The Plant Cell, Vol. 7, 1703–1712, October 1995 © 1995 American Society of Plant Physiologists

Monogenic Recessive Mutations Causing Both Late Floral Initiation and Excess Starch Accumulation in Arabidopsis

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A recessive Arabidopsis mutation, *carbohydrate accumulation mutant1* (*cam1*), which maps to position 22.8 on chromosome 3, was identified by screening leaves of ethyl methanesulfonate-mutagenized M_2 plants stained with iodine for altered starch content. Increased starch content in leaves of the *cam1* mutant was observed at the onset of flowering. This mutant also had a delayed floral initiation phenotype with more rosette leaves than the parental line. In addition, activities of several enzymes associated with starch metabolism were altered in the *cam1* mutant. The late-flowering mutant *gigantea* (*gi*) also manifested an elevated starch level in leaves. However, not all late-flowering mutants had increased leaf starch content. Double mutants *cam1 adg1* (for ADP-glucose pyrophosphorylase), *cam1 pgm* (for phosphoglucomutase), and *gi pgm* had no observable starch in leaves but showed the late-flowering phenotype, demonstrating that the elevated starch content is not the cause of late floral initiation. The pleiotropic effects of *cam1* and *gi* suggest that they may play regulatory roles in starch metabolism and floral initiation. These data suggest that starch accumulation and floral initiation may share a common regulatory pathway.

INTRODUCTION

Regulation of Starch Metabolism

Carbon fixation during photosynthesis and subsequent accumulation and utilization of starch are among the most fundamental processes in higher plants. In spite of the importance of these processes, not much is known about the regulation of the pathways of starch synthesis and degradation. For starch synthesis, the pathway via phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), ADP-glucose pyrophosphorylase (ADGase), and starch synthetase is generally considered the main pathway (for review, see Preiss, 1988). The mutual influence and regulation of photosynthesis, dark respiration, and carbon partitioning have been addressed repeatedly (Huber and Israel, 1982; Gordon et al., 1985; Rao and Terry, 1989; Rao et al., 1989, 1990; Neuhaus and Stitt, 1990; Goldschmidt and Huber, 1992), but potential regulatory mechanisms are obviously obscured by the complex situation in vivo.

The study of the starch degradative pathway is hampered by the fact that many of the enzymes (for example, amylases and starch phosphorylases) believed to be involved exist as isozymes. Moreover, most of the enzyme activities are located in the cytoplasm, whereas the starch degradation takes place inside the plastids. Although many of the enzymes involved in starch degradation are known and have been characterized (for review, see Beck and Ziegler, 1989), not much is known about their interaction, regulation, or even their role in a general degradation pathway.

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Regulation of Floral Initiation in Arabidopsis

Arabidopsis is a facultative long-day plant because a long photoperiod induces flowering in all ecotypes (Napp-Zinn, 1985). But this requirement is not absolute, and all ecotypes flower eventually under unfavorable conditions or even in the dark (Redei, 1974). Similarly, flowering is promoted by, but not dependent on, vernalization. Therefore, a floral induction mechanism independent of environmental conditions can be assumed to exist in Arabidopsis. Autonomous induction has been described for several other plant species (Bernier et al., 1981).

A variety of Arabidopsis flowering time mutants has been isolated, most of which behave as monogenic recessive plants (Koornneef et al., 1991). In some of these mutants, the late-flowering phenotype is reversible to the wild type by vernalization treatment. This shows that the mutants possess a normal cold-inducible mechanism and that the late-flowering phenotype is not due simply to a mutation affecting growth rate or vigor.

Studies of the interactions among a set of late-flowering mutations and their responses to light and cold treatments led to a causal model for floral initiation in Arabidopsis (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991; Martinez-Zapater et al., 1994). This model features an autonomous flowering induction process, which cannot be stopped and can only be modified by promoters or inhibitors.

From the study of a constitutive flowering mutant, *embryonic flower* (*emf*), the wild-type allele *EMF* appears to activate the

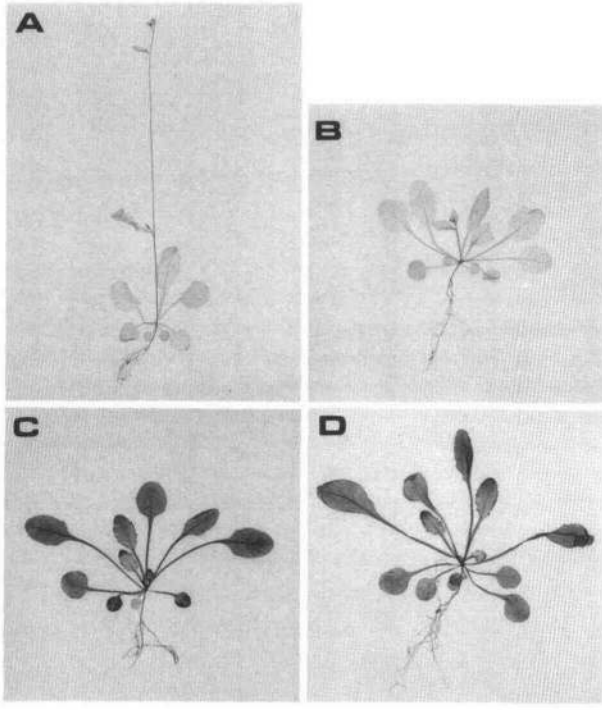

Figure 1. Late-Flowering Phenotype and Starch Accumulation in Leaves of Mutants and the Wild Type.

Plants were grown for 32 days under continuous light and left in the dark for 12 hr. Pigments of the whole plants were removed by ethanol and the leaves then stained with iodine.

(A) to **(C)** Wild-types RLD, Columbia, and *cam1*, respectively, have already entered the flowering stage.

(D) *gi1-2* mutant has not yet bolted.

vegetative state or to suppress the reproductive state of the shoot apex (Sung et al., 1992). It was proposed that the *EMF* gene product is normally produced in young plants and diminishes during development, thereby allowing the transition to the reproductive phase. This mutation is epistatic to late-flowering mutations, suggesting that a defect in this regulatory pathway that conditions the plant to embryonic flowering overrides the other controls downstream.

Several Starch Metabolic Mutants of Arabidopsis Are Delayed in Flowering

Several Arabidopsis starch metabolic mutants have been isolated (Caspar et al., 1985, 1991; Lin et al., 1988a). Two of them, *pgm* and *adg1* (for ADP-glucose pyrophosphorylase), have no apparent starch accumulation in leaves. The *adg1* mutant lacks ADGase activity, and the *pgm* mutant lacks chloroplastic PGM activity. Without leaf starch, the rate of growth and net photosynthesis of the mutant and wild type are indistinguishable when the plants are grown in constant illumination. However, in a short photoperiod, the growth of these two mutants is severely impaired and flowering is delayed.

An Arabidopsis starch excess mutant, *sex1* (previously named *sop1*; Caspar et al., 1991), has been isolated and extensively analyzed. It was suggested that *SEX1* may play a role in starch degradation. When grown in continuous light, *sex1* has a growth rate and overall appearance indistinguishable from that of the wild type. In contrast, when grown in a 12-hr photoperiod, *sex1* grows much more slowly than does the wild type. It has been hypothesized that the slower growth of the *sex1* mutant relative to the wild type 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 existence and vitality of starch null mutants (*pgm* and *adg1*) indicate that the starch metabolic pathway per se is not essential for plant growth but most likely plays a regulatory role in plant development.

Mutants are considered to be useful instruments to facilitate the analysis of starch metabolism and floral initiation. Here, we describe two late-flowering mutants, *carbohydrate accumulation mutant1* (*cam1*) and *gigantea* (*gi*), that exhibit elevated levels of leaf starch and delayed onset of flowering. We demonstrate that increased starch levels are not the cause of late flowering and that late floral initiation is not sufficient to elevate leaf starch levels. From an analysis of these two mutants, we suggest that a common regulatory pathway of starch metabolism and floral initiation may exist.

RESULTS

Isolation of Mutants with Starch Excess and Late-Flowering Phenotypes

To isolate mutants with altered starch metabolism, we screened ~10,000 M_2 plants of ethyl methanesulfonate–mutagenized seed by removing pigments from leaves with ethanol and then staining the starch with iodine. Eleven mutants with altered leaf starch content were isolated. One of the mutants, *cam1*, was isolated because its leaves were stained dark by iodine after prolonged dark treatment (12 to 48 hr) of the plants. In comparison, leaves of the parental line were not stained by iodine after 12 hr of growth in the dark, as shown in Figure 1. This dark iodine staining of *cam1* leaves indicated an increased starch content, which was later confirmed by enzymatic measurements. Elevated starch levels could not be detected in roots, flowers, and seeds, as determined by iodine staining. Thus, the phenotype is restricted to photosynthetically active tissue.

The increase in leaf starch content was observed shortly before the onset of floral initiation. Before this stage, the mutant plant is morphologically indistinguishable from the wild type and does not express elevated leaf starch content. Apart from the starch excess phenotype, the *cam1* mutant is also delayed in floral initiation, with more and larger rosette leaves than the wild type. Because the increase in starch content is

Table 1. Starch Content in Leaves

Plant	Not Flowered[a] Starch (mg/g Fresh Weight)	% of Wild Type	Flowered[b] Starch (mg/g Fresh Weight)	% of Wild Type
Col WT[c]	2.40 ± 0.03[d]	100	2.45 ± 0.24	100
Ler WT[e]	2.32 ± 0.11	100	2.49 ± 0.11	100
RLD WT[f]	2.35 ± 0.09	100	2.15 ± 0.33	100
fca (Ler)	2.31 ± 0.97	99	2.43 ± 0.27	98
fd (Ler)	3.18 ± 0.71	137	2.52 ± 0.26	101
fe (Ler)	2.08 ± 0.18	90	2.36 ± 0.38	95
ft (Ler)	2.16 ± 0.35	93	2.36 ± 0.12	95
fve (Ler)	2.84 ± 0.13	122	2.44 ± 0.17	98
fwa (Ler)	3.09 ± 0.18	133	2.67 ± 0.21	107
fy (Ler)	3.20 ± 0.25	138	2.63 ± 0.21	106
co (Ler)	2.55 ± 0.12	110	2.58 ± 0.44	104
gi1-1 (Col)	5.93 ± 0.32	247	5.30 ± 0.35	216
gi1-2 (Col)	9.32 ± 0.44	388	9.38 ± 0.49	383
gi1-3 (Ler)	6.64 ± 0.30	286	6.82 ± 1.76	274
cam1 (RLD)	2.18 ± 0.05	93	6.17 ± 0.63	287
sex1 (Col)	7.45 ± 0.36	310	6.89 ± 0.65	281
ld (Col)[g]	2.25 ± 0.06	93	ND[h]	ND

[a] Plants were grown under continuous light, and leaf samples were harvested at the four- to six-leaf stage.
[b] Plants were grown under continuous light, and leaf samples were harvested at the flowering stage.
[c] Ecotype Columbia wild type.
[d] Data represent means of at least six independent measurements.
[e] Ecotype Landsberg *erecta* wild type.
[f] Ecotype RLD wild type.
[g] The *ld* plants did not flower under continuous light for 6 months.
[h] ND, not done.

associated with the late-flowering phenotype, we examined leaf starch content in some of the other late-flowering mutants, including *fca*, *fd*, *fe*, *ft*, *fve*, *fwa*, *fy*, *constans* (*co*), and *gi*. Among the late-flowering mutants we examined, *gi* (Redei, 1962; Araki and Komeda, 1993) also had elevated leaf starch content, as shown in Table 1. In addition, the increase of starch in leaves of *gi* was observed at an early stage of growth by staining leaves from plants placed in the dark with iodine. Three alleles of *gi*, *gi1-1*, *gi1-2* (in the Columbia ecotype), and *gi1-3* (in the Landsberg *erecta* ecotype) map to position 33.3 on chromosome 1 (Koornneef, 1994), and all manifested an increase in leaf starch content, although to varying degrees. In addition, genetic analysis of the F_2 progeny of *gi1-2* × wild type showed cosegregation of the late-flowering and starch-excess phenotypes, indicating that these two phenotypes are conditioned by a monogenic recessive mutation.

Genetic Analysis of *cam1*

In reciprocal crosses of wild-type (ecotype RLD) with *cam1* plants, all F_1 and wild-type plants had identical starch content (as determined by iodine staining after dark treatment of plants) and flowering times, as shown in Table 2. The self-pollinated F_2 progeny of the cross to the RLD wild type segregated in a 3:1 Mendelian ratio (Table 2) for the phenotype, with complete or at least very tight linkage of late-flowering to starch-excess phenotype. To map the *cam1* mutation, we crossed *cam1* with three testers (CS3078, CS3079, and CS3080) having multiple phenotypic markers. In 162 F_2 plants derived from the cross of *cam1* with CS3078, we found 80 $Gl1^+$ and $Cam1^+$ plants, 35 $Gl1^+$ and $Cam1^-$ plants, 43 $Gl1^-$ and $Cam1^+$ plants, and 4 $Gl1^-$ and $Cam1^-$ plants. The estimate of the percentage of recombination between *gl1* and *cam1* by using JoinMap (Stam, 1993) is 29.2 ± 6.01. The location of *cam1* on chromosome 3 was confirmed by restriction fragment length polymorphism (RFLP) mapping. RFLPs between the RLD and the Landsberg *erecta* ecotypes were established by using the cosmid clones *g4014*, mapped at chromosome 3; 74.1 and *g4708*, mapped at chromosome 3; and 22.8 (Figure 2A; Nam et al., 1989). Thirty late-flowering, starch-excess plants from the F_2 generation of *cam1* × the CS3078 tester segregated for the *g4014* RFLP, but all showed only the *cam1* (RLD background) hybridization pattern for the *g4708* probe (Figure 2B). Therefore, we concluded that the *cam1* locus maps to chromosome 3 at position 22.8.

Starch Level and Late Flowering

To examine whether high starch levels cause late floral initiation, we crossed *cam1* with *adg1-1* and *pgm*, and *gi1-3* was crossed with *pgm*. The *adg* mutant lacked ADGase, and the *pgm* mutant lacked the plastid PGM isozyme. Neither mutant has detectable leaf starch nor delays in flowering under continuous light (Caspar et al., 1985; Lin et al., 1988a). The F_2 progeny obtained from the self-pollinated F_1 generation of the *cam1* and *adg1* cross were grown under continuous light. Table 3 shows that the F_2 progeny segregated at a 3:1 ratio for Adg^- (as determined by leaf staining and native gel activity assay). The F_2 Adg^- progeny segregated for the late-flowering phenotype, indicating that starch per se is not responsible for late flowering. Similarly, double homozygous mutants *cam1 pgm* and *gi1-3 pgm* had no detectable starch content but flowered as late as the *cam1* or *gi* mutants (Table 3).

Table 2. Genetic Analysis of Various Crosses of *cam1* Plants

Cross	$Cam1^{+,a}$	$Cam1^-$	$\chi^{2,b}$
cam1 × *CAM1*, F_1	35	0	0
cam1 × *CAM1*, F_2	591	198	0.0038
cam1/CAM1 × *cam1*	62	78	1.829

[a] $Cam1^+$ was identified by late flowering and iodine staining of leaf starch phenotypes.
[b] Calculation for an assumed 3:1 wild type-to-mutant ratio for the F_2 progeny and a 1:1 wild type-to-mutant ratio for the testcross progeny. χ^2, 0.95 = 3.84.

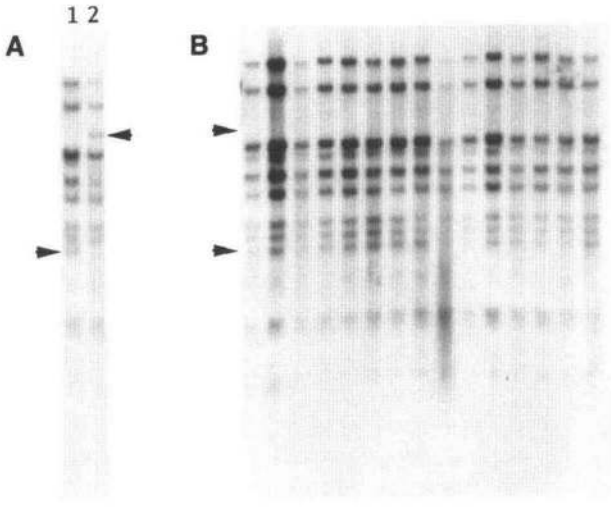

Figure 2. RFLP Analysis of F_2 Plants of *cam1* × CS3078 Indicating the Tight Linkage of *cam1* with the g4708 Marker.

(A) RFLP of HindIII-digested genomic DNA of *cam1* (RLD; lane 1) and the tester line CS3078 (Landsberg *erecta*; lane 2) probed with *g4708*.
(B) A representative blot of HindIII-digested genomic DNA of 15 randomly chosen F_2 plants with the Cam1$^-$ phenotype hybridized to the *g4708* probe.
The different bands between RLD and Landsberg *erecta* are indicated by arrowheads.

Growth Characteristics of *cam1*

Apart from the starch-excess phenotype, the *cam1* mutant was delayed in the onset of flowering. Under both a light/dark cycle and continuous light, the *cam1* mutants started to bolt ~10 to 14 days later than did the wild type, as shown in Table 4. The flowering time of the *cam1* mutant is not significantly different under either a long or short photoperiod. In comparison with the wild type grown under the same light conditions, the leaf number of the mutant increased at the time of flowering. Vernalization for 22 days at 4°C had a limited effect on the flowering time of the *cam1* mutant (Table 4). However, the leaf number at flowering time under short-day conditions is not well correlated with flowering time, perhaps due to the RLD genetic background. It has been shown that the reduction in flowering time and leaf number depends on the genotype and the photoperiodic conditions of growth (Karlsson et al., 1993). The flowering time of F_2 plants of *cam1* × *gi-3* is not later than that of either parent, suggesting that *cam1* and *gi* are in the class of late-flowering genes insensitive to photoperiod and vernalization (Martinez-Zapater et al., 1994).

Carbohydrate Content of the *cam1* Mutant

The results of iodine staining were confirmed by enzymatic measurements of the starch content in leaves. Wild-type plants and the *cam1* mutant were grown under continuous light and shifted to the dark for 48 hr. At the end of this dark period, the wild type had less starch than did the *cam1* mutant. The total amount of leaf starch, however, depended on the particular growth conditions. Table 5 shows a typical set of measurements, indicating that the starch contents in leaves of the wild type and the *cam1* mutant are different. In the cross-section of *cam1* mutant and wild-type leaves, neither the cell number nor the chloroplast number and size seemed to be significantly different.

The amount of soluble sugars accumulated during the light phase in the *cam1* mutant was greater than that of the wild type. This was true for sucrose as well as for hexoses. After growing for 2 days in the dark, both wild-type and *cam1* plants had reduced soluble sugar contents (Table 5). This decrease in total extractable sugars was due to the decrease in sucrose, whereas the level of hexoses remained basically unchanged. A time course of starch accumulation and degradation showed that the diurnal pattern was similar between young *cam1* plants and wild-type plants, as shown in Figure 3A. In older *cam1* plants, the change in starch accumulation and degradation had a pattern similar to that of the wild type, whereas the total amount of starch increased in the *cam1* mutant (Figure 3B). We also examined the diurnal change of the leaf starch level in the *gi1-2* mutant versus the wild type (Figure 3C). The diurnal pattern was similar between *gi1-2* and the wild type; however, the total amount of starch increased in the *gi1-2* mutant.

Activities of Starch Metabolism Enzymes in *cam1* Plants

To elucidate the biochemical basis for the elevated starch content in *cam1*, we measured the activities of several starch metabolism enzymes in leaf extracts of the *cam1* mutant and the wild type. The results are shown in Table 6. We also examined enzyme activities of PGI, PGM, ADGase, branching enzyme, disproportioning enzyme (D-enzyme), and starch phosphorylase by native gel electrophoresis, demonstrating that the *cam1* and *gi* mutants possess all of the isozymes

Table 3. Genetic Analyses of Double Mutants *cam1 adg1*, *cam1 pgm*, and *gi1-3 pgm*

Cross	F_2[a]			$\chi^{2,b}$
adg1 × *cam1*	Adg$^+$:Adg$^-$	214	75	0.139
	Adg$^-$/Cam1$^+$:Adg$^-$/Cam1$^-$	52	23	1.284
pgm × *cam1*	Pgm$^+$:Pgm$^-$	235	62	2.695
	Pgm$^-$/Cam1$^+$:Pgm$^-$/Cam$^-$	46	16	0.021
pgm × *gi1-3*	Pgm$^+$:Pgm$^-$	133	35	1.555
	Pgm$^-$/Gi1-3$^+$:Pgm$^-$/Gi1-3$^-$	27	8	0.086

[a] The Adg$^-$ and Pgm$^-$ phenotypes were identified by iodine staining of leaf starch and by native gel assay for enzyme activities. Testcrosses were performed to confirm the double mutants.
[b] Calculation for an assumed ratio of 3:1. χ^2, 0.95 = 3.84.

Table 4. Flowering Time and Leaf Numbers of *cam1* and RLD Wild-Type Plants under Various Growth Conditions

	Light/Dark (8/16 hr)		Continuous Light	
Treatment	RLD WT[a]	*cam1*	RLD WT	*cam1*
Not vernalized	33 ± 1 day	41 ± 3 days	18 ± 3 days	36 ± 5 days
	LN[b] = 5 ± 1	LN = 7 ± 1	LN = 4 ± 1	LN = 16 ± 3
Vernalized[c]	34 ± 2 days	38 ± 2 days	16 ± 3 days	37 ± 6 days
	LN = 4 ± 1	LN = 5 ± 1	LN = 4 ± 1	LN = 17 ± 4

[a] Ecotype RLD wild type.
[b] LN, rosette leaf number at the time of bolting.
[c] For vernalization treatment, seeds were imbibed in 4°C for 22 days before being transferred to the indicated growth conditions.

displayed by the wild type. Three enzymes associated with starch synthesis, PGI, PGM, and ADGase, show significantly higher activities in the *cam1* mutant than in the wild type (RLD ecotype), regardless of the developmental stage of the plants. In contrast, soluble starch synthetase was present at wild-type levels in young *cam1* plants (that is, before the onset of flowering), but in older plants this activity was reduced to approximately one-third of that in the wild type. The granule-bound starch synthetase activity was more than three times higher in young *cam1* plants and more than 10 times higher in older plants when compared with the wild type. Because in older wild-type plants the activity of the granule-bound starch synthetase is only a fraction of that of the soluble starch synthetase (0.727 pmol/g fresh weight per min versus 91.69 pmol/g fresh weight per min), the increase in the former cannot reverse the total decrease caused by the latter. However, these in vitro activities may not necessarily mirror what happens in vivo.

Because a variety of both starch-deficient and starch-excess mutants in *Arabidopsis* has a considerably elevated level of extrachloroplastic amylase activity (Caspar et al., 1989), we examined the *cam1* mutant for amylase activity. Using an in vitro assay based on starch digestion and staining of the remaining starch with iodine, we detected a slightly higher general amylase activity for the *cam1* mutant (data not shown). We measured a reproducible significantly decreased α-amylase activity (Table 6). This suggests a lowered α-amylase activity and an increased β-amylase activity. Native gel assays confirmed these data; the only known chloroplast amylases, A1 (β) and A2 (α) (Lin et al., 1988b), appeared unchanged. But higher activities could be detected for the extrachloroplastic β-amylase A3 and also (although less pronounced) for the A5 isozyme. The degree of changes in these latter activities varied under different growth and light conditions (data not shown). Because similar changes were observed in starchless mutants (Lin et al., 1988b; Caspar et al., 1989), this increased activity is a secondary rather than a direct effect of the primary mutation. However, the different extent of the decrease in α-amylase activity during plant development agrees well with the increasing amount of starch accumulated in the leaves.

DISCUSSION

cam1 and *gi* Mutants Exhibit Late-Flowering and Starch-Excess Phenotypes

There have been many screens for the late-flowering mutants, and multiple alleles of most late-flowering genes have been isolated (Koornneef et al., 1991). *cam1*, originally identified as

Table 5. Analysis of Carbohydrate Contents in Leaves of *cam1* and the RLD Wild Type under Different Growth Conditions

	+2 Days Continuous Light[a]		+2 Days Dark[a]	
Carbohydrate	RLD	*cam1*	RLD	*cam1*
Starch (mg/g fresh weight)	2.89 ± 0.46[b]	9.67 ± 0.45	1.44 ± 0.48	2.50 ± 0.05
Total sugars (mg sucr.equ./g fresh weight)[c]	2.5 ± 0.6	4.3 ± 0.7	1.4 ± 0.03	2.5 ± 0.06
Sucrose (mg/g fresh weight)	0.5 ± 0.3	1.1 ± 0.3	ND[d]	ND
Hexoses (mg sucr.equ./g fresh weight)	1.9 ± 0.5	3.2 ± 0.7	1.4 ± 0.2	2.5 ± 0.5

[a] Carbohydrates of the RLD wild type and the *cam1* mutant were measured after 28 days with a light/dark photoperoid of 15 hr of light/9 hr of dark plus an additional 2 days (+2) under continuous light, followed by an additional 2 days of dark treatment.
[b] Data are presented as the means ± SE of six independent measurements.
[c] mg sucr.equ./g fresh weight, milligrams of sucrose equivalent per gram fresh weight.
[d] ND, not detectable.

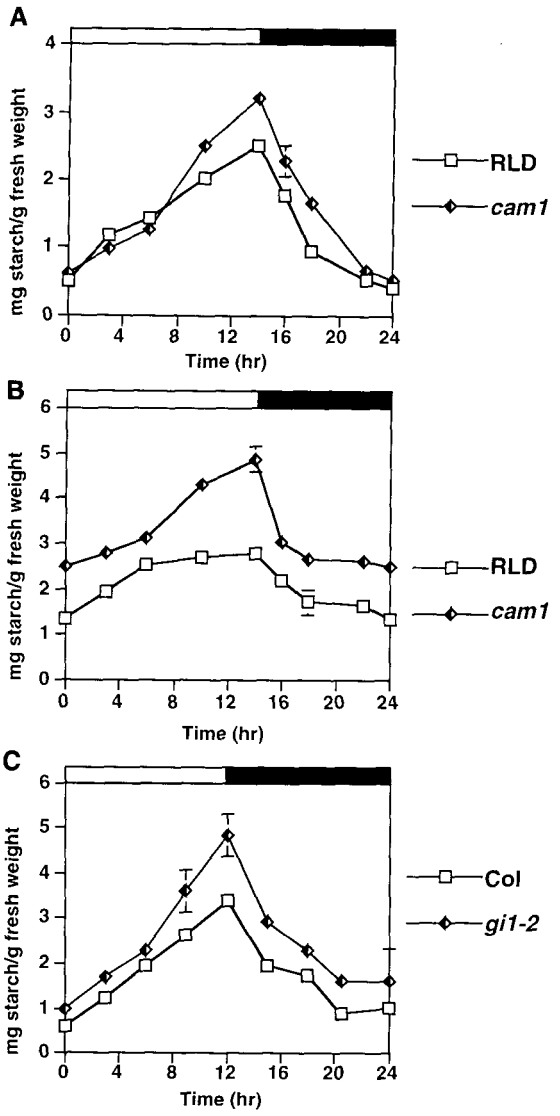

Figure 3. Leaf Starch Content Fluctuation during the Diurnal Cycle.

(A) and **(B)** Diurnal pattern of starch synthesis and degradation in *cam1* and the RLD wild type after 20 days **(A)** and 44 days **(B)** of growth under a 14/10-hr light/dark photoregime.

(C) For *gi1-2* and the Columbia (Col) wild type, the plants were grown under continuous light for 21 days and then shifted to a 12/12-hr light/dark cycle for 7 days before measurement.

The data points represent means ± SE (bars) of at least four independent measurements. Error bars not indicated are smaller than the symbols used. White and black bars above the graphs indicate the light and dark periods, respectively.

a mutant with altered leaf starch content, also has a late-flowering phenotype. By map position, *cam1* does not correspond to any of the existing late-flowering genes, perhaps due to the different genetic backgrounds, mutagens, or screening methods applied. Other late-flowering mutants, for example, *det2*, *ein2*, *etr1*, *fri*, and *flc*, have been isolated in addition to the existing ones (Martinez-Zapater et al., 1994). It is probable that many other genes involved in floral transition still remain to be identified.

We have isolated 11 mutants with altered leaf starch content by screening 10,000 M_2 plants. These 11 mutants were assigned to 10 loci on the basis of allelism tests. Three of them were isolated previously and include *adg1*, *pgm*, and *sex1*. Only one allele of the *cam1* mutant was discovered, which may have been due to the size of the population screened.

By genetic analysis, we showed that the altered starch content and late-flowering phenotype of the *cam1* mutant are caused by a monogenic recessive mutation. Delayed flowering is not significantly altered by either vernalization treatment or photoperiods. Similarly, the floral initiation of late-flowering mutants *co* and *gi* is not sensitive to vernalization and photoperiod changes (Koornneef et al., 1991). Interestingly, we observed an increase in leaf starch content in plants homozygous for the *gi* mutation but not in plants homozygous for *co*, which were isolated as late-flowering mutants. There are three *gi* alleles conditioning the late-flowering phenotype, and their mutants also have increased leaf starch content, although at different levels. It cannot be excluded that some of the allele differences are related to the ecotype differences.

The pattern of starch accumulation in leaves of the *cam1* mutant is different from that of the *gi* mutant. The increased

Table 6. Activity of Some Representative Starch Metabolism Enzymes in Leaves of *cam1* and Wild-Type Plants[a]

Enzyme	Wild-Type Activity[b]	*cam1* Mutant Activity	% of Wild-Type Activity
PGI	99.29 ± 3.87	264.34 ± 14.81	266.23
PGM	210.47 ± 6.87	273.36 ± 16.82	129.88
ADGase	0.24 ± 0.10	0.45 ± 0.07	186.67
SSS[c]	91.69 ± 28.83	27.23 ± 0.79	29.69
GBSS[d]			
A	0.91 ± 0.09	3.07 ± 0.14	388.37
B	0.73 ± 0.06	9.29 ± 0.06	1277.58
SP[e]	1.07 ± 0.03	0.88 ± 0.14	81.85
α-Amylase[f]			
A	1.56 ± 0.08	1.14 ± 0.13	72.93
B	1.44 ± 0.10	0.59 ± 0.02	40.93

[a] Plants were grown under continuous light. Enzyme activity is presented as nanomoles per milligram of protein per minute, except for starch synthetases, which is given as picomoles per gram fresh weight per minute.

[b] Data represent means ± SE of at least three independent measurements.

[c] SSS, soluble starch synthetase.

[d] GBSS, granule-bound starch synthetase. Enzyme activities differed significantly between samples from plants that did not flower (A) and those that did flower (B), so all data are shown.

[e] SP, starch phosphorylase.

[f] A and B are as given in footnote d.

leaf starch in *cam1* plants occurs upon the onset of floral initiation. In the *gi* mutant, the accumulation of starch occurs also in young plants, suggesting that the action of GI may be different from that of CAM1. Alternatively, the *cam1* mutation may be leaky such that the increased starch is observed only at a later developmental stage. In the young *cam1* plant, the activities of several enzymes of starch metabolism, for example, the granule-bound starch synthetase and starch phosphorylase, are altered (Table 6) without a significant increase of starch. This indicates that CAM1 may still affect young plants. These data indicate that *cam1* and *gi* are pleiotropic mutants and may be part of the regulatory pathway of floral initiation and carbohydrate metabolism.

cam1 Affects the Regulation of Starch Metabolism

The starch-excess phenotype of the *cam1* mutant could be caused by either overproduction or decreased degradation of starch. The latter seems unlikely because the total amount of starch degraded in the dark is no less than that of the wild type (Table 5), regardless of decreased amylase and phosphorylase activities (Table 6). Furthermore, the rate of the starch degradation in the *cam1* mutant during the diurnal cycle is similar to that in the wild type (Figure 3). The fact that most of the enzymes involved in the starch synthesis pathway have increased activities favors the overproduction hypothesis. In this case, the soluble starch synthetase activities are not rate limiting, because the increase in starch occurs despite decreased enzyme activity. However, if the mutation results only in starch overproduction, one should expect a continuous accumulation of leaf starch. In the *cam1* mutant, the starch level does not reach the maximum levels reported for Arabidopsis (Caspar et al., 1991). Furthermore, the diurnal pattern of starch accumulation and degradation persists, with an increased baseline of starch content (Figure 3B). These results indicate that the *cam1* mutation may affect the regulation of the leaf starch pool in addition to the altered starch synthesis or degradation.

Late Flowering Is Not Caused by Increased Starch Levels in Leaves

It is not known whether the altered carbohydrate metabolism causes the late-flowering phenotype or vice versa. Because another starch-excess mutant, *sex1* (Caspar et al., 1991), also flowers late under short-day conditions, a connection between starch content and floral initiation would be credible. However, the homozygous double mutants *cam1 adg1*, *cam1 pgm*, and *gi1-3 pgm* with no detectable starch in leaves are delayed in floral initiation, indicating that the increased level of starch in leaves is not the cause of the late floral initiation.

Although *cam1* and *gi* are late-flowering mutants with increased starch content in their leaves, not all the late-flowering mutants examined had such an increase (Table 1). In addition, we have isolated a mutant, *ke81*, that exhibits an elevated leaf starch level but not late flowering under either a short or long photoperiod (K. Eimert and J. Chen, unpublished data). These data indicate that the increased leaf starch content in these late-flowering mutants is not due to a secondary effect of the delay in flowering and the extended vegetative growth.

cam1 and *gi* May Be Part of a Regulatory Pathway for Starch Metabolism and Floral Initiation

Based on the existing models (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991; Martinez-Zapater et al., 1994), we assume that floral initiation occurs via an autonomous pathway. Early in this pathway, the *EMF* gene product produced in young plants could inhibit the transition. In addition, the floral transition at the apical meristem could be the result of the interaction of a few floral repression and floral promotion pathways under different environmental conditions, including vernalization and light (Martinez-Zapater et al., 1994). Apart from environmental stimuli and hormones, the availability of assimilates has been suggested as a signal for floral initiation from the previous analysis of the starch metabolic mutants *sex1* and *pgm* (reviewed in Bernier et al., 1993).

sex1, a starch-excess mutant (Caspar et al., 1991), and *pgm*, a starchless mutant (Caspar et al., 1985), are both delayed in flowering under short-day conditions. These seemingly very different starch metabolism mutants are similar in that they have an altered sugar pool. The *pgm* mutants completely lack chloroplastic PGM activity and accumulate no starch but have higher concentrations of soluble sugars than does the wild type. The *sex1* mutant has been proposed to be defective in starch degradation. After a certain elevated amount of starch is accumulated, starch synthesis is largely or completely inhibited and soluble sugars accumulate in leaves of the *sex1* mutant (Caspar et al., 1991). In both *pgm* and *sex1* mutants, there is no flux or a very low flux of carbohydrates through the starch pool, leaving the plant essentially without a buffer for freshly fixed carbon. This presumably causes an accumulation of various metabolites in the chloroplasts. One (or several) of these metabolites could either directly or indirectly mediate floral initiation. We do not know the nature of the intermediate metabolites. However, it is likely that the changed carbohydrate partitioning into soluble sugars via triose phosphate/hexose export into the cytosol is involved in the altered regulation. Similarly, the delay in floral initiation as a result of *gi* and *cam1* mutations may be due to the change in intermediate metabolites. Alternatively, GI and CAM1 are directly involved in a pathway regulating starch metabolism as well as promoting floral initiation; the mutation of these genes could result in alteration of both processes.

Based on the sensitivity to vernalization and photoperiod treatment, we can classify the *gi*, *cam1*, *pgm*, and *sex1* mutations into two groups. Floral initiation in *gi* and *cam1* mutants is delayed in long-day and short-day conditions; *pgm* and *sex1*

mutants are affected only during short-day conditions. Vernalization can promote the floral initiation of *pgm* and *sex1* grown under short-day conditions (Bernier et al., 1993; M. Wu and J. Chen, unpublished data). The late flowering of *cam1* and *gi* is insensitive to vernalization and a short photoperiod. These differences may be due to the complex interactions among factors affecting the floral initiation or represent a regulatory mechanism of *gi* and *cam1* distinct from that of *sex1* and *pgm*. Further analysis of the flowering time as well as distribution of assimilates of double mutants and determination of the gene products and function would clarify their modes of action.

From the study of yeast glycogen-deficient mutants, it is known that glycogen itself is dispensable for yeast. However, its metabolism is regulated by gene products that are also involved in other fundamentally important pathways (Cannon et al., 1994). Starch is also dispensable for plants. The regulation of starch metabolism may also interact with other developmentally regulated pathways. Although the nature of the mutation causing the Cam1 and Gi phenotypes is not known, we are confident that the mutants with these phenotypes and another set of carbohydrate metabolic mutants that have been isolated in our laboratory will enhance our insight into the regulation of carbohydrate metabolism and the interaction of carbohydrate metabolism with other processes in the plant.

METHODS

Plant Materials and Growth Conditions

The M_2 seeds of ethyl methanesulfonate–treated seeds of *Arabidopsis thaliana* (RLD ecotype) were obtained from Lehle Seeds (Round Rock, TX). Plants were grown on soil at 23°C under ~5000 Lux fluorescent light with different photoperiods or under continuous light. For the vernalization treatment, seeds were imbibed on soil at 4°C for 3 weeks before being moved to the growth conditions as given above. Arabidopsis mutants and mapping lines were obtained from the Arabidopsis Biological Resource Center at Ohio State University (Columbus, OH) and Nottingham Arabidopsis Stock Center (Nottingham, UK).

Screening of Mutants

Mutants were screened as described by Caspar et al. (1985). Plants were first screened after they developed four to six true leaves. A second screening was conducted after floral initiation. After incubating plants in the dark for the indicated time, leaf pieces were cut out, depigmented in 95% ethanol, and stained with I_2-KI (5.7 mM iodine and 43.4 mM potassium iodide in 0.2 N HCl).

Mapping

Conventional mapping was conducted using the mapping lines CS3078 (*ch1 er gl1 cer2 tt3*), CS3079 (*er bp ttg yi*), and CS3080 (*dis1 clv2 er tt5*). F_2 progeny of the cross *carbohydrate accumulation mutant1* (*cam1*) × CS3078 were scored for marker phenotypes, and the data were analyzed using the JoinMap computer program (Stam, 1993). Restriction fragment length polymorphism (RFLP) mapping was performed according to Chang et al. (1988) and Nam et al. (1989). The probes for the RFLP mapping were provided by the Arabidopsis Biological Resource Center.

Starch Measurement

Quantitative starch assays were conducted according to Ebell (1969) with the following modifications. Leaf tissue was ground in 80% ethanol and extracted three times at 80°C. The supernatant was used to measure soluble sugars. The pellet was dried, resuspended in 200 μL of 0.2 M KOH, and boiled for 30 min. After neutralization with 40 μL of acetic acid, 100 μL of the starch sample was digested with 0.5 units amyloglucosidase (Sigma) in 900 μL of 0.1 M sodium acetate buffer, pH 4.8, for 1 hr at 55°C. The reaction was stopped by immersing the tubes in boiling water for 5 min. The resulting glucose was measured by adding 37.5 μL of 0.04% *o*-phenylene dihydrochloride and 100 μL of sodium acetate buffer containing 0.1 units of glucose oxidase and 0.1 units of peroxidase to a 100-μL digested starch sample. Starch was measured at OD_{405} after incubation at 37°C for 30 min and compared with a set of digested starch standards. The method was scaled down and proved to be reliable for tissue samples as little as 10 mg.

Sugar Measurement

Soluble sugars were measured as described by Jones et al. (1977). Leaf tissue was weighed and extracted three times with 80% ethanol at 80°C. The extracts were combined, dried, resuspended in water, and assayed for sucrose and hexoses using the coupled enzyme method described by Jones et al. (1977).

Enzyme Assays

Starch synthetase was assayed according to Nelson et al. (1978). Phosphoglucomutase (PGM) and phosphoglucoisomerase (PGI) were determined by the method of Rao et al. (1990). ADP-glucose pyrophosphorylase (ADGase) and UDP-glucose pyrophosphorylase were assayed using the coupled enzyme method described by O'Brien (1976) with a pyrophosphate detection kit (Sigma). Amylase and starch phosphorylase activities were measured as described by Okita et al. (1979).

Native Gel Assays

The assays for PGI, PGM, and starch phosphorylase were conducted according to Shaw and Prasad (1969), except that 7% polyacrylamide gels were used. The ADGase and UDP-glucose pyrophosphorylase gel assays were performed following the procedure of Weaver et al. (1972). Amylases were detected as described by Lin et al. (1988b).

ACKNOWLEDGMENTS

We thank Jane Langdale and Melvin Green for reviewing the manuscript. S-M.W. and J.C. were supported by grants from the National

Science Council, Taiwan, Republic of China, and Academia Sinica, Taipei, Taiwan.

Received May 9, 1995; accepted August 18, 1995.

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