A Sepal-Expressed ADP-Glucose Pyrophosphorylase Gene (NtAGP) Is Required for Petal Expansion Growth in ‘Xanthi’ Tobacco

Man Sup Kwak, Sung Ran Min, Si-Myung Lee, Kyung-Nam Kim, Jang Ryol Liu, Kyung-Hee Paek, Jeong Sheop Shin, and Jung Myung Bae

School of Life Sciences and Biotechnology, Korea University, Seoul 136–701, Korea (M.S.K., K.-H.P., J.S.S., J.M.B.); Plant Genome Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305–806, Korea (S.R.M., J.R.L.); Biosafety Division, National Institute of Agricultural Biotechnology, Rural Developmental Administration, Suwon 441–707, Korea (S.-M.L.); and Department of Molecular Biology, Sejong University, Seoul 136–747, Korea (K.-N.K.)

In this study, a tobacco (Nicotiana tabacum ‘Xanthi’) ADP-glucose pyrophosphorylase cDNA (NtAGP) was isolated from a flower bud cDNA library and the role of NtAGP in the growth of the floral organ was characterized. The expression of NtAGP was high in the sepal, moderate in the carpel and stamen, and low in the petal tissues. NtAGP-antisense plants produced flowers with abnormal petal limbs due to the early termination of the expansion growth of the petal limbs between the corolla lobes. Microscopic observation of the limb region revealed that cell expansion was limited in NtAGP-antisense plants but that cell numbers remained unchanged. mRNA levels of NtAGP, ADP-glucose pyrophosphorylase activity, and starch content in the sepal tissues of NtAGP-antisense plants were reduced, resulting in significantly lower levels of sugars (sucrose, glucose, and fructose) in the petal limbs. The feeding of these sugars to flower buds of the NtAGP-antisense plants restored the expansion growth in the limb area between the corolla lobes. Expansion growth of the petal limb between the corolla lobes was severely arrested in ‘Xanthi’ flowers from which sepals were removed, indicating that sepal carbohydrates are essential for petal limb expansion growth. These results demonstrate that NtAGP plays a crucial role in the morphogenesis of petal limbs in ‘Xanthi’ through the synthesis of starch, which is the main carbohydrate source for expansion growth of petal limbs, in sepal tissues.

Carbohydrates play an important role in the development of floral organs. They are utilized by the plants both as nutrients to sustain the normal growth of floral organs and as signals affecting development (Clément et al., 1996; Roldán et al., 1999; Goetz et al., 2001). Carbohydrates are usually produced by photosynthesis in the green source tissues of plants, transported to the flower buds, and distributed among the various floral organs, with the partitioning within the flower buds being dependent on the strength of the organ sink. As demonstrated in ‘Enchantment’ Lilium (Clément et al., 1996), during the anther growth phase, the anther is the strongest sink among the floral organs, whereas, once anther growth is completed, the assimilates are attracted to other parts of the floral organ. Consequently, an inadequate carbohydrate supply can block the normal development of floral organs.

ADP-Glc pyrophosphorylase (AGPase; EC 2.7.7.27) is a heterotetrameric protein consisting of two large and two small subunits in higher plants. It catalyzes the synthesis of ADP-Glc, a glucosyl substrate for the synthesis of starch polymers, and inorganic pyrophosphate from Glc-1-P and ATP. It also plays an essential role in regulating starch synthesis by virtue of its allosteric properties, as it is activated by 3-phosphoglycerate and inhibited by inorganic phosphate. This allosteric regulation has recently been reported to be specified by both the small and large subunits (Cross et al., 2004; Hwang et al., 2005). Modifications of these regulatory properties result in alterations in the starch yields in several starch-accumulating plants, including potato (Solanum tuberosum), maize (Zea mays), wheat (Triticum aestivum), and rice (Oryza sativa; Müller-Röber et al., 1992; Stark et al., 1992; Giroux et al., 1996; Smidansky et al., 2002, 2003). AGPase has also been found to be activated by posttranslational redox-modification in
response to the Suc supply in potato tubers (Tiessen et al., 2002), and in response to light and sugars in the leaves of Arabidopsis (Arabidopsis thaliana), potato, and pea (Pisum sativum; Hendriks et al., 2003). It has been suggested that trehalose-6-P acts as signaling metabolite of sugar status, mediating the sugar-induced redox-regulation of AGPase in its rate-determining role in starch synthesis (Kolbe et al., 2005; Lunn et al., 2006).

To date, AGPase large subunit genes have been reported as multifamilies in Arabidopsis (Villand et al., 1993), potato (La Cognata et al., 1995), barley (Hordeum vulgare; Villand et al., 1992a), and tomato (Solanum lycopersicum; Chen et al., 1998), and as small subunit genes in bean (Vicia faba; Weber et al., 1995), maize (Giroux and Hannah, 1994; Prioul et al., 1994), sweetpotato (Ipomoea batatas; Bae and Liu, 1997), and Perilla frutescens (Choi et al., 2001). Therefore, although only a small subunit gene has been found in potato (Nakata et al., 1991), rice (Anderson et al., 1989), and Arabidopsis (Villand et al., 1993). it seems to be a common plant feature that AGPase genes are present as a multifamily. Most of large and some of small subunit AGPase genes show a tissue-specific expression pattern (Villand et al., 1992a, 1992b; Giroux and Hannah, 1994; Prioul et al., 1994; La Cognata et al., 1995; Weber et al., 1995; Chen et al., 1998). However, it has not yet been elucidated whether each isoform, in addition to showing tissue-specific expression, has a distinct role and, if so, if it is related to organ development.

Almost no information is available on the role of the AGPase genes in floral organ development. Considering that starch is the main energy source for floral organ development and that AGPase regulates starch biosynthesis, any significant alteration in the expression of AGPase genes should affect floral organ development. In support of this proposal, Lalonde et al. (1997) reported that water-stressed wheat plants produced pollen grains with little or no starch and that the expression of the AGPase gene was strongly inhibited during the second phase of pollen development. In addition, we observed that overproduction of heterologous AGPase mRNA suppressed the expression of the endogenous AGPase gene, resulting in the production of abnormal pollen grains with no starch granules (Baé et al., 2001).

In the study reported here, we further characterized the role of AGPase in floral organ development by isolating an AGPase cDNA (NtAGP) from the tobacco (Nicotiana tabacum) flower bud. Expression analysis of NtAGP showed that it was preferentially expressed in the floral organs: the sepal, stamen, and carpel. To analyze the in vivo function of NtAGP, antisense plants were produced with an NtAGP-specific DNA fragment driven by a 35S promoter. Our results demonstrated that the down-regulation of NtAGP reduced carbohydrate content in the petal limbs and arrested cell expansion in the petal limbs between the corolla lobes, indicating an essential role of NtAGP in normal petal limb growth in ‘Xanthi’ tobacco.

**RESULTS**

**Cloning of AGPase cDNA Expressed in the Flower**

To obtain a flower-expressed or -specific AGPase gene, we screened the flower bud cDNA library of ‘Xanthi’ tobacco using the sweetpotato small subunit AGPase cDNA (sTL1; Bae and Liu, 1997) as a probe. From the 3.8 × 10⁷ phages screened, 10 independent positive clones were isolated, all of which encoded an identical protein. DNA sequence analysis revealed that all of these clones were identical in terms of nucleotide sequence in the areas of overlap and that they all share high nucleotide sequence identity with the known AGPase small subunit genes. The longest cDNA was 1,769 bp in length and encoded a protein of 520 amino acids. The N-terminal end of the protein contained a putative transit peptide of 71 amino acids; this ‘Xanthi’ tobacco AGPase cDNA was termed NtAGP.

Deduced amino acid sequence comparison of NtAGP with six other plant small subunit AGPase genes showed 94%, 88%, 88%, 87%, 87%, and 86% sequence identities with potato, sweetpotato, Brassica napus, pea, Arabidopsis, and Phaseolus vulgaris, respectively (Supplemental Fig. S1). Almost no sequence identity was detected in the amino acid sequence of the N-terminal putative transit peptide (Supplemental Fig. S1). The putative transit peptide and 5’ untranslated region (from nucleotide no. 1 to nucleotide no. 205) was used as an NtAGP-specific sequence for further analysis.

**Genomic Southern-Blot Analysis of NtAGP**

Genomic Southern-blot analysis was performed with two different probes: the full-length NtAGP cDNA and the 205-bp NtAGP-specific sequence (Fig. 1). Genomic DNA was extracted from leaf tissues of ‘Xanthi’ tobacco and digested with HindIII, XbaI, and BamHI + XhoI. Nine to 10 hybridizing bands were detected with the full-length NtAGP probe, whereas only two bands were hybridized with the NtAGP-specific probe. Although some of the bands detected with the full-length probe may be due to the restriction enzyme sites present in introns, given the relatively high levels of sequence identity among small subunit AGPase genes and the low levels of sequence identity between small and large subunit AGPase genes, this result suggests that the small subunit AGPase is most likely present as a multifamily gene in the genome of ‘Xanthi’ tobacco. On the other hand, since the 205-bp NtAGP-specific sequence contains no putative intron sites (Supplemental Fig. S1; Noh et al., 2004), the detection of only two bands with this probe suggests that NtAGP is encoded by two independent loci or that NtAGP presents as two alleles in the genome of ‘Xanthi’ tobacco.

**NtAGP Is Highly Expressed in the Flower Bud**

To determine the expression pattern of NtAGP, we carried out an RNA gel-blot analysis in which total RNA was isolated from the leaf, stem, root, and flower.
bud (3–4 cm in length) and subsequently hybridized with the NtAGP-specific probe. Transcript levels were high in the flower bud and moderate in the leaf, whereas NtAGP was not transcribed in the stem and root (Fig. 2A), indicating that NtAGP is preferentially expressed in the flower bud. High levels of NtAGP transcripts were detected during all stages of flower development from young flower buds (less than 1 cm in length) to the open flowers (Fig. 2B). A tissue-specific expression analysis of NtAGP in various flower organs, including the sepal, petal, stamen, and carpel, revealed that NtAGP mRNA was most highly expressed in the sepal and moderately expressed in the stamen and carpel, while only low levels of transcripts were detected in the petal (Fig. 2C).

NtAGP-Antisense Plants Exhibit Morphological Changes in Petal Limbs

The results of the RNA gel-blot analysis suggested that NtAGP is preferentially expressed in the flower bud and that transcript levels are high in the sepal and moderate in stamen and carpel. Based on these observations, we hypothesized that NtAGP may play a role in regulating flower organ development by the synthesis of starch, the main carbohydrate source for floral organ growth. To test this hypothesis, a 205-bp NtAGP-specific fragment was fused in an antisense orientation to the cauliflower mosaic virus 35S promoter.

The antisense construct was introduced into tobacco (‘Xanthi’) plants, and 27 independent transformants were regenerated. The transfer of the antisense construct was identified by PCR analysis with neomycin phosphotransferase II (nptII) primers (data not shown). Five progenies with single-copy insertions (T2) were selected based on the segregation ratio of the kanamycin marker, and seven plants for each progeny were phenotypically analyzed. The NtAGP-antisense plants were identical to the untransformed ‘Xanthi’ plants with respect to growth rate, height, morphology of vegetative organs, and flowering time. Morphological alterations were observed in the petal limbs of 80% (28/35) of the NtAGP-antisense plants when the flowers opened and proceeded to expand their corolla tips.

Figure 1. Genomic Southern-blot analysis of NtAGP. Genomic DNA gel blots were hybridized with full-length NtAGP cDNA or the 205-bp NtAGP-specific DNA fragment. Each lane represents 50 μg of genomic DNA digested with the indicated restriction enzymes. The DNA size markers are indicated in kilobases (kb).

Figure 2. Expression pattern of NtAGP mRNA. Each lane represents 30 μg of total RNA prepared from the designated tissues. Equal loading of RNA in each lane was verified by the amount of ethidium bromide-stained rRNA shown in the bottom panel. The 205-bp NtAGP-specific DNA fragment was used as a probe. A, Tissue-specific expression of NtAGP. B, Expression of NtAGP mRNA during flower development. The five developmental stages are defined by bud size: <1 cm, bud stage 1; 1 to 2 cm, bud stage 2; 2 to 3 cm, bud stage 3; 3 to 4 cm, bud stage 4; open flower, stage 5. C, Flower organ-specific expression of NtAGP. Total RNA was isolated from the designated organs at open flower stage.
A developmental map of tobacco petals has been described (Drews et al., 1992) for ‘Samsun’ tobacco. Parts of the petal (corolla) in tobacco flowers are illustrated in Figure 3A. At stage 9, a fully elongated corolla tube bulge enlarges horizontally and the petal tips remain closed. The petal limb begins to open at stage 10, and at stage 11 the petal limbs are halfway open while the petal tips begin to expand horizontally. At stage 12, the flower is open and the corolla limb is fully expanded. In this study, we found that stage 12 of tobacco plants could be subdivided into stages 12 and 13 so that: (1) at stage 12, the expansion growth of the petal limb is still advanced and the corolla lobes are distinguishable; and (2) at stage 13, the corolla limb is fully expanded and the corolla lobes are finally indistinguishable. The morphological markers involved in petal limb growth from stages 9 to 13 are described in Table I and depicted in Figure 3B.

In ‘Xanthi’ tobacco plants, corolla lobes are not completely fused at the initiation of the flower-opening stage (stage 10), and the intercalary growth between corolla lobes, which proceeds as a horizontal expansion of the petal limbs, is quite advanced at stage 12. We found that at stage 13 the petal limbs were fully expanded and the space between the corolla lobes was filled, resulting in petal limbs with indistinguishable corolla lobes. In the NtAGP-antisense plants, however, the expansion growth of petal limbs terminated sooner than in the ‘Xanthi’ plants; consequently, the morphology of the petal limbs ended with distinctive corolla lobes (Fig. 3B). No morphological alterations were observed in the corolla tube, style, stigma, ovary, anther, filament, and sepal. NtAGP-antisense plants were fertile and set viable seeds.

**NtAGP Down-Regulation Is Responsible for Altered Morphology in the Petal Limbs**

To verify whether the altered morphology of the petal limbs correlated with the down-regulation of NtAGP, NtAGP transcript levels were determined in the five antisense lines and the ‘Xanthi’ plants. Based on the results of our RNA gel-blot analysis (Fig. 2C), which showed that transcript levels of NtAGP were high in sepal, moderate in stamen, carpel, and leaf, but very low in the petal, where the phenotypic change was observed, we examined the transcript levels of NtAGP in various flower organs of the antisense plants, including the sepal, ovary, and anther from stage 9 flowers and leaves from flowering plants.

Total RNA was isolated from five homozygous NtAGP-antisense plants (T3) and reverse transcription (RT)-PCR was conducted to detect alterations in NtAGP mRNA levels using NtAGP-specific primers (Fig. 4). In the leaf, NtAGP transcripts were severely reduced in lines 1 to 4, but the NtAGP mRNA levels were not altered in line 5. Alternatively, there were three different levels of reduced NtAGP transcripts in the sepal: The NtAGP transcripts had almost completely disappeared from this organ in antisense line 3, the mRNA levels were partially decreased in lines 1, 2, and 4, and mRNA levels in untransformed ‘Xanthi’ were detected in line 5. The NtAGP suppression pattern was identical in the ovary and anther tissues of lines 3, 4, and 5.

---

**Figure 3.** Phenotypical alteration in the NtAGP-antisense plants. A, Petal parts of the tobacco flowers are illustrated. B, The growth of petal limbs was compared between NtAGP-antisense plant and the ‘Xanthi’ plant. Each developmental stage was classified according to the morphological markers described in Table I. NtAGP, NtAGP-antisense line. [See online article for color version of this figure.]
NtAGP transcripts were detected in line 3, partially decreased mRNA levels were found in line 4, and mRNA levels detected in ‘Xanthi’ were found in line 5. Differences in the severity of the observed phenotypic changes in the petal limbs of antisense plants were examined in the three different antisense types, with antisense lines 3, 4, and 5 representing completely, partially, and nonsuppressed lines, respectively.

The intercalary growth between corolla lobes was significantly blocked in antisense line 3 and only moderately so in line 4, while the normal intercalary growth in line 5 gave rise to petal limbs in ‘Xanthi’ (Fig. 5A). The severity of the morphological changes was determined by measuring the length (distance from the top of the tube to the petal tip) of the petal limbs in flowers at stage 13. The length of the limb was longest at the middle of the corolla lobe (f in Fig. 5B) and shortest in the area between the corolla lobes (g in Fig. 5B). Both the f and g lengths were measured and compared in antisense lines 3, 4, and 5 (Fig. 5B). The g length was 2.74 ± 0.08 mm in line 3, 3.26 ± 0.24 mm in line 4, and 3.94 ± 0.37 mm in line 5, whereas the f length was essentially unchanged in the three antisense lines. Calculation of the relative ratio (g/f) showed that line 3 had the lowest ratio (36.07% ± 1.03%), line 4 had a medium ratio (44.74% ± 2.47%), and line 5 had the highest ratio (53.33% ± 5.75%; Supplemental Table S1). These results suggest that the altered morphology of the petal limbs resulted from a decrease in NtAGP transcript levels in the sepal. Antisense lines 3 and 4 were consequently selected for further analysis; the nonsilenced line 5 plant was used as a control.

Morphological Changes in the Petal Limb Correlate with a Reduction in Expansion Growth of Epidermal Cells

To determine if the morphological alteration in petal limbs correlated with the limited expansion growth of epidermal cells, we prepared sections of petal limbs (excised limb region between the corolla lobes of stage 13 flowers of antisense line 3 and control plant [line 5]) and examined these under the light microscope (Fig. 6; Table II). The epidermal cells in the antisense line were smaller than their counterparts in the control line: The adaxial epidermis cells of the antisense line were 52.1% and 67.7% smaller in width and length, respectively, than those of the control plants. Individual cells of the mesophyll cell layers of the antisense line were less expanded, with a decreased intercellular space, than those of control plants. Tangential sections of the limb adaxial epidermis area between the corolla lobes revealed that there were 28.2% more cells in an area of 50 mm² in the antisense line than in control plants. The smaller-sized cells of the antisense line, in contrast to those of control plants, were also observed in tangential sections. These results indicate that the morphological alteration in the petal limb of the NtAGP-antisense line is attributable to an arrest in the expansion growth of epidermal cells in the limb between the corolla lobes.

To examine whether morphological changes in the petal limb also resulted from differences in cell number, we calculated the total number of cells in the adaxial limb epidermis of both antisense and control plants in the area between the corolla lobes (g in Fig. 5B). Total cell numbers averaged 156.90 ± 12.76 in the control plants and 154.36 ± 3.31 in antisense line 3 plants. Consequently, there was no significant difference in total cell number between the antisense and control plants.

Down-Regulation of NtAGP in the Sepal Reduces the Amount of Carbohydrate in the Petal Limb

The AGPase found in higher plants is a heterotetramer consisting of two large and two small subunits. To determine the effect of a reduction in the number of NtAGP transcripts encoding a small subunit AGPase on AGPase activity, we conducted an AGPase activity assay in the ADP-Glc synthesizing direction using the sepal of flower buds (2 cm in length). Crude extracts were prepared from NtAGP-antisense lines 3 and 4 and the control plant. The results showed that AGPase activity was proportionally lowered with decreased NtAGP transcript levels. AGPase activity was significantly

---

Table I. Markers for tobacco flower development

| Stage | Morphological Markers |
|-------|----------------------|
| 9     | Corolla tube fully elongated; corolla tube bulge enlarging horizontally; petal tips closed |
| 10    | Corolla limb halfway open; limb light pink; petal tips begin to expand horizontally; distinctive corolla lobes |
| 11    | Flower open; corolla limb deep pink; further expansion of petal tips; distinctive corolla lobes |
| 12    | Corolla limb fully expanded and deep pink; indistinctive corolla lobes |

*aStages described by Drews et al. (1992), except that stage 12 of Drews et al. was subdivided into stages 12 and 13 in this investigation. Petal limb expansion proceeded at stage 12 and was completed at stage 13.
decreased in antisense line 3 and moderately so in line 4 in comparison with the control plant (Fig. 7A).

As AGPase directly regulates the biosynthesis of starch in plants, starch content was measured in the sepal tissues of 2-cm-long flower buds of NtAGP-antisense lines 3 and 4 and the control plants (Fig. 7B). In comparison to the control plants, the starch content in antisense lines 3 and 4 was severely and moderately decreased, respectively. This was supported by the results of iodine staining of the sepals: The staining was less intense in sepals of antisense lines 3 and 4 than in those of the control plant, indicating larger amounts of starch in the control than in the antisense lines (Fig. 7C). This result indicates that the decrease in AGPase activity lowered the levels of starch biosynthesis and, consequently, starch content was reduced in the sepal tissues of the NtAGP-antisense lines. The starch content in the petal limbs was at undetectable levels, making it impossible to determine whether there were any differences between these tissues in the antisense lines and control plants in terms of starch content. The starch content in the leaves and expansion growth of the epidermal cells of the leaves remained unchanged (Supplemental Fig. S2), although NtAGP transcript levels were significantly reduced, suggesting the possible presence of AGPase isoforms in the leaves.

A rapid expansion growth of the petal has been found to be accompanied by a rapid increase in the levels of soluble sugars in rose (Rosa hybrida; Evans and Reid, 1988), daylily (Hemerocallis hybrida ‘Cradle Song’; Bieseki, 1993), and Campanula rapunculoides (Vergauwen et al., 2000). One proposal is that these sugars act as active osmotica in petal expansion. To examine the effect of the decreased starch content in the sepals on the sugar content in the petal limbs, we...
determined the concentrations of Glc, Fru, and Suc in the petal limbs of NtAGP-antisense lines 3 and 4 at stages 10 and 13 (Table III). Fru and Glc concentrations increased dramatically from stages 10 to 13 in the control plants, and although the concentrations of these sugars also increased in lines 3 and 4 between stages 10 and 13, their levels were substantially reduced in comparison to those in the control plant at stage 10 and even more reduced at stage 13. The increase in the Suc concentration from stages 10 to 13 was relatively limited compared to the increases in Fru and Glc; however, the Suc content in lines 3 and 4 was also lower than that in the control plants. The Suc, Glc, and Fru concentrations in line 3 were lower than those in line 4 at both stages, suggesting that the reduced sugar content in the petal limb correlates with the decreased starch synthesis in the sepal.

Sugar Feeding Restores the Morphological Change in the Petal Limb

To determine if the reduced levels of carbohydrate in the petal limb actually arrest the expansion growth in the region between corolla lobes in the NtAGP-antisense lines, sugar (Suc, Glc, and Fru) feeding was carried out with flower buds of antisense line 3 at stage 9 (Fig. 8; Supplemental Table S2). Fully elongated, unopened flower buds were collected and incubated in distilled water supplemented with a 0 or 200 mM Suc, Glc, or Fru solution for 72 h. Flower buds incubated in Suc solution opened 24 h after incubation, and corolla lobes underwent full expansion growth. The morphology of the petal limbs was ultimately restored to that of the control plants with indistinctive corolla lobes and a relative ratio between g and f (g/f) of 57.49% ± 2.03%. The petal limbs of the flower buds incubated in the Glc or Fru solution proceeded to partial expansion growth, giving rise to g/f values of 47.61% ± 2.43% and 46.78% ± 2.39%. Corolla lobes of flowers incubated with 0% carbohydrate showed only limited expansion growth, and the limbs developed distinctive corolla lobes with a g/f value of 37.18% ± 3.12%. In addition, the f length as well as the g length in the flowers fed sugars were also longer than those in the nontreated or distilled water-treated flowers. This result clearly indicates that an arrest in the expansion growth of cells in the petal limbs of the antisense lines is due to an insufficient amount of carbohydrate.

Petal Limb Expansion Growth Is Dependent on the Carbohydrate Synthesized in the Sepal

To confirm the limited petal limb expansion in NtAGP-antisense lines is due to a decrease in starch synthesis, unopened flower buds were collected and incubated in distilled water supplemented with a 0 or 200 mM Suc, Glc, or Fru solution for 72 h. Flower buds incubated in Suc solution opened 24 h after incubation, and corolla lobes underwent full expansion growth. The morphology of the petal limbs was ultimately restored to that of the control plants with indistinctive corolla lobes and a relative ratio between g and f (g/f) of 57.49% ± 2.03%. The petal limbs of the flower buds incubated in the Glc or Fru solution proceeded to partial expansion growth, giving rise to g/f values of 47.61% ± 2.43% and 46.78% ± 2.39%. Corolla lobes of flowers incubated with 0% carbohydrate showed only limited expansion growth, and the limbs developed distinctive corolla lobes with a g/f value of 37.18% ± 3.12%. In addition, the f length as well as the g length in the flowers fed sugars were also longer than those in the nontreated or distilled water-treated flowers. This result clearly indicates that an arrest in the expansion growth of cells in the petal limbs of the antisense lines is due to an insufficient amount of carbohydrate.
levels in the sepal and not to the effect of less starch in other source tissues, we tested the role of the sepal on petal growth by removing the sepals from ‘Xanthi’ flower buds and observing petal growth. Sepals were carefully excised from flower buds at two different developmental stages—2 and 4 cm in length—and the flower buds allowed to continue to grow on the plants for 72 h (Fig. 9; Supplemental Fig. S3). In the 2-cm flower bud without sepal, the petal tube continued to grow and petal limbs opened successfully. However, the intercalary growth between corolla lobes was severely arrested, resulting in petal limbs with the same morphology as that observed in NtAGP-antisense flowers. The other floral organs were morphologically normal. In the 2-cm flower buds with sepal, corolla limbs fully expanded and gave rise to morphologically normal petal limbs. This result clearly shows that expansion growth of the petal limbs, especially between the corolla lobes, is dependent on the availability of carbohydrate in the sepal. In contrast, the corolla limbs from 4-cm-long flower buds (corresponding to stage 9 in Fig. 3B), with or without sepal, were morphologically identical. These results suggest that the import of carbohydrate from the sepal for petal expansion is most likely complete prior to stage 9 and that the absolute amount of sepal carbohydrate from flower buds of stages earlier than stage 9 is crucial for petal development. In addition, they show that the amount of carbohydrate in the sepals of the early-stage flower buds (no later than stage 9) directly affects the expansion growth of petal limbs.

DISCUSSION

NtAGP Is a Novel AGPase Gene Expressed in the Sepal

AGPase plays a key role in regulating the biosynthesis of starch in higher plants. Starch that is synthesized in source tissues is transported to various sink tissues in the form of Suc. Suc is then either converted to starch for accumulation in storage sink tissues or hydrolyzed to serve as a carbohydrate source for plant growth and development in the growing sinks, which consist of highly metabolically active and rapidly growing tissues. AGPase has been extensively studied as a rate-limiting regulator in starch biosynthesis in such starch-accumulating organs as seeds, tubers, and storage roots; however, relatively little attention has been paid to the role of AGPase genes in the growth and development of plant organs. In this study, NtAGP was cloned from a flower bud cDNA library to characterize the function of NtAGP in the growth of floral organs in tobacco. The expression pattern analysis indicated that it is highly expressed in flower buds, with very high levels of expression in the sepals (Fig. 2C). Most of the AGPase genes cloned to date have been preferentially expressed in storage tissues, such as the endosperms of seeds (Olive et al., 1989; Bae et al., 1990; Bhave et al., 1990; Villand et al., 1992a), potato tubers (Müller-Röber et al., 1990), and storage roots of sweet potatoes (Bae and Liu, 1997). NtAGP is the first AGPase gene to be shown to be highly expressed in floral organs, and it is possible that the sepal is one of the active source tissues among the floral organs. We consequently consider NtAGP to be a good candidate to elucidate the function(s) of AGPase in floral organ growth.

Specific Silencing of NtAGP Leads to Phenotypic Changes in Petal Limbs

We employed a transgenic approach to ascertain the role of NtAGP in floral organ growth. An NtAGP-specific
sequence was cloned in reverse orientation under the control of the 35S promoter to suppress endogenous NtAGP transcript levels. Flowers of the NtAGP-antisense plants exhibited a morphological phenotype in the petal limb characterized by an early termination of expansion growth in the region between the corolla lobes (Fig. 5; Supplemental Table S1). The arrest in expansion growth was focused in the area between the corolla lobes (g in Fig. 5B), resulting in a shorter limb length, while the limb length was essentially unchanged in the middle of the corolla lobes (f in Fig. 5B) of the NtAGP-antisense lines. This limb region-specific phenotype appears to be due to the location of a major vein in the f region and the absence of one in the g region (Supplemental Fig. S4). Therefore, the f region would be the first site to unload carbohydrates transported via a major vein, whereas the g region, which is located at the most distant area from the major vein, would be the last place for carbohydrates to be unloaded. The ultimate result is that the limb region-specific phenotype becomes prominent, especially when the amount of carbohydrate drawn into the petal limb is inadequate for normal growth.

The severity of the reduction in expansion growth was proportionally correlated with the decreased levels of NtAGP transcripts in the sepal (Figs. 4 and 5; Supplemental Table S1), suggesting that the altered morphology of the petal limbs resulted from a decrease in NtAGP mRNA levels in the sepal. No other phenotypic changes were observed in other floral organs, even though the NtAGP mRNA levels were also significantly lowered in the anther and ovary (Fig. 4A). There are two possible explanations for this observation. (1) AGPase isoforms could substitute for NtAGP in the anther and ovary, and, consequently, starch biosynthesis would be unaffected in the anther and ovary tissues of antisense lines. However, we found that the role of NtAGP is critical and that no isoforms replace NtAGP in the sepal. (2) The sink strength of the anther and ovary might be much greater than that of the petal. In this case, if there were any shortage in carbohydrate quantity in the sepal, the shortage could be counterbalanced by carbohydrate supplied from other source tissues, such as the leaves. This would mean that the growth and development of the anther and ovary are not sensitive to the reduced levels of carbohydrate in the sepal of the NtAGP-antisense lines. In comparison, the sink strength of the petal might be relatively weak so that the carbohydrate content in the petal could be influenced primarily by starch biosynthesis in the sepal, the nearest source tissue. Thus, the expansion growth of the corolla limbs would be, at least partially, dependent on the amount of carbohydrate supplied from the sepal. In support of this latter explanation, Clément et al. (1996) reported that, during the anther growth phase, the anther retains the highest sink strength among the floral organs, attracting assimilates mainly through the filament. We have also shown that expansion growth of the petal limb was directly affected by the amount of sepal carbohydate but that the growth of other floral organs, including the anther and ovary, was unaffected (Fig. 9; Supplemental Fig. S3), suggesting

![Figure 8. Phenotype rescue by sugar feeding. Flower buds of NtAGP-antisense line 3 at stage 9 were collected and incubated in distilled water supplemented with 0 or 200 mM Suc, Glc (Glu in B), or Fru. Three different flower buds were used for each sugar and each sugar concentration. The rescue test was repeated three times. A, Morphology of the petal limbs fed the three sugars; pictures were obtained 72 h after incubation initiation. B, Comparison of the length of the petal limbs fed various sugars. Limb length was measured at 72 h after incubation initiation. The petal lengths described in Figure 5 are represented as f and g. NT, Nontreated. [See online article for color version of this figure.]](image_url)

### Table III. Sugar content in the petal limbs of NtAGP-antisense lines

| Sugar | Stage 10 | Stage 13 | Stage 10 | Stage 13 | Stage 10 | Stage 13 |
|-------|----------|----------|----------|----------|----------|----------|
| Glc   | 2.87 ± 0.03 | 9.53 ± 0.39 | 3.09 ± 0.12 | 12.61 ± 0.33 | 3.13 ± 0.60 | 15.97 ± 0.60 |
| Fru   | 2.45 ± 0.21 | 14.22 ± 0.36 | 2.66 ± 0.12 | 18.15 ± 0.18 | 3.21 ± 0.06 | 21.11 ± 0.36 |
| Suc   | 2.69 ± 0.01 | 3.03 ± 0.13 | 3.23 ± 0.29 | 3.43 ± 0.13 | 3.49 ± 0.07 | 4.51 ± 0.06 |

*Concentration (mg g fresh weight⁻¹) measured on pooled petal limbs from at least five individual plants. Data are means ± SD of three independent measurements.*
that the anther and ovary have a greater sink strength than the petal.

Observations of the petal limb area by light microscopy revealed that phenotypic differences in that the antisense expression of NtAGP affected cell expansion but not cell division in the region between the corolla lobes (Fig. 6; Table II). In petunia (Petunia hybridoides), cell division in the developing petals was terminated in advance of flower opening (Reale et al., 2002), and the unfolded flower was only 40% of its final length when the cells stopped dividing (Martin and Gerats, 1993). The phenotypic alteration in the NtAGP-antisense plants commences after the flowers have opened. This also excludes the possibility that the shorter limb length in the area between the corolla lobes of NtAGP-antisense plants accounts for the differences in cell proliferation between control and antisense plants.

Down-Regulation of NtAGP Reduced Carbohydrate Content in the Petal Limb and Arrested the Expansion Growth

The sepal elimination test with ‘Xanthi’ flower buds verified that expansion growth of the petal limb between the corolla lobes was entirely dependent on the availability of carbohydrate in the sepal. In NtAGP-antisense lines, AGPase activity and starch content were reduced in the sepal, and, consequently, carbohydrate levels were diminished in the petal limb (Fig. 7; Table III). The reduction in AGPase activity and starch content in the sepal and the carbohydrate content in the petal limb were proportional to the NtAGP transcript levels in the sepal, suggesting that the down-regulation of NtAGP led to the reduction in the amount of carbohydrate in the petal limb through a decrease in starch synthesis in the sepal. The petal limbs of the antisense plants exhibited morphological alterations due to the limited expansion growth of the petal limbs between corolla lobes. These morphological changes were completely rescued by feeding Suc and partially rescued by feeding Glc or Fru to flower buds of the NtAGP-antisense plants (Fig. 8; Supplemental Table S2), which shows that the arrest in the expansion growth of petal limbs in the area between corolla lobes of NtAGP-antisense plants is attributable to an insufficient supply of carbohydrate. Suc feeding appeared to be more effective than either Glc or Fru feeding in restoring expansion growth. Although this may simply be due to a 2-fold higher concentration of hexose in Suc-fed flowers than that in Glc- or Fru-fed flowers, this result suggests two other possibilities. The first is that the partial restoration of expansion growth by Glc or Fru feeding might be due to a less efficient uptake of Glc and Fru than Suc under our experimental conditions. This possibility is supported by earlier observations that Suc feeding is more effective in increasing Glc concentration than an equimolar Glc feeding in sugar beet (Beta vulgaris) and Arabidopsis leaves (Lee and Daie, 1997; Sokolov et al., 1998). The second possibility is that an increase in both Glc and Fru concentrations simultaneously by feeding Suc is more effective than an increase in Glc or Fru content alone for the expansion growth of petal limbs. The observation that both Glc and Fru concentrations were dramatically elevated when expansion growth of the petal limbs was in progress in the control tobacco plant suggests that both of these possibilities are plausible.

In the rose plant, rapid expansion of the petal was accompanied by decrease in starch and increases in soluble sugars in the petals (Evans and Reid, 1988). The osmotic potential of the petal increased from −790 to −690 kPa (equivalent to a change from 0.340–0.304 osmol) during expansion. It was concluded that starch hydrolysis during petal growth was important for the maintenance of the expanded cell size but that it was not the factor triggering cell expansion. Alternatively, Biesleski (1993) reported that rapid fructan hydrolysis commenced just prior to flower bud opening and that Fru plus Glc accounted for more than 80% of the total soluble carbohydrate. The osmolarity of petal cell sap increased significantly during fructan hydrolysis (from 0.300–0.340 osmol), suggesting that the onset of fructan hydrolysis, with a concomitant large increase in osmoticum, is an important event driving flower.
expansion in the ‘Cradle Song’ daylily. Vergauwen et al. (2000) also observed a rapid breakdown of fructose in the sepal during flower opening in C. rapunculoides. These researchers also proposed that the resulting increase in Glc and Fru concentrations in the petal is an osmotic driving force in petal expansion. Although the cause of petal expansion—extensibility and/or osmolarity—has not been agreed upon, rapid hydrolysis of storage carbohydrate with a simultaneous increase in soluble sugars has always been found to be accompanied by petal expansion. Zenoni et al. (2004) reported that the down-regulation of a petunia expansin gene (PhEXP1) reduced the amount of crystalline cellulose in cell walls and led to a decrease in the size of the petal limbs. The reduced size of the petal limbs was found to correlate with a decrease in the expansion growth of the epidermal cells. Soluble sugars in petal limbs could also be used by epidermal cells to synthesize and assemble the crystalline cellulose in the cell walls, thereby enabling the cells to expand and reach their final size. Thus, a sufficient amount of soluble sugars might be required for epidermal cells to develop ample amounts of crystalline cellulose in expanding petal limbs. These findings are in agreement with the proposal that NtAGP plays an essential role in the normal expansion growth of petal limbs by synthesizing an adequate amount of starch, which would then generate the main driving or maintaining force in ‘Xanthi’ petal limb expansion, in the sepal.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Tobacco (Nicotiana tabacum ‘Xanthi’) and transgenic plants were grown in large [27 x 27 x 24 (height)] cm pots supplemented with fully fertilized soil in a greenhouse under a 16/8-h (light, 25°C/dark, 20°C) regimen. Various tissue samples for RNA extraction and for the analyses of AGPase activity, starch, and sugar content were collected at 7 h after the initiation of the light period.

Cloning of NtAGP

An NtAGP cDNA was isolated by screening a ZA2P11 flower bud cDNA library of ‘Xanthi’ tobacco. A sweetpotato (Ipomoea batatas) small subunit AGPase cDNA (tGTU; Bae and Liu, 1997) was used as a probe. The probe was [32P]labeled using the Random Primed DNA Labeling kit (Boehringer Mannheim). Approximately 3.8 x 106 phages were screened. After phage lifting, the membranes (Hybond-N; Amersham) were hybridized overnight at 55°C in 6x SSC, 0.5% (w/v) SDS and 100 µg/ml denatured salmon sperm DNA and then washed first for 15 min at room temperature in 2x SSC, 0.5% (w/v) SDS and then for 2 h at 55°C in 1x SSC, 0.1% (w/v) SDS. The positive phage clones were converted to phagemids following the manufacturer’s instructions (Stratagene).

RNA Gel-Blot Analysis

Total RNA was prepared using Tri-Reagent (Invitrogen), following the manufacturer’s instructions. Approximately 30 µg of total RNA was electrophoresed on 1% agarose gel containing 15% (v/v) formaldehyde and then blotted onto nylon membranes (Zeta-Probe GT genomic-test blotting membranes; Bio-Rad) in 20x SSC. As a probe, the N-terminal 205 bp cDNA fragment (NtAGP-specific probe) was PCR amplified with gene-specific primers (forward, 5'-AGGAATACGTCAGTGAG-3'; reverse, 5'-TGAATTCGCGCTCCTAGG-3') and then labeled with [32P]dCTP using the Random Primed DNA Labeling kit (Boehringer Mannheim). Prehybridization and hybridization were carried out in 0.25 x sodium phosphate, pH 7.2, and 7% (w/v) SDS solution at 55°C overnight. The membranes were washed twice in 20 x sodium phosphate, pH 7.2, and 5% (w/v) SDS at room temperature, then washed a third time in 20 x sodium phosphate, pH 7.2, and 1% (w/v) SDS at 55°C for 15 min.

Genomic Southern-Blot Analysis

Genomic DNA was isolated from tobacco leaf tissues following the method of Shure et al. (1983). A 50 µg aliquot of genomic DNA was digested with HindIII, XhoI, and BamHI + XhoI and separated on 0.8% agarose gels at 25 V overnight. The gels were then soaked for 30 min in a denaturation solution (1.5 M NaCl and 0.5 M NaOH), followed by neutralization for 30 min in a neutralization solution (1.5 M NaCl and 0.5 M Tris-HCl, pH 8.0). The DNA was then blotted onto nylon membranes (Zeta-Probe GT genomic-test blotting membranes) in 10x SSC and the membranes hybridized under the same conditions as those used for the RNA gel-blot analysis. The 1.8-kb full-length NtAGP cDNA or 205-bp NtAGP-specific DNA fragment was used as a probe.

Generation and Identification of NtAGP-Antisense Tobacco Plants

The 5'-205-bp NtAGP-specific fragments were amplified by PCR with NtAGP-specific primers (forward, 5'-AGGGAGCTCAGAAGATAGCTAGTGAGG-3'; reverse, 5'-GATTATATCTGGAATGCCTGCTTGAGG-3'). Sacl and EcoRV restriction sites were introduced at the ends of the forward and reverse primers, respectively, in order to facilitate subcloning. The PCR products were then digested with Sacl and EcoRV and fused to the 35S promoter in an antisense orientation by insertion of the 205-bp NtAGP-specific fragments at the Smal and Sacl sites of pMBP1. The resulting binary vector of pMBP1-NtAGP was transformed into Agrobacterium tumefaciens GV3101 by the direct DNA uptake method (An, 1987). A. tumefaciens GV3101 carrying pMBP1-NtAGP was cocultivated with leaf discs of the tobacco plants (‘Xanthi’). After shoots had regenerated from the cut edges of the leaf discs on the selectable Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1 mg/L 6-benzyladenine, 0.1 mg/L α-naphthaleneacetic acid, 300 mg/L carbenicillin, and 100 mg/L kanamycin, the young shoots were cut, transferred onto the selectable MS medium, and cultured in a growth chamber with light. Roots were induced on selectable MS medium containing 300 mg/L carbenicillin and 100 mg/L kanamycin without additional hormones, and the plantlets were then grown in a greenhouse. Transgenic tobacco plants were identified by PCR amplification of nptII DNA, which was the selectable marker gene used in the pMBP1 construct. Genomic DNA was isolated from the leaves of kanamycin-resistant tobacco plants as described by Edwards et al. (1991). PCR was performed with 0.1 µg of DNA and nptII primers (forward, 5'-AGGGATCTCCGAGATTATA-3'; reverse, 5'-AATCCGACGGCGCATACCTGA-3') under the following conditions: 30 sequential cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min, followed by 5 min of final extension at 72°C. The PCR products were run on 1% agarose gels.

Microscopic Observation

Petal limbs at stage 13 were collected and fixed overnight at 4°C in a solution containing 2% (w/v) paraformaldehyde and 2.5% (w/v) glutaraldehyde in 25 mM phosphate buffer, pH 7.0. The samples were dehydrated in a graded ethanol series and embedded in acrylic resin (LR White Resin; London Resin Company). The resin-embedded samples were sliced into 2-µm sections with an ultra-microtome (Bromma 2088; LKB) and stained with Safranin O. The tissue sections were observed under light microscopy (BX51 TRF; Olympus).

AGPase Activity Assay

The sepal of flower buds (2 cm in length) were ground to a fine powder in liquid nitrogen and homogenized with a mortar and pestle in an extraction buffer containing 50 mM phosphate buffer, pH 7.2, 10 mM EDTA, 0.1% Triton X-100, and 0.1% sarcosyl. AGPase activity was assayed in the direction of ADP-Glc synthesis (Kleczkowski et al., 1993). The reaction mixture contained 200 µM HEPES, pH 7.0, 10 mM MgCl2, 0.05% bovine serum albumin, 1 mM ATP, 0.5 mM [U-14C]Glc-1-P (specific radioactivity: 900 cpm mmol-1), and 5 units of inorganic pyrophosphatase in a final volume of 0.1 mL. The reaction...
was initiated by adding the sample and stopped after 10 min at 37°C by boiling for 5 min. Alkaline phosphatase (0.5 units) was added to each reaction and incubated at 37°C for 1 h. The reaction sample was blotted onto DEAE-81 ion-exchange chromatography paper and washed three times with distilled water. The filter was dried and the product formation counted in a liquid scintillation spectrometer.

**Determination of Starch Content**

Approximately 100 mg of sepal tissue from 2-cm-long flower buds was macerated in liquid nitrogen, transferred into a test tube, and incubated at 80°C for 5 min with 80% ethanol, following which a second aliquot of 5 mL of the 80% ethanol solution was added. After centrifugation for 10 min at 1,000g, the supernatant was discarded and the pellet resuspended in 10 mL of the 80% ethanol solution, followed by centrifugation for 10 min at 1,000g. The supernatant was then carefully poured off and discarded. Dimethyl sulfoxide (2 mL) was added to each sample and the samples incubated for 5 min in a boiling water bath. Starch concentrations were determined using a starch assay kit (Sigma) according to the manufacturer’s instructions.

**Determination of Sugar Content**

Petals were macerated in liquid nitrogen. One gram of tissue powder was then transferred into conical tubes, 5 mL of distilled water was added, and the samples were incubated for 10 min at room temperature. The extracts were transferred into microcentrifuge tubes and centrifuged at 12,000 rpm for 10 min. Suc and Glc concentrations were determined in the supernatant using a Suc assay kit (Sigma) according to the manufacturer’s instructions. Fru concentration was determined using a Fru assay kit (Sigma).

**RT-PCR**

Sepal, anther, and ovary tissues from flowers at stage 9 and fully expanded leaf tissues from flowering tobacco plants were collected for assessment of their NtAGP mRNA levels. Total RNA was extracted from each sample using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer’s instructions. Six micrograms of total RNA was reverse transcribed using 0.5 μM oligo(dT) and 200 units of SuperScriptIII (Invitrogen) in a 20-μL reaction volume. The resulting cDNA solution was then diluted with 30 μL of TE (10 mM Tris-HCL, pH 8.0, 1 mM EDTA). NtAGP-specific primers (forward, 5′-AGAATACCCTGAGTGGAC-3′; reverse, 5′-TGGAATGCGCTGCTGAG-3′) were used to amplify the 5′-end 205 bp of NtAGP. Tobacco β-tubulin DNA was amplified as an internal equal loading control using β-tubulin primers (forward, 5′-GGCAAGTTACCGAGGCTGA-3′; reverse, 5′-GCATGTAGTCTTCCAGGAT-3′). One microliter of the cDNA solution and 10 pmol each primer were used for semiquantitative RT-PCR in a total volume of 20 μL. The PCR conditions consisted of 22 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a 5-min final extension at 72°C with sepal, anther, and ovary cDNA and 26 cycles with leaf cDNA. The amplified products were electrophoresed on a 1.2% agarose gel.

**Sugar Feeding**

Fully elongated, unopened flower buds (stage 9) were collected by cutting at 5 mm below the top of the peduncle and incubated in distilled water supplemented with 0 or 200 mM Suc, Glc, or Fru for 72 h at 25°C under a 16/8-h (light/dark) regimen.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Alignment of deduced NtAGP and other plant AGPase proteins.

**Supplemental Figure S2.** Starch content and growth of the leaves of NtAGP-antisense plants.

**Supplemental Figure S3.** The role of the sepal in petal limb growth.

**Supplemental Figure S4.** Carbohydrate transport from sepal to petal.

**Supplemental Table S1.** Phenotype of NtAGP-antisense plants in petal limbs.

**Supplemental Table S2.** Phenotype rescue by sugar feeding.

**ACKNOWLEDGMENT**

We are grateful to Dr. Ki-Joong Kim for his valuable suggestions in the characterization of the phenotype in this study.

Received May 9, 2007; accepted July 16, 2007; published July 27, 2007.

**LITERATURE CITED**

An G (1987) Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. Plant Physiol 81: 86–91

Anderson JM, Hnilo J, Larson R, Okita TW, Morell M, Preiss J (1989) The encoded primary sequence of a seed ADP-glucose pyrophosphorylase subunit and its homology to the bacterial enzyme. J Biol Chem 264: 12238–12242

Bae JM, Giroux M, Hannah LC (1990) Cloning and characterization of the brittle-2 gene of maize. Maydica 35: 317–322

Bae JM, Harn CH, Tae KH, Suh MC, Shin JS, Liu JR (2001) Partial male sterility induced in tobacco by overproduction of mRNA of sweet potato small subunit ADP-glucose pyrophosphorylase. J Plant Physiol 158: 1273–1279

Bae JM, Liu JR (1997) Molecular cloning and characterization of two novel isoforms of the small subunit of ADP-glucose pyrophosphorylase from sweet potato. Mol Gen Genet 254: 179–185

Bhave MR, Lawrence S, Barton C, Hannah LC (1990) Identification and molecular characterization of shrunken-2 cDNA clones of maize. Plant Cell 2: 581–588

Biesleski RL (1993) Fructan hydrolysis drives petal expansion in the ephemeral daylily flower. Plant Physiol 103: 213–219

Chen BY, Janes HW, Gianfagna T (1997) Molecular cloning and characterization of the NtAGP-subunit and its homology to the bacterial enzyme. J Biol Chem 272: 3793–3800

Choi SB, Kim KH, Kavakli IH, Lee SK, Okita TW (2001) Transcriptional expression characteristics and subcellular localization of ADP-glucose pyrophosphorylase in the oil plant Perilla frutescens. Plant Cell Physiol 42: 146–153

Clément C, Burrous M, Audran JC (1996) Floral organ growth and carbohydrate content during pollen development in Lilium. Am J Bot 83: 459–469

Cross JM, Clancy M, Shaw JR, Greene TW, Schmidt RR, Okita TW, Hannah LC (2004) Both subunits of ADP-glucose pyrophosphorylase are regulatory. Plant Physiol 135: 137–144

Drews GN, Beals TP, Bul AQ, Goldberg RB (1992) Regional and cell-specific gene expression patterns during petal development. Plant Cell 4: 1383–1404

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19: 1349

Evans RY, Reid MS (1988) Changes in carbohydrates and osmotic potential during rhythmic expansion of rose petals. J Am Soc Hortic Sci 113: 884–888

Giroux MJ, Hannah LC (1994) ADP-glucose pyrophosphorylase in shrunken-2 and brittle-2 mutants of maize. Mol Gen Genet 243: 400–408

Giroux MJ, Shaw J, Barry G, Cobb BG, Greene T, Okita TW, Hannah LC (1996) A single mutation that increases maize seed weight. Proc Natl Acad Sci USA 93: 5824–5829

Goetz M, Godt DE, Guivarc'h A, Kahmann U, Chriqui D, Roitsch T (2001) Induction of male sterility in plants by metabolic engineering of the shrunken-2 gene of maize. Maydica 459–469

Hendriks JHM, Kolbe A, Gibon Y, Stitt M, Geigenberger P (2003) ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. Plant Physiol 133: 839–849

Hwang SK, Salamone PR, Okita TW (2005) Allosteric regulation of the higher plant ADP-glucose pyrophosphorylase is a product of synergy between the two subunits. FEBS Lett 579: 983–990
