The homozygous T-DNA mutant of the AMY1 gene in Arabidopsis was identified and importantly, shown to cause an early flowering phenotype. We found that the disruption of AMY1 enhanced expression of CO and FT. The expression analyses of genes related to starch metabolism revealed that expression of the AGPase small subunit APS1 in the wild type was higher than in the amy1 mutant. However, there were no significant differences in expression levels of the AGPase large subunit genes ApL1, AMY2, or AMY3 between wild type and the amy1 mutant. Expression profiling showed that AMY1 was highly expressed in leaves, stems, and flowers, and expressed less in leafstalks and roots. Furthermore, the level of AMY1 mRNA was highly elevated with age and in senescing leaves. RT-PCR analyses showed that the expression of AMY1 was induced by heat shock, GA, and ABA, while salt stress had no apparent effect on its expression. [BMB reports 2009; 42(2): 101-105]

RESULTS

Analyses of amy1 mutants

The CS25129 T-DNA insertion within the AMY1 gene in Arabidopsis was identified from the Salk collection. Homozygous individuals were identified by PCR and RT-PCR analyses. Sequencing of the T-DNA flanking region in amy1 showed that the insertion was localized 679 nucleotides downstream from
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Fig. 1. Identification, by PCR, of the amy1 with T-DNA insertion. A. Genomic organization of the AMY1 gene and localization of the T-DNA insertions in amy1 mutants. The numbering begins at the ATG translation start codon. Arrows denote the direction and position of the PCR primers. LBpROK2 = the T-DNA left border primer. B. PCR products amplified from genomic DNA with various primer combinations around the AMY1 region are shown. Lanes 1-9 correspond to PCR products generated using the primers 3' and LBpROK2. Lanes 10-18 correspond to PCR products generated using the primers 5' and 3'; Lane M, DNA Maker. C. RT-PCR analyses shows absence of amy1 mutant transcript in genotypes. Actin-2 was used as an internal control.

Fig. 2. The phenotype analyses of wild type and amy1 mutant of Arabidopsis thaliana under long-day conditions at different growth stages: A. 22-day-old plants; B. 30-day-old plants; C. 36-day-old plants.

The expression of AMY1 in different organs of Arabidopsis
Total RNA were extracted from different organs of 4-week-old wild type plants. RT-PCR analyses showed that the higher levels of AMY1-mRNA were observed in leaves, stems, and flowers, and expressed at slightly lower levels in leafstalks and roots (Fig. 3A).

AMY1 gene expression in Arabidopsis plants with varying ages
Total RNA were extracted from the 3-, 5- and 8-week-old plant leaves. RT-PCR analyses (Fig. 3B) revealed that the AMY1 mRNA level was highly elevated with age and in senescing leaves, suggesting that AMY1 expression is differentially regulated at the level of transcription as plants age.

Expression analyses of related genes to starch metabolism
The expression patterns of the AGPase small subunit gene, Aps1 and AGPase large subunit genes (ApL1), AMY2, and AMY3 in the amy1 mutant and wild type were studied. The results indicated that the expression level of the small subunit of AGPase (APS1) in wild type was higher than that in the amy1
mutant. There was no significant difference in the expression level of the APL1, AMY2, or AMY3 genes between wild type and amy1 mutants (Fig. 3C).

Expression analyses of flowering-related genes
Because the amy1 mutant flowered earlier than the wild type (Fig. 2), we decided to investigate the expression levels of flowering-related genes in amy1 mutants compared to the wild type. Specifically, we measured the expression of the floral-promotion long-day (LD) pathway genes including CO and FT by RT-PCR. The results indicated that disruption of AMY1 enhanced the expression level of both of these genes (Fig. 3D).

Expression of AMY1 in response to various stresses
The expression patterns for the AMY1 gene were considerably different when plants were subjected to different environmental stresses (Fig. 4). The 100 μM GA, 0.6 μM ABA, and heat shock at 37°C induced an increase in mRNA levels of the AMY1 gene, but the characteristics of expression of AMY1 gene demonstrated that the response of this gene to heat was much slower than to GA and ABA. The increase in AMY1 expression could be observed after 1 h with GA treatment and after only 1/2 h with ABA treatment, but not until after 2 h when treated with heat at 37°C (Fig. 4A, C, and D). The 200 mmol/L NaCl treatment had no effect on the expression of the AMY1 gene in these experiments (Fig. 4B). These results showed that the AMY1 gene was likely to be an important point of regulation in heat shock, GA and ABA signaling pathways.

DISCUSSION
The AMY1 T-DNA mutant showed an early flowering phenotype when grown in 16-h-light/8-h-dark cycle, so we investigated the expression changes of flowering-related genes. RT-PCR results indicated that the disruption of AMY1 enhanced the expression level of CO and FT. A simple explanation could be that AMY1 is an upstream gene of FT and CO in the flowering signal pathway. When AMY1 is disrupted, CO is expressed at high levels, and expression of the FT gene is induced. FT activates downstream flowering genes, committing the plant to the flowering state.

Transcriptional and allosteric regulation of AGPase plays a major role in the regulation of starch synthesis. AGPase catalyzes the conversion of Glc-1-P and ATP to ADP-Glc and inorganic pyrophosphate (PPi), which is the first committed step in the pathway of starch synthesis (11-13). The regulatory subunit APL1 and the smaller catalytic subunit APS1 play a key role in the regulation of starch synthesis (14), while the major functions of other subunits are: i) increasing the compatibility between the smaller subunit and the activating factor, and ii) reducing the affinity between the smaller subunit and inhibiting factors (15, 16). Previous studies have shown that disruption of any or all of the three predicted α-amylase genes in Arabidopsis had no effect on transitory starch metabolism (17). Delatte et al. also identified that there was only a minor role for a chloroplastic α-amylase in Arabidopsis (18). In the present study, we found that the expression level of the small subunit of AGPase (APS1) in wild type was higher than that in the amy1 mutant. Therefore, we presumed that the disruption of the AMY1 gene may have an effect on regulatory factors of the starch metabolism pathway, and specifically, that the APS1 and AMY1 genes may regulate this pathway.

RT-PCR analyses revealed that the AMY1 mRNA level increased from 3- to 8-weeks-old plants, suggesting that AMY1 expression is related to senescence and is regulated at the level of transcription. Leaf senescence is an organized developmental program influenced by plant age as well as environmental conditions such as nutrient supply or temperature (19-22). Phytohormones such as ABA or ethylene can also induce senescence (19). Certain stresses and hormones such as ABA are also able to induce genes that are directly responsive to those treatments (23). Moreover, some senescence associated genes (SAGs) are regulated by ABA (19, 24). Therefore, we investigated the effects of heat shock, salt, GA, and ABA on the level of AMY1 mRNA. RT-PCR analyses showed that the GA, ABA, and heat (37°C) treatments induced an increase in mRNA levels of the AMY1 gene, while the NaCl had no detectable effect on the expression of this gene. These results suggested that the AMY1 gene was likely to be a point of regulation in heat shock, GA, and ABA signaling pathways of Arabidopsis.

MATERIALS AND METHODS
Plant materials and growth conditions
Arabidopsis thaliana T-DNA insertion lines (CS25129) of the AMY1 gene (At4g25000) were obtained from the Salk Institute Genomic Analyses Laboratory. For in vitro culture, seeds were surface-sterilized by treatment in 70% ethanol for 10 min, followed by 7% NaClO for 7 min, and finally, five washes with sterile distilled water. The seeds were stored in the dark at 4°C for 3 d. The wild type Arabidopsis thaliana ecotype Columbia (Col-0) plants and CS25129 T-DNA insertion lines were grown
at low density in 16-h-light/8-h-dark cycles at a temperature of 22°C under 100 to 150 μmol m⁻² s⁻¹ of light.

**AMY1 insertion mutant**

To identify individuals homozygous for the T-DNA insertion, genomic DNA was obtained from kanamycin-resistant seedlings and subjected to PCR-based genotyping using the following primers: 5′-CTGCCGCAATGACCTAA; 3′-TGGTTCACGTAGTGGGCAATC-3′. For the T-DNA left border primer of the pROK2 vector, we used LBPpROK2 (5′-TGGTTCACGTAGTGGGCAATC-3′). The primers were designed according to the expected genome fragments from Signal (http://signal.salk.edu/isectprimers.html). Mutant identification was made by using the two-paired reactions method. Flowering time-analyses were performed with *Arabidopsis* insertion mutants and wild type plants grown under 16-h-light conditions at 22°C. Flowering time was measured by scoring the number of leaves on the main stems or the number of days from sowing until flower buds were visible by eye at the center of the rosette. The flowering phenotype was observed three times for each plant type (mutant and wild type).

**Abiotic stress**

Plants were grown on Murashige and Skoog plates supplemented with 1.5% Suc. After two weeks, *Arabidopsis* seedlings were treated by heat shock at 37°C, 200 mmol NaCl, 100 μmol m⁻² s⁻¹ of light. Flowering time-analyses were performed with *Arabidopsis* insertion mutants and wild type plants grown under 16-h-light conditions at 22°C. Flowering time was measured by scoring the number of leaves on the main stems or the number of days from sowing until flower buds were visible by eye at the center of the rosette. The flowering phenotype was observed three times for each plant type (mutant and wild type).

**Semi-quantitative RT-PCR analyses**

Total RNA was isolated using Puprep RNAeasy mini kit (Ambiogen Life Tech Ltd). DNA-free RNA was obtained following RQ1 DNase I treatment according to the manufacturer’s instructions (Promega, Madison, WI, USA). The levels of mRNA were analyzed using semi-quantitative reverse transcription (RT)-PCR as described (24). The cDNA was generally diluted 10-fold, and 1 μL of diluted cDNA was used in a 20 μL PCR reaction. PCR primer sequences used in this study are as follows: Actin-2F (5′-CATCTGTGCAATCAGGAGGT-3′), Actin-2R (5′-CACAACCAGGCTTGAAACAG-3′); AMY1F (5′-AAAGAGTGTTCGGAATG-3′), AMY1R (5′-ATCCACTGTTTGAGACC-3′); ApL1F (5′-TCCACTGCGAGGTCTCAT-3′), ApL1R (5′-AGGCCATATTGATCTAC-3′); FTR (5′-TACACTGTTTGCCTGCCAAG-3′), FTF (5′-CTGGAACAACCTTTGCAA-3′); AMY2F (5′-AAGCCGTAAAGGGTCAGT-3′), AMY2R (5′-TCTTGGAGACCC-3′); Aps1F (5′-TCCACTCGGAGGTTTCAT-3′), Aps1R (5′-GAAAGAGTTGTCCGAATG-3′), AMY1R (5′-ATCCACTGTTTGAGACC-3′); Actin-2R (5′-CACAAACGAGGGCTGGAACAAG-3′); AMY1F (5′-AAAGAGTGTTCGGAATG-3′), AMY1R (5′-ATCCACTGTTTGAGACC-3′); ApL1F (5′-TCCACTGCGAGGTCTCAT-3′), ApL1R (5′-AGGCCATATTGATCTAC-3′); FTFR (5′-TACACTGTTTGCCTGCCAAG-3′).

PCR was generally performed with a 5 min denaturation at 95°C followed by 24 to 35 cycles consisting of the following steps: 95°C for 30 s, 48 to 60°C for 30 s, and 72°C for 30 s. The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide (10 μg/mL) as described (26). RT-PCR reactions for each experiment were repeated at least three times, and representative gel images are shown. The expression level of the actin-2 gene was used as the internal control to normalize expression levels of genes tested.

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