AtHAP3b Plays a Crucial Role in the Regulation of Flowering Time in *Arabidopsis* during Osmotic Stress

Nai-Zhi Chen, Xiu-Qing Zhang, Peng-Cheng Wei, Qi-Jun Chen, Fei Ren, Jia Chen and Xue-Chen Wang*
State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100094, China

Received 21 March 2007, Accepted 1 June 2007

The HAP complex has been found in many eukaryotic organisms. HAP recognizes the CCAAT box present in the promoters of 30% of all eukaryotic genes. The HAP complex consists of three subunits - HAP2, HAP3 and HAP5. In this paper, we report the biological function of the *AtHAP3b* gene that encodes one of the HAP3 subunits in *Arabidopsis*. Compared with wild-type plants, *hap3b-1* and *hap3b-2* mutants exhibited a delayed flowering time under long-day photoperiod conditions. Moreover, the transcription levels of *FT* were substantially lower in the mutants than in the wild-type plants. These results imply that *AtHAP3b* may function in the control of flowering time by regulating the expression of *FT* in *Arabidopsis*. In a subsequent study, *AtHAP3b* was found to be induced by osmotic stress. Under osmotic stress conditions, the *hap3b-1* and *hap3b-2* mutants flowered considerably later than the wild-type plants. These results suggest that the *AtHAP3b* gene plays more important roles in the control of flowering under osmotic stress in *Arabidopsis*.

**Keywords:** *Arabidopsis*, *AtHAP3b*, Flowering time, HAP complex, Osmotic stress

---

**Introduction**

The CCAAT box is one of the most common elements in eukaryotic promoters. It is present in 30% of all eukaryotic genes and is located 80-100 bp upstream of the transcription start site (Bucher, 1990; Maity and de Crombrugghe, 1998; Mantovani, 1999). The HAP complex (also known as CBF, NF-Y, or CP1) was isolated as a CCAAT-binding protein complex, and it is an evolutionarily conserved transcription factor that occurs in a wide range of organisms from yeast to humans. It includes three subunits: NF-YA (CBF-B or HAP2), NF-YB (CBF-A or HAP3), and NF-YC (CBF-C or HAP5). They are all required for DNA binding (Mantovani, 1999). A fourth subunit, HAP4 is present in yeast; it does not bind to DNA but is required for complex formation (Forsburg and Guarente, 1989; McNabb et al., 1997). In yeast and humans, each subunit of the HAP complex is encoded by a single gene, whereas in plants, each subunit of the HAP complex is encoded by multiple genes. In *Arabidopsis*, there are 10, 10 and 9 genes encoding the HAP2, HAP3 and HAP5 subunits, respectively. Further, 11 putative HAP3 genes and 13 putative HAP5 genes have been recognized in rice (Edwards et al., 1998; Gusmaroli et al., 2001; Gusmaroli et al., 2002; Miyoshi et al., 2003).

In recent years, several HAP subunits have been identified in plants. The first was isolated from maize as a HAP3 homolog (Li et al., 1992). The *Bn CBF-B* subunit was identified in *Brassica napus* as a plant homolog of HAP2 (Albani and Robert, 1995). *MtHAP2-1* in the model legume *Medicago truncatula* plays a key role in nodule development (Combier et al., 2006). There are 3 OsHAP3 subunits (OsHAP3A, OsHAP3B, and OsHAP3C) and they function during chloroplast development (Miyoshi et al., 2003). The *Arabidopsis* HAP3 subunits LEC1 and LEC1-LIKE (L1L) play important roles in embryogenesis (Lotan et al., 1998; Kwong et al., 2003; Lee et al., 2003).

Interestingly, the HAP subunits can function not only by complex formation but also by individual interaction with other proteins. It was reported that the AtHAP3 and AtHAP5 subunits can interact with the CCT domain of the CONSTANS (CO), CONSTANS-LIKE, and TOC1 proteins (Wenkel et al., 2006). HAP2 contains two important domains: NF-YA1 interacts with the HAP3/HAP5 dimer during the formation of the trimeric HAP complex, while NF-YA2 interacts with the CCAAT box. The CCT domain is highly similar to the NF-YA2 domain in terms of structure (Xing et al., 1994; Robson et al., 2001; Romier et al., 2003). Recent reports have revealed...
that the CCT domain protein CO forms a heterotrimeric CO/AtHAP3/AtHAP5 complex by interacting with the AtHAP3/AtHAP5 dimer, similar to the AtHAP2 subunit. CO functions in the photoperiodic flowering pathway. Consequently, the heterotrimeric CO/AtHAP3/AtHAP5 complex also plays an important role in this pathway. Overexpression of the AtHAP3a and AtHAP2a transients in Arabidopsis has been shown to delay flowering (Wenkel et al., 2006).

In this study, we investigated the biological function of AtHAP3b in Arabidopsis. We examined the expression pattern of AtHAP3b and obtained two AtHAP3b T-DNA insertion mutants. Our result showed that the hap3b-1 and hap3b-2 mutants flowered later than wild-type plants under long-day photoperiod conditions. In addition, we found that AtHAP3b was induced by osmotic stress. Further, the difference of flowering time between the hap3b mutants and wild-type plants was substantially higher under osmotic stress conditions. In conclusion, our results indicated that AtHAP3b is required for the regulation of flowering time under osmotic stress conditions.

Materials and Methods

Plant materials and growth conditions. Arabidopsis thaliana ecotype Columbia seeds were surface-sterilized in a solution of 0.5% NaClO for 15 min, washed five times with sterile distilled water and stratified at 4°C for 2 d in the dark. Following this treatment, the plants were germinated and grown on Munshire and Skoog medium, supplemented with 3% (w/v) sucrose and 0.8% agar, pH 5.7. Germination was executed in a growth chamber at 22°C.

Histochemical analysis of GUS expression. To examine the tissue-specific expression of AtHAP3b, 1066bp of the AtHAP3b promoter region was amplified using 5'-CTGCAGTGTTCCCTACCTTTCA-3' and 5'-GAA TTCCTTTGCTGCCTCTCT-3', and quantitative PCR analysis was performed as described previously (Chai et al., 2006) with specific primers of AtHAP3b gene 5'-AGGGTGCTCCTCCGTAATC-3' and 5'-TACGTGACAGCCCCAGCAGAAA-3'. And quantitative real-time RT-PCR analysis was performed using the specific primers of FT gene 5'-CGCCAGAATTCACAGCTCG-3' and 5'-TCTTTCTCCCGACCCACT-3', or the specific primers of AP1 gene 5'-ACAATATGGCTCCCCCTC-3' and 5'-TCTTTCTGACAGCAGCCCCAC-3'. The ACTIN2 mRNA expression was amplified as an internal control for real-time RT-PCR analysis using the specific primers 5'-GGAAACATGGCTGCTCGTGCGG-3' and 5'-CAGCAGGTTATCCATGGC-3'. And quantitative PCR experiments were repeated three independent times.

Identification of the T-DNA insertion mutants for the AtHAP3b gene. T-DNA insertion lines of the AtHAP3b gene (At5g47640), named as hap3b-1 (SALK_001306) and hap3b-2 (SALK_026666) (http://signal.salk.edu) were obtained from NASC. The AtHAP3b homozygous mutants were screened by PCR amplification using the following primers. hap3b-1 LP: 5'-GAATGCAGCTCGACCAGCAGGAGAG-3', RP: 5'-CCTGCACTGCTCTGTGGCAG-3', hap3b-2 LP: 5'-GACGGTCCAGCCCCACGTAAG-3', RP: 5'-TGCGTCCGGAAAAATGGCGTGG-3', and the primer from T-DNA region Lb1: 5'-CCGIGGAAAGAAAAGCAATGCTGCTACA-3'. The expression of the AtHAP3b gene in hap3b-1 and hap3b-2 mutant plants was examined by reverse transcript PCR analysis.

Complementation of the T-DNA insertion mutants hap3b-1 and hap3b-2. The AtHAP5 genomic region was amplified using Pfu DNA polymerase from Arabidopsis genomic DNA with the primers 5'-CTGCAGTGTTCCCTACCTTTCA-3' and 5'-GGAAACATGGCTGCTCGTGCGG-3' (enzyme digestion site underlined) primer sequences. The amplified product was cloned into the the PstI and EcoRI sites of pCAMBIA1391 GUS activity assay constructs were performed as follows: transgenic plants were inoculated in S-bromo-4-chloro-3-indolyl/β-D-glucuronide (X-gluc) buffer [100 mM sodium phosphate buffer pH 7.0, 0.1 mM EDTA, 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6, 1 mM X-gluc and 0.1% Triton X-100] at 37°C for 12 h seedlings were washed to clear chlorophyll with 70% ethanol. For GUS expression analysis under various stress conditions, 10-day-old AtHAP3b::GUS transgenic plants grown on MS medium were treated by various stresses and then incubated in X-gluc buffer at 37°C for 1 h or 3 h.

RT-PCR and quantitative real-time RT-PCR analysis. Total RNA was extracted from 4-week-old seedlings with TRIzol Reagent (Invitrogen). Reverse transcription reactions were performed using the M-MLV Reverse Transcriptase (Promega) with the oligo(dT)12 primers. The AtHAP3b gene was amplified using gene-specific primers 5'-TCTTTCCACACTCAACCC-3' and 5'-CACCTCCACTGTCGCTACC-3', and 5'-ACGGTGGCTACC-3'. The ACTIN2 mRNA expression was amplified as an internal control using the specific primers 5'-TCTTTCCACACTCAACCC-3' and 5'-CACCTCCACTGTCGCTACC-3'.

Quantitative real-time RT-PCR was performed as described (Chai et al., 2006) with specific primers of AtHAP3b gene 5'-AGGGTGCTCCTCCGTAATC-3' and 5'-TACGTGACAGCCCCAGCAGAAA-3'. And quantitative real-time RT-PCR analysis was performed using the specific primers of FT gene 5'-CGCCAGAATTCACAGCTCG-3' and 5'-TCTTTCTCCCGACCCACT-3', or the specific primers of AP1 gene 5'-ACAATATGGCTCCCCCTC-3' and 5'-TCTTTCTGACAGCAGCCCCAC-3'. The ACTIN2 mRNA expression was amplified as an internal control for real-time RT-PCR analysis using the specific primers 5'-GGAAACATGGCTGCTCGTGCGG-3' and 5'-CAGCAGGTTATCCATGGC-3'. And quantitative PCR experiments were repeated three independent times.

Identification of the T-DNA insertion mutants for the AtHAP3b gene. T-DNA insertion lines of the AtHAP3b gene (At5g47640), named as hap3b-1 (SALK_001306) and hap3b-2 (SALK_026666) (http://signal.salk.edu) were obtained from NASC. The AtHAP3b homozygous mutants were screened by PCR amplification using the following primers. hap3b-1 LP: 5'-GAATGCAGCTCGACCAGCAGGAGAG-3', RP: 5'-CCTGCACTGCTCTGTGGCAG-3', hap3b-2 LP: 5'-GACGGTCCAGCCCCACGTAAG-3', RP: 5'-TGCGTCCGGAAAAATGGCGTGG-3', and the primer from T-DNA region Lb1: 5'-CCGIGGAAAGAAAAGCAATGCTGCTACA-3'. The expression of the AtHAP3b gene in hap3b-1 and hap3b-2 mutant plants was examined by reverse transcript PCR analysis.

Complementation of the T-DNA insertion mutants hap3b-1 and hap3b-2. The AtHAP5 genomic region was amplified using Pfu DNA polymerase from Arabidopsis genomic DNA with the primers 5'-CTGCAGTGTTCCCTACCTTTCA-3' and 5'-GGAAACATGGCTGCTCGTGCGG-3' (enzyme digestion site underlined) primer sequences. The amplified product was cloned into the the PstI and EcoRI sites of pCAMBIA1391. The construct was introduced into Agrobacterium strain GV3101 and transformed into hap3b-1 and hap3b-2 mutants.

Measurement of flowering time. The flowering time was measured as total leaf number as described previously (Koornneef et al., 1998). The total leaf number was recorded including the number of rosette leaves and the number of cauline leaves on the main stem when the first flower opened.

Analysis of the flowering time phenotype under osmotic stress conditions. For quantitative real-time RT-PCR analysis, 2-week-old seedlings grown on MS medium were immersed in a solution containing 300 mM mannitol for 1, 3, 6, 12 and 24 h, with distilled water as a control. To analyze the effect of osmotic stress on the flowering time phenotype, 10-day-old seedlings grown on MS medium were transferred to MS medium containing 100 mM mannitol. The plants were photographed after 49 days of growth.
Results and Discussion

Expression pattern of AtHAP3b in different tissues and at different developmental stages. To determine the expression of AtHAP3b in various tissues, we constructed the AtHAP3b promoter::GUS fusion gene to transform wild-type Arabidopsis plants. The GUS signal was observed in almost the entire 10-day-old seedling, although the roots exhibited a weaker signal (Fig. 1(a) A) and the fusion gene was not expressed at all in the root tip (Fig. 1(a) F). However, in mature seedlings, the GUS activity in the roots was reduced and/or nearly absent (Fig. 1(a) G). A strong signal was observed in the rosette and cauline leaves, particularly the vascular tissues, of 4-week-old seedlings (Fig. 1(a) B). The GUS signal was absent in mature stems (Fig. (a) E) and present at both ends of mature siliques (Fig. 1(a) C). Among the floral organs, only calyces and filaments exhibited strong staining (Fig. 1(a) D).

To quantitatively examine the mRNA expression levels of AtHAP3b in various tissues, RNA was extracted from various tissues including the roots, stems, flowers and rosette and cauline leaves. We found that the flowers and cauline leaves exhibited the highest transcription level of AtHAP3b (Fig. 1(b)). Previous studies also showed that most HAP subunits are expressed during flower development (Edwards et al., 1998; Gusmaroli et al., 2001; Gusmaroli et al., 2002).

AtHAP3b expression during the developmental stages was also examined by quantitative real-time RT-PCR analysis. RNA was extracted from 4-, 7-, 14-, 21- and 38-day-old seedlings. The results showed that the level of AtHAP3b mRNA peaked in 38-day-old seedlings (Fig. 1(c)). At this stage, 30-50% of the flower buds had opened. In other words, the transcription level of AtHAP3b during the flowering stage was higher than that during other stages.

Expression pattern of the AtHAP3b gene in response to osmotic stress. The expression pattern of the AtHAP3b gene in response to osmotic stress was analyzed using quantitative real-time RT-PCR. AtHAP3b expression was strongly induced by osmotic stress. The expression level of AtHAP3b peaked after 1 h of osmotic stress treatment (Fig. 2(a)). These results indicate that AtHAP3b may be involved in the response to osmotic stress.

We further analyzed the pattern of GUS expression driven by the AtHAP3b promoter under osmotic stress conditions in AtHAP3b::GUS transgenic plants. As expected, after 1-h incubation in X-gluc buffer at 37°C, GUS staining revealed that the transcript levels of the GUS reporter gene were obviously increased under osmotic stress. Further, no GUS signal was observed in both transgenic Arabidopsis plants treated with distilled water and plants grown on MS medium (Fig. 2(b)). This result confirmed that AtHAP3b was induced by osmotic stress. After 3-h incubation in X-gluc buffer at 37°C, GUS staining could be detected in all materials; however, the GUS signal observed in the transgenic plants grown on MS medium was much weaker than that in plants grown under osmotic stress conditions. Compared with the transgenic plants grown on MS medium, those treated with distilled water also exhibited increased GUS expression (Fig. 2(c)). This result indicates that AtHAP3b may also be induced by waterlogging stress. In summary, these data further confirmed the inducible expression of AtHAP3b under stress conditions. Consistent with our results, it has been shown that
AtHAP3b expression is not detectable in liquid-cultured plants, while high expression is observed in soil-grown plants; this data also suggested the possible role of AtHAP3b in regulation of the osmotic stress response (Edwards et al., 1998).

Identification and complementation of the T-DNA insertion mutants hap3b-1 and hap3b-2. To examine the function of the AtHAP3b gene in plants, two mutants with a T-DNA insertion in the AtHAP3b genomic sequence were obtained. In the hap3b-1 mutant, the T-DNA was inserted in the 5'-untranslated region and in the hap3b-2 mutant, it was inserted in the coding region (Fig. 3(A)). RT-PCR analysis showed that AtHAP3b mRNA was not detectable in the hap3b-1 and hap3b-2 mutants (Fig. 3(B)). This result indicated that AtHAP3b expression was lost in hap3b-1 and hap3b-2 plants. Two homozygous lines of hap3b-1 and hap3b-2 were used for further analysis.

The two homozygous lines Com1 and Com2 were selected as complementation lines of hap3b-1 and hap3b-2 plants, respectively. Quantitative real-time RT-PCR analysis revealed that the transcript levels of AtHAP3b in the Com1 plants were slightly higher than those in wild-type Arabidopsis plants. However, the transcript levels of AtHAP3b in the Com2 plants were similar to those in wild-type Arabidopsis plants (Fig. 3(C)).

Flowering time phenotypes of hap3b-1 and hap3b-2 mutants and their complementation lines. The observed AtHAP3b expression patterns suggest that AtHAP3b may function in flowering control. To further determine the effect of AtHAP3b on flowering time, we observed the phenotype of hap3b-1 and hap3b-2 mutants and wild-type plants under long-day photoperiod conditions. We found that under these conditions, the two AtHAP3b knockout mutants hap3b-1 and hap3b-2 flowered later than wild-type plants. Furthermore, the flowering time phenotypes of hap3b-1 and hap3b-2 could
AtHAP3b Plays a Crucial Role in Regulation of Flowering Time

be rescued by complementation of AtHAP3b. Under long-day photoperiod conditions, plants of the Com1 and Com2 exhibited similar flowering time phenotypes, although slightly earlier than wild-type plants (Fig. 4(A)). Flowering time also is indicated by the total number of leaves (Fig. 4(B)).

To further confirm the above results, we analyzed the expression patterns of several genes related to flowering control by real-time RT-PCR in hap3b-1 and hap3b-2 mutants and their respective complementation lines (Fig. 5). The transcription levels of FT and AP1 were considerably lower in the mutants than those in the wild-type plants (Fig. 5). As expected, the expression levels of these genes in the complementation lines were dramatically higher than that in the hap3b mutants. Moreover, the FT and AP1 transcripts were more abundant in the complementation lines than in the wild-type plants. However, the expression levels of FLC, CO, FVE, and GI did not obviously differ between the mutant and wild-type plants. Additionally, it is known that AP1 is an important gene for floral transition and that it acts downstream of FT (Ruiz-García et al., 1997; Wigge et al., 2005). Taken together, these results suggest that AtHAP3b plays an important role in flowering control by regulating FT expression.

However, unlike the AtHAP3b mutation, the AtHAP3a mutation did not cause a change in the flowering time from that in the wild-type (Wenkel et al., 2006). AtHAP3b may be an important factor distinct from AtHAP3a. Different HAP genes probably have different functions in the control of flowering time.

Effect of osmotic stress on the phenotype of hap3b-1 and hap3b-2 mutants and their complementation lines. As observed from the above results, AtHAP3b was strongly induced by osmotic stress (Fig. 2(a)), and under normal growth conditions, its knockout mutants flowered later than wild-type plants (Fig. 4(A)). To further examine the function of the AtHAP3b gene under osmotic stress conditions, we assessed the osmotic stress tolerance. 6-day-old seedlings were transferred to MS plates containing 350, 400, and 450 mM mannitol. Next, the plates were placed in the vertical position, and the root tips were oriented upward for 9 days. The AtHAP3b-knockout mutants hap3b-1 and hap3b-2 were very slightly sensitive to osmotic stress compared to the wild-type plants (data not shown). This result suggests that AtHAP3b may be not necessary for the response to severe osmotic stress.

To further examine the effect of osmotic stress on the flowering time phenotype, 10-day-old seedlings of hap3b-1, hap3b-2, the two complementation lines Com1 and Com2, and wild-type plants were transferred to MS medium containing 100 mM mannitol. After 33 days of growth under osmotic stress conditions, most of the wild-type plants flowered, but
is required to identify the precise molecular mechanism by which AtHAP3b regulates flowering time.

Acknowledgments We thank the Arabidopsis Biological Research Center for kindly providing the T-DNA insertion mutant seeds. This work was supported by grants from the National Basic Research Program of China (No. 2006CB10100 and No. 2003CB114500) and the National Natural Science Foundation of China (No. 30370129 and No.30421002)

References


