Induction of *ApL3* Expression by Trehalose Complements the Starch-Deficient Arabidopsis Mutant *adg2-1* Lacking ApL1, the Large Subunit of ADP-Glucose Pyrophosphorylase[1]

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The disaccharide trehalose has strong effects on plant metabolism and development. In Arabidopsis seedlings, growth on trehalose-containing medium leads to an inhibition of root elongation, an accumulation of starch in the shoots, an increased activity of ADP-Glc pyrophosphorylase (AGPase), and an induction of the expression of the AGPase gene, *ApL3* (A. Wingler, T. Fritzius, A. Wiemken, T. Boller, R.A. Aeschbacher [2000] Plant Physiol 124: 105–114). We used Arabidopsis mutants deficient in starch synthesis to examine whether the primary effect of trehalose was to affect carbohydrate allocation by the induction of AGPase in the photosynthetic tissue. In a mutant lacking the large AGPase subunit, ApL1, (*adg2-1* mutant) growth on trehalose restored AGPase activity and led to a strong accumulation of starch in the shoots. In contrast, starch synthesis could not be induced in a mutant lacking the small AGPase subunit, ApS, (*adg1-1* mutant) or in a mutant lacking plastidic phosphoglucomutase (*pgm1-1* mutant). These results indicate that ApL3 can substitute for ApL1 in the AGPase complex. In addition, root elongation in the mutants, especially in the *adg1-1* mutant, was partially resistant to trehalose, suggesting that the induction of *ApL3* expression and the resulting accumulation of starch in the shoots were partially responsible for the effects of trehalose on the growth of wild-type plants.

ADP-Glc pyrophosphorylase (AGPase) catalyzes the synthesis of ADP-Glc, the glucosyl donor used by starch synthases, from Glc-1-P and ATP; AGPase activity is therefore required for starch biosynthesis in photosynthetic, as well as in non-photosynthetic tissues of plants (Preiss, 1982). Two starch-deficient mutants of Arabidopsis have been shown to carry mutations in genes encoding subunits of AGPase. Plant AGPases are heterotetramers typically composed of two small subunit proteins and two large subunit proteins. An Arabidopsis mutant lacking the small subunit protein, ApS, (*adg1-1* mutant; Lin et al., 1988a; Wang et al., 1998) has no detectable AGPase activity. An Arabidopsis mutant affected in the large subunit protein, ApL1, (*adg2-1* mutant; Lin et al., 1988b; Wang et al., 1997) retains about 5% of the wild-type AGPase activity. This residual activity has been ascribed to the formation of enzymatically active homotetramers of small subunits (Li and Preiss, 1992). In addition, the small residual AGPase activity in *adg2-1* could be due to the expression of the two other Arabidopsis genes encoding large subunit proteins, *ApL2* and *ApL3* (Villand et al., 1993), although the function of the ApL2 and ApL3 proteins is unclear (Kleczkowski et al., 1999).

The disaccharide trehalose occurs in a large variety of microorganisms that form mutualistic or pathogenic interactions with plants. It has recently been shown that plants such as Arabidopsis also possess genes encoding functional enzymes of trehalose synthesis (Blázquez et al., 1998; Vogel et al., 1998; Goddijn and van Dun, 1999), and trehalose itself has been detected in axenically grown Arabidopsis plants (A. Wingler, O. Fiehn, T. Boller, and A. Wiemken, unpublished data). Thus, plants are probably not only exposed to trehalose formed by microorganisms in plant-microbe interactions, but also endogenously from trehalose that could act as a signal in plant metabolism, particularly in the context of sugar sensing and assimilate partitioning. For example, it has been shown that trehalose induces enzymes of fructan synthesis in barley (Wagner et al., 1986; Müller et al., 2000) and Suc synthase activity in soybean (Müller et al., 1998). In Arabidopsis, growth on trehalose-containing medium results in an inhibition of root elongation (Aeschbacher et al., 2000), which is probably due to a strong accumulation of starch in the shoots, leading to a reduced supply of carbon to the roots (Wingler et al., 2000). The trehalose-induced accumulation of starch is accompanied by an increase in the activity of AGPase and by an induction of the expression of *ApL3*. To study this effect more closely and to analyze the role of ApL3 in starch synthesis we tested the effect of trehalose on AGPase activity

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and on starch synthesis in the mutants *adg2-1* and *adg1-1*, as well as in the *pgm1-1* mutant in which starch synthesis is impaired due to the lack of plastidic phosphoglucomutase (Caspar et al., 1985).

RESULTS

Trehalose-Induced Expression of the *ApL3* Gene in Starch-Deficient Arabidopsis Mutants

Previous experiments with wild-type Arabidopsis have shown that growth on trehalose-containing medium leads to a strong induction of the expression of the AGPase gene, *ApL3*, in the shoots of seedlings (Wingler et al., 2000). We found that the *ApL3* gene was similarly induced by trehalose in the three different mutants deficient in starch synthesis, *adg1-1*, *adg2-1*, and *pgm1-1* (Fig. 1). As previously observed for wild-type plants, trehalose did not influence the expression of *ApS*, *ApL1*, or *ApL2*. In accordance with Wang et al. (1997; 1998), no effect of the *adg1-1* and *adg2-1* mutations on the amounts of *ApS* or *ApL1* mRNAs was detectable.

AGPase Activity

We measured AGPase activity in the wild-type plants and in the mutants after growth in the absence or presence of 30 mM trehalose. In wild-type plants, the activity of AGPase was doubled in the presence of trehalose (Fig. 2). An increase in AGPase activity was also found in the *pgm1-1* mutant. The *adg1-1* mutant did not show any significant AGPase activity in the absence or in the presence of trehalose. In contrast, the in vitro activity of AGPase in the *adg2-1* mutant increased at least 20-fold after growth in the presence of trehalose. The measured activity was, however, still lower than that in the wild type grown without trehalose.

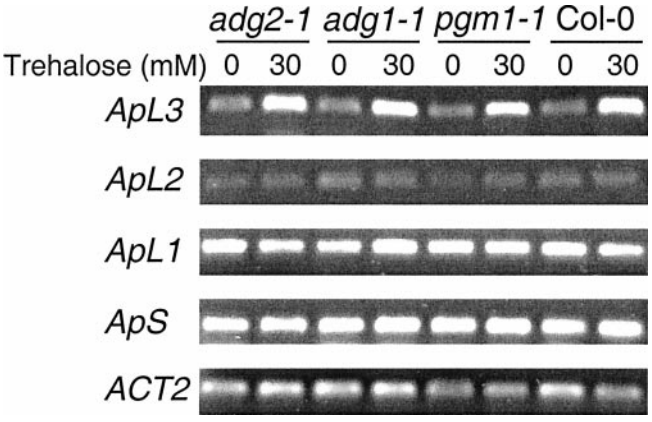

Figure 1. Reverse transcriptase-PCR analysis of the expression of the AGPase genes *ApS*, *ApL1*, *ApL2*, and *ApL3* in wild-type Arabidopsis seedlings (Col-0) and in mutants deficient in starch synthesis (*adg2-1*, *adg1-1*, and *pgm1-1*), grown for 14 d on one-half-strength Murashige and Skoog medium containing 0 or 30 mM trehalose.

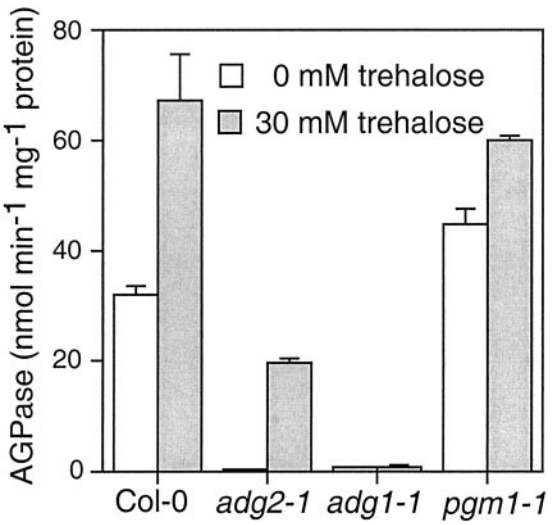

Figure 2. Activity of AGPase in wild-type Arabidopsis seedlings (Col-0) and in mutants deficient in starch synthesis (*adg2-1*, *adg1-1*, and *pgm1-1*), grown for 14 d on one-half-strength Murashige and Skoog medium containing 0 or 30 mM trehalose. Data are means of plants from three separate agar plates ± SE.

Starch Formation

Starch contents in the shoots of wild-type plants increased strongly with increasing trehalose concentrations, resulting in a 20-fold higher starch content in the presence of 60 mM trehalose than in the absence of trehalose (Fig. 3). Although the *adg2-1* mutant contained less starch than the wild type in the absence of trehalose, starch strongly accumulated in this mutant when it was grown on trehalose-containing medium. At 60 mM trehalose, the starch content in the *adg2-1* mutant was almost 15 times

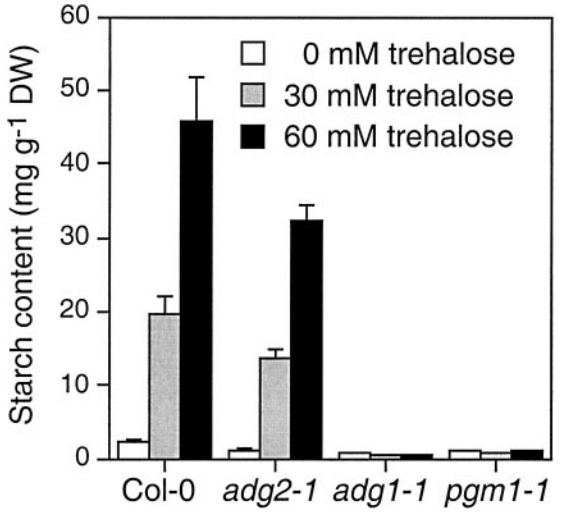

Figure 3. Starch content in wild-type Arabidopsis seedlings (Col-0) and in mutants deficient in starch synthesis (*adg2-1*, *adg1-1*, and *pgm1-1*), grown for 14 d on one-half-strength Murashige and Skoog medium containing 0, 30, or 60 mM trehalose. Data are means of plants from four separate agar plates ± SE.

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higher than in the wild type in the absence of trehalose. As expected, starch contents in the *adg1-1* and *pgm1-1* mutants were very low, i.e. close to the detection limit, in the absence of trehalose, and there was no effect of trehalose on starch formation.

To study the distribution of starch we stained the seedlings with KI/I_2 solution (Fig. 4). Whereas no accumulation of starch was visible in the absence of trehalose, starch strongly accumulated in the cotyledons and in the developing leaves when the *adg2-1* mutant or the wild type were grown in the presence of trehalose. One of the cotyledons was typically smaller and more strongly stained than the other cotyledon. No accumulation of starch could be observed in the *adg1-1* or *pgm1-1* mutants.

Trehalose affected starch synthesis not only in seedlings of Arabidopsis, but also in mature plants. Rosette leaves of wild-type plants grown for 4 weeks in the presence of 25 mM trehalose contained 95.7 ± 10.2 mg starch g^{-1} dry weight (mean of three plants ± SE) compared with 14.7 ± 0.1 mg starch g^{-1} dry weight in plants grown without addition of trehalose.

Root Growth

Previous experiments have shown that in parallel with the accumulation of starch in the shoots of Arabidopsis seedlings, trehalose strongly inhibits the elongation of roots and the expansion of leaves (Aeschbacher et al., 2000; Wingler et al., 2000). Our hypothesis was that the enhanced accumulation of photosynthates in the form of starch in the shoots caused a reduced allocation of carbon to the roots and, thereby, a starvation of the root tissue. If this hypothesis is correct, mutants that are incapable of converting assimilated carbon into starch should be resistant to the trehalose-dependent inhibition of root elongation. In wild-type plants, root length was already reduced to less than one-half in the presence of 5 mM trehalose and was further decreased at higher trehalose concentrations (Table I). The mutants deficient in starch synthesis, *adg1-1*, *adg2-1*, and *pgm1-1* also showed a trehalose-dependent decrease in root elongation. However, this decrease was not as pronounced as in wild-type plants. At 5 and at 15 mM trehalose, all of the mutants had longer roots than the wild-type plants, whereas at 30 mM trehalose only the roots of *adg1-1* mutant were longer than those of the wild type.

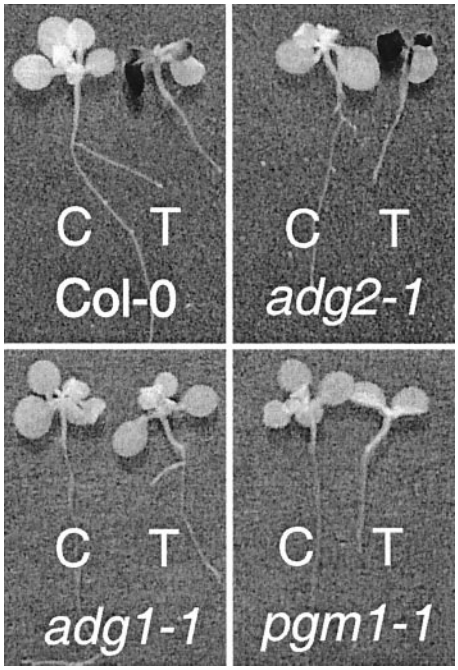

Figure 4. Distribution of starch in wild-type Arabidopsis seedlings (Col-0) and in mutants deficient in starch synthesis (*adg2-1*, *adg1-1*, and *pgm1-1*), grown for 14 d on one-half-strength Murashige and Skoog medium containing 0 (C) or 30 mM trehalose (T). The seedlings were stained for starch with KI/I_2 solution.

Photosynthesis

To check if trehalose application affects the rate of photosynthesis in wild-type plants we determined chlorophyll fluorescence parameters. There was no evidence of chronic photoinhibition, as would have been indicated by a decrease in the F_v/F_m ratio (Table II). In a similar manner, the quantum efficiency of photosystem II electron transport (ΦPSII) was not reduced by trehalose. These data indicate that photosynthetic function was not affected by growth on trehalose-containing medium.

DISCUSSION

In a previous study we have shown that application of trehalose has strong effects on root development and on carbohydrate metabolism in Arabidopsis seedlings (Wingler et al., 2000). Trehalose inhibits the elongation of roots and leads to an accumulation of starch, enhances AGPase activity, and induces the expression of *ApL3* in the shoots. We have also demonstrated that these effects are not caused by the cleavage of trehalose to Glc and cannot be ascribed to an increase in osmotic pressure. Here we chose a genetic approach using mutants deficient in starch synthesis for analyzing the causal relationship between the induction of *ApL3* expression, the accumulation of starch, and the inhibition of root elongation. In particular, our aim was to investigate whether or not AGPase activity and starch synthesis in the *adg2-1* mutant can be complemented by the trehalose-dependent induction of *ApL3* expression.

Effect of Trehalose on Carbon Allocation and on Root Elongation

In wild-type plants, trehalose caused a strong reduction in root length (Table I). Since root elongation

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Table I. *Root length of wild-type Arabidopsis seedlings (Col-0) and of mutants deficient in starch synthesis (adg2-1, adg1-1, and pgm1-1) after growth for 14 d on one-half-strength Murashige and Skoog medium containing between 0 and 30 mM trehalose*

Data are means of between 35 and 72 plants ± SE.

Trehalose	Root Length in Millimeters and as a Percentage of Col-0						
	Col-0	*adg2-1*		*adg1-1*		*pgm1-1*	
	mm		%	*mm*	%	*mm*	%
0 mM	21.4 ± 0.6	24.7 ± 1.3	115	29.5 ± 1.4	138	27.7 ± 1.1	129
5 mM	9.3 ± 0.3	28.8 ± 2.0	311	17.5 ± 0.3	189	22.1 ± 0.8	239
15 mM	6.6 ± 0.2	10.4 ± 0.6	157	14.5 ± 0.7	220	14.4 ± 1.0	218
30 mM	4.6 ± 0.4	4.4 ± 1.7	95	13.2 ± 0.4	286	5.4 ± 0.4	116

can be restored when Glc or Suc are provided together with trehalose (Wingler et al., 2000), we assumed that the effect of trehalose was mainly metabolic and was due to a starvation of the root tissue. The simplest explanation for such a starvation would have been that trehalose interfered with photosynthetic CO_2 assimilation. Our results on chlorophyll fluorescence do, however, exclude such an effect of trehalose (Table II). Even at a relatively high concentration of 30 mM trehalose, there were no indications of chronic photoinhibition or of decreased rates of photosynthetic electron transport. The accumulation of starch in the shoots of the seedlings (Figs. 3 and 4) also rules out that decreased rates of photosynthesis were responsible for the inhibition of root elongation. Instead, it appeared likely that the accumulation of photosynthetically fixed carbon in the form of starch was the primary effect of trehalose and that this accumulation was resulting from the higher AGPase activity (Fig. 2), diverting more assimilated carbon to ADP-Glc and subsequently to starch. As a result, less carbon would be available for export to the roots. In mutants with impaired starch formation, one would, consequently, expect the effect of trehalose on root elongation to be less severe than in wild-type plants. At low concentrations of trehalose (5 or 15 mM), all three starch-deficient mutants, *adg1-1*, *adg2-1*, and *pgm1-1*, showed increased resistance to trehalose. Since starch synthesis can also be induced by trehalose in the *adg2-1* mutant, it is not surprising that root elongation in this mutant was equally affected as in wild-type plants at a higher concentration of 30 mM trehalose. In contrast, the *adg1-1* mutant, which does not accumulate starch, showed increased resistance at all trehalose concentrations tested, supporting the hypothesis that accumulation of excess starch is partially responsible for the inhibition of root elongation. In an alternate manner, it is possible that higher sugar contents in the starch-deficient mutants might compete with trehalose at its site of action and thereby alleviate the effect of trehalose. At high concentrations of trehalose, root length was also reduced in the *adg1-1* and *pgm1-1* mutants, suggesting that in addition to inducing starch formation, trehalose has other effects on Arabidopsis. Such effects could act directly at the level of the root cells or they could result from additional alterations of shoot metabolism, e.g. from an inhibition of Suc synthesis.

Complementation of AGPase Activity in the *adg2-1* Mutant by Trehalose-Induced Expression of *ApL3*

Based on mutation analysis, the presence of ApS and ApL1 was up to now considered to be required for AGPase activity (Lin et al., 1988a, 1988b). ApL3, in contrast, was not considered to contribute significantly to the overall AGPase activity in the leaves (Sokolov et al., 1998). However, our results indicate that ApL3, induced by trehalose (Fig. 1), can substitute for ApL1 in the *adg2-1* mutant (Figs. 2–4). Because trehalose application does not complement the *adg1-1* mutant, the effect of trehalose obviously requires the intact ApS protein. In the *adg2-1* mutant, ApS protein is present, but its amount is reduced (Lin et al., 1988b). It is possible that by inducing *ApL3* expression in the presence of trehalose, increased amounts of ApL3 protein can stabilize the ApS protein. Besides, heterozygous plants of a cross of wild-type Arabidopsis with the *adg1-1* mutant contained the same AGPase activity as the wild type (Lin et al., 1988a), suggesting that ApS protein is normally in excess. This also implies that the amount of ApS protein in the wild type is adequate to support the increased AGPase activity we found in plants grown in the presence of trehalose. Based on our results we propose that ApL3 and ApS can form a functional AGPase complex in the same way as ApL1 and ApS (see model in Fig. 5). ApL3 on its own is, however, incapable of forming a functional AGPase.

Plant AGPases are generally activated by 3-phosphoglycerate and inhibited by P_i (Preiss, 1982). Although the small subunits of plant AGPases are

Table II. *F_v/F_m and ΦPSII in wild-type Arabidopsis seedlings (Col-0) grown for 13 d on one-half-strength Murashige and Skoog medium with or without addition of 30 mM trehalose*

Data are means of seedlings from three to five different agar plates ± SE.

Trehalose	F_v/F_m	ΦPSII
0 mM	0.746 ± 0.005	0.543 ± 0.016
30 mM	0.749 ± 0.003	0.563 ± 0.006

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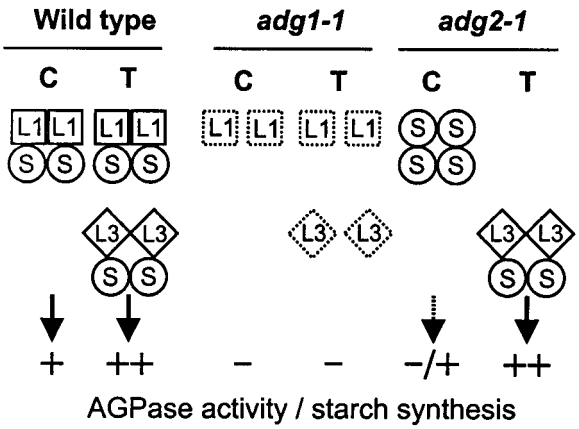

Figure 5. Model for the effect of trehalose on the subunit composition of AGPase and on starch synthesis in wild-type Arabidopsis plants and in mutants deficient in ApS (*adg1-1*) or in ApL1 (*adg2-1*). Dotted boxes indicate that the ApL1 and ApL3 proteins are probably degraded in the absence of ApS. C, Without trehalose; T, plus trehalose; S, ApS; L1, ApL1; L3, ApL3.

considered to be mainly responsible for the catalytic activity, the large subunits are considered to modulate the regulatory properties of the enzyme complex (Ballicora et al., 1995; Greene et al., 1998). It is, therefore, possible that ApL3 and ApL1 confer different regulatory properties to AGPase. Our results showing that starch formation in the *adg2-1* mutant grown in the presence of trehalose was very high, even though the activity of AGPase determined in the in vitro assay was comparatively low, also suggest such differences in the regulation of AGPase activity. For example, a complex composed of ApS and ApL3 could be relatively independent of activation by 3-phosphoglycerate and insensitive to P_i and, consequently, very active in vivo. In an alternate manner, it is possible that the in vivo activity of AGPase was stimulated because of an increased plastidic 3-phosphoglycerate/P_i ratio, which would, for example, result from an inhibition of Suc synthesis in the presence of trehalose. Replacing ApL1 with ApL3 by growing the *adg2-1* mutant on trehalose should make it possible to study how ApL3 affects the catalytic properties of the enzyme complex. Since trehalose does not only induce starch synthesis in the shoots of seedlings, but also in the rosette leaves of older plants, it should be possible to produce sufficient material for the purification and characterization the AGPase containing ApL3 instead of ApL1.

The complementation of the *adg2-1* mutant in the presence of trehalose suggests that an induction of *ApL3* expression could have a significant impact on photosynthetic starch metabolism. In addition to trehalose, Glc and Suc have been shown to induce the expression of *ApL3*, whereas feeding of sugars can lead to a decreased expression of *ApL1* (Sokolov et al., 1998). Accumulation of sugars could, therefore, alter the subunit composition of AGPase in Arabidopsis and, in turn, lead to an altered in vivo activity. Furthermore, we have recently shown that trehalose synthesis occurs in Arabidopsis (A. Wingler, O. Fiehn, T. Boller, and A. Wiemken, unpublished data). Even though the overall content of trehalose is low, high concentrations may occur in specific cells where they could possibly induce *ApL3* expression and starch synthesis. Thus, trehalose-regulated expression of *ApL3* may be an important element of assimilate partitioning in plants.

MATERIALS AND METHODS

Plant Material

Seeds of wild-type (Col-0) Arabidopsis plants and of starch-deficient mutants (*pgm1-1*, Caspar et al., 1985; *adg1-1*, Lin et al., 1988a; and *adg2-1*, Lin et al., 1988b) were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK). The plants were grown on one-half-strength Murashige and Skoog medium without Suc (Sigma-Aldrich, Buchs, Switzerland) solidified with 1% (w/v) agar. The agar plates were oriented vertically and were incubated in a daily cycle of 18 h of light (80 μmol m^{-2} s^{-1}) at 22°C and 6 h of darkness at 18°C. After 14 d, the shoots of the seedlings were harvested at a time between 6 and 8 h into the photoperiod. For further growth, the plants were transferred into Phytacon plant cell culture vessels (Sigma-Aldrich) containing the same medium and were harvested after a total time of 4 weeks.

Determination of Starch

For a visual analysis of starch distribution, whole seedlings were fixed and destained for 1 h in 95% (v/v) ethanol, stained in 43.4 mM KI/5.7 mM I_2, and washed in water. For quantitative analysis of starch contents, soluble sugars were extracted in 80% (v/v) ethanol at 80°C, and starch was digested and measured as Glc (Wingler et al., 2000).

Assay of AGPase Activity

Enzyme extracts were prepared as described by Neuhaus and Stitt (1990), but with addition of 10 mg mL^{-1} insoluble polyvinylpyrrolidone (Polyclar AT, Serva, Heidelberg, Germany), and AGPase activity was assayed according to Sowokinos (1976). Protein concentrations in the extracts were determined with the Bio-Rad (Bio-Rad Laboratories, Glattbrugg, Switzerland) protein assay according to Bradford (1976).

Determination of Chlorophyll Fluorescence Parameters

Modulated chlorophyll fluorescence was measured according to Schreiber et al. (1986) using a Mini-PAM system (Walz, Effeltrich, Germany). After dark treatment for at least 20 min, the quantum efficiency of excitation capture by open photosystem II centers (F_v/F_m) was determined.

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Saturating light pulses of a duration of 0.8 s were then applied every 90 s, and actinic light was set at 250 μmol m^{-2} s^{-1}. The ΦPSII was calculated according to Genty et al. (1989).

Reverse Transcriptase-PCR

Total RNA was extracted from shoots of the Arabidopsis seedlings using the RNeasy kit (Qiagen, Basel) and was treated with DNAse (MessageClean kit, GenHunter, Nashville, TN). One microgram of RNA was reverse transcribed using a reverse transcription kit (Boehringer, Mannheim, Germany) with random as well as oligo(dT) primers. PCR on the first strand cDNA was performed under the conditions described in Wingler et al. (2000). The genes, primers, and fragment sizes were the following: *ApS* (accession no. X73365) 5′-GATGTAATGCTAGACTTACTAC-3′ and 5′-GTCAGTAACATCAGCATCAAG-3′ (278 bp); *ApL1* (accession no. X73367) 5′-TCTATGTGAATGCTTATCTCTC-3′ and 5′-CTATGCTCAATCAAGCAGTTGG-3′ (237 bp); *ApL2* (accession no. X73366) 5′-TTCTAAGGTCAAGTTATCCTAC-3′ and 5′-TCCTGAAGCTCTACTCCAGAC-3′ (351 bp); *ApL3* (accession no. X73364) 5′-ATGTTCAAGGATACATCTACAG-3′ and 5′-CTGAAGCTCAACACCATAGTCA-3′ (285 bp); and *ACT2* (accession no. U41998) 5′-GGAAGGATCTGTACGGTAAC-3′ and 5′-TGTGAACGATTCCTGGACCT-3′ (247 bp). The number of cycles was 23 cycles for *ACT2*, 28 cycles for *ApS*, *ApL1*, and *ApL2*, and 30 cycles for *ApL3*.

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