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Title:

A putative CCAAT-binding transcription factor is a regulator of flowering timing in *Arabidopsis*

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Abbreviations:  HAP, Heme Activator Protein;  NF-Y, NUCLEAR FACTOR-Y; FT, FLOWERING LOCUS T;  SOC, SUPPRESSOR OF OVEREXPRESSSION OF CONSTANS 1; CO, CONSTANS; FLC, FLOWERING LOCUS C; VRN2, VERNALIZATION 2; GA, gibberellic acid; TOC1, TIMING OF CAB1; SPB, SQUAMOSA PROMOTER BINDING
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Abstract

Flowering at the appropriate time of year is essential for successful reproduction in plants. We found that HAP3b in Arabidopsis (Arabidopsis thaliana), a putative CCAAT-binding transcription factor gene, is involved in controlling flowering time. Overexpression of HAP3b promotes early flowering while hap3b, a null mutant of HAP3b, is delayed in flowering under a long-day photoperiod. Under short-day conditions, however, hap3b did not show a delayed flowering compared to wild-type based on the leaf number, suggesting that HAP3b may normally be involved in the photoperiod-regulated flowering pathway. Mutant hap3b plants showed earlier flowering upon gibberellic acid or vernalization treatment, which means that HAP3b is not involved in flowering promoted by GA or vernalization. Further transcript profiling and gene expression analysis suggests that HAP3b can promote flowering by enhancing expression of key flowering time genes such as FT and SOC1. Our results provide strong evidence supporting a role of HAP3b in regulating flowering in plants grown under long day conditions.

Keywords: CCAAT binding protein, HAP3b, flowering time, photoperiod
Introduction

Flowering time in plants is controlled by environmental stimuli such as day length (photoperiod pathway), light quality, exposure to low temperatures (vernalization pathway), and internal factors such as plant age or stage of development (autonomous and gibberellic acid (GA) pathways). These different pathways converge to regulate a small set of genes, such as FT (FLOWERING LOCUS T, a small protein with similarity to RAF-kinase inhibitor) and SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1, a MADS transcription factor). For example, the photoperiod pathway promotes flowering through CO (CONSTANS, a zinc finger transcription factor) to upregulate FT and SOC1. However, a similar upregulation of FT and SOC1 through the vernalization and the autonomous pathways occurs through a mechanism that suppresses a floral suppressor, FLC (FLOWERING LOCUS C, a MADS transcription factor) (reviewed by Mouradov et al., 2002; Putterill et al., 2004; Amasino, 2005).

Recent studies indicated that several members in a large transcription factor family called HAP are involved in regulating flowering timing. HAP in plants encodes a putative CCAAT-binding transcription factor (CBF) similar to Heme Activator Protein (HAP) or nuclear factor-Y (NF-Y) in yeast and vertebrate (Lotan et al., 1998; Mantovani, 1999). In yeast and mammalian systems, it is known that HAPs form a complex and regulate gene expression. The complex forms through an initial interaction between HAP3 and HAP5, which then allows the formation of a heterotrimer with HAP2. The heterotrimer then binds to a CCAAT element with very high specificity and affinity (Romier et al., 2003). There is seemingly only one gene for each HAP factor in yeast and animals (Mantovani, 1999). In plants, however, these factors are all encoded in a gene family (Edwards et al., 1998; Yang et al., 2005). There are at least 10 annotated members in each family in Arabidopsis and also multiple members in each HAP family in rice (Kwong et al., 2003; Yang et al., 2005). To distinguish different members in the same family in Arabidopsis, each member is labeled alphabetically. For instance, in the HAP3 family, At2g38880 is named as HAP3a, and At5g47640 as HAP3b (Wenkel et al., 2006).

Several HAP members have been studied in plants. LEC1 encodes a HAP3 in Arabidopsis. Mutant lec1 is defective in embryo development during the seed
development stage, causing a leafy cotyledon phenotype. Ectopically over-expressing LEC1 can induce embryo development in vegetative cells (Lotan et al., 1998). Recently, another HAP3 (L1L) which is closely related to LEC1, was also found to be involved in embryo development (Kwong et al., 2003). In rice, an OsHAP3 is involved in chloroplast biogenesis. Suppression of gene expression of OsHAP3 using RNAi reduced expression of the gene and affected chlorophyll and chloroplast development (Miyoshi et al., 2003).

In regulating flowering timing, Ben-Naim et al. reported that overexpression of a tomato HAP5a in Arabidopsis caused early flowering (Ben-Naim et al., 2006). However, overexpression of Arabidopsis HAP3a and HAP5a delayed flowering (Wenkel et al., 2006). These conflicting results from overexpression hinder a clear conclusion for the roles of these HAPs in controlling flowering timing due to lack of supporting evidence from loss-of-function mutants. In this study, we provide evidence from both loss-of-function mutant and overexpression plants to support the role of HAP3b in controlling flowering. Up-regulation of HAP3b promotes early flowering, whereas a hap3b knockout results in delayed flowering in a long day photoperiod.

Results

Altered flowering timing in mutant hap3b and HAP3b-overexpression plants

A genetic approach was taken to examine the role of a group of HAP in plant growth/development and response to stress. Among the insertional mutants identified from the SALK T-DNA insertion collection (http://signal.salk.edu), an insertion mutant for HAP3b showed delayed flowering phenotype compared to its wild-type (wt) plants grown at a long day (16h/8h light/dark) photoperiod (Figs. 1A and 1D). The mutant plants developed on average about four more leaves than wild-type plants before flowering (i.e., about a 33% delay which equals approximately 7 days). The hap3b mutant (SALK_025666) has a T-DNA insertion at 9 bp after the first ATG (Fig. 1B) and no full-length transcript was detected using RT-PCR, suggesting a loss of function mutation (Fig. 1C). A null mutation was further confirmed by the microarray data (see below), which showed no evidence for the accumulation of a truncated HAP3b transcript.
To confirm that the mutant phenotype was not an artifact of a second site mutation, we set up a complementation test by expressing a wild-type copy of the *HAP3b* cDNA under the control of CaMV 35S promoter. When the *hap3b* mutant was transformed with the *P_{35S}:HAP3b-GFP* vector, the delayed flowering phenotype was reversed, indicating that HAP3b-GFP fusion protein was functional and capable of rescuing the loss-of-function *hap3b* mutant (Supplemental Fig. 1).

Not only did the *HAP3b-GFP* overexpression lines show a reversal of the *hap3b* mutant phenotype, they also provided evidence that the up-regulation of the *HAP3b* gene could promote premature flowering (Supplemental Fig. 1). An overexpression of *HAP3b* in wild-type plants promotes early flowering even more. As shown in Figure 1D, a representative *HAP3b*-overexpression line (*P_{actin}:HAP3b*) from the five lines characterized reached flowering with four leaves less (Fig. 1D) than the control plants that were overexpressing a GUS gene (C1).

The predicted *HAP3b* protein (191 amino acid) consists of three domains (Lee et al., 2003). The central domain, comprised of more than 90 amino acids, showed significant sequence identity with HAP3 or subunit B of NF-Y in yeast and rat that is required for DNA binding and interactions with other HAP proteins (Lee et al., 2003). A HAP3b-GFP fusion protein was predominately localized in nuclei of leaf (Fig. 2a) and root (Fig. 2b) cells, consistent with *HAP3b*’s predicted role as a transcription factor.

Using a *uidA* reporter gene (encoding the reporter enzyme glucuronidase or GUS) fused to the predicted promoter region from *HAP3b*, we observed that the *HAP3b* promoter is active in leaves, vascular tissues, flower stem, cauline leaves and flowers, which support the information in public databases (the Genevestigator database). In addition, we also revealed some more detailed expression patterns, such as in leaf trichome, filaments, and transmitting tissues in the style (Supplemental Fig. 2A-E). Interestingly, *HAP3b* expression is highly upregulated by salt and osmotic (mannitol) stress in both leaves and roots of *Arabidopsis* 3h after treatment (Kreps et al., 2002). The upregulation of *HAP3b* by stress is supported by the information in public databases where *HAP3b* transcript levels are also upregulated by drought, heat and abscissic acid (ABA) treatment in addition to salt and an osmotic stress (Supplemental Table I, the TAIR gene expression database, [http://www.arabidopsis.org](http://www.arabidopsis.org)). Nutrient deficiency and
UV treatments also moderately upregulated the transcript level of \textit{HAP3b} (data not shown, the Genevestigator database, https://www.genevestigator.ethz.ch/at/index.php). Other hormones, including indole-acetic acid (IAA), gibberellic acid (GA), cytokinin, and brassinolide had no effect on \textit{HAP3b} gene expression (data not shown, the Genevestigator database).

\textit{Effect of photoperiod, GA and vernalization on flowering timing of hap3b}

Many genes are known to regulate flowering timing through their activity in four major pathways, i.e. photoperiod, vernalization, GA, and autonomous pathways. In order to understand whether HAP3b is related to these known pathways, wild-type, \textit{hap3b}, and \textit{HAP3b}-overexpression plants were grown under different photoperiods, treated with gibberellic acid, and subjected to vernalization.

Mutant \textit{hap3b}, wild-type, \textit{HAP3b}-overexpression and overexpression control plants (C1) were sprayed with 20 μM GA. Both mutant \textit{hap3b} and wild-type plants flowered earlier (with fewer rosette leaves) compared to their non-GA-treatment controls (Fig. 3A). \textit{HAP3b}-overexpression plants did not show earlier flowering in response to GA. It is probable that the \textit{HAP3b}-overexpression plants flowered so early that it masked any potential flower promoting effect from GA. Since the \textit{hap3b} mutation did not affect a GA response, HAP3b is not involved in GA-induced flowering pathway.

A function for HAP3b in the vernalization pathway was also excluded, since the \textit{hap3b} mutant and \textit{HAP3b}-overexpression plants showed earlier flowering after vernalization treatment (Fig. 3B). Wild-type plants did not show significant response to vernalization treatment, while C1 showed significant reduction in leaf number by vernalization. That vernalization treatment has no effect on flowering timing in Columbia wild-type was also reported before (Lim et al., 2004).

In contrast to the flowering phenotype demonstrated under long-day photoperiod conditions (Fig. 2D), \textit{hap3b} and overexpression plants flowered with the same leaf number as their respective controls under a short day photoperiod (16h dark/8h light) (Fig. 3C). However, \textit{HAP3b}-overexpression plants flowered significantly earlier when compared with the regular wild-type control. These phenotypes resemble those of \textit{gi} and \textit{co} mutants in the photoperiod pathway.
Transcript profiling of hap3b and HAP3b-overexpression plants using microarray

To identify potential candidate genes that are affected by HAP3b, a microarray experiment using the Arabidopsis whole genome array (Affymetrix ATH1 chip) was carried out with hap3b knockout and HAP3b-overexpression plants. The arrays were used here as a discovery tool, with significant changes independently confirmed by quantitative RT-PCR. To identify potentially important changes, we grouped genes that show an opposite response (at least 25% change in gene expression level based on mean values of signal intensity) in hap3b and HAP3b-overexpression plants in comparison with wild-type plants. We identified 15 candidate genes that were downregulated in hap3b but upregulated in overexpression plants (Table I). The transcript level of HAP3b showed the greatest increase (~38 fold) in the overexpression plants and was not detected in hap3b, as expected.

Among the other 15 genes that were upregulated in overexpression plants but downregulated in hap3b, were two genes known to regulate flowering. SOC1 transcripts were downregulated 1.5 fold in hap3b plants, but upregulated 1.8 fold in the overexpression plants. Two SQUAMOSA PROMOTER BINDING (SPB) protein-like genes, SPL3 and SPL4 are noteworthy since overexpression of SPL3 resulted in early flowering (Cardon et al., 1997). Expression of SPL4 mirrored the expression pattern of SPL3, peaking at inflorescence and flower development stages (data from the Genevestigator database). Other known flowering-related genes are listed in supplemental Table II, and are either expressed at very low levels (signal absent or marginal) or showed no consistent opposite pattern (supplemental Table II) in hap3b and the overexpression plants.

Also in the list (Table I) are a putative cell wall protein gene (At2g20870), a putative cytochrome P450 gene (At3g10570) and a GDSL-motif lipase/hydrolase family protein gene (At5g33370) which all showed expression predominantly in floral organs. A vegetative storage protein 1 gene (At5g24780), a jacalin lectin family protein gene (At2g39330) and a UDP-glucose 4-epimerase gene (At1g12780) were expressed at the highest level in floral organs as well as in stem apex or cauline leaves (the Genevestigator database).
database). These results suggest that the majority of the genes affected by HAP3b in the list are involved in reproductive growth.

Several major flowering genes were selected for quantitative PCR analysis. $SOC1$ was upregulated in overexpression plants and downregulated in $hap3b$, confirming the expression pattern in the array analysis. $FT$ which was not detected on the array in wild-type and mutant plants was detected by qPCR and showed the same pattern as $SOC1$ (Fig. 4). This supports a model in which $HAP3b$ normally promotes flowering through a pathway involving the up-regulation of $SOC1$ and $FT$. Expression levels of other major flowering-related genes, $TOC1$ ($TIMING$ $OF$ $CAB1$), $CO$ and $FLC$, were not significantly affected (supplemental Fig. 3).

Discussion

In this study we provide genetic evidence for the function of $HAP3b$, which encodes a CCAAT-binding transcription factor, in controlling flower timing.

HAP3b regulates flower timing through a photoperiod pathway

Evidence presented here from $hap3b$ mutants and $HAP3b$-overexpression clearly shows that HAP3b contributes to the regulation of flower timing under long day photoperiod conditions. We found no evidence to link HAP3b to flower timing under short day conditions. Similar long day specific phenotypes have also been observed for $co$ and $gi$ mutants, which are the key players in the photoperiod pathway. Since $hap3b$ plants show a normal response to GA and vernalization treatments, the results exclude a role of HAP3b in the GA and the vernalization flowering pathways. An involvement of HAP3b in the autonomous pathway is also unlikely, since the $FLC$ transcript level, which is upregulated in the mutants of the autonomous pathway (Mockler et al., 2004), is not affected in the $hap3b$ plants. Our other analyses on the $hap3b$ plants did not reveal other visible phenotypes related to morphology and development (data not shown). The combined results suggest the stress-modifying function of HAP3b is probably restricted to regulating flowering in the long-day photoperiod pathway. A recent study demonstrated that HAP3b can directly interact with CO and COL15 in the yeast two-
hybrid analysis (Wenkel et al., 2006). Thus, HAP3b may promote early flowering in the long-day photoperiod by affecting activity of CO or COL proteins.

Many HAPs in *Arabidopsis* are found to interact with CO or COL and overexpression of HAP3a and HAP5a has been shown to delay flowering (Wenkel et al., 2006), implicating complexity of regulation in the photoperiod pathway. However, loss-of-function of HAP3a did not affect flowering timing. The role of HAPs in controlling flowering timing is further complicated when a tomato HAP5 promotes flowering when overexpressed in *Arabidopsis* (Ben-Naim et al., 2006). Thus the significance of these HAPs in controlling flowering timing requires further study. Our results are the first to provide clear genetic evidence to show HAP is indeed involved in control of flowering time and HAP3b plays a critical role in regulating flowering timing in the long-day photoperiod pathway.

**Model for HAP3b Mode of Action**

In yeast and animal systems, HAPs form a heterotrimer for transcription activation. Wenkel et al. (2006) showed that HAP3a and HAP5a in *Arabidopsis* were able to interact in vivo. Thus, it is very possible that HAPs in plants work in a similar way in animal and yeast, i.e. by forming a HAP complex during transcription activation. However, a binding of plant HAP complex to the CCAAT element has not been demonstrated. Plant promoters don’t have a consistent location for CCAAT elements (Lotan et al., 1998; Wenkel et al., 2006). In animals, HAPs regulated genes usually have a CCAAT-box located at the -60 to -100 location in the promoter (Mantovani, 1998). Our analysis of the top ~20 HAP3b-affected genes from the array experiment also showed a random pattern of CCAAT distribution in the promoters, even though a majority of these genes have one or two CCAAT boxes within -1 to -500 bp (data not shown). Thus, HAPs in plants may bind to an element or sequence that differs from CCAAT.

Overexpression of HAP3a and HAP3b, two members in the same family, resulted in opposite results in flowering timing control, raising an interesting question of how these different HAP3s achieve an opposite effect. One of the possibilities is that HAP3a and HAP3b form different complexes with their own HAP5 and HAP2 so that the
complexes function differently. Alternatively, HAP3a and HAP3b form of a complex involving the same HAP5 and HAP2, since they both can interact with CO and COL in the yeast-two hybrid system. In this case, a competition of HAP3a with HAP3b for binding CO will decrease the number of CO-HAP3b-containing complexes and delay flowering. Thus, a fine balance of HAP3a and HAP3b will determine the flowering timing in plants, which may represent a novel mechanism in regulating flowering timing in the photoperiod pathway.

In conclusion, our results provide strong genetic evidence supporting a model in which HAP3b play a role in regulating flowering in plants grown under long day conditions (Fig. 5). The promotion of flowering is achieved probably through an interaction with CO or COL proteins. However, an interaction of CO/COL with HAP3b in vivo needs to be demonstrated. Interestingly, HAP3b shows a very similar expression pattern, with one of the highest levels in leaf vascular tissues (Supplemental Fig. 2, also see the Genevestigator database), to CO (An et al., 2004). Since CO is known to activate FT expression mainly in the leaf phloem companion cells (Takada and Goto, 2003; An et al., 2004), the co-localization of CO and HAP3b may be required for the interaction of these two proteins which will further activate expression of FT or other genes identified in Table 1 from the array analysis. It needs to be mentioned that some genes such as At2g20870 and At3g10570 which are downregulated in hap3b in Table 1 are also downregulated in ft mutants, while others such as At2g39330 and At1g12780 which are downregulated in hap3b mutants are not affected in ft mutants (the Genevestigator database). Thus, some of the genes affected by the hap3b mutation in Table 1 are potentially regulated through FT while some may be regulated by HAP3b in a different mechanism. Future work also is needed to address whether or which HAP2/HAP5 are involved in forming a HAP complex with HAP3b to regulate flowering in vivo. Since several environmental stresses up-regulate HAP3b, these results raise a possibility that HAP3b provides a pathway by which abiotic stress response pathways may help promote early flowering.
Methods

Plant Materials and Growth Conditions:

Seeds of Arabidopsis thaliana (Columbia 0 ecotype background, either wild-type, mutant or overexpression transgenic plants) were sown in well-watered potting mix (Enriched Potting Mix, Miracle-Gro Lawn Products, Inc., Marysville, OH), and kept in a cold room (4°C) for two days. Seeds were germinated and seedlings were grown on a light shelf or in a growth chamber under a 16h/8h light/dark cycle, except for the short day photoperiod experiments. Light was supplied by cool-white florescent bulbs, reaching an intensity of approximately 120 µmol m⁻² s⁻¹ on the surface of the shelf. For some experiments, seedlings were cultured in a square Petri dish (10 x 10 x 1.5 cm³) containing 35 mL of sterile solid medium consisting of 0.5X MS salt, 0.5% sucrose, 10 mM MES and 0.6% Phytagel (Sigma, St. Louis, MO) at pH 5.8. Seeds were first surface sterilized and arranged on the surface of the solid medium and were given a cold treatment at 4°C for 48-72 h. Plants were grown in a growth chamber under the conditions described above.

The T-DNA insertion mutant lines (Alonso et al., 2003) of At5g47640, SALK_025666, SALK_105662 and SALK_105664, in the Columbia 0 ecotype background were obtained from the ABRC stock center at Ohio State University. Insertion mutant information was obtained from the SIGnAL website at http://signal.salk.edu and verified by PCR and RT-PCR methods. Only SALK_025666 was confirmed as a true insertional mutant.

Plasmid Constructs and Plant Transformation

The promoter (1.5 kb before 5’UTR) or the transcribed portion including 5’UTR and 3’UTR of HAP3b were PCR-amplified from Arabidopsis genomic DNA separately and cloned into the Zero Blunt PCR Cloning vector (Invitrogen, Carlsbad, CA). All PCR amplifications were carried out with high-fidelity DNA polymerase (PfuUltra DNA polymerase, Stratagene, La Jolla, CA). The sequence of the cloned promoter or transcribed portion was verified by DNA sequencing and subcloned into modified pBI121 binary vectors. For promoter analysis, the promoter was subcloned into pBI121 to drive expression of a uidA or GUS gene (PHAP3b:GUS). For the overexpression
experiments ($P_{\text{actin}}$:HAP3b), the promoter of ACT2 (At3g18780) was used to drive expression of HAP3b cDNA. The ACT2 promoter was also used to drive GUS expression (C1, $P_{\text{actin}}$:GUS) as a control for overexpression plants. For making HAP3b-GFP fusion protein ($P_{35S}$:HAP3b-GFP), HAP3b was cloned with the stop codon removed and fused to GFP in frame at the N-terminus of the GFP protein. The CaMV 35S promoter is used to drive the expression of the fusion protein. Plants were transformed with *Agrobacterium tumefaciens* using the floral dipping method (Clough and Bent, 1998). The transformants were selected on agar plates containing 30 µg/mL basta or kanamycin and verified using PCR with construct-specific primers. All the overexpression plants ($P_{\text{actin}}$:HAP3b, $P_{\text{actin}}$:GUS, $P_{35S}$:HAP3b-GFP) were selected for two more generations and homozygous transgenic plants (T3) were used for further characterization.

**GUS Staining and GFP Localization:**

T1 and T2 transgenic plants carrying the $P_{\text{HAP3b}}$:GUS construct were assayed for GUS color reaction following a method described by Stangeland and Salehian (2002). For HAP3b-GFP localization, roots and leaves of young seedlings were used for examination using a laser scanning confocal microscope (Bio-Rad MRC 1024, BioRad, CA).

**Genomic DNA Extraction and T-DNA Insertional Mutant Screening:**

Homozygous T-DNA insertional mutants were identified by following the protocol described at the SALK Insertion Sequence Database (http://signal.salk.edu/tabout.html) using a PCR method. Leaf tissues of soil-grown seedlings were first collected from individual plants. Genomic DNA was extracted using a quick CTAB method (Rogers and Bendich, 1988) and used for PCR reactions with the primers recommended in the SALK protocol.

**Flowering Time:**

Seeds of wild-type, hap3b mutant, HAP3b-overexpression transgenic plants ($P_{\text{actin}}$:HAP3b) and overexpression control plants (C1: $P_{\text{actin}}$:GUS) were germinated in the
same flat containing well-watered potting mix. After 2-day cold treatment, plants were grown under different conditions until flowering. The rosette leaf numbers were counted after all the plants flowered (Koornneef et al., 1991). For the long-day experiment, plants were grown under a 16h/8h light/dark photoperiod. For the short-day experiment, plants were grown under a 16h/8 dark/light photoperiod. The entire plant of 15-d old seedlings grown in Petri-dishes or leaf tissue of 18-d old soil-grown plants was harvested for gene expression analysis. For gibberellic acid (GA) treatment, the plants were separated into control and GA treatment groups when approximately 4-5 leaves were emerged. Leaf number was counted and the GA treatment plants were sprayed with 20 µM GA twice a week. Five applications of GA were performed (Lim et al., 2004). For vernalization treatment, seeds were germinated on Phytagel plates and kept in a cold room (4°C) in the dark for various amounts of time (30 and 2 days). Seedlings were then taken out, transplanted to soil, and grown under a 16h/8h light/dark photoperiod until flowering (Mockler et al., 2004).

Microarray and Gene Expression:

Seeds of wild-type, hap3b mutant and HAP3b-overexpression transgenic plants were germinated in the same flat containing well-watered potting mix. Leaves of 18-d old plants grown in soil under a 16h/8h light/dark photoperiod were harvested 6 h after lights were on. RNA was extracted using Tri-reagent (Ambion, Austin, TX). The array labeling, hybridization, scanning and initial data processing were conducted as a service by the Center of Integrated BioSystems at Utah State University. A total of five arrays (Affymetrix ATH1 chip, Cat#:900385) were processed: two chips for wild-type plants, two for mutant plants (hap3b) and one for overexpression plants (P_{actin}:HAP3b). RNA used for the chip experiment was from five independent biological samples from two independent experiments. Each sample represented a collection of leaves from 12 plants.

To confirm expression of selected genes from the microarray experiments using a quantitative PCR, seeds of wild-type, hap3b mutant, HAP3b-overexpression transgenic plants and overexpression control plants (C1= P_{actin}:GUS) were germinated in a single MS-Phytagel plate. Fifteen-day-old seedlings were harvested for RNA extraction. A quantitative PCR method was performed by following a method described by Wang et al.
(2003) with the following modifications. Quantitation of the transcript level was first normalized with values from an actin gene (ACT2). The normalized transcript levels in hap3b or HAP3b-overexpression plants were then divided by that of their corresponding wild-type or C1 plants to obtain fold change.

**Primers**

The primers used for cloning HAP3b and the promoter, and for mutant screening and gene expression are listed in supplemental Table III. Most of the gene-specific primers for expression study were located in exons flanking an intron. The resulting PCR products were larger if genomic DNA was present in cDNA samples.

**Statistical Analysis**

All the experiments were performed at least three times. A standard t-test was used to determine significance with a 95% confidence interval. P-values reported are two tailed analyses.
Supplemental Materials

Supplemental Table I. Increase in HAP3b transcript level under stress

The upper half of the table shows the fold increase in transcript level of HAP3b from our previous study (Kreps et al., 2002). The lower half of the table lists the fold increase of HAP3b levels (representing approximately the greatest change in a time course experiment performed by Kudla, Puchta, Bartels, Harter and Nover) under various stresses reported by other groups in the Arabidopsis microarray database (http://www.arabidopsis.org/). Only the treatments that showed at least a two-fold increase in HAP3b transcript level for at least two time-points in the time-course experiment are listed in the table.

| Affy ID /Gene | 300 mM Mannitol 3h, Leaf | 300 mM Mannitol 3h, Root | 150 mM NaCl 3h, Leaf | 150 mM NaCl 3h, Root |
|---------------|--------------------------|--------------------------|----------------------|----------------------|
| At5g47640     | 6                        | 6                        | 8                    | 2                    |
| 20437_at      | 11.92                    | 3.48                     | 7.04                 | 2.64                 |

From Kreps et al., 2002.

| Drought | Osmotic | Salt | Heat | ABA |
|---------|---------|------|------|-----|
| At5g47640 | 6       | 6    | 8    | 2   |
|         |         |      |      | 11  |

From microarray expression database (www.Arabidopsis.org)
Supplemental Table II. Change in transcript level of genes known to be associated with flowering time control in hap3b and HAP3b-overexpression (ox = P_actin:HAP3b) plants grown under normal growth conditions.

The genes that were labeled as absent or marginal signal on arrays are shown in blue.

| Gene    | Affy ID      | Mean signal in wt | Mean signal in hap3b | Fold change (hap3b / wt) | Signal in ox | Fold change (ox / wt) | Gene ID |
|---------|--------------|-------------------|----------------------|--------------------------|--------------|-----------------------|---------|
| FLK     | 258581_at    | 8.05              | 8.30                 | -                        | 7.53         | -                     | At3g04160 |
| TOC1    | 247525_at    | 680.09            | 614.58               | 0.90                     | 673.97       | 0.99                  | At5g61380 |
| HOS1    | 245120_at    | 84.29             | 85.96                | 1.02                     | 81.51        | 0.97                  | At2g39810 |
| HOS9    | 266354_at    | 6.84              | 7.16                 | -                        | 6.49         | -                     | At2g01500 |
| CO      | 246525_at    | 9.07              | 9.07                 | -                        | 9.44         | -                     | At5g15840 |
| VRN1    | 256944_at    | 165.36            | 161.32               | 0.98                     | 147.22       | 0.89                  | At3g18990 |
| VRN2    | 245280_at    | 19.12             | 12.30                | -                        | 24.00        | 1.25                  | At4g16845 |
| FT      | 264638_at    | 6.73              | 6.82                 | -                        | 14.56        | -                     | At1g65480 |
| FLC     | 250476_at    | 49.85             | 58.14                | -                        | 43.88        | -                     | At5g10140 |
| Agebet  | 249210_at    | 71.37             | 67.30                | 0.94                     | 70.23        | 0.98                  | At5g42670 |
| AG      | 254595_at    | 5.94              | 6.97                 | -                        | 5.70         | -                     | At4g18960 |
| AP1     | 259372_at    | 5.56              | 5.34                 | -                        | 13.13        | -                     | At1g69120 |
| AP2     | 246217_at    | 37.00             | 41.34                | -                        | 35.97        | -                     | At4g36920 |
| AP3     | 251898_at    | 4.48              | 4.50                 | -                        | 5.45         | -                     | At3g54340 |
| CLV1    | 262728_at    | 799.78            | 819.52               | 1.02                     | 857.66       | 1.07                  | At1g75820 |
| CLV2    | 264183_at    | 48.64             | 50.33                | 1.03                     | 67.37        | 1.39                  | At1g65380 |
| CLV3    | 265624_at    | 4.22              | 4.23                 | -                        | 4.17         | -                     | At2g27250 |
| FCA     | 245489_at    | 28.41             | 28.41                | -                        | 28.41        | -                     | At4g16280 |
| FLD     | 258944_at    | 20.37             | 20.05                | -                        | 20.37        | -                     | At3g10390 |
| FLM     | 264949_at    | 17.28             | 16.29                | -                        | 16.62        | -                     | At1g77080 |
| FRI     | 255634_at    | 13.18             | 10.18                | -                        | 10.21        | -                     | At4g00650 |
| FVE/ACG | 265946_s_at  | 600.03            | 690.30               | 1.15                     | 756.17       | 1.26                  | At2g19520 |
| FWA     | 245239_at    | 5.39              | 5.27                 | -                        | 5.19         | -                     | At4g25530 |
| FY      | 245848_at    | 51.81             | 49.44                | 0.95                     | 46.45        | 0.90                  | At5g13480 |
| HEN4    | 247276_at    | 33.19             | 30.46                | 0.92                     | 32.34        | 0.97                  | At5g64390 |
| HAU1    | 257695_at    | 119.19            | 104.52               | 0.88                     | 98.00        | 0.82                  | At3g12680 |
| HAU2    | 249877_at    | 44.99             | 46.34                | 1.03                     | 44.99        | 1.00                  | At5g23150 |
| LD      | 255444_at    | 26.86             | 24.47                | 0.91                     | 24.47        | 0.91                  | At4g02560 |
| LEY     | 247490_at    | 4.37              | 4.36                 | -                        | 4.37         | -                     | At5g61850 |
| PI      | 246072_at    | 7.16              | 6.32                 | -                        | 17.47        | -                     | At5g20240 |
| SU      | 257915_at    | 6.93              | 6.97                 | -                        | 6.93         | -                     | At3g23130 |
| TFL1    | 250869_at    | 4.50              | 4.55                 | -                        | 5.63         | -                     | At5g03840 |
| TFL2    | 250060_at    | 55.40             | 55.25                | 1.00                     | 55.25        | 1.00                  | At5g17690 |
| AGL24   | 254130_at    | 56.09             | 53.40                | 0.95                     | 56.53        | 1.01                  | At4g24540 |
| APRR3   | 247668_at    | 51.44             | 51.44                | 1.00                     | 51.28        | 1.00                  | At5g60100 |
| APRR5   | 249741_at    | 438.84            | 460.16               | 1.05                     | 455.20       | 1.04                  | At5g24470 |
| APRR7   | 250971_at    | 123.41            | 90.27                | 0.73                     | 108.29       | 0.88                  | At5g02810 |
| APRR9   | 266720_s_at  | 8.13              | 8.13                 | -                        | 8.13         | -                     | At2g46790 |
| Gene  | Accession | Time 1 | Time 2 | Ratio 1 | Ratio 2 | Accession  |
|-------|-----------|--------|--------|---------|---------|------------|
| CCA1  | 266719_at | 11.50  | 11.20  | -       | 11.54   | -          | At2g46830 |
| GI    | 264211_at | 687.24 | 720.71 | 1.05    | 685.67  | 1.00       | At1g22770 |
| LHY   | 261569_at | 8.83   | 9.28   | -       | 8.97    | -          | At1g01060 |
| RVE2  | 249606_at | 45.36  | 76.12  | 1.68    | 89.47   | 1.97       | At5g37260 |
| PFT1  | 255725_at | 51.49  | 49.42  | 0.96    | 49.61   | 0.96       | At1g25540 |
### Supplemental Table III. Primers for cloning and gene expression

| Primer Sequences (5’-3’) | Applications |
|--------------------------|--------------|
| GGCGCGCCAAGGTGTGGAATCGCATGG | 5’-upstream, to clone HAP3b promoter |
| TTAATTAATATTCTGAAAATTACAAAGGAATAAAAAA | 3’-downstream, to clone HAP3b promoter |
| GGCGCGCCATGGGGGATTCCGACA | 5’-upstream, to clone HAP3b for HAP3b-GFP |
| GGCGCGCCCCCATGGGGGATTCCGACA | 3’-downstream, to clone HAP3b for HAP3b-GFP |
| TTTAATTTAAATGCGGGATTCCGACA | 5’-upstream, to clone HAP3b for overexpression |
| TGGCGGCGCTTAAATGTCCGTCTACC | 3’-downstream, to clone HAP3b for overexpression |
| GCCACTTCTTCTTCTTGCTGTTACT | TOC1-5 for RT-qPCR |
| TATTGCTCGTGCTCCCTCTCTTC | TOC1-3, for RT-qPCR |
| CTGGGCGATCTAAGGATCGACG | SOC1-5, for RT-qPCR |
| GAACAAGGTAAACCCCAATGAA | SOC1-3, for RT-qPCR |
| AGACGTTCCTTGATCCGTATTA | FT-5, for RT-qPCR |
| GTGATATCTCAGCAAAACTTCGC | FT-3, for RT-qPCR |
| TTCCCCACTTAACTCAACCCCAAA | HAP3b-5, for RT-qPCR of wt and mutant |
| CTTCCCCTCTCTCCCTCAACCT | HAP3b-3, for RT-qPCR of wt and mutant |
| CTTGGATCGATTGGAACACG | FLC-5, for RT-qPCR |
| CTAGTCACGGAGAGGGCAGTC | FLC-3, for RT-qPCR |
| AGGAGGTTGCTTGCTGCTTGTC | CO-5, for RT-qPCR |
| CTTTGGGCGTTTCTTGGTGTA | CO-3, for RT-qPCR |
| GTGTCACCACCTGAAAGGAAG | ACT2-5, (At3g18780) for RT-qPCR |
| CAATGGGACTAAAAACGCAAAA | ACT2-3, (At3g18780) for RT-qPCR |
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Figure Legends

**Figure 1.** Delayed flowering in hap3b mutants and early flowering in HAP3b-overexpression plants grown under a 16h/8h light/dark photoperiod. A. delayed flowering in hap3b; B. hap3b has an a T-DNA insertion at 9 bp after the first ATG; C. no transcript was detected in hap3b using RT-PCR using two independent plants; there is no intron in HAP3b, and the PCR products are the same when using genomic DNA as a template (gDNA); D. hap3b developed more leaves before flowering compared with wild-type plants (wt); overexpression plants (Pactin:HAP3b in wild-type background) flowered earlier with less leaves compared with control plants (C1: Pactin:GUS in wild-type background). The data are means ± SE (n ≈ 30) from three independent experiments. ** indicates p < 0.001 compared with wt.

**Figure 2.** Localization of HAP3b in nuclei using GFP as a reporter gene: leaf epidermal cells (A) and root tip cells (B).

**Figure 3.** Flower timing assays showing that HAP3b function is restricted to modifying a long day photoperiod flowering timing pathway. Panels A and B show that the hap3b mutants delay in flowering (under long–day conditions) is not reversed by GA (panel A) or vernalization (panel B), whereas mutant hap3b and wild-type plants grown under a short day photoperiod show no differences. * indicates p < 0.01 and ** indicates p < 0.001 compared with minus GA control (panel A) or minus vernalization control (panel B) or wt (panel C).

**Figure 4.** SOC1 (A) and FT (B) genes were down-regulated in hap3b but up-regulated in HAP3b-overexpression plants. The transcript level of each gene was determined using a qPCR method and was then normalized with the transcript level of ACT2. The fold change was calculated by dividing the normalized transcript level of hap3b by that of wild-type plants (wt) or the normalized transcript level of overexpression plants (Pactin:HAP3b) by that of C1 (Pactin:GUS) plants. The data are means ± SE of three independent experiments. The pattern was reproducible in each experiment.
Figure 5. A proposed model for how HAP3b expression can promote early flowering

Supplemental Figure 1. Altered flowering time in hap3b and promotion of flowering in hap3b plants that were transformed with P35S:HAP3b-GFP construct. Hap3b developed more leaves before flowering compared with wild-type plants (wt); overexpression of a HAP3b-GFP fusion protein (P35S:HAP3b-GFP) in hap3b reversed the mutant phenotype and resulted in earlier flowering than wt plants under long day photoperiod conditions. The data are means ± SE (n ≈ 30) from three independent experiments. * indicates p < 0.01 and ** indicates p < 0.001 compared with wt.

Supplemental Figure 2. Tissue localization of HAP3b expression. Expression pattern of HAP3b in Arabidopsis using a GUS reporter gene: leaves at different stages (A), leaf veins (B), trichomes (C), flowers, cauline leaf and silique (D), and sepals and filaments (E).

Supplemental Figure 3. Change in transcript level of TOC1 (A), CO (B), and FLC (C) genes in hap3b and overexpression plants. The transcript level of each gene was determined using a qPCR method and was then normalized with the transcript level of ACT2. The fold change was calculated by dividing the normalized transcript level of hap3b by that of wild-type plants (wt) or the normalized transcript level of overexpression plants (Pactin:HAP3b) by that of C1 (Pactin:GUS) plants. The data are means ± SE of four independent experiments.
Table I. Genes that were affected in \textit{hap3b} and \textit{HAP3b}-overexpression plants

The mean signals of two arrays for wild-type plants (wt), two for mutant plants (\textit{hap3b}), or one for overexpression plants (\textit{ox} = \textit{P}_{\text{actin}:HAP3b}) were listed in the table and used for calculation of fold change. The genes listed in the table were all labeled as “present” on the arrays, except for \textit{HAP3b} gene whose transcript was not detected in both \textit{hap3b} samples (or arrays), and showed at least 25\% change in transcript level in both \textit{hap3b} and overexpression plants. “^A” indicates the signal on arrays is labeled as “Absent”.

Table I: Genes upregulated in *HAP3b*-overexpression plants but downregulated in *hap3b* and quantification of their RNA levels.

| Affy ID  | Mean signal in wt | Mean signal in hap3b | Fold change (hap3b / wt) | Signal in ox | Fold change (ox / wt) | Gene ID   | Annotation                                      |
|----------|-------------------|----------------------|-------------------------|--------------|-----------------------|-----------|-----------------------------------------------|
| 248764_at| 355.04            | 32.91^A              | 0.00                    | 13652.40     | 38.45                 | At5g47640 | HAP3b or CCAAT-binding protein                 |
| 265441_at| 185.77            | 37.47                | 0.20                    | 1071.70      | 5.77                  | At2g20870 | cell wall protein precursor                    |
| 256597_at| 224.29            | 85.86                | 0.38                    | 720.43       | 3.21                  | At3g28500 | 60S acidic ribosomal protein P2 (RPP2C)       |
| 249645_at| 167.81            | 100.60               | 0.60                    | 758.15       | 4.52                  | At5g36910 | thionin (THI2.2)                              |
| 261375_at| 104.63            | 65.80                | 0.63                    | 144.91       | 1.38                  | At1g53160 | squamosa promoter-binding protein-like 4 (SPL4) |
| 266989_at| 47.55             | 30.94                | 0.65                    | 62.98        | 1.32                  | At2g39330 | jacalin lectin family protein                 |
| 258962_at| 84.52             | 55.09                | 0.65                    | 126.06       | 1.49                  | At3g10570 | cytochrome P450 putative                      |
| 245928_s_at| 2558.78         | 1674.04              | 0.65                    | 5327.26      | 2.08                  | At5g24780 | vegetative storage protein 1 (VSP1)           |
|          |                   |                      |                         |              |                       |           | vegetative storage protein 2 (VSP2)           |
| 246396_at| 66.10             | 43.98                | 0.67                    | 96.49        | 1.46                  | At1g58180 | carbonic anhydrase family protein             |
|          |                   |                      |                         |              |                       |           | carbonate dehydratase family protein          |
| 267509_at| 56.24             | 37.80                | 0.67                    | 98.72        | 1.76                  | At2g45660 | MADS-box protein (AGL20) = SOC1               |
| 254573_at| 79.43             | 55.78                | 0.70                    | 113.39       | 1.43                  | At4g19420 | pectinacetyltransferase family protein        |
| 261211_at| 339.79            | 238.71               | 0.70                    | 434.96       | 1.28                  | At1g12780 | UDP-glucose 4-epimerase / UDP-
|          |                   |                      |                         |              |                       |           | galactose 4-epimerase / Galactowaldenase      |
| 246687_at| 111.23            | 78.28                | 0.70                    | 205.75       | 1.85                  | At5g33370 | GDSL-motif lipase/hydrolase family protein    |
| 264146_at| 1023.99           | 769.14               | 0.75                    | 1383.38      | 1.35                  | At1g02205 | CER1 protein;                                 |
| 261601_at| 90.02             | 67.81                | 0.75                    | 124.87       | 1.39                  | At1g49670 | ARP protein, oxidoreductase, dehydrogenase    |
| 267460_at| 78.53             | 59.20                | 0.75                    | 117.37       | 1.49                  | At2g33810 | squamosa promoter-binding protein-like 3 (SPL3) |
**A** Wild-type and hap3b plants.

**B** Schematic of the T-DNA insertion site.

**C** Gel electrophoresis showing the expression of the HAP3b gene and AtACT2 control.

**D** Bar graph showing leaf number at flowering:
- wt
- hap3b
- C1
- P actin:HAP3b

Leaf Number at Flowering:
- wt: 12 ± 1
- hap3b: 16 ± 2
- C1: 14 ± 2
- P actin:HAP3b: 8 ± 1

**Note:** At least two biological replicates were performed. **p < 0.01**.
Figure 2
Leaf Number at Flowering

**Figure legend**

(A) C1 P actin:HAP3b

(B) 2d 4 °C

(C) 8h/16h light/dark

* and ** indicate statistical significance compared to the control.
Figure 4

A. *SOC1*

B. *FT*
Figure 5

Long day pathway

CO

HAP2?

HAP3b

HAP5?

FT

SOC1

Vegetative

Reproductive