African Journal of Biotechnology Vol. 11(28), pp. 7151-7157, 5 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3718
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Regulation of the flowering time of *Arabidopsis thaliana* by thylakoid ascorbate peroxidase

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Accepted 19 March, 2012

Flowering time of higher plants is precisely controlled by various exogenous and endogenous factors. Recent researches implied that H_2O_2 is a potential flowering initiation factor. In order to confirm this hypothesis, thylakoid ascorbate peroxidase (*tAPX*) overexpressing *Arabidopsis*, the mutant line containing a T-DNA insertion and the wild type have been analyzed in this study, since APX was an important enzyme scavenging H_2O_2 in plant cells. It was found that during the vegetative growth stage there was no phenotypic difference among the three lines under common conditions, but 3,3'-diaminobenzidinetetrahydrochloride (DAB) staining showed that the endogenous H_2O_2 content varied: the mutant line had the highest content; the wild type took the second place, while the *tAPX*-overexpressing line had the lowest H_2O_2 content. This trend was in accordance with the bolting and flowering time during the following reproductive growth stage: the mutant bolted and flowered first, followed by the wild type, and the overexpressing line bolted and flowered last. This correlation confirmed the previous hypothesis that "H_2O_2 is a possible factor in flowering induction".

Keywords: Ascorbate peroxidase, *Arabidopsis thaliana*, flowering time, hydrogen peroxide.

INTRODUCTION

Researches about ascorbate peroxidase (APX, EC 1.11.1.11) started from 1976 (Foyer and Hailiwell, 1976) and so far, more than 30 years has passed. Previous researches about APX from higher plants almost focused on its redox status and enhancing tolerances to various stresses. So far, it is known that APX catalyzed the reduction of H_2O_2 to H_2O and O_2 using ascorbic acid (ASA) as specific electron donor (Asada, 1999), and it is the most important H_2O_2-eliminating enzyme in chloroplast (Asada, 1992). Its activity was detected not only in higher plants and eukaryotic algae, but also in *Cyanobacteria* and some kinds of insects. Murgia et al. (2004) found that *Arabidopsis* overexpressing *tAPX* was more resistant to photo-oxidative stress induced by Paraquat (Pq) and reduced the symptoms of cell death induced by nitric oxide. Overexpression of thylakoid *APX* from *Solanum lycopersicum* (*StAPX*) in tobacco increased early seedling tolerance to salinity and osmotic stresses (Sun et al., 2010). Our previous studies found that *BnAPX* from *Brassica napus* enhanced salt and drought tolerances in *Arabidopsis* (Chai et al., 2011). Moreover, Xu et al. (2008) attributed the enhanced salt-tolerance of transgenic *Arabidopsis* carrying a peroxisomal *APX* gene (*HvAPX1*) from barley to the reduction of oxidative stress injury. Since APX eliminated H_2O_2, it could also change some other phenotype caused by H_2O_2.

Recent researches showed that H_2O_2 was a possible factor in flower induction. Lokhande et al. (2003) studied four *Arabidopsis* ecotypes from different latitudes and found that flowering started from low-latitude to high-latitude ecotypes. Since the lower-latitude ecotypes suffered higher oxidative stress, the study proposed the hypothesis that "H_2O_2 is one of the possible factors in flower induction". Furthermore, Moharekar et al. (2007)

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Abbreviations: APX, Ascorbate peroxidase; **ASA,** ascorbate; **CTAB,** cetyltrimethylammonium bromide; **DAB,** 3,3'-diaminobenzidinetetrahydrochloride; **H_2O_2,** hydrogen peroxide; **PCR,** polymerase chain reaction; **ROS,** reactive oxygen species

found that in *Arabidopsis*, flowering time was negatively correlated with irradiance, which led to oxidative stress. Hence they proposed that an increase in oxidative stress induces earlier flowering. So it can be inferred that if H_2O_2-eliminating gene APX was overexpressed or suppressed in *Arabidopsis*, the flowering time should be delayed or advanced compared with the wild type, respectively. Although some precious study reported increased ROS during the transition to flowering (Banuelos et al., 2008; Lokhande et al., 2003; Moharekar et al., 2007), studies on *APX*-transgenic plants or mutants affecting the flowering time seemed seldom.

In this study, *tAPX*-overexpressing *Arabidopsis*, the wild type and the mutant line containing a T-DNA insertion were studied and it was found that the bolting and flowering time was negatively related with the endogenous H_2O_2 content. In other words, the higher the endogenous H_2O_2 content, the earlier the *Arabidopsis* bolted and flowered. This result confirms the hypothesis that H_2O_2 initiated flowering (Lokhande et al., 2003).

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana, accession Columbia was used in this study: the homozygous transgenic *Arabidopsis* overexpressing *thylakoid-APX* (*tAPX*) gene was obtained from Professor I. Murgia (2004). The mutant *Arabidopsis* line containing a T-DNA insert in the exon of the *tAPX* gene (SALK_027804.54.20) was obtained from the Arabidopsis Biological Resource Center (ABRC). The wild type was kept in our laboratory. After three days jarovization at 4°C, seeds of *Arabidopsis* were first surface-sterilized in 75% ethanol for 1 min, followed by immersion in 0.1% $HgCl_2$ for 10 min, and rinsed at least three times with sterile distilled water. Then the seeds were germinated in soils-mixture of vermiculite: peat (1:1) in an environmentally climatic chamber at 22°C with 16/8 h of light/dark cycle and 70% humidity.

The screening of the homozygous mutant *Arabidopsis* line

The primer designing and the protocol were according to information online (http://signal.salk.edu/tdnaprimers.2.html). Three primers (LBb1.3, LP and RP) were used for the screening. They were LBb1.3 (5'-ATTTTGCCGATTTCGGAAC-3'), LP (5'- ACAA-GATCAAACCCACGAATG-3') and RP (5'- TACTTCACCAAGAT-GGGATGG-3'). Amplification for wild type should produce a long band from LP to RP; homozygous lines should produce a small band from RP to LBb1.3, and for heterozygous lines they got both bands. Total DNA was extracted from three-week-old *Arabidopsis* with cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990) and used as the template for the polymerase chain reaction (PCR). The PCR cycling procedure consisted of 5 min at 95°C, followed by 36 cycles for 30 s at 95°C, 30 s at 61°C and 60 s at 72°C, a final 8-min extension at 72°C, and termination of the reaction at 4°C.

The semi-quantitative and quantitative PCR analysis of the level of transcriptional level of *tAPX*

Total RNA was prepared from leaves of three-week-old over expressing line, the wild type and the mutant seedlings using Trizol reagent according to the instructions. RNA samples were then treated with RNase-free DNase I. The quality and concentration of RNA were accurately determined by a UV–visible light spectrophotometer. Reverse transcription was performed by reverse transcriptase in accordance with the instructions. The transcription level of *tAPX* was determined by both semi-quantitative and quantitative real-time PCR (qPCR) analysis using the specific primers for APX (APX-1: 5′- TCTGGTGTTA-CCCACTGATG -3′, APX-2: 5′- TTTCCCGTAGAATACTTTGC-3′). The *β-ACTIN* gene (ACT-1: 5′- TCTCGTTGTCCTCCTCACTT -3′; ACT-2: 5′- TATCATCAGCCTCAGCCATT -3′) was used as an internal control and also amplified simultaneously from each sample. The semi-quantitative PCR cycling procedure consisted of 3 min at 95°C, 27 cycles for 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, and a final 5-min extension at 72°C. The qPCR cycling consisted of 1 min at 95°C, 40 cycles of 10 s at 95°C, 40 s at 56°C, 45 s at 72°C (data collection), followed by a melting curve procedure of 1 min at 95°C, 1 min at 56°C, 78 cycles for 56°C ramping to 95°C at the rate of 0.5°C/10 s. The qPCR was performed on a Bio-Rad iCycler fluorescence thermocycler (Bio-Rad, Hercules, CA). The fluorescence master mix reagent for the reaction was Sybr Green (Toyobo). All of the cycle threshold (Ct) values of *tAPX* amplification were normalized by the corresponding *β-ACTIN* Ct values. Three parallel repeats were done and the results were summarized as averages and the standard deviation (SD). The data were analyzed and plotted using Microsoft Office software.

H_2O_2 staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB)

The H_2O_2 content was determined by the modified DAB method (Thordal-Christensen et al., 1997; Guan and Scandalios, 2000; Zhou et al., 2000). Leaves of *Arabidopsis* were cut and submerged into test tubes containing about 10 ml DAB solution (1 mg/ml DAB with NaOH, pH 3.8), and then the petioles fully immersed in the solution were cut again. After about 12 h in dark, the leaves were washed with water and then transferred into clean test tubes with cotton plug. EtOH (95%) was added into the test tubes, and the tubes were heated in boiling water for about 18 min. And the leaves were transferred into fresh EtOH when chlorophyll was removed completely.

Measuring the flowering time

Seeds of the overexpressing line, the wild type and the mutant line were kept in 4°C of three days and the process of jarovization could remove the dormancy to make sure the seeds germinated and grew synchronously. The seeds grew in soil under normal conditions (22°C, 16/8 h light/dark and 70% humidity), and when the first bolting began, the inflorescence heights were recorded and measured day by day. Three replicates were carried out and standard deviations were calculated. The data were analyzed and plotted in Microsoft Office software.

RESULTS

The homozygous mutant *Arabidopsis* line

The seeds of mutant line (SALK_027804.54.20) were gotten from ABRC, which had a T-DNA insertion into the first exon of *tAPX* gene. So the homozygous lines could be identified with three specificity primers. Figure 1a shows that amplification for the wild type got a unique

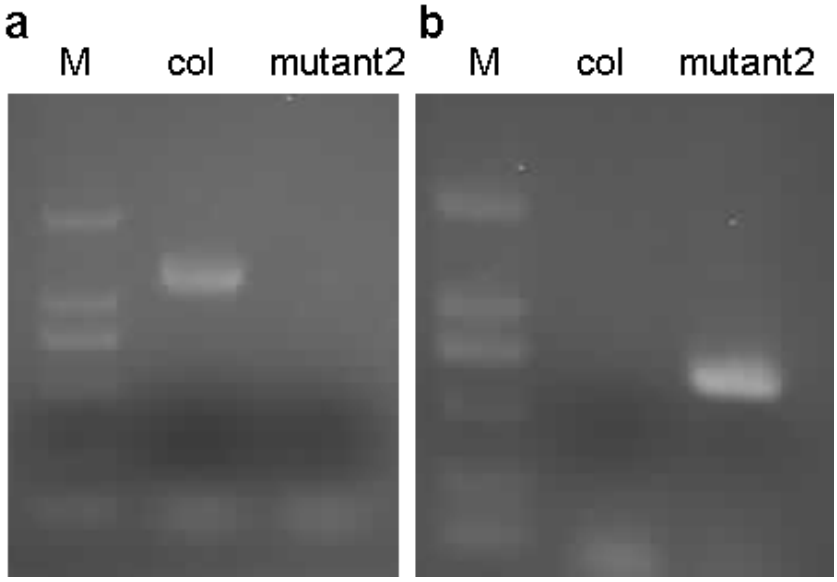

Figure 1. The screening of the homozygous mutant line. a) PCR for wild type produced a unique long product of 1200 bp from LP to RP, while for mutant 2 nothing was amplified; b) PCR for mutant 2 produced only a short band of 550 bp from RP to LBb1.3, while for the wild type there was no amplification. M, DNA marker DL 2000; col, wild type; mutant2, mutant line 2.

large product of 1200 bp from LP to RP, and amplification for mutant 2 got only a small band of 550 bp from RP to LBb1.3 (Figure 1b). PCR for some other mutant plants obtained both bands (data not shown), because they had one DNA chain with the T-DNA insertion and the other chain without it and they were identified as heterozygous. So according to the method of screening the homozygous line, mutant 2 was considered homozygous and its seeds were used in the following researches.

tAPX regulated the H_2O_2 content of the leaves

Semi-quantitative and quantitative real-time PCR (qPCR) were used to verify the *tAPX* transcriptional level. Figure 2a shows that the expression of *tAPX* was enhanced in overexpressing line, while it was knocked down in the mutant. The relative expression value of *tAPX* mRNA observed in overexpressing line was over 52-fold higher than that observed in wild-type plants, while *tAPX* was seldom expressed in the mutant line (Figure 2b). Therefore, it was assumed that the transformed sense sequence driven by CaMV 35S promoter was effectively overexpressed and the T-DNA insertion in the mutant line knocked out the *tAPX*. Although there were no remarkable phenotypic differences among the over-expressing line at the vegetative growth stage, the wild type and the mutant line (data not shown) under normal conditions, exhibited some internal changes since the transcriptional level of *tAPX* altered so enormously among the three lines. In consideration of its main function which was to scavenge the H_2O_2, endogenous levels of H_2O_2 in overexpressing line, the wild type and the mutant line were determined. DAB was involved in the staining, which reacted with H_2O_2 *in situ* and bronzing spots consequently appeared (Guan and Scandalios, 2000; Zhang et al., 2009). Therefore, as shown Figure 2c, leaves from transgenic line had the lightest color, while leaves from mutant line were dyed the most. In other words, the transgenic lines suffered the least H_2O_2, while the mutant suffered the most H_2O_2. Even in normal physiological and biochemical process, ROS including H_2O_2 was produced (Asada and Takahashi, 1987), and in this study, the level of H_2O_2 was eliminated more or less effectively in the overexpressing or mutant line respectively. Since the H_2O_2 content differed among the three lines, so the bolting and flowering time should be determined to test the hypothesis H_2O_2 regulated flowering time.

tAPX regulated the bolting and flowering time of *Arabidopsis*

To test whether H_2O_2 regulated the flowering initiation, seeds germinating contemporarily after jarovization were grown in soil. In the vegetative growth stage, there seemed no phenotypic difference between the over-expressing line, the wild type and the mutant line. On the 29th day after germination, the first bolting appeared

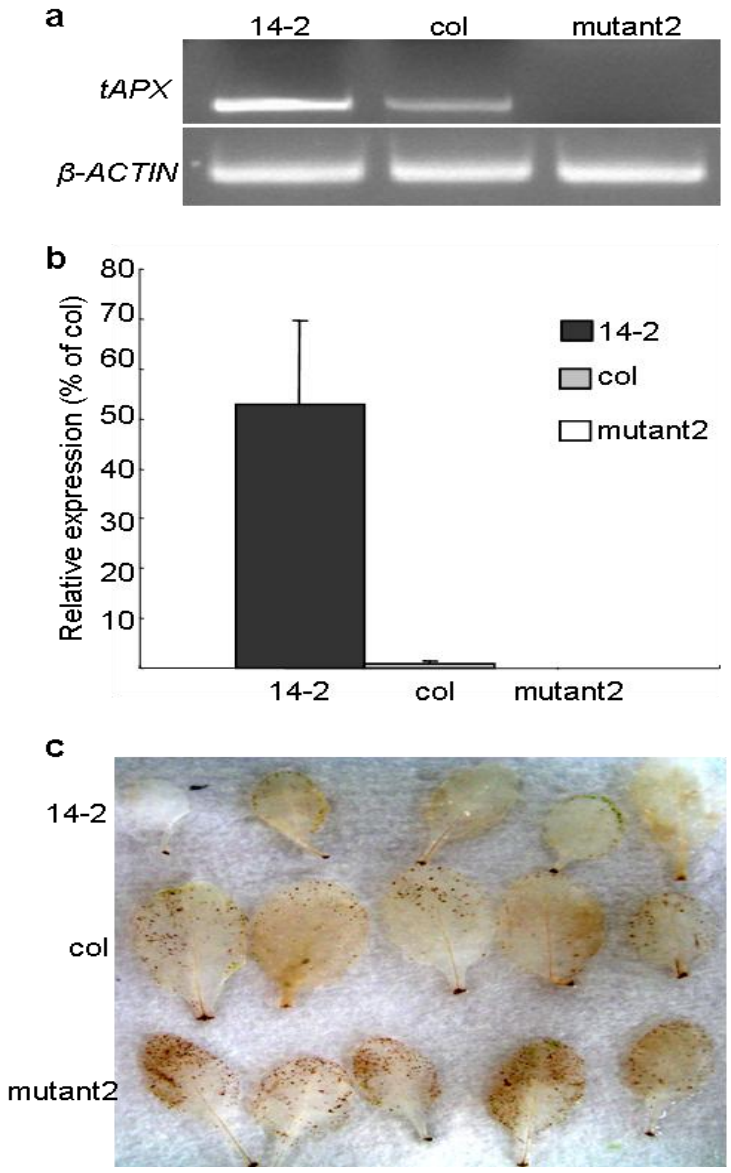

Figure 2. *tAPX* transcriptional level and H_2O_2 content. a) The determination of transcriptional level by semi-quantitative PCR. b) Quantitative real-time PCR analysis of *tAPX* transcriptional level in overexpressing line, the wild type and the mutant. Total RNA was prepared from three-weeks-old seedlings. *β-ACTIN* was used as the internal control. The *tAPX*/*ACTIN* ratio of overexpressing line or the mutant was normalized to that of wild type. c) H_2O_2 content determined by DAB method of the overexpressing line, the wild type and the mutant respectively. 14-2, The overexpressing line; col, wild type; mutant2, mutant line 2. Results represent the mean of three replicates. Bars correspond to the standard deviation. DAB, 3,3'-Diaminobenzidinetetrahydrochloride.

in the mutant line, and then followed by the wild type. As to the overexpressing line, there was no bolting until the 35th day. And from the 29th day on, the inflorescence heights were measured and recorded every day. It was indicated in Figure 3a and b that the overexpression of *tAPX* altered the flowering time more remarkably than the repression of *tAPX* did. For example, it was on the 35th day after germination that the inflorescence height of mutant line reached 5 cm, only 1 day earlier than the wild type. However, the overexpressing line got that height till 42 day (5 days later than the wild type). As reported by Murgia at al. (2004), seeds of 14-2 line germinated a little

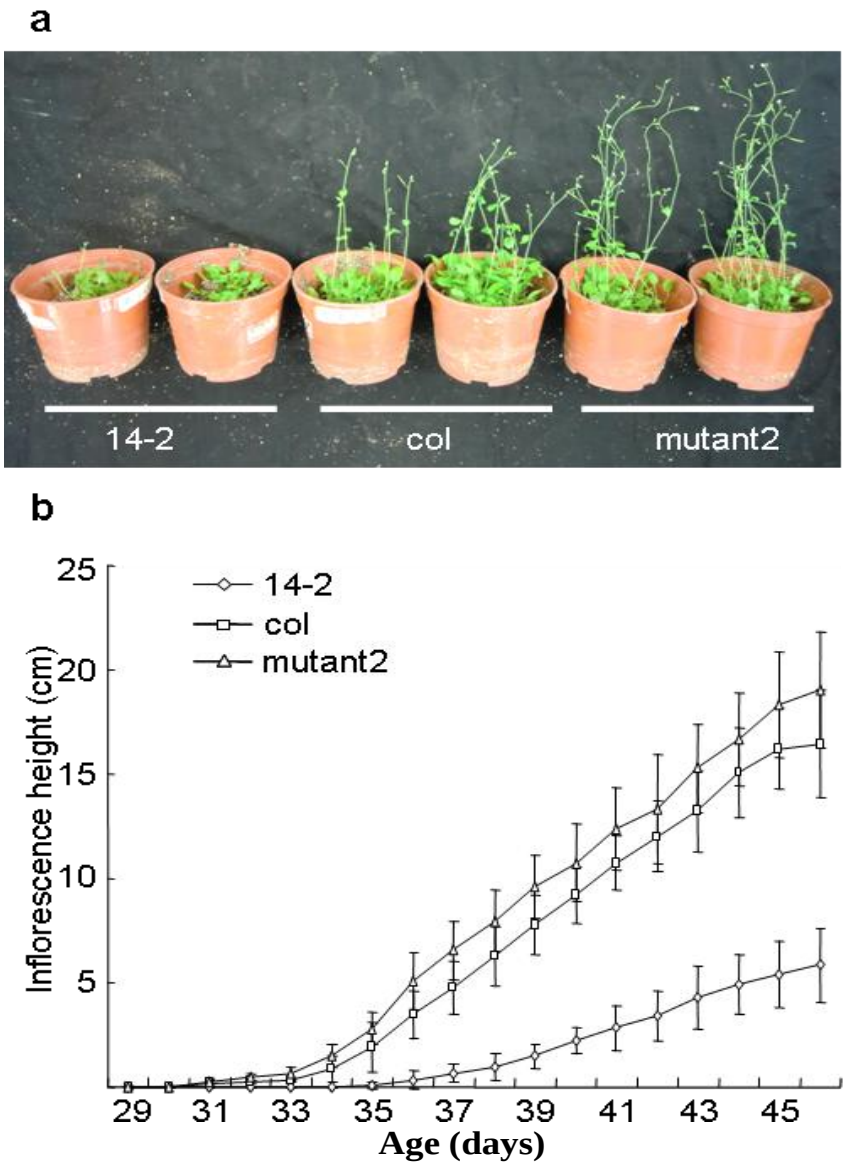

Figure 3. *tAPX* regulated the flowering time. a) Inflorescence heights of the overexpressing line, the wild-type and the mutant line in the 38th day after germination. b) Inflorescence height was measured for each seedling during growth under controlled conditions. 14-2, The overexpressing line; col, the wild type; mutant2, mutant line 2. Results represent the mean of three replicates. Bars correspond to the standard deviation.

late than the wild type and the mutant, but it was because of the insertion site rather than the function of the *tAPX* gene and it was almost unnoticeable. So it did not affect the experiment results.

DISCUSSION

Flowering process is a complex life phenomenon in higher plants. It is an important transition from vegetative growth stage to reproductive growth stage (Yong et al., 2000). The correct timing of flowering is therefore necessary to maximize reproductive success (Bernier, 1988; Simpson and Dean, 2002).

To explain this mysterious situation, researchers carried out a lot of studies physiologically and biochemically, and various hypotheses were proposed in succession; for example, the florigen hypothesis (Lang, 1952; Evans, 1971), the nutrient diversion hypothesis (Bernier, 1988) and the multifactorial control model (Koornneef et al., 1998; Mouradov et al., 2002; Simpson and Dean, 2002). And the last one was the most widely

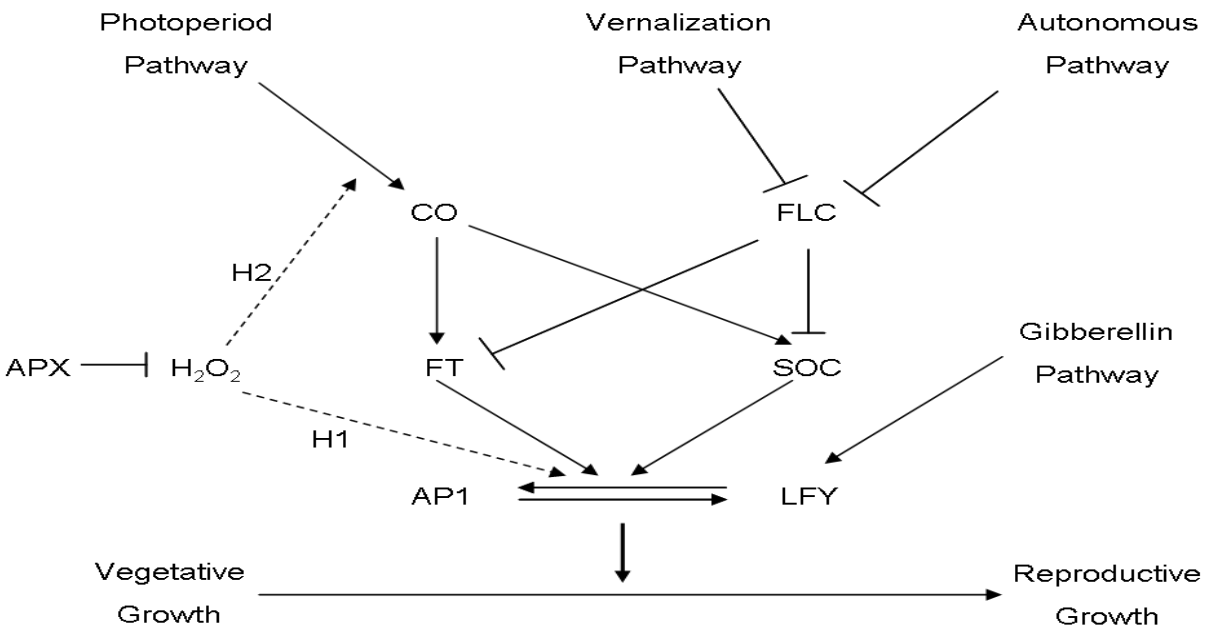

Figure 4. Outline of the proposed potential pathways of how H_2O_2 regulated the flowering time. Solid lines indicate the known pathways; dotted lines indicate the pathways proposed in this study. H1, Hypothetical pathway 1; H2, hypothetical pathway 2.

acknowledged nowadays, which regarded the nutrient accumulation as an aspect of floral induction, and held that there were kinds of inducing factors and suppressing factors besides in flowering process. Recent studies focused on model plant *Arabidopsis* obtained a series of genes controlling flowering time and confirmed this hypothesis. In *Arabidopsis*, there were four pathways controlling the flowering time: the photoperiod pathway, the vernalization pathway, the autonomous pathway and the gibberellin pathway (Komeda, 2004). Besides, factors such as overcrowding, nutrient deficiency, heat, drought, salicylic acid and microRNA were already reported regulating flowering time (Simpson and Dean, 2002; Zhang and Zuo, 2006). In addition, Lokhande et al. (2003) and Moharekar et al. (2007) found that flowering time was negatively correlated with H_2O_2 content, so they proposed a hypothesis that H_2O_2 is a possible factor in flower induction. But these regulations of H_2O_2 content were passive to a certain degree, for the alteration of H_2O_2 content was carried out by stress treatment or comparison among *Arabidopsis* ecotypes from different latitudes.

In this study, we took measures to actively control the H_2O_2 content. The transgenic *Arabidopsis* overexpressing *tAPX* and mutant line deficient in *tAPX* were obtained and the transcriptional level analysis indicated that the relative expression ratio of *tAPX* was 52:1:0 in the over-expressing line, the wild type and the mutant line, respectively (Figure 2b). As a result, the H_2O_2 content was lowest in the overexpressing line and highest in the mutant line (Figure 2c) under common conditions, and this alteration finally led to the difference in flowering time (Figures 3a and b). The significance of this study was that H_2O_2 content was regulated on purpose by upstream *tAPX* gene, rather than altered by environmental stress factors. Further, the hypothesis that H_2O_2 was one of the possible factors in flower induction (Lokhande et al., 2003) was confirmed. It was also indicated in Figure 3b that the number of delayed flowering days in *tAPX*-overexpressing line compared with the wild type was obviously more magnificent than the number of advanced flowering days in the mutant line. It was known that there were kinds of APX isozymes in the *Arabidopsis*, so the mutant line lost tAPX which accounted for a small part of the total APX; while the overexpressing line increased the total APX because the 35S promoter started the *tAPX* gene absolutely and strongly. So the decrement of APX in the mutant was less than the increment of APX in the overexpressing line; and we considered it as the reason why the delayed flowering time in overexpressing line was more than the advanced flowering time in the mutant.This study provided a method in delaying or advancing the flowering time in *Arabidopsis*. Applying this technology to ameliorating the ornamental plants will be more practically significant. As to the mechanism, how some kinds of plant hormones promote flowering was revealed.

For example, the gibberellins (Blázquez et al., 1998) promoted the flowering of *Arabidopsis* by activating the promoter of *LEAFY* gene. So we hypothesized that H_2O_2 activated some floral induction genes or repress some genes that deterred flowering (Figure 4). On the other

hand, according to the water-water cycle in chloroplasts, the $\cdot O_2^-$ produced by photosystem was disproportionated to H_2O_2 and O_2 by SOD, then the H_2O_2 was reduced to water by APX subsequently (Asada, 1999). Thus, APX established an indirect contact with the photosystem, and we proposed another hypothesis that H_2O_2 might have potential impacts on the photoperiod pathway (Figure 4). Further researches on this are currently going on in our laboratory.

ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (30971557, 30971816).

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