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Mutants of *Arabidopsis* with Altered Regulation of Starch Degradation[1]

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ABSTRACT

Mutants of *Arabidopsis thaliana* (L.) Heynh. with altered regulation of starch degradation were identified by screening for plants that retained high levels of leaf starch after a period of extended darkness. The mutant phenotype was also expressed in seeds, flowers, and roots, indicating that the same pathway of starch degradation is used in these tissues. In many respects, the physiological consequences of the mutations were equivalent to the effects observed in previously characterized mutants of *Arabidopsis* that are unable to synthesize starch. One mutant line, which was characterized in detail, had normal levels of activity of the starch degradative enzymes α-amylase, β-amylase, phosphorylase, D-enzyme, and debranching enzyme. Thus, it was not possible to establish a biochemical basis for the phenotype, which was due to a recessive mutation at a locus designated *sex1* at position 12.2 on chromosome 1. This raises the possibility that hitherto unidentified factors, altered by the mutation, play a key role in regulating or catalyzing starch degradation.

The synthesis of starch has been intensively studied, and the properties and regulation of the enzymes involved are quite well understood. By contrast, knowledge of starch degradation is relatively rudimentary. Although many of the enzymes presumed to be involved have been studied in detail, the overall pathway has not been unequivocally defined (reviewed in refs. 2, 22, 23). Similarly, although there is physiological evidence that starch degradation is regulated (*e.g.* ref. 6, reviewed in ref. 23), neither the enzymes involved nor the mechanism of the regulation has been identified.

Several inherent problems are associated with elucidating the starch degradative pathway. First, plants contain many different enzymes that can degrade starch or products derived from starch. Therefore, these enzymes cannot be characterized in detail until they have been purified away from the competing activities. Furthermore, the presumed intermediate products of starch degradation often can be derived, in principle at least, by alternate pathways. Thus, it is possible that there may be multiple starch degradative pathways operating in a given plant or that different plants may utilize different pathways. Second, many of the enzymes that are thought to be involved in starch degradation exist as multiple isoforms, which may have differing properties or roles in the plant (22). Also, many of the well-studied presumptive starch degradative enzymes (*e.g.* α- and β-amylase and phosphorylase) are located primarily outside of the plastid (2). Since leaf starch is synthesized and accumulates only inside plastids, the role of the abundant extra-chloroplastic forms of these enzymes in starch degradation is questionable. Finally, neither specific inhibitors nor mutations that block starch degradation have been described previously.

To facilitate the study of starch degradation, we have isolated a series of mutants of *Arabidopsis thaliana* that affect this process. One class of these mutants, described here, is highly deficient in starch degradation in leaves and at least partially deficient in other tissues. The presence of normal levels of activity of the enzymes thought to be associated with starch degradation suggests that this new class of mutants may be particularly useful in elucidating new information about the regulation of starch metabolism.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The mutants were isolated from the Columbia WT[4] *Arabidopsis thaliana* (L.) Heynh. following mutagenesis with ethyl methane sulfonate as described previously (5, 8). Except where otherwise specified, all plants were grown at 22°C with fluorescent illumination of about 120 μmol m^{-2} s^{-1} (PAR) on a perlite:vermiculite:sphagnum (1:1:1) mixture irrigated with a mineral nutrient solution (8). Mutant lines that had been backcrossed at least once to the WT and reselected in the resulting F_2 generation were used for the physiological and biochemical experiments reported here. A more advanced line, designated TC265, constructed by backcrossing TC26 to the WT five times, is qualitatively similar to the once-backcrossed line on the basis of its starch content, growth rate, and morphology.

A line of *Arabidopsis* designated W1 was obtained from Maarten Koornneef (Wageningen). This line carries mapped mutations in the *an, er, py, gl1, cer2,* and *ms1* genes (12).

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[4] Abbreviation: WT, wild type.

Enzyme Assays

Fresh or frozen leaves were ground in ice-cold buffer and clarified by centrifugation (5 min at 14,000 *g* at 4°C), and the supernatant was used for enzyme assays. The grinding buffer was 50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM EDTA for starch synthase, and ADPglucose pyrophosphorylase. The grinding buffer for debranching enzyme was 50 mM Tris-Cl (pH 7.3), 4 mM $CaCl_2$, 4 mM $MgCl_2$, 4 mM $MnCl_2$, and 10 mM DTT. All other enzymes were extracted in 100 mM Tris-Cl (pH 7.5). Amylase (16), starch phosphorylase (13), D-enzyme (15), soluble starch synthase (9), ribulose bisphosphate carboxylase (20), UDPglucose pyrophosphorylase (19), ADPglucose pyrophosphorylase (13), and acid invertase (5) were assayed as described previously. Debranching enzyme (pullulanase) was assayed in 50 mM Hepes-NaOH (pH 7.0), 4 mM $CaCl_2$, 4 mM $MgCl_2$, 4 mM $MnCl_2$, 10 mM DTT, and 5 mg/mL pullulan. Reducing sugars produced were measured with dinitrosalicylic acid reagent (21). Protein concentration was determined with bicinchoninic acid (Pierce Chemical Co.) using BSA as a standard.

Zymograms

Plants were grown in a greenhouse for 3 weeks, then placed in growth cabinets in a 12 h photoperiod at 23°C. Twelve days later, plants were harvested, frozen in liquid N_2, and stored at −80°C until use. Plants were harvested 8 h after the beginning of the photoperiod. For the preparation of extracts, 1 g of leaf tissue was homogenized with a mortar and pestle in 4 mL of 50 mM Tris-HCl (pH 7.5) containing 2 mM EDTA, 4 mM $CaCl_2$, and 1 mM DTT. The homogenate was clarified by centrifugation at 23,400*g* for 15 min before use. Amylolytic activities were detected in nondenaturing acrylamide gels following electrophoretic transfer through a starch-containing gel as described previously (11). An amount of extract equivalent to 40 mg of fresh weight of *Arabidopsis* leaves was loaded in each lane.

Phosphorylase activities were resolved by the same nondenaturing gel electrophoretic procedure as for amylolytic activities. After electrophoresis, the gels were stained for activity by immersing in 50 mM sodium citrate (pH 5.0) containing 20 mM glucose-1-phosphate and 0.8% (w/v) glycogen and incubating overnight at 28°C (25). Carbohydrate produced by phosphorylase activity in the synthetic direction was detected by staining with 0.02% I_2, 0.2% KI, 200 mM sodium acetate (pH 4.8). An amount of extract equivalent to 40 mg of fresh weight of *Arabidopsis* leaves was loaded in each lane.

Starch and Sugar Measurements

Qualitative starch assays of leaves, seeds, roots, and flower tissues were carried out by decolorizing the tissue in 95% (v/v) ethanol at room temperature, then staining with I_2-KI as described previously (5). For quantitative measurements of starch and soluble sugars, leaves were harvested, weighed, ground in 80% (v/v) ethanol, and extracted twice more with 80% (v/v) ethanol at 70°C. The ethanol extracts were combined, dried, dissolved in water, and enzymatically assayed for sucrose, glucose, and fructose using invertase, hexokinase, phosphoglucoisomerase, and glucose 6-phosphate dehydrogenase (10). The ethanol-insoluble residue was assayed for starch as described previously (13). Glucose derived from the digested starch was analyzed enzymatically using hexokinase and glucose-6-P dehydrogenase (10). Quantitative hydrolysis of the starch to glucose under these conditions was confirmed by the complete hydrolysis of amylopectin added to the sample solution.

RESULTS

Isolation of Mutants

Mutants with altered regulation of starch degradation were isolated from a mutagenized M_2 population of *Arabidopsis* in two ways. Since it was considered possible that a plant unable to degrade starch might be inviable due to overaccumulation of starch if grown under normal conditions, the first approach was designed to allow the identification of potential mutants in conditions that would reduce this likelihood. Preliminary experiments indicated that WT plants did not accumulate starch if grown under very low irradiance (about 20 μmol m^{-2} s^{-1}). Therefore, M_2 plants were grown in these conditions for about 4 weeks until they were large enough for screening. The plants were then shifted to higher irradiance (about 100 μmol m^{-2} s^{-1}) and an atmosphere enriched in CO_2 (1% v/v) for 1 d, resulting in the accumulation of large quantities of leaf starch. The plants were then shifted to darkness for 3 d and then leaves from individual plants were stained with iodine to qualitatively assay starch content. In these conditions, WT plants normally degrade essentially all of their starch within 12 h of darkness (5, 13, 14). A single mutant line, designated TC26, isolated from 577 M_2 plants screened in this way, showed no detectable starch degradation during the 72-h dark period. Figure 1 shows a reconstruction experiment in which the WT and TC26 were grown in identical conditions to those used for screening of the mutants as described above. The leaves were removed and stained with iodine at the end of each step.

The second screening approach did not involve any precau-

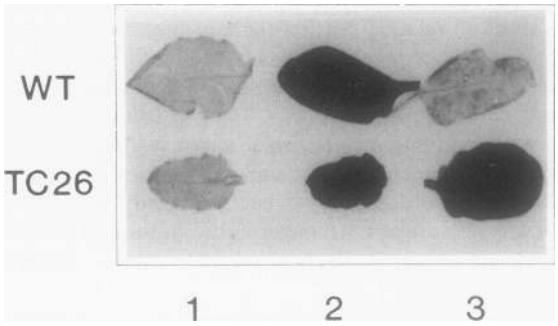

Figure 1. Qualitative assay of leaf starch in WT and mutant. WT and TC26 plants were grown in the conditions used to isolate TC26 and leaves were stained with iodine. Column 1; Plants were grown for about 4 weeks in continuous low light (20 μmol m^{-2} s^{-1}). Column 2; Plants were then shifted to high light (100 μmol m^{-2} s^{-1}) and high atmospheric CO_2 (1% v/v) and leaves stained after 24 h. Column 3; Plants were then shifted to complete darkness, ambient CO_2 for 3 d and the leaves were then stained.

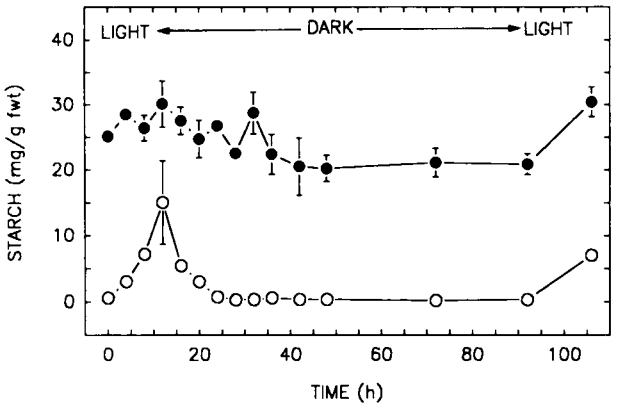

Figure 2. Starch content of WT and mutant plants in a 12-h photoperiod and extended darkness. WT and TC26 plants were grown until they reached the mature rosette stage (28 d for WT, 40 d for TC26) in a 12-h photoperiod with 200 μmol m^{-2} s^{-1} PAR. At the end of a normal 12 h dark period, the plants were placed in continuous darkness for an additional 72 h and then returned to the light for 12 h. Unshaded, mature leaves from individual plants were harvested at each time point. Symbols represent the mean (±SE) starch content of three individual plants. Error bars not indicated are smaller than the symbols. ○, WT; ●, TC26.

tions to reduce the possibility of hyperaccumulation of starch leading to lethality. Plants were grown in a greenhouse under natural light. In these conditions, WT plants completely degraded their starch during the normal dark period. Single leaves from approximately 4580 M_2 plants were stained for starch at the end of the dark period and three lines, designated TL50, TL52, and TL54, were retained. TL52 was subsequently found to be allelic to TC26 (see below) and is qualitatively indistinguishable from it by all the criteria described herein. Thus, only the results for TC26 are presented. However, since TL52 and TC26 were isolated from independently mutagenized lots of M_2 seed, they represent independent mutational events. The mutants TL50 and TL54 are nonallelic to each other and to TC26/TL52 and have not yet been fully characterized. The overall frequency of mutant phenotypes (*i.e.* four mutations at three loci from 5157 M_2 plants) was comparable with that observed in the isolation of mutations affecting other metabolic pathways (17).

The presence of starch in the leaves of TC26 after extended dark periods could be due either to a reduced rate of starch breakdown in the dark or to a greatly increased accumulation of starch in the light, such that degradation is not completed within the dark period. To differentiate between these possibilities and to verify the results from the qualitative iodine stains with quantitative starch assays, WT and TC26 plants were grown in a 12 h photoperiod. At the end of the dark period, the plants were left in darkness for an additional 72 h and then returned to the light for 12 h. Measurements of leaf starch content during these treatments (Fig. 2) confirmed that TC26 retained leaf starch even after 84 h of darkness, whereas in the WT it was entirely degraded within the normal 12-h dark period. Furthermore, TC26 showed little or no variation in starch content during a normal light dark cycle (hours 0–24 in Fig. 2) and only a 30% decrease in starch content during the additional 72 h dark period (h 24–96, Fig. 2). To determine whether the plants remained viable and, thus, potentially capable of degrading starch during the 84 h dark period, plants were illuminated for 12 h following the extended dark period. This 12 h treatment caused both the WT and the mutant to increase their starch content, demonstrating that the reduced rate of starch degradation in the mutant during the extended dark period is not attributable to any loss of viability.

WT plants grown with a photoperiod have very low levels of starch at the end of a dark period (5, 13, 14). Thus, the amount of starch in WT leaves during the photoperiod largely reflects the rate of synthesis during that photoperiod. By contrast, the results in Figure 2 show that at the end of a photoperiod the mutant has relatively high levels of residual starch. Thus, if the mutant accumulated new starch at the same rate as the WT during a photoperiod, but degraded it at a slower rate during a dark period, the amount of starch in the leaves would be expected to accumulate over time to much greater levels than those found in the WT. The observation that the mutant has only about twice as much starch as the WT requires an explanation.

To determine if the amount of starch in the mutant could be altered by varying the growth conditions, the starch content in leaves of WT and TC26 was measured in conditions of varying light and CO_2 concentration. The results (Table I) indicate that the starch content of TC26 remains invariant during a 12-h light/dark photoperiod at both low (50 μmol m^{-2} s^{-1}), intermediate (200 μmol m^{-2} s^{-1}), and high (400 μmol m^{-2} s^{-1}) irradiances. However, the amount of starch accumulated in both the mutant and the WT was responsive to the light level. This effect was particularly pronounced in continuous light. Elevated atmospheric CO_2 (2% v/v) also caused an increased starch content in both the WT and TC26,

Table I. *Starch Content of WT and TC26 Leaves Grown in Various Conditions*

WT and TC26 plants were grown in continuous light (24 h/0 h) or a 12 h photoperiod (12 h/12 h) in an ambient or elevated (2% v/v) CO_2 atmosphere with an irradiance of 50, 200, or 400 μmol m^{-2} s^{-1} PAR for 19 to 27 d and then leaves were removed and assayed for starch. Values are the mean of two to six independent samples.

Condition	Irradiance	Starch	
		WT	TC26
	μmol m^{-2} s^{-1}	mg/g fresh wt ± SEM	
24/0	50	1.0 ± 0.1	2.7 ± 0.3
	200	4.7 ± 0.1	11.3 ± 0.7
	400	14.4 ± 1.4	26.9 ± 0.8
24/0 + 2% CO_2	50	1.3 ± 0.2	3.8 ± 0.2
	200	11.1 ± 1.0	13.8 ± 0.3
	400	53.8 ± 7.6	33.1 ± 7.1
Onset of photoperiod	50	0.2 ± 0.1	15.3 ± 1.4
End of photoperiod	50	2.6 ± 0.1	15.4 ± 0.5
Onset of photoperiod	200	0.7 ± 0.2	32.8 ± 4.1
End of photoperiod	200	11.8 ± 1.3	34.8 ± 2.0
Onset of photoperiod	400	0.7 ± 0.2	30.9 ± 0.2
End of photoperiod	400	9.7 ± 0.6	30.9 ± 0.4

but this effect was much more pronounced in the WT. CO_2 supplementation increased the starch content in TC26 by 40, 22, and 23% at 50, 200, and 400 μmol m^{-2} s^{-1}, respectively, whereas under the same light conditions, CO_2 supplementation increased the starch content of the WT by 29, 137, and 274%, respectively. This increase was so marked in the WT that at 2% CO_2 and 400 μmol m^{-2} s^{-1}, the WT accumulated more starch the mutant. The basis for the greater effect of CO_2 on the WT than TC26 is not known and will require a detailed analysis of the photosynthetic characteristics of the mutant. However, the overall conclusion from these results of growth in varying light and CO_2 conditions is that the starch content in TC26 leaves is determined by a mechanism that is sensitive to environmental conditions.

Genetic Analysis

The genetic basis for the lack of starch degradation in the mutant lines TC26, TL50, TL52, and TL54 was determined by crossing each with the WT and with the other mutants. The resulting F_1 and F_2 progeny were scored for the presence or absence of high levels of leaf starch following a 12 h dark period. Qualitative starch assays using the iodine stain showed that all F_1 individuals produced by crossing the WT with the mutants were able to degrade starch. The F_2 progeny from a cross of TC26 × WT gave a ratio of WT to mutant phenotypes of 3.4, which is a satisfactory fit to the 3:1 hypothesis (χ^2 = 0.34, P = 0.56). Taken together, these results are consistent with the starch degradation phenotype in TC26 being caused by a single, recessive, nuclear mutation. We have designated the gene identified by this mutation *sex1* (*s*tarch *ex*cess) and the allele contained in TC26 as *sex1-1*. When TL52 was crossed with TC26, all of the F_1 and F_2 plants were unable to degrade starch, indicating that the mutation in TL52 is an allele of the *sex1* gene (allele designated *sex1-2*). In contrast, F_1 plants from crosses of TC26 with TL50 and TL54 were able to degrade starch, indicating that the mutations in these lines are in other genes, provisionally named *sex2* in TL50 and *sex3* in TL54 pending further characterization. The *sex* mutants were designated by the provisional gene symbol *sop* in an earlier publication (4).

The *sex1* gene was mapped by crossing TC26 with the multiply marked line W1 and scoring the phenotypes of 322 F_2 individuals. Analysis of linkage of *sex1* to the chromosome 1 marker *an* (phenotypic classes: $an^+/sex1^+$ = 171; $an^+/sex1^-$ = 68; $an^-/sex1^+$ = 82; $an^-/sex1^-$ = 1) indicated that they were closely linked (contingency χ^2 = 27.2; P ≥ 0.001). The recombination frequency between *sex1* and *an* was determined to be 12.2 ± 5.5% using the Linkage-1 microcomputer program (24). Since *an* is the most distal marker yet found on the relatively well-marked area of this chromosome (12), the evidence indicates that *sex1* is proximal to *an* at position 12.2 on the genetic map of Koornneef *et al.* (12).

Effects on Starch in Other Tissues

Since starch metabolism may differ in autotrophic and heterotrophic cells, it was of interest to know whether the *sex1-1* mutation would affect starch metabolism in parts of the plant other than leaves. In the WT, starch accumulates to a great extent primarily in photosynthetic tissues, including leaves, stems, and immature seeds, and the root cap (ref. 3 and T Casper, unpublished observations). A survey of the starch content of various tissues indicated that roots, petals (results not presented), anthers, and dry seeds of the mutant contained significantly more starch than the WT (Fig. 3). The increased amount of starch in the anthers of the mutant was visible throughout these tissues but was always most heavily localized in a distinct zone where the anther joins the filament (Fig. 3A). Similarly, flower petals of the mutant contained high levels of starch that was primarily localized in the basal half, whereas the petals in the WT contained little or no starch (results not presented). In the WT, starch accumulated to high levels in the columella cells of the root cap but was nearly absent in the cells that were detaching from the cap (Fig. 3B; ref. 3), indicating that a very rapid breakdown of starch occurs as the cells detach. By contrast, in the mutant,

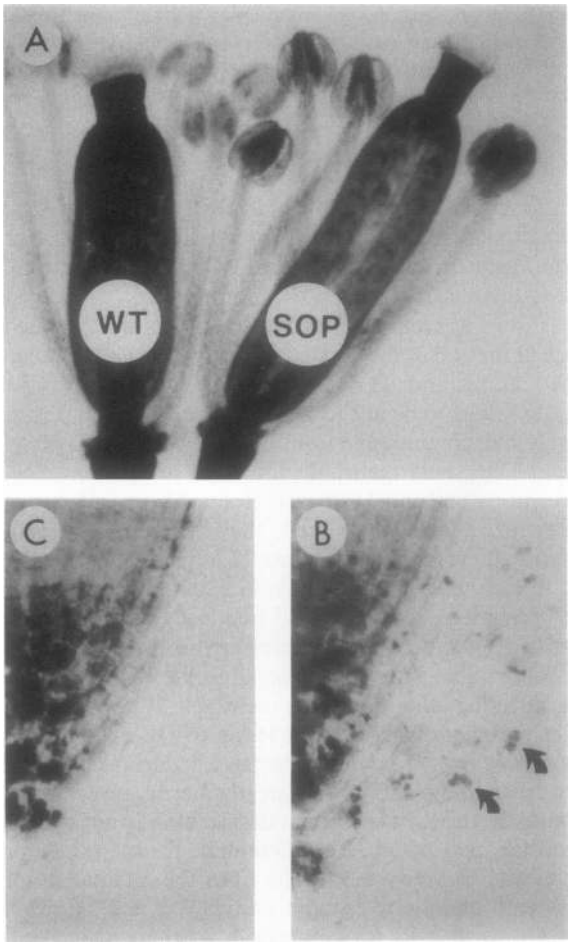

Figure 3. Histochemical staining of starch in flowers and roots of WT and mutant *Arabidopsis*. Tissues were removed from plants grown in continuous light, decolorized with 95% ethanol, and stained with iodine. A, Flowers at anthesis from which the sepals and petals have been removed. B and C, Root tips of 5 d old seedlings grown in continuous light on agar medium (GM) containing 1% sucrose. Starch grains in the sloughed cells of the root cap of TC26 are indicated with arrows.

the detaching cells retained large amounts of starch (Fig. 3C). In addition, the body of the root of the mutant contained higher levels of starch than the WT (results not presented).

When seeds were stained for starch at various times during development, there were no differences until relatively late in development when the starch content of the WT declined but the mutant did not (Fig. 4). This developmental pattern of starch content in the WT is similar to that observed in the closely related mustard *Brassica napus* (18), in which starch initially accumulates in developing seeds and is then degraded later in development when lipids and proteins are synthesized. The starch in the mature seeds of the mutant accumulated to high levels (4.3 mg/g fresh weight in TC26 seeds *versus* undetectable levels in WT seeds) and was present in both the seed coat (testa) and the embryo. The seed coat is a maternal tissue, and in reciprocal crosses between TC26 and the WT, the presence of starch in the seed coat was dependent on the genotype of the maternal parent (results not shown).

Thus, in addition to photosynthetic leaf tissue, a wide range of nonphotosynthetic tissues utilize the *sex1* gene product for starch degradation. The absence of starch in these tissues in the WT presumably reflects the fact that the starch synthesized in these tissues is normally rapidly turned over.

Biochemical Analysis

In an effort to identify the enzymatic basis of the inability of TC26 to degrade starch, the activities of four starch degrading enzymes and five other enzymes associated with starch and sucrose metabolism were measured in the mutant and WT. The results (Table II) show that activity for phosphorylase, D-enzyme, and debranching enzyme (pullulanase) was present in the mutant at about the same levels as in the WT. As described previously (4), the level of total amylase activity was equal to or higher than the WT, depending on the growth conditions. Since this elevated activity occurs in starchless mutants as well as in TC26, it must be a secondary effect of the altered carbohydrate metabolism caused by these various mutations and is not related to the primary defect in any of these mutants. Similarly, differences between mutant and WT in the amount of activities of starch synthase, ADPglucose pyrophosphorylase, UDPglucose pyrophosphorylase, acid invertase, and ribulose bisphosphate carboxylase were also observed in comparisons of the starchless mutants and WT (T Caspar, unpublished observations), suggesting that the differences are due to secondary effects of the altered carbohydrate metabolism.

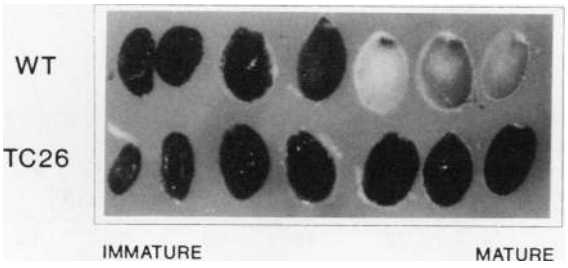

Figure 4. Seeds dissected from siliques at various stages of maturity from a single flower stalk of WT and TC26 plants grown in continuous light and stained with iodine. The least mature seed was from the youngest silique, which had begun to elongate. Thereafter, every third silique on the flower stalk was sampled. The most mature seeds came from fully developed siliques that had not yet begun to turn yellow. The mature seeds are about 0.5 mm in length.

Table II. *Activities of Enzymes Involved in Leaf Carbohydrate Metabolism*

Plants were grown for three to five weeks in a 12 h/12 h photoperiod. The presence of SEs indicates that the values represent the means of at least three independent assays.

Enzyme	Enzyme Activity	
	WT	TC26
	nmol/min/mg protein	
Phosphorylase	29 ± 0.7	23 ± 1.3
D-enzyme	19 ± 0.6	20 ± 1.0
Amylase	44 ± 14	273 ± 95
Debranching enzyme	1.4	1.3
Starch synthase[a]	1.8	0.7
ADP-glucose pyrophosphorylase	55	36
UDP-glucose pyrophosphorylase[b]	41 ± 2.2	14 ± 4.7
Acid invertase	100 ± 6	160 ± 7
Rubisco[c]	180 ± 15	120 ± 5

[a] Starch synthase activity may be lower in TC26 because of the higher amylase activity in this extract, which interferes with the starch synthase assay. [b] The activity of UDP-glucose pyrophosphorylase was lower in both TC26 and TL52 (24 nmol/min/mg) than the WT, but when grown in continuous light the mutants had higher activities than the WT (*i.e.* WT = 16, TC26 = 42, TL52 = 36 nmol/min/mg protein). [c] Ribulose bisphosphate carboxylase (Rubisco) activity was also lower in TL52 and the starchless mutants TC7, TL25 than in the WT (4).

Since five amylase isoforms have been demonstrated in *Arabidopsis* (4, 16), an amylase zymogram was run to determine whether any isoforms were absent in TC26. Figure 5 shows that each of the forms present in the WT is also present in TC26 in approximately equal activities. Previous studies have shown that the α-amylase designated A1 (Fig. 5) is the only detectable chloroplast amylase (16). Since the mutant and the WT exhibited similar levels of activity of this enzyme on zymograms of three independent extracts (Fig. 5 and results not presented), it seems unlikely that a defect in this activity can account for the mutant phenotype.

Many plants contain multiple isozymes of phosphorylase (22). Therefore, to assess the possibility that a deficiency in a specific isozyme could be responsible for the mutant phenotype, the activity was measured on zymograms. The results of this analysis indicated that both mutant and WT *Arabidopsis* have two isoforms (Fig. 5), and that the mutant had at least as much activity as the WT.

Effects of the Mutation on Sugar Metabolism

In addition to starch, the soluble sugars represent the other major transitory storage form of carbon in *Arabidopsis* leaves. Figure 6 shows the soluble sugar levels in leaves of the WT and TC26 when grown in a 12-h photoperiod and then placed in an extended dark period. During the first 4 h of the photoperiod, the mutant accumulates sugar more rapidly that

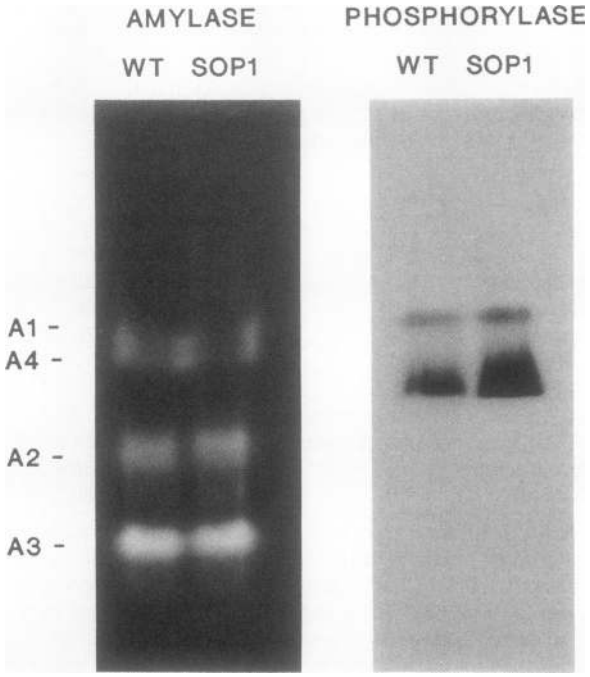

Figure 5. Amylase and phosphorylase activities in extracts from WT and mutant leaves harvested 8 h after the onset of the 12 h photoperiod.

the WT. However, later in the day, sugar accumulation stops in the mutant but continues at a uniform rate in the WT such that, by the end of the day, the sugar content in the WT and mutant leaves is comparable. This rapid rise and subsequent plateau in sugar content in the mutant is probably caused by the lack of starch synthesis (see Fig. 2) as a sink for recently fixed carbon. The comparable sugar content at the end of the day contrasts with the results of similar comparisons of the WT and a starchless mutant deficient in phosphoglucomutase activity (5) in which soluble sugar levels were found to be greatly increased during the photoperiod. This may be related to the fact that the experiments reported here were conducted using plants grown at higher light intensities than in previous experiments. Light intensity apparently exerts an effect on the amount of soluble sugars that accumulate in the starchless mutants (M. Stitt, personal communication). There were no significant differences in the soluble sugar levels between mutant and WT during the normal or extended period of darkness.

Effects of the Mutation on Growth

The mutants TC26 and TL52 are both healthy and vigorous. However, their growth rate relative to the WT is dependent on the photoperiod under which they are grown. When grown in a 12-h photoperiod, TC26 grows much more slowly than the WT (Fig. 7A). In contrast, when grown in continuous light, TC26 has a growth rate and overall appearance indistinguishable from the WT (Fig. 7B). The independently isolated allelic mutant TL52 has qualitatively identical growth responses to photoperiods as TC26, which confirms that the effects on growth rate are due to mutations in the *sex1* locus. This effect of photoperiod on growth rate is similar to that previously observed for starchless mutants deficient in either phosphoglucomutase (5) or ADPglucose pyrophosphorylase (13).

DISCUSSION

To facilitate the study of the pathway of starch degradation, we have isolated mutants with altered regulation of this process. The first of these mutants to be analyzed, TC26 and TL52, had a low rate of starch depletion in leaves, either during a normal 12-h dark period or an extended 72-h dark period. In contrast, the WT completely depleted its starch reserves by the end of the normal 12-h photoperiod. In addition, the mutation affected starch content (presumably via an effect on starch degradation) in roots, flowers, and seeds, suggesting that these organs share with leaves a common step in starch degradation. Despite this deficiency in a primary energy and carbohydrate releasing pathway, the mutants were vigorous and completely fertile. This is consistent with the conclusions of studies with starchless mutants of *Arabidopsis* (5, 13) and tobacco (7) that starch is not required for plant growth or development in controlled growth conditions. The low level of starch degradation in the *sex* mutants was associated with a reduced growth rate in a 12-h photoperiod, relative to the WT, whereas the growth rate of the mutants in continuous light was the same as the WT. It is unlikely that the over-accumulation of starch *per se* is directly responsible for this decreased growth rate since even in continuous light, where the mutant grows as rapidly as the WT, the mutant accumulates more starch. Furthermore, a similar effect of photoperiod on growth rate is observed in the starchless *Arabidopsis* mutants (5, 13). Rather, since the starch pool in TC26 is essentially at equilibrium—having neither net synthesis in the light nor net degradation in the dark—it is effectively eliminated as a diurnal carbohydrate storage pool that can act as a buffer for other cellular metabolites. The result is that the starch degradation mutants TC26 and TL52

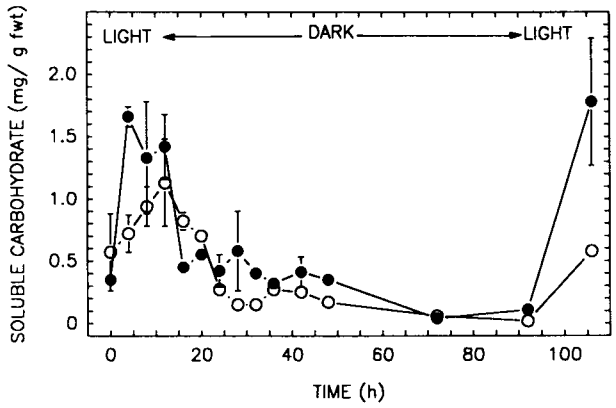

Figure 6. Soluble sugar content of leaves of WT and mutant plants in a 12 h photoperiod and extended darkness. WT and TC26 plants were grown and harvested as described in Fig. 2. Symbols represent the mean (±SE) soluble sugar (sucrose, glucose, and fructose) content of three individual plants. Error bars not indicated are smaller than the symbols. ○, WT; ●, TC26.

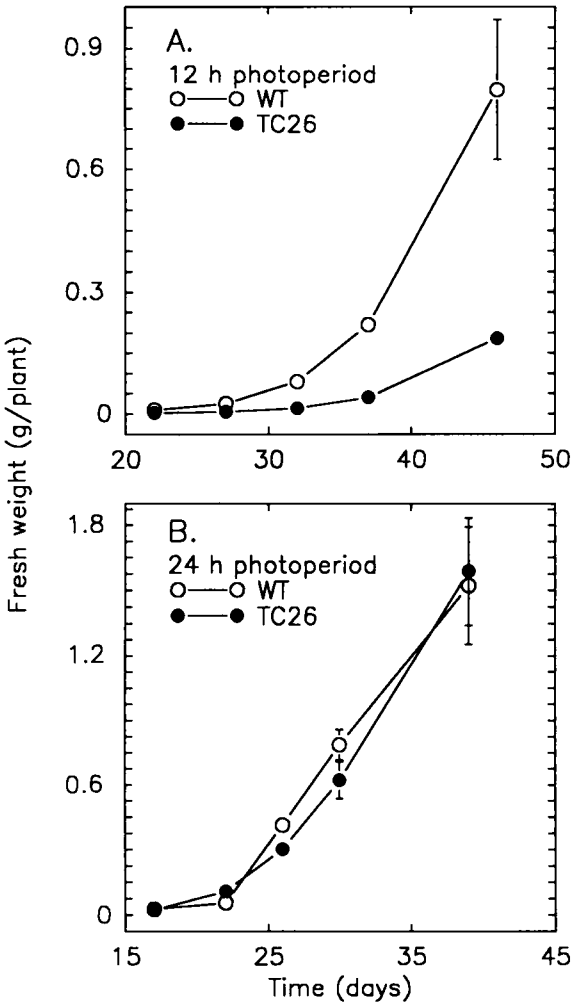

Figure 7. Growth rate of WT and mutant plants. Plants were grown in (A) a 12 h photoperiod at 115 μmol m^{-2} s^{-1} PAR or (B) continuous light at 110 μmol m^{-2} s^{-1} PAR for the indicated times and then harvested and the aerial portions weighed. Symbols represent the mean ±SE (n = 5).

are functionally equivalent to the starchless mutants. In TC26, as well as the starchless phosphoglucomutase mutant TC7, sugar levels rise rapidly at the beginning of the light period and fall rapidly at the beginning of the dark period (see Fig. 6, this paper, and Fig. 4 in ref. 5). We suggest that the slower growth of the mutants relative to the WT in photoperiodic growth conditions is caused by the lack of effective metabolic buffering by the starch pool during the alternating light and dark conditions.

The size of the starch pool in TC26 provides information on the mechanism(s) that regulates starch accumulation in the WT in conditions when chloroplasts are fully loaded with starch (*e.g.* growth in long days or continuous light). Despite the deficiency in starch degradation, leaves from TC26 plants grown in a wide range of conditions accumulate at most only severalfold more starch than the WT (Table I). However, the starch levels in the mutant, as in the WT, are sensitive to environmental factors (irradiance and CO_2 concentration) that affect photosynthesis. Thus, the amount of starch accumulated in the leaves of the mutant is not the maximum that an *Arabidopsis* leaf can accumulate. The fact that net starch synthesis ceases in the mutant at these relatively modest levels is most easily explained by the existence of a system that modulates starch synthesis in response to the size of the starch pool. That is, we propose that as the starch pool progressively increases in size because of the lack of starch degradation, the starch synthesis rate progressively drops due to the proposed regulatory system. Eventually, the starch pool reaches a level at which starch synthesis is largely or completely inhibited. However, the implications of this heuristic model conflict with results obtained previously with a leaky starch synthesis mutant of *Arabidopsis* (14). When grown in continuous light, this mutant accumulated only 40% as much starch as the WT. To explain this observation, we previously proposed the existence of a system that measures the total capacity for starch synthesis, rather than the size of the starch pool as proposed above, and then regulates synthesis and degradation to achieve an overall regulation of starch accumulation. A resolution of this apparent contradiction is not obvious. It is possible that starch degradation occurs to a sufficient extent in TC26 to allow regulation of the starch pool by a dynamic balancing of synthesis and degradation. However, this hypothesis implies that the starch degradation rate in TC26 in the light would have to be much higher than the low rate we observed during the dark. At present, this possibility cannot be excluded. However, if starch degradation is active in the mutant in the light, this would indicate a difference in the mechanism of starch degradation in light *versus* dark. Although this is a possibility, we favor an explanation based on several systems that operate together to sense and regulate the size of the starch pool. It is possible that normally regulation of the starch pool is based on the rate of starch synthesis. When the starch pool is larger than the norm for a particular set of environmental conditions (*e.g.* as in TC26), perhaps a second regulatory system is utilized that measures the actual size of the starch pool, or some constituent of starch such as the number of nonreducing ends. Clearly, the regulation of the size of the starch pool in steady-state conditions is a complex phenomenon of which we have only a rudimentary knowledge.

Since the starch degradation pathway has been difficult to elucidate using standard physiological and biochemical analyses, one of the most valuable uses of starch degradation mutants such as TC26 should be to allow a conclusive demonstration of the enzymes and regulatory steps involved in this pathway. TC26 was shown to have normal activities of five enzymes—D-enzyme, α- and β-amylase, phosphorylase, and debranching enzyme—thought to be involved in starch degradation. These results indicate a requirement for other enzymes or, perhaps, specific (low activity) forms of these enzymes for starch degradation in *Arabidopsis* that have not been detected or characterized. At least five isoforms of α- and β-amylase and two isoforms of phosphorylase are present in *Arabidopsis*, and each is present in TC26 at levels comparable with those in the WT. D-enzyme is also known to exist as multiple isoforms in various plants (15, 22) and further work will be required to determine whether a specific form of D-enzyme is deficient in TC26.

We also compared the gross chemical properties of the starch in mutant and WT. The absorption maximum of a starch/iodine complex is a measure of the chain length of the starch (1). Using this somewhat coarse assay, no difference in the chain length of the amylose between the WT and TC26 was detected (G Kakefuda, unpublished results). Detailed analysis of the structure of starch granules from the mutant may be a useful avenue for future attempts to elucidate the basis for the properties of this mutant.

It is also possible that the enzymatic lesion in TC26 could be in other enzymes thought to be involved with secondary steps in starch mobilization such as the further metabolism of the soluble sugars released from starch, their export from the chloroplast, and further metabolism in the cytosol. Such a result, however, would require the action of a feedback regulatory mechanism to explain how the blockage in a downstream reaction could inhibit the early steps of starch degradation. Physiological evidence for regulation of starch degradation exists. For example, in barley, starch degradation in the dark was shown to be delayed until sucrose levels dropped below a critical value (6). Although the biochemical basis for this response is not understood, this example nevertheless demonstrates that feedback type regulatory systems affecting starch degradation may exist. The lesion in the mutant TC26 does not affect the specific sort of system suggested by the barley results since the mutant degrades soluble sugars at least as fast as the WT (Fig. 6) and fails to degrade starch even when the soluble sugar levels have dropped essentially to zero.

The eventual elucidation of the biochemical defect in TC26 obviously requires more study, but when accomplished, it should greatly enhance our understanding of starch degradation. Furthermore, the fact that TC26 is healthy and completely viable indicates that the absence of starch degradation is tolerated by *Arabidopsis,* and thus it should be possible to extend this approach to other mutants with similar phenotypes. In fact, our initial screening of about 5000 M_2 plants identified two other loci, *sex2* and *sex3,* with similar phenotypes. The fact that only two of the four mutants isolated belong to the same complementation group suggests that still more loci may exist that are required for starch degradation. Isolation and biochemical characterization of other loci that regulate starch degradation should be a useful approach to the study of this aspect of metabolism.

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LITERATURE CITED

1. **Bailey JM, Whelan WJ** (1961) Physical properties of starch: I. Relationship between iodine stain and chain length. J Biol Chem **236:** 969–973
2. **Beck E, Ziegler P** (1989) Biosynthesis and degradation of starch in higher plants. Annu Rev Plant Physiol Plant Mol Biol **40:** 95–117
3. **Caspar T, Pickard BG** (1989) Gravitropism in a starchless mutant of *Arabidopsis:* implications for the starch-statolith theory of gravity sensing. Planta **177:** 185–197
4. **Caspar T, Lin TP, Monroe J, Bernhard W, Spilatro S, Preiss J, Somerville CR** (1989) Altered regulation of β-amylase activity in mutants of *Arabidopsis* with lesions in starch metabolism. Proc Natl Acad Sci USA **86:** 5830–5833
5. **Caspar T, Huber SC, Somerville C** (1986) Alterations in growth, photosynthesis and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) Heynh deficient in chloroplast phosphoglucomutase activity. Plant Physiol **79:** 1–7
6. **Gordon AJ, Ryle GJA, Webb G** (1980) The relationship between sucrose and starch during "dark" export from leaves of uniculm barley. J Exp Bot **31:** 845–850
7. **Hanson KR, McHale NA** (1988) A starchless mutant of *Nicotiana sylvestris* containing a modified plastid phosphoglucomutase. Plant Physiol **88:** 838–844
8. **Haughn GW, Somerville CR** (1986) Sulfonylurea resistant mutants of *Arabidopsis thaliana.* Mol Gen Genet **204:** 430–434
9. **Hawker JS, Ozbun JL, Ozaki H, Greenberg E, Preiss J** (1974) Interaction of spinach leaf adenosine diphosphate glucose 1,4–1,4-glucosyl transferase and α-1,4-glucan, 1,4-glucan-6-glucosyl transferase in synthesis of branched α-glucan. Arch Biochem Biophys **160:** 530–551
10. **Jones MGK, Outlaw WH, Lowry OH** (1977) Enzymic assays of 10^{-7} to 10^{-14} moles of sucrose in plant tissue. Plant Physiol **60:** 379–383
11. **Kakefuda G, Duke SH** (1984) Electrophoretic transfer as a technique for the detection and identification of plant amylolytic enzymes in polyacrylamide gels. Plant Physiol **75:** 278–280
12. **Koornneef M, van Eden J, Hanhart CJ, Stam P, Braaksma FJ, Feenstra WJ** (1983) Linkage map of *Arabidopsis thaliana.* J Hered **74:** 265–272
13. **Lin TP, Caspar T, Somerville C, Preiss J** (1988) Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh, lacking ADPglucose pyrophosphorylase activity. Plant Physiol **86:** 1131–1135
14. **Lin TP, Caspar T, Somerville CR, Preiss J** (1988) A starch deficient mutant of *Arabidopsis thaliana* with low ADPglucose pyrophosphorylase activity lacks one of the two subunits of the enzyme. Plant Physiol **88:** 1175–1181
15. **Lin TP, Preiss J** (1988) Characterization of D-enzyme (4-α-glucanotransferase) in *Arabidopsis* leaf. Plant Physiol **86:** 260–265
16. **Lin TP, Spilatro SR, Preiss J** (1988) Subcellular localization and characterization of amylases in *Arabidopsis* leaf. Plant Physiol **86:** 251–259
17. **McCourt P, Somerville CR** (1987) The use of mutants for the study of plant metabolism. *In* DD Davies, ed, The Biochemistry of Plants, Vol 13. Academic Press, New York, pp 34–64
18. **Norton G, Harris JF** (1975) Compositional changes in developing rape seed (*Brassica napus* L.). Planta **123:** 163–174
19. **Ozbun JL, Hawker JS, Greenberg E, Lammel C, Preiss J, Lee EYC** (1973) Starch synthase, phosphorylase, ADPglucose pyrophosphorylase and UDPglucosepyrophosphorylase in developing maize kernels. Plant Physiol **51:** 1–5
20. **Pierce JW, McCurry SD, Mulligan RM, Tolbert NE** (1982) Activation and assay of ribulose-1,5-bisphosphate carboxylase/oxygenase. Methods Enzymol **89:** 47–55
21. **Rick W, Stegbauer HP** (1974) α-Amylase: measurement of reducing groups. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis, English Ed 2, Vol 2. Academic Press, New York, pp 885–903
22. **Steup M** (1988) Starch degradation. *In* J Preiss, ed, Biochemistry of Plants, Vol 14. Academic Press, New York, pp 255–296
23. **Stitt M, Steup M** (1985) Starch and sucrose degradation. *In* R Douce, DA Day, eds, Encyclopedia of Plant Physiology, New Series, Vol 18. Springer-Verlag, New York, pp 347–390
24. **Suiter KA, Wendel JF, Case JS** (1983) Linkage 1: a PASCAL computer program for the detection and analysis of genetic linkage. J Hered **74:** 203–204
25. **Tandecarz JS, Sivak MN, Cardini CE** (1978) A primer independent form of potato tuber phosphorylase. Biochem Biophys Res Commun **82:** 157–164