sealed 500-ml chamber and incubated in the light (200 $\mu E/m^2$ per s) in air containing 0.035% (v/v) $^{14}CO_2$ at 55.8 mCi/mmol for a 30-min pulse. Then leaves from one wild-type and one mutant plant were removed, killed in 80% (v/v) ethanol and analysed. The remaining plants were transferred to air in 500-ml chambers, one plant per chamber, and placed in the dark. The leaves of one wild-type and one mutant plant were analysed after 12 h in the dark and the leaves of the remaining plants were analysed after 72 h in the dark. The label present in the various fractions is expressed as a percentage of the total ^{14}C metabolized at the end of the pulse: this value was obtained by summing the ^{14}C recovered in the water-soluble and insoluble fractions of the pulse samples. Values are the means ± S.E.M. of estimates from three independent experiments, each with plants from a different population.

	^{14}C per fraction (% of total ^{14}C metabolized during the pulse)					
	Pulse		12-h chase		72-h chase	
Fraction	Wild type	Mutant	Wild type	Mutant	Wild type	Mutant
CO_2			35.4 ± 5.3	31.2 ± 1.1	64.8 ± 10.6	55.0 ± 3.1
Insoluble substances	31.6 ± 3.6	23.9 ± 6.1	16.3 ± 2.1	17.6 ± 5.2	17.4 ± 4.7	17.5 ± 1.9
Starch	21.4 ± 2.9	10.0 ± 3.2	0.5 ± 0.3	1.6 ± 0.8	0.3 ± 0.1	1.1 ± 0.5
Water-soluble substances	68.4 ± 3.6	76.1 ± 6.1	19.5 ± 2.0	26.4 ± 4.6	18.0 ± 5.2	16.2 ± 0.5
Total starch (mg/g fresh wt.)	6.0 ± 0.6	48.3 ± 11.1	2.7 ± 0.9	36.7 ± 5.9	1.1 ± 0.7	28.7 ± 3.7
Mass of leaves (mg)	138 ± 41	120 ± 28	148 ± 11	133 ± 33	145 ± 30	152 ± 50
Total ^{14}C metabolized						
10^6 × d.p.m./g fresh wt.	314 ± 117	240 ± 38				
% of ^{14}C supplied/g fresh wt.	33 ± 12	25 ± 4				

Measurement of glucose in isolated chloroplasts

Replicate samples of chloroplasts, 50–75 μg of chlorophyll in 200 μl of resuspension medium, were layered above 120 μl of silicone oil (AR 200) in 0.4-ml centrifuge tubes, which were then incubated in the dark at 20 °C for 60 min. Samples were taken at intervals and the incubation was stopped by centrifugation at 9650 ***g*** for 45 s. The chloroplasts passed through the oil and were separated from the incubation medium, which remained above the oil. The medium was removed. The tips of the tubes were cut off with a hot scalpel, the chloroplast pellets were resuspended in 0.5 ml of water and the resulting suspension was centrifuged at 11000 ***g*** for 15 min. The supernatant was assayed for glucose and the value obtained is taken as a measure of the glucose content of the chloroplasts. Glucose was assayed as described under measurement of starch.

RESULTS AND DISCUSSION

Caspar et al. (1991) concluded that the mutant of *Arabidopsis* TC26 showed little or no variation in leaf starch content during a normal 24-h light/dark cycle. We found that the relatively high starch content of the leaves of the mutant made it difficult to detect small differences between samples. Our use of a larger number of samples than Caspar et al. (1991) probably accounts for the fact that we were able to demonstrate that the starch content of the leaves of the mutant rose during the day and fell during the night in plants grown in a 12-h photoperiod (Table 1). The diurnal variation in starch content of the leaves of the mutant was less than that in the wild type, but it was demonstrable. In the leaves of the wild type starch had almost completely disappeared by the end of the night; the maximum content measured during the day was slightly less than that reported by Caspar et al. (1991). Conversely, the amounts of starch that we detected in the leaves of the mutant were appreciably higher than those found by Caspar et al. (1991).

In order to confirm that the leaves of the mutant could break down starch at an appreciable rate we labelled starch with $^{14}CO_2$ in a pulse in the light and then observed whether the label was lost from starch in a subsequent chase in air in the dark. The plants were grown in a 16-h photoperiod and were exposed to $^{14}CO_2$ for 30 min after 4 h of that photoperiod (Table 2). Our data show that there was appreciable incorporation of label into starch during the pulse in both wild type and mutant, but

Table 3 Uptake of P_i by chloroplasts isolated from wild-type and mutant plants

Suspensions of chloroplasts were prepared from protoplasts and 100 μl (10–20 μg of chlorophyll) was layered above silicone oil in a microfuge tube. Assays were started by adding [^{32}P]P_i or [U-^{14}C]sorbitol. The final volume of the reaction mixtures was 200 μl; the concentrations of [^{32}P]P_i and [U-^{14}C]sorbitol were 0.3 mM and 300 mM, respectively, and their specific radioactivities were 16.7 mCi/mmol and 0.008 mCi/mmol respectively. The assays were performed at 4 °C, and terminated by centrifuging the chloroplasts through the silicone oil. Labelling by [^{32}P]P_i was used to estimate the absolute amount of P_i recovered in the chloroplast fraction. Labelling by [U-^{14}C]sorbitol was used as a measure of the amount of added labelled solute that was recovered in the chloroplast fraction but was external to the inner chloroplast membrane. Label from [U-^{14}C]sorbitol recovered in the chloroplast fraction was expressed as a percentage of the amount supplied. A similar percentage of the [^{32}P]P_i supplied was assumed to be in the external space of the chloroplast fraction. From this percentage we calculated the amount of P_i recovered in the chloroplast fraction that was in the external space. These values are subtracted from the absolute amounts of P_i in the chloroplast fractions to give estimates of P_i uptake. Values for the wild type are means ± S.E.M. of 15–18 assays from six independent experiments; the values for the mutant are from 7–9 assays from three independent experiments. Each experiment used chloroplasts from a different population of plants.

	Wild type			Mutant		
	P_i in chloroplast fraction (nmol/mg of chlorophyll)		Uptake of P_i (nmol/mg	P_i in chloroplast fraction (nmol/mg of chlorophyll)		Uptake of P_i (nmol/mg
Time (min)	Total	External space	of chlorophyll)	Total	External space	of chlorophyll)
0.25	17.3 ± 1.2	5.5 ± 0.4	11.8	18.4 ± 1.9	7.9 ± 0.5	10.5
0.50	19.8 ± 1.6	5.3 ± 0.4	14.5	19.8 ± 2.0	8.5 ± 0.5	11.3
1.00	20.9 ± 1.8	5.5 ± 0.4	15.4	20.5 ± 1.7	8.4 ± 0.6	12.1
2.00	27.2 ± 2.9	5.6 ± 0.4	21.6	24.5 ± 1.9	8.6 ± 0.6	15.9
5.00	36.2 ± 3.2	5.8 ± 0.4	30.4	34.4 ± 3.4	9.1 ± 0.7	25.3

incorporation by the mutant was only half that found for the wild type. Transfer to darkness resulted in almost complete loss of label from starch in the wild type after 12 h. In the mutant, 84 % of the label was lost from starch during the same time. The loss of label from the starch in the mutant was also accompanied by a net decrease in starch content (Table 2).

The close agreement between our measurements of starch content and the behaviour of the labelled starch leads us to conclude that mutant TC265 of *Arabidopsis* does break down starch at night at an appreciable rate relative to the wild type. This view is supported by the fact that Caspar et al. (1991) found that in leaf extracts the activities of the enzymes normally regarded as being responsible for starch breakdown were not reduced by the mutation. We suggest that the primary lesion in mutant TC265 of *Arabidopsis* is not in the capacity of its leaves to break down starch.

As suggested by Caspar et al. (1991), the lesion in mutant TC265 might be in the mechanism responsible for the transport of the products of starch degradation from the chloroplast to the cytosol. We investigated this hypothesis by comparing the activities of the triose-phosphate translocator in chloroplasts isolated from leaves of wild-type and mutant plants. We isolated the chloroplasts from protoplasts prepared from mature leaves. The chloroplasts from leaves of the wild type had an intactness of 89 ± 3 % and a rate of ferricyanide-dependent O_2 evolution of 134 ± 18 μmol of O_2/h per mg of chlorophyll. The corresponding values for chloroplasts from the mutant were 78 ± 4 % and 115 ± 23 μmol of O_2/h per mg of chlorophyll. Values are means ± S.E.M.; n = 17 for wild type and 10 for the mutant.

The activity of the triose-phosphate translocator was measured essentially as described by Heldt (1980). Chloroplast suspensions were layered above silicone oil and incubated with [^{32}P]P_i. The incubations were terminated by centrifuging the chloroplasts through the silicone oil and the amount of ^{32}P in the chloroplast fraction was determined. Replicate samples of chloroplasts were incubated with [U-^{14}C]sorbitol and the amount of ^{14}C recovered in the chloroplast fraction was taken as a measure of the amount of added labelled solute that was recovered in the chloroplast fraction, but which was external to the inner membrane of the chloroplast envelope. The former value was corrected by subtraction of the latter to give the estimates of P_i uptake into the chloroplasts (Table 3). All our preparations of chloroplasts showed appreciable time-dependent uptake of P_i into the sorbitol-impermeable space. We found no difference between chloroplasts from leaves of wild-type plants and chloroplasts from the mutant. We plotted the estimates of P_i uptake shown in Table 3 as a graph and estimated the rate of P_i uptake to be 12 μmol of P_i/h per mg of chlorophyll. This value is closely comparable with that reported for spinach chloroplasts, 11 μmol of P_i/h per mg of chlorophyll (Fliege et al., 1978). We suggest that we had isolated chloroplasts from both wild type and mutant that retained at least some of their transport properties and the activity of the triose-phosphate translocator was similar in the wild type and the mutant. We conclude that the lesion in the mutant TC265 is not in the triose-phosphate translocator.

Schäfer et al. (1977) demonstrated glucose transport by spinach chloroplasts. We compared chloroplasts from the wild type and mutant TC265 in this respect. The methods used were identical with those used to measure the activity of the triose-phosphate translocator. We checked that the chloroplasts that we used to study glucose uptake were physiologically active by demonstrating that they were capable of P_i uptake. In each experiment we used samples from the same preparations of chloroplasts to measure first glucose uptake and then P_i uptake. The data obtained are those shown in Tables 4 and 3 respectively. Label from [U-^{14}C]glucose was found in the sorbitol-impermeable space of chloroplast preparations from the wild type, but not from the mutant (Table 4). The rate of uptake found for chloroplasts from the wild type was 1.6 μmol of glucose/h per mg of chlorophyll. This is lower than the values of 5.4 and 3 μmol of glucose/h per mg of chlorophyll found for spinach chloroplasts by Schäfer et al. (1977) and Herold et al. (1981) respectively.

The data in Table 4 suggest that the mutant is deficient in the ability to transport glucose across the chloroplast envelope. If this hypothesis is correct then we would expect that during starch breakdown by isolated chloroplasts those from the mutant would have a higher content of glucose. We prepared chloroplasts from wild-type and mutant leaves and incubated them in the dark to promote starch breakdown. At intervals samples of the chloroplasts were isolated by centrifugation through silicone oil and their glucose content was determined (Figure 1). The glucose

Table 4 Uptake of glucose by chloroplasts isolated from wild-type and mutant plants

Suspensions of chloroplasts were prepared from protoplasts and 150 μl (20–75 μg of chlorophyll) were incubated with [U-^{14}C]glucose and [U-^{14}C]sorbitol as described in Table 3 except that the temperature was 20 °C and that the final concentration and specific radioactivity of the [U-^{14}C]glucose were 2 mM and 1.25 mCi/mmol respectively. The amount of [U-^{14}C]glucose in the external space of the chloroplasts was determined from the labelling by [U-^{14}C]sorbitol as described in Table 3. Values for wild type are means ± S.E.M. of 18 assays from six independent experiments, except that there were only six assays at 0.5 min and 12 at 10 min; the values for the mutant are from nine assays from three independent experiments. Each experiment used chloroplasts from a different population of plants. A two-tailed, paired, Student's *t*-test was used to analyse whether, at each time point, uptake of [U-^{14}C]glucose differed from uptake of [U-^{14}C]sorbitol (glucose versus sorbitol column), and whether the net uptake of [U-^{14}C]glucose increased with respect to the uptake of [U-^{14}C]glucose at the first time point (glucose versus glucose column).

	Wild type			Mutant			Statistical analysis (*P*)			
	Glucose content (nmol/mg of chlorophyll)		Glucose uptake (nmol/mg	Glucose content (nmol/mg of chlorophyll)		Glucose uptake (nmol/mg	Glucose v sorbitol		Glucose v glucose	
Time (min)	Total	External space	of chlorophyll)	Total	External space	of chlorophyll)	Wild type	Mutant	Wild type	Mutant
0.5	46.4 ± 0.8	43.2 ± 2.3	3.2	–	–	–	0.2031	–	–	–
1	46.4 ± 2.1	42.0 ± 2.1	4.4	50.7 ± 4.6	49.4 ± 2.4	1.3	0.0001	0.1438	0.0335	–
2	47.7 ± 2.5	42.9 ± 2.1	4.8	48.7 ± 2.3	49.8 ± 2.0	−1.1	0.0002	0.3136	0.0274	0.6312
5	55.4 ± 2.9	45.8 ± 2.0	9.6	53.6 ± 2.2	50.4 ± 2.2	3.2	0.0001	0.0751	0.0121	0.5230
10	57.2 ± 5.1	48.5 ± 2.8	8.7	53.1 ± 2.2	52.8 ± 2.9	0.3	0.0121	0.8954	0.0090	0.4963
20	64.6 ± 5.8	50.7 ± 1.7	13.9	54.2 ± 2.6	51.6 ± 1.9	2.6	0.0095	0.3908	0.0195	0.5623

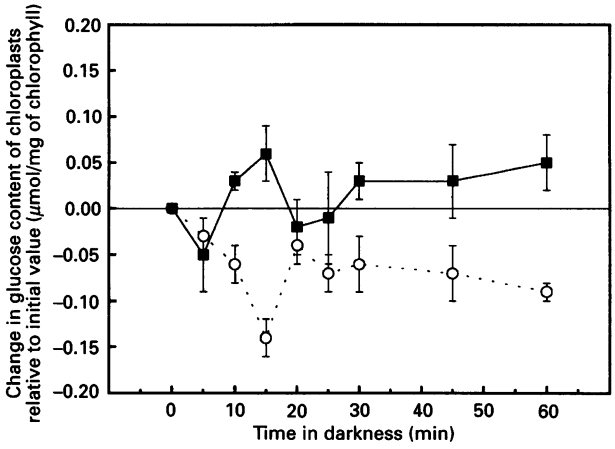

Figure 1 Effect of incubation in the dark on the glucose content of chloroplasts from wild-type and mutant plants

Suspensions of chloroplasts were prepared from protoplasts and 200 μl samples (50–75 μg of chlorophyll) were layered on silicone oil in a microcentrifuge tube, which was incubated in the dark at 20 °C. Incubations were stopped by centrifugation and the amount of glucose recovered in the chloroplast fraction was determined. In each experiment, at each time point, the change in glucose content relative to that at the start of the incubation was determined (○, wild type; ■, mutant TC265). The initial glucose content of wild-type chloroplasts was 0.27 ± 0.03 μmol/mg of chlorophyll and that of mutant chloroplasts was 0.56 ± 0.05 μmol/mg of chlorophyll. Each value is the mean ± S.E.M. of between five and nine measurements from three independent experiments, each with plants from a different population.

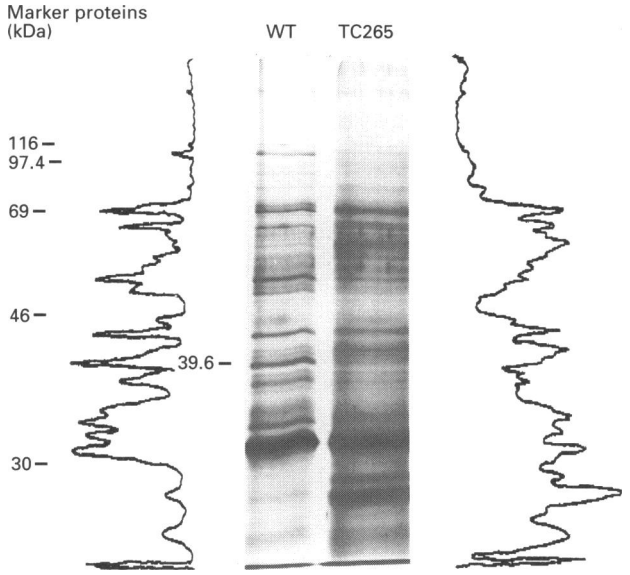

Figure 2 Protein composition of the envelope membranes of chloroplasts from wild-type and mutant plants

Chloroplasts were isolated from protoplasts, lysed and fractionated on a sucrose gradient to yield an envelope fraction, which was analysed by PAGE. Protein loadings were standardized with respect to the band at 31 kDa (the triose-phosphate translocator) and were 1 μg for the wild type and 1.5 μg for the mutant. Silver staining revealed the pattern of the proteins and the relative intensities of the different bands were measured using a scanning densitometer. The marker proteins are β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), BSA (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

content of freshly isolated chloroplasts from the leaves of the mutant was 0.56 ± 0.05 μmol/mg of chlorophyll, a value appreciably higher than that found for the chloroplasts from the wild type, 0.27 ± 0.03 μmol/mg of chlorophyll; values are means ± S.E.M. (n = 3). The glucose content of the chloroplasts from the wild type fell during the incubation in the dark; in the chloroplasts from the mutant there was a small increase in glucose.

We investigated whether we could detect any differences in the protein constituents of the chloroplast envelopes from wild-type and mutant plants. We isolated chloroplasts from protoplasts, lysed the chloroplasts, fractionated the lysate by sucrose-density-gradient centrifugation according to the procedure of Somerville and Somerville (1985), and obtained a chloroplast envelope fraction. The proteins in this fraction were separated by SDS/PAGE, stained with silver, and the relative intensities of the bands were estimated with a scanning densitometer (Figure 2). The pattern shown by the envelope fractions from the wild-type plants is sufficiently similar to those found in other studies to establish that we had isolated a chloroplast envelope fraction

(Somerville and Somerville, 1985). The latter workers identified three bands with molecular masses close to 40 kDa in envelope preparations from wild-type *Arabidopsis*. Somerville and Somerville assigned values of 42.4, 39.6 and 38 kDa to these bands, and concluded that the band at 42.4 kDa was the dicarboxylate translocator. We found a similar pattern for the envelope preparations from the wild type. Comparison with preparations from mutant TC265 revealed a number of differences (Figure 2), the most obvious and consistently demonstrated was the relative lack of the middle of the above three bands. We suggest that this middle band is equivalent to the band of molecular mass 39.6 kDa that was demonstrated by Somerville and Somerville (1985). We analysed three preparations of chloroplast envelopes; each preparation was made from a different batch of plants. The relative lack of the 39.6 kDa protein was seen in all three preparations.

We conclude that the primary lesion in the *Arabidopsis* mutant TC265 is not in an enzyme directly responsible for the catalysis of starch breakdown. The net decrease in starch content in the dark that we detected in the mutant, together with the rapid loss of label from starch made in the pulse in $^{14}CO_2$, show that the mutant approached the wild type in these respects. Our inability to detect glucose uptake by chloroplasts from the mutant strongly suggests that this is the major lesion. The possibility that this negative result is an artefact due to selective inactivation of the glucose transporter during the isolation of the chloroplasts is lessened by our demonstration that each preparation of chloroplasts that lacked the ability to take up glucose was subsequently shown to take up P_i. Additional evidence that the mutant is deficient in respect of glucose transport is provided by the higher initial content of glucose in freshly isolated chloroplasts from the mutant, and by the fact that this glucose content did not decline during incubation of the chloroplasts in the dark. Finally, there is the further evidence provided by the relative lack of a protein of approximate molecular mass 40 kDa from the chloroplast envelope of the mutant. We suggest that *Arabidopsis* mutant TC265 is deficient in a hexose transporter of the chloroplast envelope, and that this observation provides substantial support for the view that the products of starch breakdown that are destined for conversion into sucrose are exported from the chloroplast to the cytosol via this transporter.

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