PHYSIOLOGICAL AND MOLECULAR FUNCTIONS
OF HAP3B IN FLOWERING TIME REGULATION
AND COLD STRESS RESPONSE

by

Mingxiang Liang

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of the requirements for the degree

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In

Plant Science

Approved:

___________________  ___________________
Dr. David Hole    Dr. Yajun Wu
Major Professor   Research Advisor

___________________  ___________________
Dr. Daryl B. DeWald Dr. Jennifer MacAdam
Committee member   Committee member

___________________  ___________________
Dr. John Carman    Dr. Byron R. Burnham
Committee member   Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

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ABSTRACT

Physiological and Molecular Function of HAP3b in Flowering Time Regulation and Cold Stress Response

by

Mingxiang Liang, Doctor of Philosophy
Utah State University, 2010

Major Professor: Dr. David Hole
Department: Plants, Soils and Climate

Heme-activated proteins (HAPs) are transcription factors that have multiple roles in plant growth and development, such as embryogenesis, flowering time control, and drought tolerance.

In the present study I found that HAP3b was also involved in controlling response to cold stress. Transcript profiling and gene expression analyses indicated that HAP3b repressed the CBF3 regulon under normal growth conditions. As a result, plants with HAP3b-overexpressed showed decreased survival rates while plants homozygous for the null allele hap3b showed an improved freezing tolerance compared to wild-type plants.

To understand the mechanism of HAP3b in Arabidopsis, i.e. whether it also acts through forming a heterotrimer, the yeast two-hybrid system and the protein coimmunoprecipitation method were used to identify the proteins that could interact
with HAP3b. From yeast two-hybrid analyses, it was found that HAP3b could interact with one (At3g14020) of ten HAP2s and all ten HAP5s tested. Further analyses showed that the newly identified HAP2 protein could only interact with two HAP5 proteins, those encoded by \textit{At5g63470} and \textit{At1g56170}.

To address whether HAPs also play important roles in major crop plants, \textit{HAP3} genes in barley (\textit{Hordeum vulgare} L.) were identified and characterized. From database sequence analyses, cloning, and sequencing, it was found that barley plants have at least six full-length members in the \textit{HAP3} family. Phylogenetic analyses showed that each barley HAP3 was different, forming its own cluster with the HAP3s from other plant species. Each barley \textit{HAP3} also showed its own expression pattern in different tissues, at different developmental stages and under various environmental stresses. In particular, \textit{TC176294} showed the highest sequence similarity to \textit{HAP3b} in \textit{Arabidopsis} and its high expression was associated with flowering. In addition, \textit{TC176294} was upregulated by various abiotic stresses and by abscisic acid (ABA). Thus, \textit{TC176294} might be a barley ortholog of \textit{HAP3b}. \textit{TC191694} showed the highest sequence similarity to \textit{HAP3c} and might be a barley ortholog of \textit{HAP3c}. \textit{TC191694} overexpression plants were early flowering compared to \textit{HAP3b}-overexpression and wild-type plants while overexpression of \textit{TC176294} plants were not.
I would first like to give my sincere thanks to my research advisor, Dr. Yajun Wu. I am really impressed by his work ethic and great passion for science and research. I greatly appreciated his financial support, his patient edification, and his personal exemplary guidance. I am fortunate to have had the opportunity to learn from him both research skills and life attitude. I am so grateful to Dr. David Hole for his financial support and his help in my research projects, especially with the barley project. Great thanks also to my committee members, Dr. Jennifer MacAdam, Dr. Daryll DeWald, and Dr. John Carman, who spent so much time reviewing my proposal and dissertation and providing necessary guidance for my research program. I am grateful to Ying Qian and Elizabeth Davis for their instruction and help in the lab. Special thanks to Jenny Ballif for her careful editing and proofreading of my dissertation and to Chris Parry for help with mutant screening. I would like to thank Xiaoning Cai, Qingsong Zheng, Peizhi Yang, and Hongmei Zheng for their great company. I also would like to give my great appreciation to my wife, Li Xu, for her constant encouragement and motivation. I also thank Dr. Jeanette Norton for supporting my wife as a visiting scientist, making her stay with me possible. Finally, I owe so much to my parents who gave their selfless love and comfort to their son no matter how far it was.

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UAS  UPSTREAM ACTIVATION SITE
VIP  VERNALIZATION INDEPENDENCE GENES
VRN1  VERNALIZATION 1
VRN2  VERNALIZATION 2
VIN3  VERNALIZATION INSENSITIVE 3
WSOC1  SOC1 ortholog in wheat
WT  wild-type

Capitalized abbreviations refer to the protein (ex. SIF), capitalized italicized abbreviations refer to the gene or non-protein gene product (ex. SIF), and lowercase italicized abbreviations refer to the mutant (ex. sif).
CHAPTER 1  
INTRODUCTION

Heme-Activated Protein (HAP), also known as nuclear factor Y (NF-Y) or CCAAT-Binding Factor (CBF), was first identified from yeast because mutations in either gene (HAP2 or HAP3) blocked expression of mitochondrial proteins (such as CYC1, encoding iso-1-cytochrome c) and prevented growth on lactate medium (Pinkham and Guarente, 1985; Hahn and Guarente, 1988). The CYC1 promoter is comprised of two upstream activation sites (UASs). One of which (UAS2) contains an inverted CCAAT motif that is required for transcription. Activation of transcription from UAS2 requires both HAP2 and HAP3 (Pinkham and Guarente, 1985; Pinkham et al., 1987; Hahn and Guarente, 1988), which form a CCAAT-box-binding complex. McNabb et al. (1995) identified another component, HAP5, in the CCAAT-binding complex. HAP5 is required for the assembly and DNA-binding activity of the complex (McNabb et al., 1995). In a hap5 mutant, CCAAT-binding activity of the complex is missing in an in vitro analysis. Furthermore, purified recombinant HAP2, HAP3, and HAP5 are able to reconstruct CCAAT-binding activity through mobility shift analysis. Another subunit of the complex, HAP4, exists in yeast, which contains an acidic domain that strongly activates transcription (Forsburg and Guarente, 1989). In a strain with a deletion in the HAP4 gene, the CCAAT box is not activated. However, by transferring the HAP2-GAL4 fusion vector, a hap4 could partially grow on lactate (Olesen and Guarente, 1990), indicating that HAP4 might not be essential for the binding of HAP2/HAP3/HAP5 to CCAAT. All these data suggest that the
HAP2/HAP3/HAP5 complex represents a DNA-binding factor in which all three subunits are required for downstream gene activation.

HAPs have been shown to be functionally conserved over evolution. Clones of HAP counterparts have been isolated from yeast (Olesen et al., 1991), plant (Edwards et al., 1998; Thirumurugan et al., 2008), mouse (Vanhuijsduijnen et al., 1990), rat (Maity et al., 1990), and human (Becker et al., 1991) sources. While the CCAAT box occurs commonly in eukaryotic promoters, among the various DNA interacting proteins that bind to this box, only HAP2/HAP3/HAP5 has been shown to require all 5 nucleotides (Mantovani, 1998). There were some exceptions such as von Willebrand factor in humans where NF-Y interacted not only with the CCAAT element but also the CCGNNNCC sequence as an activator and a repressor (Peng and Jahroudi, 2002).

HAP genes in plants are involved in embryo development (Lotan et al., 1998; Kwong et al., 2003), chloroplast biogenesis (Miyoshi et al., 2003; Nelson et al., 2007), nodule development (Combier et al., 2006), stress response (Kreps et al., 2002; Li et al., 2008), root elongation (Ballif, 2007), and flowering regulation (Cai et al., 2007; Kumimoto et al., 2008). In contrast to the situation in yeast and animals, where each subunit is encoded by a single gene, multiple genes exist for each of the HAP2, HAP3, and HAP5 subunits in plants, providing the potential for multiple alternative forms of HAP complexes in plants (Edwards et al., 1998; Ito et al., 2005). In Arabidopsis there are at least 10 annotated members in each HAP family (Gusmaroli et al., 2001, 2002; Siefers et al., 2009). In rice, there are at least 10 HAP2 genes, 11 HAP3 genes and 7
$HAP5$ genes (Thirumurugan et al., 2008). HAP4 genes have not been identified in the plant kingdom. More gene members in the same family could indicate gene redundancy or function differentiations.

Concerning flowering, Ben-Naim et al. (2006) reported that overexpression of a tomato $HAP5$ in $Arabidopsis$ caused early flowering. In contrast, flowering was delayed by overexpression of a $HAP2a$ (At5g12840) or a $HAP3a$ (At2g38880) in $Arabidopsis$ (Wenkel et al., 2006). Flowering time in $hap2a$ and $hap3a$ mutants, however, was not affected. Cai et al. (2007) reported that overexpression of $Arabidopsis$ $HAP3b$ (At5g47640) promoted early flowering while $hap3b$, a null mutant of $HAP3b$, resulted in delayed flowering under a long-day photoperiod but not under short-day conditions. This suggests that $HAP3b$ might normally be involved in the long-day photoperiod-regulated flowering pathway. NF-YB3 (HAP3c, At4g14540), the most closely related $Arabidopsis$ protein to HAP3b, shares similar activities with HAP3b. Both HAP3b and HAP3c are necessary and sufficient for the promotion of flowering in response to inductive photoperiodic long-day conditions. This is supported by the fact that double mutant $hap3b$ $hap3c$ showed significant delay in flowering time compared to either single mutant. HAP3b and HAP3c probably regulate flowering time by the direct activation of the key floral regulator Flowering Locus T (FT) (Kumimoto et al., 2008).

It is unclear as to how different HAPs achieve different physiological functions in plants or whether they forms a heterotrimer as they do during transcription activation. The $LEAFY COTYLEDON1$ ($LEC1$) was the first $HAP3$ cloned and studied
in plants (Yamamoto et al., 2009). It controls fatty acid biosynthesis during embryo development (Mu et al., 2008). A recent study showed LEC1 could recruit bZIP67, an ABA-response element binding factor, to form a complex to activate *CRUCIFERIN C* and control seed development (Yamamoto et al., 2009). In another study, LEC1 or LEC1-like was found to function with NF-YA5 (At1g54160) or NF-YC4 (At5g63470) *in vitro* to mediate the blue light or ABA response (Warpeha et al., 2007). Thus, it appears that HAP may form a heterotrimer only during certain activities.

In HAP-mediated flowering time control, Wenkel et al. (2006) showed that HAP3a and HAP5a in *Arabidopsis* were able to interact *in vivo*. They also showed that CONSTANS (CO) proteins could interact with HAP3a and HAP5a *in vitro*. Since CO share some sequence similarity to HAP2, it was thus postulated that HAPs also regulate flowering time through formation of a heterotrimer complex.

It is not known whether HAP3b promotes flowering under long day conditions through a similar mechanism as HAP3a, i.e. by forming a heterotrimer, and why overexpression of *HAP3a* and *HAP3b* resulted in opposite results, one delaying flowering and the other promoting flowering. There are two main hypotheses: One is that HAP3a and HAP3b may compete in the same trimer complex. HAP3b in the complex would promote flowering and replacement of HAP3b with HAP3a would delay flowering. The other hypothesis is that HAP3a and HAP3b may form different complexes with their own HAP5 and HAP2 so that the complexes function differently; HAP3a and HAP3b may both interact with CO and COL (CONSTANS-Like) and compete for binding CO which would decrease the number of CO-HAP3b-containing
complexes and delay flowering. Thus, the ratio of HAP3a-CO and HAP3b-CO would determine the timing of flowering in plants, which may represent a novel mechanism in regulating flowering timing in the photoperiod pathway. To distinguish these two hypotheses, identification of proteins that can interact with HAP3b is required.

1.1 *Arabidopsis* Flowering Pathways

The developmental transition from vegetative to reproductive growth is essential for successful reproduction and requires the proper integration of external stimuli such as day length or low temperature and endogenous signals. In terms of *Arabidopsis* floral transition, environmental control is mostly modulated by photoperiod and vernalization pathways, whereas endogenous stimuli are regulated by the autonomous and gibberellic acid (GA) pathways (Simpson and Dean, 2002).

1.1.1 GA pathway

GAs (gibberellic acid or gibberellin) are diterpenoid hormones found in plants, fungi, and bacteria. At least 136 naturally occurring GAs have been identified. Endogenous GAs can influence a large number of developmental processes. Gibberellins can stimulate stem growth, especially in rosette species (such as *Arabidopsis*) and in the Poaceae (grass) family. Rosette species are plants in which the first-formed internodes do not elongate under certain growing conditions. This results in a compact cluster or rosette of leaves. Gibberellins also promote seed germination and regulate the transition from young to adult phase, i.e. floral initiation. GAs can bypass the long-day requirement for flowering in many plants, especially rosette species. GA is first recognized by a receptor GIBBERELLIN INSENSITIVE
DWARF1 (GID 1) which forms a complex that inactivates DELLA protein, a protein that normally inhibits plant growth (Harberd et al., 2009). GA can also induce flowering by bypassing \textit{FT} and \textit{FLC (FLOWERING LOCUS C)} to activate expression of \textit{LFY (LEAFY)} and \textit{SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS)} (Blazquez et al., 1998; Moon et al., 2003). The activation of \textit{LFY} by GA is mediated by the transcription factor GAMYB, which is also negatively regulated by DELLA proteins (Gocal et al., 2001). In addition, GAMYB levels are also modulated by a DELLA-dependent microRNA (MiR159) that promotes the degradation of the \textit{GAMYB}-like transcripts (Achard et al., 2004).

\subsection*{1.1.2 Autonomous pathway}

Plants that do not require a particular day-length or hormone or vernalization treatment to start flowering are called autonomous flowering. The autonomous pathway may contain inhibition or induction pathways. Inhibition of the flowering time is primarily achieved through the overall regulation of \textit{FLC}. Any upstream gene that induces \textit{FLC} could result in late flowering. Dominant \textit{FRIGIDA (FRI)} is such a case. FRI promotes \textit{FLC} expression by enhancing \textit{FLC} transcription and splicing, resulting in delayed flowering (Geraldo et al., 2009). Other genes including \textit{FRIGIDA LIKE 1 (FRL1)} (Michaels et al., 2004), \textit{EARLY IN SHORT DAYS (ESD1)} (Gomez-Mena et al., 2001), \textit{PHOTOPERIOD INDEPENDENT EARLY FLOWERING (PIE1)} (Noh and Amasino, 2003) and \textit{VERNALIZATION INDEPENDENCE GENES (VIP)} (Zhang and Van Nocker, 2002) can also repress flowering by promoting \textit{FLC} expression.
Other genes that inhibit FLC and are excluded from other flowering pathways are classified in the autonomous induction pathway. The proteins encoded by these genes promote flowering. These genes include FCA (Macknight et al., 1997); FY, a homolog of the yeast RNA 3’ processing factor Pfs2p (Simpson et al., 2003); FPA (Schomburg et al., 2001), FLK (Lim et al., 2004); FLD, a homolog of the human lysine-specific demethylase 1 (He et al., 2003); FVE (Ausin et al., 2004) and LUMINIDEPENDENS (LD) (Lee et al., 1994) and others. FCA and FLD transcriptionally silence FLC through dimethylation (Liu et al., 2007) while FVE represses FLC transcription through histone deacetylation (Ausin et al., 2004).

1.1.3 Photoperiod pathway

Under the photoperiod flowering pathway, plant photoreceptors first perceive light (wavelength, duration, direction, and intensity). Three known class of receptors are phytochromes, cryptochromes and phototropins. Phytochromes, responsible for sensing red and far-red light, usually form the most important part of photomorphogenesis. Cryptochromes sense UV-light/blue light and phototropins respond to blue light. The interaction of the cryptochromes, phytochromes and phototropins ensures that all the different light regimes are identified. How phytochromes transduce the light signal to a physiological reaction is elusive. Activated phytochromes may cause protein translocation, transcription regulation, protein stability and protein phosphorylation (Han et al., 2007). Many downstream proteins interacting with phytochromes have been identified, such as PIF3, NDPK2 and PKS1. Another downstream gene CONSTITUTIVE PHOTOMORPHOGENESIS
1 (COP1) may operate under the downstream action of both phytochrome and cryptochromes (Subramanian et al., 2004). The COP1 protein functions as an E3 ubiquitin-protein ligase. The role of COP1 is to target proteins for degradation via ubiquitin, and other components are involved in actual protein degradation. Some reports have suggested that proteins similar to HY5 may be the direct targets of COP1-regulated degradation (Osterlund et al., 2000). Other than perceiving the quality of daylight, photoperiodic induction of flowering depends on the ability of plants to measure time. The *Arabidopsis* circadian clock is based on the activities of three main proteins: CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY) and TIMING OF CAB EXPRESSION 1 (TOC1). TOC1 upregulates expression of *LHY* and *CCA1* indirectly. The LHY and CCA1 protein have been shown to bind directly to the promoter of the *TOC1* gene, where they repress its transcription. Of course, other components in the circadian clock are continually identified (Long et al., 2008; McClung, 2009).

PSUEDO-RESPONSE REGULATORs (PRR), TOC1-like genes, might be involved in this central oscillator (Jones, 2009). Although photoreceptors have a key role in entraining circadian rhythm, the mechanism remains unclear. EARLY FLOWERING 3 and 4 (ELF 3/4) or XAP5 CIRCADIAN TIMEKEEPER may be good candidates to link light with the circadian oscillator (Jones, 2009). It is now believed that signals from the circadian clock and the phytochromes are integrated by the *GIGANTEA* (*GI*) and *CONSTANS* (*CO*) genes. *CO* expression is regulated by the endogenous circadian clock on a roughly 24-hour cycle that peaks during the night. The CO protein directly
causes floral induction by activating expression of FT (Putterill et al., 1995). CO activates SOC1 through FT (Yoo et al., 2005). FT and SOC1 are integrators of four major known flowering pathways (Moon et al., 2003). FT protein is a long distance “florigen” in Arabidopsis flowering (Jaeger and Wigge, 2007). The GI gene acts upstream of CO and is important for promoting CO transcription (Fowler et al., 1999). GI forms a complex with FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) to bind the promoter of CO to regulate its expression (Sawa et al., 2007). GI also affects the CCA1 and LHY genes, which are components of the circadian clock in Arabidopsis that regulate CO expression (Fowler et al., 1999). EARLY FLOWERING 3 (ELF3) also acts upstream of CO, but as a negative regulator, ensuring CO transcript levels are not too high (Hicks et al., 1996). Degradation of CO was also caused by SUPPRESSOR OF PHYAs (SPA). One member of SPA, SPA1, could also interact with COP1 (Laubinger et al., 2006).

Several other genes, such as recently identified stress-responsive HAP3b, also function in the photoperiod pathway but the position of this player in this hierarchy is still not clear (Cai et al., 2007). Interestingly, HAP3c, which shows the greatest similarity to HAP3b in the Arabidopsis HAP3 gene family, has an additive effect with HAP3b in promoting flowering. This indicates that HAP3b and HAP3c play an important role in controlling flowering onset in the long-day photoperiod pathway (Kumimoto et al., 2008). HAP3b and HAP3c in Arabidopsis promoted flowering by increasing the transcript levels of FT but the mechanism behind this is elusive. HAP3b and HAP3c are two of the 13 HAP3 members in Arabidopsis (Siefers et al.,
2009).

1.1.4 Vernalization pathway

In the vernalization pathway of floral induction, plants must perceive low temperature and ‘remember’ that perception in order to induce flowering. Low temperatures are perceived by cells in the shoot and root apical meristem. The VERNALIZATION (VRN) proteins are involved in the memory aspect of vernalization. VRN1 protein binds *FLC in vitro*. VRN1 is needed for methylation in *FLC* to stably repress its expression and VRN2 functions by inhibiting the expression of the *FLC* gene after a cold treatment (Levy et al., 2002; Yan et al., 2004).

VERNALIZATION INSENSITIVE 3 (VIN3) transiently repressed *FLC* transcript levels by histone deacetylation (Sung and Amasino, 2004). LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) is also required to maintain repression of *FLC* after exposure to cold (Mylne et al., 2006). Other proteins, such as AGL19, induce expression of floral meristem identity gene *SOC1*, bypassing both FLC and the flowering time integrators FT (Schonrock et al., 2006). *AGL24* is positively regulated by vernalization but not by FLC, which demonstrates an FLC-independent pathway (Michaels et al., 2003). Overexpression of *AGL24* induced *SOC1* expression and *vice versa*.

1.2 Cold Stress Signaling Pathways

Low temperature stress, such as chilling and freezing, is one of the major abiotic stresses that has a direct negative impact on agricultural production. Research with the model plant *Arabidopsis* has revealed that plants have developed complicated
mechanisms to fine-tune gene regulation under cold stress so as tolerate stress. During cold treatment a number of genes are induced, including C-repeat binding factor CBF/DRE-binding proteins (DREBs) transcription factors and their effector genes like *RD29A* (or *COR78* or *LTI78*), *KIN1*, *KIN2* (or *COR6.6*), *COR15A*, and *COR47* (or *RD17*) (Gilmour et al., 1998; Thomashow, 1999). *CBF/DREB1* genes were rapidly induced (within 15 minutes) by cold stress and subsequently activated the expression of their target genes (Liu et al., 1998). CBFs are now known as some of the major components of the cold stress signaling pathway. Overexpression of *CBF/DREB1* genes in plants was demonstrated to consistently improve freezing resistance even in the absence of a cold acclimation treatment (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000).

CBFs comprise a multigene family and are regulated in a complex manner. For the CBF3 pathway, ICE1 is so far the most upstream transcription factor. ICE1 positively regulates *CBF3* expression (Chinnusamy et al., 2003). ICE1 itself is regulated by sumoylation by SIZ1 and ubiquination by HOS1 (Dong et al., 2006; Miura et al., 2007). ICE2 plays a similar role by activating *CBF1* expression as part of the cold response (Fursova et al., 2009). MYB15, ZAT12 and FVE are negative regulators of the CBF pathways (Chinnusamy et al., 2003; Kim et al., 2004; Vogel et al., 2005; Agarwal et al., 2006). MYB15, a R2R3-MYB protein, binds to the promoters of *CBF1*, *CBF2*, and *CBF3* to negatively control expression of *CBF* genes. It was also shown that the *Arabidopsis* ZAT12, a zinc-finger protein, plays a role in cold stress response (Vogel et al., 2005). Transcript levels of *CBF1-3* decreased
rapidly in ZAT12-overexpressing plants compared to wild-type plants in response to cold. The downstream genes such as COR78 and COR6.6, however, were only slightly lower in the overexpression plants. In five plants, the expression level of CBFs and downstream regulons, such as, COR15A and COR47, were higher than wild type plants which implies that this protein, a putative retinoblastoma-associated protein, negatively regulates this cold signaling pathway (Kim et al., 2004).

Several lines of evidence suggested the existence of additional signal pathways or CBF-independent pathways involved in regulation of the low temperature response in plants. For example, esk1, a cold tolerant mutant in Arabidopsis, accumulated high levels of proline but did not show increased expression of cold-regulated genes in the CBF regulon (Xin and Browse, 1998; Xin et al., 2007). HOS9 and HOS10, homeodomain transcription factors, are other examples. Cold treatment quickly induced the COR15A and KIN1 but expression of CBFs were not changed in hos9 and hos10 mutant plants (Zhu et al., 2004). In addition, CBFs were induced in the ada2b mutants as in wild-type plants under low temperature, but transcription of COR genes were reduced in this mutant in cold acclimation suggesting the existence of CBF-independent freezing tolerance (Viachonasios et al., 2003).

1.3 Cross-talk Between the Flowering Pathways and Cold Stress

More and more evidence shows that the floral promotion pathways closely interact with plant cold response mechanisms. For example, in the autonomous pathway, mutating FVE, a putative retinoblastoma-associated protein, causes a delay
in flowering and enhances the cold response (Kim et al., 2004). Transcript levels of 
COR15a and COR47 were much higher in mutants than in wild-type plants.

CBF/DREB1 transcripts in response to cold in wild-type plants and mutant plants 
were similar but CBF/DREB1 expression occurred earlier in five mutant plants. In the 
photoperiod pathway, mutation in the GI gene delayed flowering under long days 
(Fowler et al., 1999) and showed decreased cold tolerance. However, no significant 
differences were detected in the transcript levels of CBF/DREB1 genes and their 
targeted genes RD29A, COR15A, KIN1, and KIN2 between wild-type and gi-3 plants 
in response to cold stress (Cao et al., 2005). Another important floral promoter in the 
photoperiod pathway, co-2, a mutant of CO, however showed increased cold tolerance 
(Yoo et al., 2007). LOV1 (Yoo et al., 2007), a NAC (NAM, ATAF1, ATAF2, and 
CUC2)-domain transcription factor, negatively regulates CO expression. The lov1 
mutant was not tolerant to cold temperature, whereas a gain-of-function allele was 
resistant to cold stress. This freezing tolerance was attributed to the upregulation of 
COR15A and KIN1 without altering expression of the CBF/DREB1 gene.

1.4 Flowering Pathway in Crops

Flowering time-regulating components similar to Arabidopsis have been 
identified from rice, the model plant for the short-day photoperiod in monocots. In the 
photoperiod flowering pathway, OsGI, a rice ortholog of the Arabidopsis GI had a 
similar expression pattern (Hayama et al., 2002), and aberrant expression of OsGI 
cause late flowering under long day conditions (Hayama et al., 2003). OsGI 
functions to upregulate Hdl1 (the CO ortholog). The difference between long day and
short day plants is that $Hd1$ has a dual role in regulating $Hd3a$ (the $FT$ ortholog) depending on the photoperiod. Under the long day $Hd1$ represses $Hd3a$ expression, while under the short day $Hd1$ enhances $Hd3a$ expression. $OsSOC1$ is expressed in similar tissue and at a similar development stage as $SOC1$ in $Arabidopsis$ (Tadege et al., 2003). HAP genes were characterized in rice but their role in flowering control has not been examined (Thirumurugan et al., 2008). Since rice is not sensitive to vernalization, it is perhaps not surprising that orthologs of $FLC$, $FRI$, $VRN1$ or $VRN2$ have not been identified so far. For the autonomous pathway, $OsFCA$ and $OsFVE$ were recently identified (Lee et al., 2005; Baek et al., 2008).

Some major cereal crops, such as wheat and barley, are long-day photoperiod plants. Wheat TaHd1-1 was identified to have the same role as CO in $Arabidopsis$ (Nemoto et al., 2003). Wheat TaGI1 (the GI ortholog) functioned in flowering time control just like GI in $Arabidopsis$ (Zhao et al., 2005). Barley HvCO and HvGI were recently identified (Griffiths et al., 2003; Dunford et al., 2005). The flowering integrators wheat TaFT and barley HvFT also were found to have a similar function to FT in $Arabidopsis$ (Yan et al., 2006; Li and Dubcovsky, 2008; Kikuchi et al., 2009). The other integrator wheat WSOC1 (SOC1 ortholog in wheat) was found to play roles in both photoperiod and vernalization pathways (Shitsukawa et al., 2007). Under the vernalization pathway, HvVRN1 was controlled by vernalization while HvVRN2 was regulated by day-length (Trevaskis et al., 2006; Sasani et al., 2009). Thus, it appears that these long-day photoperiod cereal plants have similar mechanisms for regulating flowering time as $Arabidopsis$. However, the $HAP$ family of genes in cereal crop
plants have not been studied with regard to their roles in regulating flowering time.

1.5 Major Hypothesis

In yeast and mammalian systems, HAP2, HAP3, and HAP5 form a trimer during transcription activation. I hypothesize that *Arabidopsis* HAPs act the same. In particular, that HAP3b in *Arabidopsis* will form a complex with HAP2 and HAP5 and then regulate flowering time. Since *HAP3b* transcripts were upregulated by stress, I propose HAP3b plays an important role in stress adaptation. I further hypothesize that the orthologs of *Arabidopsis* HAP3b and HAP3c in barley will be conserved in function in flowering time control and stress response.

1.6 Major Objectives

The objectives of this study were to:

1. Investigate the role of HAP3b in stress response. Since transcript level of *HAP3b* increases under cold stress, HAP3b might function in CBF-dependent or independent pathways. Freezing tolerance of the *hap3b* mutant, *HAP3b* overexpression and wild-type plants were determined and expression of genes in the CBF regulon were examined.

2. Identify other components in the HAP3b complex and investigate their function in flowering time control, stress response and root elongation. To address this objective, yeast two-hybrid and protein coimmunoprecipitation methods were used to identify the proteins that can interact with HAP3b. Genetic approaches were then used to test the role of the identified proteins in flowering time control, cold
stress tolerance and root elongation.

3. Identify the HAP3b and HAP3c orthologs in barley, study its gene expression pattern, and examine gene functions, through overexpression of barley ortholog(s) in *Arabidopsis*, in flowering time control and cold stress response.

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Figure 1: Illustration of the main flowering pathways in *Arabidopsis*. Adapted from (Corbesier and Coupland, 2006; Ballif, 2007). Lines with bars indicate gene repression, and lines with arrows represent induction.
CHAPTER 2

HAP3b IS A NEGATIVE REGULATOR OF CBF3 IN COLD RESPONSE

2.1 Abstract

Heme-activated proteins (HAPs), also known as nuclear factor Ys (NF-Ys) or CCAAT-binding factor proteins (CBFs), are transcription factors and have multiple roles in plant growth and development. In a previous study, we demonstrated that HAP3b in Arabidopsis (*Arabidopsis thaliana*) regulates flowering time through the long day photoperiod. We report in this study that HAP3b is also involved in controlling plant cold stress response. Transcript profiling and gene expression analysis indicated that HAP3b repressed the CBF3 regulon under normal growth conditions. As a result, *HAP3b*-overexpression plants showed decreased survival rates while *hap3b*, a null allele mutant line, showed an improved freezing tolerance compared to wild-type plants. Since *HAP3b* is upregulated by multiple abiotic stresses and promotes flowering, HAP3b could be an important link between flowering time control and low temperature response pathways, and it could provide *Arabidopsis* with an evolutionary advantage, i.e. completing reproductive growth under stress by efficiently using energy and resources.

2.2 Introduction

Low temperature stress from chilling and freezing is a major abiotic stress that negatively impacts agricultural production. Research with the model plant *Arabidopsis* has revealed that plants have developed sophisticated mechanisms to
tolerate stress by fine-tuning gene regulation under cold stress. During cold treatment a number of genes are induced including C-repeat binding factor (CBF)/DRE-binding proteins (DREBs), transcription factors, and transcription factor effector genes, e.g. \textit{RD29A} (or \textit{COR78} or \textit{LTI78}), \textit{KIN1}, \textit{KIN2} (or \textit{COR6.6}), \textit{COR15A}, and \textit{COR47} (or \textit{RD17}) (Gilmour et al., 1998; Thomashow, 1999). \textit{CBF/DREB1} genes were rapidly induced (within 15 minutes) by cold stress and subsequently activated the expression of their target genes (Liu et al., 1998). CBFs are now known as some of the major components of the cold stress signaling pathway. Overexpression of \textit{CBF/DREB1} genes in plants was demonstrated to consistently improve freezing resistance even in the absence of a cold acclimation treatment (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000).

CBFs comprise a multigene family and are regulated in a complex manner. For the CBF3 pathway, ICE1 (Inducer of CBF Expression 1) is so far the most upstream transcription factor. ICE1 positively regulates \textit{CBF3} expression (Chinnusamy et al., 2003). ICE1 itself was regulated by sumoylation by SIZ1 and ubiquination by HOS1 (Dong et al., 2006; Miura et al., 2007). ICE2 was also identified to have a similar role by activating \textit{CBF1} expression in response to cold (Fursova et al., 2009). MYB15, ZAT12 and FVE were negative regulators of the CBF pathways (Chinnusamy et al., 2003; Kim et al., 2004; Vogel et al., 2005; Agarwal et al., 2006). MYB15, a R2R3-MYB protein, binds to the promoters of \textit{CBF1}, \textit{CBF2} and \textit{CBF3} to negatively control expression of \textit{CBF} genes. It was also shown that the \textit{Arabidopsis} \textit{ZAT12}, a zinc-finger protein, played a role in cold stress response (Vogel et al., 2005).
Transcript levels of CBF1-3 were decreased rapidly in ZAT12-overexpressing plants compared to wild-type plants in response to cold. The downstream genes such as COR78 and COR6.6, however, were only slightly lower in the overexpression plants. In five plants, the expression level of CBFs and downstream regulon, such as COR15A and COR47, were higher than wild type plants which implied that this protein, a putative retinoblastoma-associated protein, negatively regulates this cold signaling pathway (Kim et al., 2004).

Several lines of evidence suggested the existence of additional signal pathways or CBF-independent pathways involved in the plant regulation of the low temperature response. For example, esk1, a cold tolerant mutant in Arabidopsis, accumulated high levels of proline but did not show increased expression of cold-regulated genes in the CBF regulon (Xin and Browse, 1998; Xin et al., 2007). HOS9 and HOS10, homeodomain transcription factors, are other examples. Cold treatment quickly induced the expression of COR15A and KIN1 but expression of CBFs were not changed in hos9 and hos10 mutant plants (Zhu et al., 2004). In addition, CBFs were induced in the ada2b mutants and in wild-type plants under low temperature, but transcription of COR genes were reduced in this mutant in cold acclimation, suggesting the existence of a CBF-independent freezing tolerance mechanism (Viachonasios et al., 2003).

The developmental transition from vegetative to reproductive growth is essential for successful reproduction and requires proper integration of external stimuli such as day length, temperature, and endogenous signals. In terms of Arabidopsis floral
transition, environmental control is mostly modulated through the photoperiod and vernalization pathways, whereas endogenous stimuli are regulated through the autonomous and gibberellin pathways (Simpson and Dean, 2002). More and more evidence shows that the floral promotion pathways closely interact with plant cold response. In the autonomous pathway, for example, mutation in FVE caused a delay in flowering time and enhanced the cold response (Kim et al., 2004). Transcript levels of COR15A and COR47 were much higher in mutants than in wild-type plants. The expression of CBF3/DREB1a transcripts in response to cold was similar between wild-type plants and mutant plants; however the induction of CBF3/DREB1a expression occurred earlier in fve mutant plants, leading to better freezing tolerance.

In the photoperiod pathway, mutation in the GI gene delayed flowering under long days but not under short days (Fowler et al., 1999) and showed decreased cold tolerance. However, no significant differences were detected between wild-type and gi-3 plants in the transcript levels of CBF/DREB1 genes and their targeted genes RD29A, COR15a, KIN1, and KIN2 in response to cold stress (Cao et al., 2005). A mutant of CONSTANS (CO), co-2, which is another important floral promoter in the photoperiod pathway, however, showed increased cold tolerance (Yoo et al., 2007). Lov1, a NAC (NAM, ATAF1, ATAF2, and CUC2)-domain transcription factor, negatively regulates CO expression (Yoo et al., 2007). The lov1 mutant was not tolerant to cold temperature, whereas a gain-of-function allele was resistant to cold stress. This freezing tolerance was attributed to the upregulation of COR15A and KIN1 without altering expression of CBF/DREB1 genes. HAP3b, a CCAAT
transcription factor, was found to be induced by osmotic stress, cold stress, and ABA (Kreps et al., 2002) and recently HAP3b was shown to regulate flowering through the long day photoperiod pathway (Kreps et al., 2002; Cai et al., 2007). However, the role of HAP3b in stress response is still not clear. The objective of this study was to demonstrate that HAP3b is a negative regulator of the CBF3-dependent regulon. This further demonstrated the close link between flowering time pathways and cold stress response.

2.3 Materials and Methods

2.3.1 Plant materials and growth conditions

Soil planting: Seeds of Arabidopsis thaliana (Columbia 0 ecotype background), wild-type, hap3b or overexpression of HAP3b plants (Cai et al., 2006) 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 2 days. Seeds were germinated and seedlings were grown on a light shelf or in a growth chamber under a 16h/8h light/dark cycle at 23°C. 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.

Plate planting: Seeds (wild-type, hap3b, overexpression of HAP3b plants, gi (At1g22770), co (At5g15840), ld (At4g02560) in Columbia 0 ecotype background; flc (At5g10140), fve (At2g19520) in Landsberg ecotype background) were first surface sterilized and germinated, and seedlings were grown in a Petri dish (150 mm in diameter) containing 55 mL of sterile solid medium consisting of 0.5X MS salt, 0.5% sucrose, 1/2 × MES (BIOPLUS, 765081, Gibbstown, NJ) and 0.6% Phytagel or 0.8%
agar (Sigma, St. Louis, MO) at pH 5.8. Plates containing seeds were kept in a cold room (4°C) for 2 days and then moved in a growth chamber. Plants grew under the conditions described above. Eight-day-old seedlings were harvested for RNA extraction and quantitative gene expression analysis as described below.

2.3.2 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. Plants were grown under the conditions described above. Leaves of 18-d-old plants 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, catalog no. 900385, Santa Clara, CA) were processed: two chips for wild-type plants, two for mutant plants (hap3b), and one for overexpression plants (Pactin: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, a quantitative PCR was used. Seeds of wild-type, hap3b mutant, HAP3b-overexpression transgenic plants, and overexpression control plants (C1 = Pactin:GUS) were germinated in a single Murashige and Skoog-Phytigel plate. Fifteen-day-old seedlings were harvested for RNA extraction. A quantitative PCR method was performed by following a method described by Cai et al. (2006) with the following modifications.
Quantification 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.

### 2.3.3 Freezing stress test

**A. Survival test:** Seeds of wild-type, *hap3b*, *HAP3b*-overexpression plants, *gi*, *co*, *hap3c* (At4g14540, obtained from Dr. O. J. Ratcliffe, Kumimoto et al., 2008), double mutant *hap3b* × *hap3c* (also obtained from Dr. O. J. Ratcliffe), were first surface sterilized and arranged on the solid MS medium. The plates containing seeds were kept in a cold room (4°C) for 2 d and then moved into a growth chamber. Seeds were germinated and seedlings were grown under the conditions described above. Two-week-old seedlings were subjected to freezing by incubating the plate plants in a freezer at -20°C for 30 or 50 min depending on the experiment. The plates were then moved to a dark cold room at 4°C for 2 h, and returned to the growth chamber for recovery. The survival rates were scored 7 d after treatment (Ishitani et al., 1998; Zhu et al., 2004).

**B. Membrane leakage analysis.** The fully-expanded leaves of *hap3b* mutant, *HAP3b*-overexpression line, and wild-type plants at the 8-leaf stage were harvested for freezing treatment in a controlled chamber. Plants were grown in soil under the light conditions described above. For the freezing test, chamber (TPS, Tenney, Series 942, PA) temperature was decreased from 0°C to -14°C within 7 h (RAM = -2°C/hr). Leaves in test tubes were taken out of the chamber every one hour and membrane
leakage was measured. To measure membrane leakage, leaves were immersed in 10 mL distilled water and shaken gently over night at 4°C. Conductivity of the resulting solution was measured using an electric conductance meter. The tube and solution with tissues were then autoclaved for 15 min. The solution, after cooling down, was measured for 100% leakage of the tissues. A percentage of membrane leakage was then calculated (Gilmour et al., 1988; Xin and Browse, 1998).

2.3.4 Cold Stress treatment and gene expression analysis

Expression of CBF1, CBF2, CBF3, COR47, RD29A in hap3b mutant, HAP3b-overexpression lines and wild-type plants were conducted using the semi-quantitative RT-PCR method as described in Liang et al. (Liang et al., 2006). For expression of these genes under cold stress, 2-week-old plants grown on the solid medium were treated at 0°C for 3 h under light. Total RNA was extracted using RNAwiz (Ambion, #9736, Austin, TX) and DNase treatment of RNA samples was applied by RQ1 RNase-Free DNase I (Promega, #M6101, Madison, WI). DNase-treated RNA was first tested in a PCR reaction to ensure no genomic DNA contamination and then reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen, #18080-093, Carlsbad, CA) by following the manufacturers’ protocols.

2.4 Results

2.4.1 HAP3b-overexpression plants are more sensitive to freezing stress

Since the HAP3b transcript level was upregulated by multiple abiotic stresses,
we examined whether overexpression of HAP3b would enhance the plant tolerance to stresses. Surprisingly, HAP3b-overexpression plants were more sensitive to a brief freezing stress (30 min) compared to wild-type plants (Figure 2.1). The survival rate of HAP3b-overexpression plants was slightly over 40% while wild-type plants showed a 75% survival rate. No significant difference was observed between mutant hap3b and wild-type plants in freezing tolerance. To determine whether there was a subtle difference in freezing response between wild-type and mutant plants, we used a more sensitive method, i.e. membrane electrolyte leakage assay, to examine membrane integrity of mutant hap3b and wild-type plants under a series of freezing temperatures. Wild-type plants and mutant hap3b showed no difference in membrane leakage until freezing temperature reached -12°C (Figure 2.2). Despite large variation in each data point, it was clear that hap3b plants showed overall less membrane damage than wild-type plants at more severe freezing stress, suggesting mutant plants may perform better under severe stress. Another experiment was then conducted to treat wild-type and hap3b plants with more severe freezing stress by incubating plants at -20°C for a longer time (50 min). Under these conditions, the mutant hap3b showed a significantly higher survival rate than wild-type plants, demonstrating an improved freezing tolerance in hap3b mutant (Fig 2.6).

2.4.2 HAP3b negatively regulates the CBF3-regulon

Since CBF3 is known as a major regulator of freezing tolerance in Arabidopsis (Gilmour et al., 1998, 2000), we hypothesized that HAP3b might affect freezing tolerance through controlling CBF3 expression. We investigated expression of CBF3
and several genes in its regulon. Without cold treatment, the CBF3 transcript level was increased in the hap3b mutant and decreased in the HAP3b-overexpression plants compared to wild-type plants (Fig 2.3). The reduction in CBF3 transcript level in HAP3b-overexpression plants was more pronounced than the increase found in hap3b mutants. RD29A, a downstream gene of the CBF3 regulon, showed changes similar to CBF3 transcripts in the hap3b mutant and HAP3b-overexpression plants when compared with wild-type plants (Fig 2.3). However, COR47, another CBF3-regulated gene, showed significantly increased transcript levels in hap3b mutant plants and no change in HAP3b-overexpression plants (Fig 2.3).

The change in transcript levels of CBF3 and its regulon was supported by the data of our previous microarray experiment which compared wild-type, hap3b and HAP3b-overexpression plants. Array data analysis revealed that several known cold-response genes, including CBF3/DREB1a, KIN1, RD29A and COR15a, were among the top 14 genes that were upregulated in hap3b but down-regulated in the overexpression plants (Table 2.1). An analysis of known CBF3/DREB1a-regulated genes (Maruyama et al., 2004) showed that 12 (~40%) of them had the same expression pattern as CBF3/DREB1a although the relative change is much less significant (Table 2.2), suggesting that HAP3b may normally suppress the CBF3 regulon.

The transcript level of ICE1 (Chinnusamy et al., 2003; Lee et al., 2005), a regulator of CBF3/DREB1a expression, was not affected by HAP3b expression (Table 2.3). These results indicate that HAP3b acts as a negative regulator of the
CBF3/DREB1a pathway. HAP3b could act downstream of ICE1 (see discussion below).

CBFs comprise a multigene family and CBF1 and CBF2 contribute in different degrees to freezing tolerance (Jaglo-Ottosen et al., 1998; Chinnusamy et al., 2006). As shown in Figure 2.4, the transcript levels of CBF1 and CBF2 were greater in hap3b plants but were not affected in overexpression plants compared to wild-type plants. The data suggest that HAP3b may not have a significant effect on the expression of CBF1 and CBF2.

2.4.3 Induction of CBF3 transcription by low temperature was not suppressed by HAP3b expression

Since the data suggested that HAP3b normally suppresses the CBF3 regulon in Arabidopsis, the next question was whether HAP3b will suppress the induction of the CBF3 regulon in low temperature. Low temperature drastically increased CBF transcript levels in all the plants (> one hundred thousand fold, data not shown). Although the hap3b mutant maintained the highest level and HAP3b-overexpression lines showed the lowest CBF3 transcript levels compared to wild-type plants, the difference in transcript levels among these plants were not statistically significant (Fig. 2.3). Moreover, the cold-induced CBF3 transcript levels for all the plants were so great compared to their own levels at room temperature, it makes the slight difference in CBF3 transcript level at low temperature among the genotypes even less significant. Thus, the results suggest that HAP3b does not suppress the CBF3 regulon under low temperature.
2.4.4 CBF3 expression in other flowering mutants under room temperature

Several studies have indicated cross-talk between flowering pathways and low temperature response (Kim et al., 2004; Cao et al., 2005; Yoo et al., 2007). In the photoperiod pathway alone, several genes were reported to have various impacts on the low temperature response (Cao et al., 2005; Yoo et al., 2007). This raises the question whether the interaction between flowering time control and low temperature response is up to a specific gene in each pathway or involves multiple components in a pathway. Flowering-time mutants in the photoperiod (co and gi), vernalization (flc), and autonomous pathways (ld and fve) were chosen to compare with wild-type for the expression of CBF3 under normal growth conditions (grown at room temperature). Our results showed that CBF3 transcript levels were not changed in co or ld and were slightly increased in gi and fve mutant plants. CBF3 transcript level showed the greatest increase in flc plants (Fig. 2.5).

2.4.5 Mutant hap3c did not show improved freezing tolerance

In a recent report, HAP3c, a gene showing the greatest similarity to HAP3b in the Arabidopsis HAP3 gene family, had an additive effect with HAP3b in promoting long-day photoperiod flowering (Kumimoto et al., 2008). We hypothesized that HAP3c may also be involved in freezing tolerance. Mutant hap3c and double mutant hap3c×hap3b were examined in a freezing test. Mutant hap3c grown at room temperature showed no difference in freezing tolerance compared to wild-type plants. Surprisingly, double mutant hap3c×hap3b also showed no improvement in freezing
tolerance, while the hap3b single mutant showed significantly greater freezing
tolerance than the wild type (Fig. 2.6).

2.5 Discussion

Our previous studies showed that HAP3b regulates flowering time in
Arabidopsis through the long day photoperiod pathway. In this study, we found that
HAP3b reduces freezing tolerance in plants by suppressing the CBF3 regulon.

2.5.1 HAP3b suppresses the CBF3 regulon

Our genetic evidence strongly indicates that expression of HAP3b reduces
freezing tolerance in plants, which is achieved by repressing the CBF3 regulon under
room temperature growth conditions.

CBF3 is a member in the CBF multigene family and plays a critical role in
determining freezing tolerance in Arabidopsis. Overexpression of CBF1-3 led to
increased levels of proline and sugar and an increase in freezing tolerance for both
non-acclimated and cold-acclimated plants (Gilmour et al., 2004). However,
overexpression of CBFs also resulted in dwarf plant phenotype and growth retardation
(Gilmour et al., 2004). Thus, various mechanisms, both positive and negative controls,
have been developed in plants to regulate expression of CBF genes (Lee et al., 2001;
Chinnusamy et al., 2003). Among them, ICE1, is a major upstream regulator of the
CBF3 regulon and positively regulates expression of CBF3 under low temperature
(Chinnusamy et al., 2003). Based on a published microarray data, HAP3b was down
regulated in ice1 mutant, suggesting a positive regulation of ICE1 on HAP3b
expression (Lee et al., 2005). Thus, ICE1 could both directly upregulate CBF3 and
downregulate CBF3 through HAP3b. Interestingly, all these genes are induced by cold treatment. Thus, the results may indicate that HAP3b represents a feedback loop to prevent overaccumulation of CBF transcripts during low temperature response.

HAP3b clearly represses the CBF3 regulon based on the expression data of both HAP3b-overexpression plants and hap3b mutants. The effect of HAP3b on CBF1 and CBF2 transcripts was unclear due to large variations in transcript level from mutant plants and the fact that overexpression of HAP3b did not change the transcript level of CBF1 and CBF2 compared to wild-type plants. Thus HAP3b may have a specific effect on the CBF3 regulon.

The specificity of regulation of CBF was reported in other studies. For example, ICE1 specifically upregulates CBF3 (Lee et al., 2005). HOS9 however showed greatly preferred upregulation on CBF2 compared to CBF3. These specific regulations may differentiate expression patterns of each CBF and may be required for their different functions. Indeed, CBF1 and CBF3 were recently identified to have different expression patterns from CBF2 and they positively control cold response by activating the same subset of CBF-target genes (Novillo et al., 2007). The cbf2 mutant plants were found to have increased CBF1 and CBF3 transcripts and improved freezing tolerance before and after cold acclimation, which implied that CBFs were subject to autoregulation and each CBF may have different functions (Novillo et al., 2004).

2.5.2 HAP3b-regulated genes and relation to HAP3c

Since HAP3b is a CCAAT binding transcription factor, an important question is whether HAP3b binds directly to this element within the CBF3 promoter. This
possibility of binding is partially supported by the fact that the CBF3 promoter has several CCAAT sequences in reverse and forward directions (Table 2.4). Other than CBF3, the top 10 genes having similar expression patterns to CBF3 (reduced transcript level in HAP3b-overexpression plants and increased in hap3b mutant plants) all had several CCAAT sites in the putative promoter regions. Even though the CCAAT sequence was commonly identified in the promoter region, only ~30% of over 500 unrelated eukaryotic promoters have this element (Bucher, 1990). The higher frequency of CCAAT in these top 10 genes implies that HAP3b might regulate some of these downstream genes via cis-element binding. However, formation of a HAP2/HAP3/HAP5 heterotrimer and specific binding to the CCAAT motif have not yet been demonstrated in plants. A recent study showed that two other members in the HAP3 family, LEC1 and LEC1-like proteins, could recruit bZIP67, an ABA-response element binding factor, to form a complex to activate the promoter of CRUCIFERIN C and control seed development (Yamamoto et al., 2009). Thus, the significance of over-representation of CCAAT in the promoters of the genes affected by HAP3b expression remains to be determined.

Other than the genes in the CBF3 pathway, several HAP3b-regulated genes (Table 2.2) have also been associated with cold stress response or involved in response to other stresses. At3g16450, a jacalin lectin family protein, was induced by glycine-rich RNA-binding protein 2 (GRP2), a positive cold response regulator (Kim et al., 2007). Transgenic overexpression of GRP2 enhanced seed germination and seedling growth during cold and salt stress. Overexpression of At2g37130, a
peroxidase, and At2g38870, a protease inhibitor, increased resistance to invasion of the plant fungus *Botrytis cinerea* (Chassot et al., 2007). At2g43000, encoding a harpin-induced protein, is probably also associated with plant resistance to pathogens. Interestingly, HAP3b also negatively regulated two cell wall proteins, arabinogalactan-proteins (At2g22470) and expansin (At4g17030). While these proteins are generally believed to be involved in plant growth and development (Showalter, 2001; Cosgrove, 2005), the reason why only these two specific genes were suppressed by HAP3b is unclear. Overall, it appears that HAP3b inhibits other stress responses besides cold-stress response, probably for more efficient use of energy and resources for reproductive growth.

HAP3c, the most closely related to HAP3b in the HAP3 family, also regulates flowering time through the long day photoperiod pathway and plays an additive role with HAP3b in flowering time control (Kumimoto et al., 2008). However, HAP3c seems not to function in the cold stress response pathway since the *hap3c* mutant plants performed the same as wild-type plants during freezing tests. More surprisingly, *hap3b hap3c* double mutant plants only showed a slightly higher survival rate than wild-type plants but a significantly lower survival rate than the *hap3b* single mutant. Thus, the results suggest that HAP3c may actually function oppositely from HAP3b to offset HAP3b’s role in regulating the cold stress response, adding another layer of complexity to the cold stress response pathway.

**2.5.3 Crosstalk between plant flowering and cold Stress response in *Arabidopsis***

The fact that HAP3b promotes flowering through the long day photoperiod
pathway while suppressing low temperature response suggests crosstalk between the flowering pathway and freezing tolerance. Other players in the long day photoperiod pathway have also been implicated in freezing tolerance. Mutant co plants were more resistant to freezing stress compared to wild-type plants (Yoo et al., 2007). Consistent with the results, overexpression of the LOV1 gene, a CO repressor in the long day photoperiod flowering, increased tolerance to cold temperature (Yoo et al., 2007). A recent study showed that mutant plants of SOC1, a downstream gene of CO and HAP3b and an integrator of four known flowering pathways, had improved survival rates under freezing (Seo et al., 2009). Therefore, one may speculate that all these genes regulate SOC1 expression, and thus CBF expression and freezing tolerance. In addition, mutant plants fve in the autonomous pathway had lower expression of SOC1 due to higher expression of FLC compared to wild-type plants and increased levels of COR15 and faster induction of CBF3 under cold, resulting in greater tolerance to freezing (Kim et al., 2004). Again the results support the notion that there is a general negative relationship between flowering time regulation and freezing tolerance. The relationship may be achieved by the key regulator SOC1 in the flowering pathways that acts as a negative regulator of the CBF3 regulon.

However, some results from this study and evidence from other studies suggest that the interaction between flowering time regulation and freezing tolerance may be more complicated. Mutation of the GI gene, the direct upstream positive regulator of CO, had lower SOC1 transcript levels but showed decreased constitutive freezing tolerance in plants (Cao et al., 2005). In gi mutants and control plants there were no
significant differences in expression levels of *CBF1-3* and their targeted genes, including *RD29A, COR15A, KIN1*, and *KIN2* (Cao et al., 2005). In addition, mutant *flc*, which would result in an enhanced expression of *SOC1*, actually showed increased expression of *CBF3* in our study. Moreover, *fve* and *ld*, mutants in the autonomous pathway, showed opposite results. Both genes repress *FLC* and thus promote expression of *SOC1*; however, under non-cold stress conditions, one showed an enhanced expression of *CBF3* and the other did not. Thus, more studies are necessary to address whether there is a real converging point where a negative interaction between flowering time regulation and freezing tolerance occurs and to reveal the comprehensive network of interaction between flowering time regulation and low temperature response.

In summary, we provided strong genetic evidence demonstrating that HAP3b constitutively acts as a repressor of the CBF3 pathway. Since *HAP3b* is upregulated by multiple abiotic stresses and promotes flowering, HAP3b could be an important link between flowering time control and low temperature response pathways, potentially representing an adaptational mechanism for *Arabidopsis* to complete reproductive growth under stress by effectively using resources and energy.

2.6 References


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HOS9, mediates cold tolerance through a CBF-independent pathway.
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America 101: 9873-9878
### Table 2.1. Genes Downregulated in Overexpression Plants But Upregulated in hap3b Mutant on Affymetrix Genechip

<table>
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<tr>
<th>Affy ID</th>
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<th>Mean signal for hap3b</th>
<th>Fold change (hap3b/wt)</th>
<th>Mean signal for ox</th>
<th>Fold change (ox/wt)</th>
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Note: wt, wild type; ox, HAP3b-overexpression plants; hap3b, mutant.
### Table 2.2. Changes in Transcript Levels of Known CBF3/DREB1a-regulated Genes in *hap3b* Mutant and Overexpression Plants Grown Under Normal Growth Conditions

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<td>898.29</td>
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<td>Farnesylated protein (ATTPs)</td>
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Note: wt, wild type; ox, HAP3b-overexpression, *hap3b*, mutant. The genes that were labeled as absent or marginal signal on arrays are shown in blue. -, indicates fold change was not calculated due to low signal.
Table 2.3  \textit{Changes in Transcript Levels of ICE1, Other CBFs/DREBs and Some Non-CBF3/DREB1a-regulated Genes in hap3b Mutant and Overexpression Plants Grown Under Normal Growth Conditions}\n
<table>
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<tr>
<th>Gene</th>
<th>Affy ID</th>
<th>Mean signal for wt</th>
<th>Mean signal for hap3b</th>
<th>Fold change (hap3b / wt)</th>
<th>Signal for ox</th>
<th>Fold change (ox / wt)</th>
<th>Gene ID</th>
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<td>CBF2/DREB1c</td>
<td>254075_at</td>
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<td>51.97</td>
<td>-</td>
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<td>CBF4</td>
<td>248389_at</td>
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<td>10.82</td>
<td>-</td>
<td>9.54</td>
<td>-</td>
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<td>DREB2B</td>
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Note: wt, wild type; ox, \textit{HAP3b}-overexpression; hap3b, mutant. The genes that were labeled as absent or marginal signal on arrays are shown in blue. -, fold change was not calculated due to low signal.
Table 2.4  CCAAT Motif Location in Top 10 Genes That Are Negatively Regulated by HAP3b and in Additional Genes in the CBF Family

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<th>800–1000</th>
<th>1000–1600</th>
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<td>559(+)</td>
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Note: Promoter sequence was identified as intergenic sequence, starting from the end of 3'UTR of one gene to the beginning of 5'UTR of the gene of interest. The numbers in each column associated with specific region of promoters indicate CCAAT locations in the predicted promoter sequences relative to the beginning of 5’UTR. The smaller the number, the closer the CCAAT element to the gene. “+” and “−” in the parenthesis indicate “forward” and “reverse” orientation of the CCAAT element, respectively.
Table 2.5 Primers for Gene Expression Analysis of Cold Response Genes

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<th>Primer Sequences (5’-3’)</th>
<th>Descriptions</th>
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<td>3’-downstream, CBF1, gene expression</td>
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<td>3’-downstream, CBF2, gene expression</td>
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<tr>
<td>TTCAGCAAACCATAACCAACAAAAA</td>
<td>5’-upstream, CBF3, gene expression</td>
</tr>
<tr>
<td>GCACTCTCAA ACATCGCCTCATC</td>
<td>3’-downstream, CBF3, gene expression</td>
</tr>
<tr>
<td>GAATCACCACGACGACACA</td>
<td>5’-upstream, COR47, gene expression</td>
</tr>
<tr>
<td>CTCCACCACACTCTCCGACACT</td>
<td>3’-downstream, COR47, gene expression</td>
</tr>
<tr>
<td>ATGAGAATGGTGGCCTAAGATG</td>
<td>5’-upstream, RD29A, gene expression</td>
</tr>
<tr>
<td>TGACAATTTCGGACAGAGGATG</td>
<td>3’-downstream, RD29A, gene expression</td>
</tr>
</tbody>
</table>
Figure 2.1 Decreased survival rate of \(HAP3b\)-overexpression (\(Pactin:HAP3b\)) plants under freezing stress compared to wild-type plants (WT) and \(hap3b\) mutant. Data are means ± SE from five independent experiments. *, indicates P<0.05 compared with wild type.
Figure 2.2 Difference in membrane leakage of wild-type plants and mutant hap3b under freezing temperatures. Data are means ± SE of three independent experiments, and each experiment consisted of measurements of three samples (individual leaves) for both wild-type and hap3b plants at each freezing temperature.
**Figure 2.3** Relative *CBF3* transcript levels in *hap3b* mutant and *HAP3b*-overexpression plants grown at room temperature or treated with low temperature compared to wild-type plants. Data are means ±SE of three independent experiments.
Figure 2.4 Relative transcript levels of CBF genes and their regulated genes in hap3b mutant and HAP3b-overexpression plants compared with wild-type plants grown at room temperature. Data are means ±SE of three independent experiments.
Figure 2.5 Relative transcript levels of CBF3 in various flowering time mutants compared to wild-type plants grown at room temperature. CBF3 transcript level in each genotype was first normalized using an actin gene and the normalized transcript level was then compared with the level in wild-type plants. Data are means ±SE of three independent experiments.
Figure 2.6 Survival rate of wild-type plants and various flowering time mutants after freezing treatment. Two-week-old plants grown on solid medium in Petri dishes were incubated at -20°C for 50 min. Survival plants were scored 10 d after treatment. Data are means ± SE of three independent experiments.
CHAPTER 3
IDENTIFICATION OF HAP SUBUNITS THAT ARE ASSOCIATED WITH HAP3b

3.1 Abstract

Heme-activated proteins (HAPs) are transcription factors and activate transcription through forming a heterotrimer consisting of HAP2, HAP3, and HAP5 in yeast and mammalian systems. However, whether plant HAPs function through forming a heterotrimer remains elusive. We previously showed that HAP3b in Arabidopsis (Arabidopsis thaliana) promotes flowering through the long day photoperiod pathway while it suppresses the cold response pathway. In this study, we used the yeast two-hybrid system and protein coimmunoprecipitation method to identify the proteins that could interact with HAP3b. From the yeast two-hybrid analysis, it was found that HAP3b could interact with one (At3g14020) of the ten HAP2 and all ten HAP5s tested in Arabidopsis. Further analysis showed that the newly identified HAP2 could only interact with two HAP5 (At5g63470 and At1g56170). Thus, HAP3b in Arabidopsis may also form a heterotrimer and HAP2 might determine the specificity of the heterotrimer. Protein coimmunoprecipitation analysis, however, revealed a totally different set of proteins that interacted with HAP3b. The reasons for the discrepancy of these results are discussed. To provide supporting evidence for the protein-protein interaction data, a genetic approach was used to examine the functions of some of the identified proteins in flowering time control and freezing tolerance.
3.2 Introduction

HAP (Heme-Activated Protein), also known as NF-Y (Nuclear Factor Y) or CBF (CCAAT-Binding Factor) genes, were first identified from yeast because mutations in either gene (HAP2 or HAP3) blocked expression of mitochondrial proteins (such as, CYC1, encoding iso-1-cytochrome c) and prevented growth on lactate medium (Pinkham and Guarente, 1985; Hahn and Guarente, 1988). The CYC1 promoter is comprised of two upstream activation sites (UASs), one of which (UAS2) contains an inverted CCAAT motif that is required for transcription. Activation of transcription from UAS2 requires both HAP2 and HAP3 (Pinkham and Guarente, 1985; Pinkham et al., 1987; Hahn and Guarente, 1988), which form a CCAAT-box-binding complex. McNabb et al. (1995) identified another component, HAP5, in the CCAAT-binding complex. HAP5 is required for the assembly and DNA-binding activity of the complex (McNabb et al., 1995). In a hap5 mutant, CCAAT-binding activity of the complex is missing in an in vitro analysis. Furthermore, purified recombinant HAP2, HAP3, and HAP5 are able to reconstruct CCAAT-binding activity through mobility shift analysis. Another subunit of the complex, HAP4, exists in yeast, which contains an acidic domain that strongly activates transcription (Forsburg and Guarente, 1989). In a strain with a deletion in the HAP4 gene, the CCAAT box is not activated. However, a hap4 could partially grow on lactate by transferring the HAP2-GAL4 fusion vector (Olesen and Guarente, 1990), indicating that HAP4 might not be essential for the binding of HAP2/HAP3/HAP5 to CCAAT. All these data suggest
that the HAP2/HAP3/HAP5 complex represents a DNA-binding factor in which all three subunits are required for downstream gene activation.

HAPs are shown to be functionally conserved over evolution. Clones of HAP counterparts have been isolated from yeast (Olesen et al., 1991), plant (Edwards et al., 1998; Thirumurugan et al., 2008), mouse (Vanhuijsduijnen et al., 1990), rat (Maity et al., 1990), and humans (Becker et al., 1991). While the CCAAT box occurs commonly in eukaryotic promoters, among the various DNA interacting proteins that bind to this box, it appears that only HAP2/HAP3/HAP5 has been shown to require all 5 nucleotides (Mantovani, 1998). There were some exceptions such as in the human von Willebrand factor where NF-Y interacted not only with the CCAAT element as an activator and but also CCGNNNCCC sequence to be a repressor (Peng and Jahroudi, 2002).

HAPs in plants are involved in embryo development (Lotan et al., 1998; Kwong et al., 2003), chloroplast biogenesis (Miyoshi et al., 2003; Nelson et al., 2007), nodule development (Combier et al., 2006), stress response (Kreps et al., 2002; Li et al., 2008), root elongation (Ballif, 2007), and flowering regulation (Cai et al., 2007; Kumimoto et al., 2008). In contrast to the situation in yeast and animals, where each subunit is encoded by a single gene, multiple genes exist for each of the HAP2, HAP3, and HAP5 subunits in plants, providing the potential for multiple alternative forms of HAP complexes in plants (Edwards et al., 1998; Ito et al., 2005). In Arabidopsis there are at least 10 annotated members in each HAP family (Gusmaroli et al., 2001, 2002; Sievers et al., 2009). In rice, there are at least 10 HAP2 genes, 11 HAP3 genes and 7
HAP5 genes (Thirumurugan et al., 2008). HAP4 have not been identified in the plant kingdom. More gene members in the same family could mean gene redundancy or function differentiations.

Regarding flowering, Ben-Naim et al. (2006) reported that overexpression of a tomato *HAP5* in *Arabidopsis* caused early flowering. In contrast, flowering was delayed by overexpression of a *HAP2a* (At5g12840) or a *HAP3a* (At2g38880) in *Arabidopsis* (Wenkel et al., 2006). Flowering time in *hap2a* and *hap3a* mutants, however, is not affected. Cai et al. (2007) reported that overexpression of *Arabidopsis* *HAP3b* (At5g47640) promoted early flowering while *hap3b*, a null mutant of *HAP3b*, showed delayed flowering under a long-day photoperiod but not under short-day conditions, suggesting that HAP3b might normally be involved in the long-day photoperiod-regulated flowering pathway. NF-YB3 (HAP3c, At4g14540), the most closely related *Arabidopsis* protein to HAP3b, shares similar activities with HAP3b. Both HAP3b and HAP3c are necessary and sufficient for the promotion of flowering in response to inductive photoperiodic long-day conditions. This is supported by the fact that the double mutant *hap3b hap3c* showed a significant delay in flowering time compared to either single mutant. HAP3b and HAP3c likely regulate flowering time by the direct activation of the key floral regulator Flowering Locus T (FT) (Kumimoto et al., 2008).

How different HAPs achieve different physiological functions in plants or whether plant HAPs form a heterotrimer as yeast and animal HAPs do during transcription activation remains inconclusive. The *LEC1* gene was the first *HAP3*
gene cloned and studied in plants (Lotan et al., 1998). It controls fatty acid
biosynthesis to induce embryo development (Mu et al., 2008). A recent study showed
LEC1 could recruit bZIP67, an ABA-response element binding factor, to form a
complex to activate the promoter of CRUCIFERIN C and control seed development
(Yamamoto et al., 2009). In another study, LEC1 or LEC1-like was found to function
with NF-YA5 (At1g54160) and NF-YC4 (At5g63470) in vitro to mediate blue light or
ABA response (Warpeha et al., 2007). Thus, it appears that HAP may form a
heterotrimer only during certain activities.

In HAP-mediated flowering time control, Wenkel et al. (2006) showed that
HAP3a and HAP5a in Arabidopsis were able to interact in vivo. They also
demonstrated that CONSTANS proteins could interact with HAP3a and HAP5a in
vitro. Since CO shares some sequence similarity with HAP2, it was thus postulated
that HAPs also regulate flowering time through formation of a heterotrimer complex.

The questions are whether HAP3b promotes flowering under long day conditions
through a similar mechanism as HAP3a, i.e. by forming a heterotrimer, and why
overexpression of HAP3a and HAP3b resulted in opposite results, one delaying
flowering and the other promoting flowering. There are two main hypotheses: One is
that HAP3a and HAP3b may compete in the same trimer complex. HAP3b in the
complex would promote flowering and replacement of HAP3b with HAP3a would
delay flowering. The other hypothesis is that HAP3a and HAP3b may form different
complexes with their own specific HAP5 and HAP2 so that the complexes function
differently; HAP3a and HAP3b may both interact with CO and COL and compete for
binding CO which would decrease the number of CO-HAP3b-containing complexes and delay flowering. Thus, the ratio of HAP3a-CO and HAP3b-CO would determine the timing of flowering in plants, which may represent a novel mechanism in regulating flowering timing in the photoperiod pathway. To distinguish these two hypotheses, identification of proteins that can interact with HAP3b is required.

The objectives of this study were to identify proteins that interact with HAP3b and determine whether these proteins are also involved in long-day flowering control. In addition, HAP3b was demonstrated as a negative regulator of the CBF3-mediated cold stress response pathway (Chapter 2) and promoting root elongation (Ballif, 2007). Thus, potential functions of these HAP3b-interacting proteins in cold stress tolerance and root elongation were also examined.

3.3 Materials and Methods

3.3.1 Plant materials and growth conditions

Soil planting: Seeds of Arabidopsis thaliana (Columbia 0 ecotype background), either wild-type, mutant or overexpression transgenic plants (Kumimoto et al., 2008) were sown in well-watered potting mix (Enriched Potting Mix, Miracle-Gro Lawn Products, Inc., Marysville, OH), and kept in a cold room (4ºC) for 2 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.
Plate planting (screening for overexpression transformants): Seeds were first surface sterilized and germinated, and seedlings were grown in a Petri dish (150 mm in diameter) containing 55 mL of sterile solid medium consisting of 0.5X MS salt, 0.5% sucrose, 1/2X MES (BIOPLUS, 765081, Gibbstown, NJ) and 0.6% Phytagel or 0.8% agar (Sigma, St. Louis, MO) and antibiotics or herbicide (kanamycin, 50 μg mL⁻¹ or Basta, 50 μg mL⁻¹ depending on the binary vector used, plus carbinicillin, 100 μg mL⁻¹) at pH 5.8. Plants were grown in a growth chamber under the conditions described above.

The T-DNA insertion mutant lines for identified HAPs: SALK_006559 for At1g54160, SALK_111422c for At1g56170, SALK_028169c and SALK_143369c for At3g14020, SALK_086334c for At3g48590 and SALK_132910C for At5g63470 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 T-DNA insertion sites were verified by PCR methods. T-DNA insertional mutant SALK_025666 for HAP3b (At5g47640) was identified in the same way as described above and reported by Cai et al. (2007).

3.3.2 Plasmid Constructs

3.3.2.1 Constructs for yeast two-hybrid (YTH) analysis

The CDS portion of HAP3b, all HAP5 (At5g08970, At1g54830, At1g56170, At5g63470, At5g50480, At5g50470, At5g38140, At3g48590, At5g50490, and At5g27910) and all HAP2 (At1g54160, At1g30500, At2g34720, At3g20910, At5g12840, At1g17590, At1g72830, At3g14020, At3g05690, and At5g06510): one
HAP3 (At1g21970) were PCR-amplified from Arabidopsis cDNA 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 cloned sequences were verified by DNA sequencing and subcloned into the pGAD424 AD vector (bait) or pGBT9 DNA BD vector (prey).

3.3.2.2 Constructs for HAP3b domain analysis using yeast two-hybrid analysis

Since all HAP3 proteins can generally be divided into three domains, it is necessary to identify which domain is in charge of specific protein interaction. The HAP3b CDS sequence was manually divided into three fragments based on the domains in the protein: the sequence encoding the N-terminal domain (1-66 bp), the center sequence encoding the conserved domain (66-369 bp) and the sequence encoding C-terminal domain (370-573 bp). Primers were designed to amplify and clone the N-terminal and the C-terminal domains. A combination of two domains – N-terminal domain plus the center conserved domain (1-369 bp) and the center conserved domain plus C-terminal domain (370-573 bp) – was also designed for cloning. Partial HAP3b CDS fragments were PCR-amplified, cloned, and sequenced by following the same procedure described above. The DNA fragments were then sub-cloned into pGAD424 AD vector.

3.3.2.3 Construct for interaction of CO with HAP3b

To test interaction between HAP3b and CO (At5G15840), the DNA sequence
encoding the CCT domain (amino acids 306 to 373, Wenkel et al., 2006) of CO was cloned into the pGBT9 DNA BD vector, yielding an in-frame fusion with the Gal4-DNA binding domain.

3.3.3 Yeast two-hybrid screening

All HAP5s, all HAP2s and one HAP3 (At1g21970) were cloned into the pGBT9 DNA BD vector. All bait and prey constructs were transformed in the HF7C yeast strain and tested for autoactivation of individual constructs before co-transformation of the HAP plasmids. Yeast transformation was performed following the Bio-Rad MicroPulser™ electroporation procedure (#165-2100). The screen was performed on SD ampicillin medium lacking His, Leu, and Trp plus 5 mM 3-aminotriazole (Clontech, #630412, Mountain View, CA). Yeast clones were investigated after 3-4 d and PCR confirmed.

3.3.4 Yeast colony PCR

Yeast cells were sampled by gently touching a single colony with the tip of a sterile toothpick. The yeast cells were mixed in 25 μL PCR mixture on ice by swirling the toothpick tip in the PCR mixture. PCR mixture of each reaction consists of: 0.5 μL Taq-polymerase (5U μL⁻¹), 2.5 μL 10×buffer, 1 μL 25× dNTPs (2 mM), 1 μL each primer (100 pmol μL⁻¹), and 20 μL H₂O. PCR was run with the following parameters: 1. 95°C, 5 min; 2. 95°C, 30 sec; 3. 50-55°C, 30 sec; 4. 72°C, 1 min kb⁻¹; for 35 cycles from step 2 to 4; 5. 72°C, 3 min. RCR results were examined on an agarose gel.
3.3.5 Overexpression of HAPs in plants

To generate At3g14020, At5g63470, At1g30500 and At3g48590 over-expression lines, the CaMV 35S promoter and PBI121 binary vector were used. Plants were transformed with \textit{Agrobacterium tumefaciens} using the floral dipping method (Clough and Bent, 1998). The transformants were selected on agar plates containing 50 \(\mu\)g mL\(^{-1}\) carbinicillin and kanamycin and verified using PCR with construct-specific primers. All the overexpression plants were selected for two more generations and homozygous transgenic plants (T3) were used for further characterization.

3.3.6 Protein extraction and \textit{in vitro} coimmunoprecipitations

Seedlings (15 g, fresh weight), wild-type or \textit{HAP3b}-overexpression line (overexpression of \textit{HAP3b}-Green Fluorescence Protein fusion protein, Cai et al., 2007) grown in soil were ground in liquid nitrogen, and thawed in 30 mL of extraction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% triton x-100, 1 mM PMSF, and 1× protease inhibitor cocktail). The mixture was kept at 4 °C for 30 min, filtered through four layers of Miracloth (Calbiochem, #475855, Gibbstown, NJ), and centrifuged at 12,000 g for 10 min at 4 °C. The protein concentration in the supernatant was determined by EZQ assay (Molecular Probes, R33200, Carlsbad, CA). Antibody immobilization follows the ProFound Kit (Pierce, 23600, Rockford, IL). One hundred microliters of coupling gel was applied to cross-link 200 \(\mu\)g of anti-green fluorescent protein multiclonal antibody (Invitrogen, A11122, Carlsbad, CA). Extracts containing similar amounts of total protein were incubated with 100 \(\mu\)L IgG beads overnight at 4 °C with gentle rotation. The IgG beads were collected after
centrifugation at 3000 g for 1 min at 4 °C. Proteins were eluted by adding 50 µL elution buffer to the gel in the spin column. The final eluent was concentrated by regular TCA-Doc precipitation protocol and then used for protein identification using HPLC-MS. The protein mixtures were first digested according to Waters Protein Expression System Manual. The digested protein samples (3 mL each) were introduced into a Symmetry® C18 trapping column (180 µM × 20 mm) by NanoACQUITY Sample Manager (Waters, Manchester, UK) washed by H₂O in one minute at 15 mL min⁻¹. The peptides were eluted from the Trapping column over a 100 µm ×100 mm BEH 130 C₁₈ column with a 140 min gradient (1-5% solvent B in solvent A over 0.1 min, 5-25% solvent B over 89.9 min, 25-35% solvent B for 5 min, 35-85% solvent B for 2 min, 85% solvent B over 13 min, 85-100% solvent B for 2 min and 100% solvent B for 28 min) at 1.2 µL min⁻¹ flow rate using an NanoACQUITY UPLC (Waters, Manchester, UK). For this system, solvent A was composed of 99.9% H₂O, 0.1% formic acid. Solvent B was composed of 99.9% acetonitrile and 0.1% formic acid. The mass spectrometry (MS) was set to a parallel fragmentation mode (MSE) with scan times of 1.0 second. The low fragmentation energy was 5 volts and the high fragmentation was from 17 to 35 volts. (GLU1)-Fibrinopeptide B was used as an internal calibration standard with LockSpray. Waters ProteinLynx Global SERVER Version 2.3 was used to analyze the ms dataset.

3.3.7 Genomic DNA extraction and T-DNA insertional mutant screening

Leaf tissues of soil-grown seedlings were first collected from individual plants.
Genomic DNA was extracted using a quick CTAB method (Rogers and Bendich, 1985) and used for PCR reactions with the primers recommended in the SALK protocol.

### 3.3.8 RNA extraction and reverse transcription

Total RNA was extracted using RNaWiz (Ambion, #9736, Austin, TX). DNase treatment of RNA samples were applied by RQ1 RNase-Free DNase I (Promega, #M6101, Madison, WI). DNase-treated RNA was first tested in a PCR reaction to ensure no genomic DNA contamination and then reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen, #18080-093, Carlsbad, CA) following the manufacturers’ protocols.

### 3.3.9 Flowering time

Seeds of wild-type, mutant, and overexpression transgenic plants were germinated in the same flat containing well-watered potting mix. After a 2-d cold treatment, seeds were germinated and 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 an 8h/16h light/dark photoperiod.

### 3.3.10 Double mutant generation and screening

Four double mutants were generated by crossing SALK_025666 (*hap3b* for At5g47640) with SALK_006559 for At1g54160, SALK_086334c for At3g48590 and SALK_132910c for At5g63470, respectively. Subsequent genotyping using PCR
confirmed that F1 plants were hemizygous for T-DNA insertions (data not shown). Hemizygous plants were allowed to self-pollinate for another generation and homozygous double mutants were identified using a PCR method and used for further analysis.

3.3.11 Primers

The primers used for cloning full-length or partial-length HAP3b, all HAP5s, all HAP2s, and for screening mutant plants and overexpression plants are listed in Table 3.6.

3.3.12 Freezing stress test

Survival test: Seeds of wild-type, HAP2 (At3g14020) or HAP5 (At5g63470)-overexpression plants, hap5 mutant, were first surface sterilized and arranged on the solid MS medium. The plates containing seeds were kept in a cold room (4°C) for 2 d and then moved into a growth chamber. Seeds were germinated and seedlings were grown under the conditions described above. Two-week-old seedlings were subjected to freezing by incubating the plate plants in a freezer at -20°C for 30 or 50 min depending on the experiment. The plates were then moved to a dark cold room at 4°C for 2 h, and returned to the growth chamber for recovery. The survival rates were scored 7 d after treatment (Ishitani et al., 1998; Zhu et al., 2004).

3.4 Results

3.4.1 HAP3b interacts with Arabidopsis HAP2 and HAP5 in yeast two-hybrid analysis

In yeast, HAP3 first forms a complex with HAP5 and then recruits HAP2 into
the complex which then binds to DNA (Hahn and Guarente, 1988). In \textit{Arabidopsis}, however, there are more than 10 members in each of the HAP2 and HAP5 families; by contrast in yeast there is only one HAP2 and one HAP3. To examine whether HAP3b would interact with any of the HAP2s and HAP5s, 10 HAP2s and 10 HAP5s were cloned and each co-transformed paired with HAP3b into yeast for protein expression and interaction. As a positive control, the CCT domain of CO protein was cloned and co-transformed with HAP3b into the yeast since their interaction had been reported previously (Wenkel et al., 2006). All the constructs were also transformed into yeast individually, without pairing with HAP3b, to identify potential autoactivation of transcription, which was not observed from expressing any of these proteins alone. Of all the HAP2s tested, only one HAP2 (At3g14020) could weakly interact with HAP3b. All the HAP5s, however, showed strong interaction with HAP3b. Interesting, HAP3b did not interact with the CCT domain of the CO protein (Table 3.1) which contradicting a previous study showing a positive interaction of CO with several HAP3s including HAP3b (Wenkel et al., 2006). The weak interaction was again verified after the vector swap between identified HAP2 and HAP3b (data not shown).

### 3.4.2 Conserved center domain of HAP3b controls the interaction with HAP5s

Since HAP3b could interact with all HAP5s, this non-specific interaction could be due to the protein-protein interaction domain in HAP3b. All the HAP3 proteins have a conserved protein binding domain in the middle and non-conserved N-terminal and C-terminal domains (Lee et al., 2003). Thus, the specificity of the interaction may
be determined by the terminal domains. To test this hypothesis, the HAP3b gene was
divided into three segments (domains), cloned and used in the yeast two-hybrid
analysis for interaction with each HAP5. Different combinations between the center
domain and one of the terminal domains were also used in the yeast two-hybrid
analysis.

Results showed that neither the N-terminal domain nor the C-terminal domain
alone interacted with any HAP5 (Table 3.2). Any combination that contains the center
domain showed positive interaction with all HAP5s (Table 3.2). Thus, it appears that
HAP3b does not show specificity to HAP5s.

3.4.3 At1g56170 and At5g63470 could interact with HAP2

Since HAP3b did not control specificity in protein interaction, it was further
hypothesized that the interaction between HAP2 and HAP3b might determine the
specificity in the heterotrimer formation in plants. HAP2 (At3gl4020) was thus used
in the yeast two-hybrid analysis with each HAP5. Among all the HAP5 tested, only
At1g56170 and At5g63470 showed interaction with this HAP2 (Table 3.3).

3.4.4 HAP3b interacts with non-HAP proteins in protein co-immunoprecipitation

To confirm that the HAP2 and two HAP5s identified from the yeast two-hybrid
analysis were real components in the HAP3b complex in vivo, a protein
co-immunoprecipitation (Co-IP) experiment was conducted. HAP3b was
overexpressed as a fusion protein with green fluorescence protein (GFP) in the hap3b
mutant background. Overexpression of HAP3b-GFP fusion protein reversed the
mutant late flowering phenotype, suggesting that the HAP3b-GFP fusion protein is functioning correctly (Cai et al., 2007). The anti-GFP polyclonal antibody was used to bind HAP3b-GFP and thus isolate other proteins that were associated with HAP3b in vivo. There were no HAP proteins detected from Co-IP analysis. Several identified proteins from the Co-IP included beta-thioglucoside glucohydrolase 2 (TGG2), peroxisomal NAD (+)-malate dehydrogenase 2 (PMDH2) and ribosomal protein L12 (RPL12) (Table 3.4).

3.4.5 HAP2-overexpression plants show enhanced cold tolerance

To examine the functions of identified HAP2 and HAP5, we overexpressed the genes in Arabidopsis. The identified HAP2-overexpression and HAP5-overexpression plants were further subjected to freezing test. Interestingly, HAP2-overexpression plants showed enhanced freezing resistance while HAP5-overexpression plants did not show improved freezing tolerance compared to WT (Fig. 3.1).

3.5 Discussion

3.5.1 HAP3b interacts specifically with HAP2 but not with HAP5

Previous studies indicated that HAP3b regulates flowering time through the long day photoperiod pathway in Arabidopsis (Cai et al., 2007). To understand its working mechanism, we used the yeast two-hybrid analysis to identify HAP2 and HAP5 proteins that could interact with HAP3b. Identification of HAP2 and HAP5 proteins could provide strong evidence that plants may use the same mechanism (i.e. forming a heterotrimer) found in yeast and mammalian systems for transcription activation. In
addition, since HAP2, HAP3, or HAP5 in plants is each encoded by a gene family, identification of individual members in the HAP2 and HAP5 families that interact with HAP3b would provide insight into the specificity of heterotrimer formation. The results of this study showed that HAP3b indeed had specific interaction with a HAP2 but no specificity to HAP5. The results, together with those from other studies (Ben-Naim et al., 2006; Wenkel et al., 2006), support the notion that plant HAPs may also form a heterotrimer for transcription activation.

HAP3 in yeast and mammalian cells interacts with both HAP2 and HAP5 during the trimer formation and the specific regions in HAP3 involved in the interactions have been mapped out. Two segments of CBF-A (HAP3 homologue in mammalian) between residues 63 and 102 and between residues 109 and 142 are essential for interactions with CBF-C (the HAP5 homologue in mammals) and CBF-B (HAP2 homologue in mammals) based on different cbf-a mutant analyses (Sinha et al., 1996). Residues within the segment of CBF-C between positions 59 and 108 are necessary for interaction with CBF-A. In fact, HAP5 and HAP3 interacted with each other through histone fold motifs (Romier et al., 2003). Residues 42 to 60 and residues 105 to 113 are necessary for interactions between the CBF-A/CBF-C heterodimer with CBF-B (Kim et al., 1996). HAP2 was identified to have a 65-amino acid core sequence (154-218 residues) responsible for DNA-binding and HAP2/HAP3/HAP5 trimer assembly (Olesen and Guarente, 1990). In summary, all HAP2/HAP3/HAP5 proteins have the domain for both reciprocal interactions. Plant HAPs contain these
conserved domains (Siefers et al., 2009), providing an explanation for interactions of HAP3b with HAP2 and HAP5.

The multiplicity of the HAP genes in plants makes determining specificity of HAP2/HAP3/HAP5 uniquely difficult, this cannot be studied in yeast and mammalian cells due to a single gene for each subunit. Each member in the HAP3 family in Arabidopsis can be divided into three domains, N-terminal, C-terminal and a central domain (Lee et al., 2003). A domain swapping study in Arabidopsis using domains from different HAP3 members indicated that a specific amino acid in the central domain conferred the unique activity of LEC1 in embryogenesis, differentiating its function from other HAP3b genes (Lee et al., 2003). There are different possible explanations for how this particular amino acid determines the specific action of LEC1. One of the possibilities is that the particular amino acid may mediate specific interaction with other HAPS, thus determining the functionality of the HAP complex. The similar domain analysis approach was used in this study to determine whether a specific domain in HAP3b is the key to the decision of the specificity. However, our results suggested that the specificity of the trimer might not be determined by a certain domain in HAP3b, besides demonstrating that the center domain is indeed important for protein-protein interaction. The specificity of interaction between HAP3b and one of the HAP5s might be determined through a different mechanism such as by controlling co-expression of a HAP5 in the same tissue or cell as HAP3b. Alternatively, the specificity of the HAP3b and a HAP5 in a trimer could be
determined by other components such as HAP2 in the final trimer assembly process (see discussion below).

HAP3b did not interact with the CCT domain of CO which was considered a HAP2-like protein. The results contradicted a previous study showing a positive interaction of CO with several HAP3s including HAP3b (Wenkel et al., 2006). The discrepancy may be due to the difference in the yeast two-hybrid systems used in the two groups. Or, perhaps CO did not interact with HAP3b directly. Other proteins such as HAP5 might scaffold these two proteins which makes examination of the direct interaction in our yeast two-hybrid system impossible. Our genetic evidence that a co hap3b double mutant showed an additive effect on delaying flowering (Wu, unpublished data); however, this evidence suggested that HAP3b works independently from CO, supporting the notion that HAP3b and CO might not interact with each other (see discussion below).

3.5.2 HAP2 specifically interacts with two HAP5s

In yeast, HAP3 first forms a complex with HAP5 then this complex recruits HAP2. In the filamentous fungus Aspergillus nidulans, only HAPB (ortholog for HAP2) had a nuclear localization signal. After single HAPC and HAPE proteins (orthologs for HAP3 and HAP5, respectively) had formed a heterodimer, the complex was then transported via HAPB into the nucleus (Steidl et al., 2004). Protein fusion of human NF-YA and A. nidulans HapB could complement the yeast hap2 mutant which implied that the nuclear localization signal of HAP2 might be conserved in yeast and mammalian cells (Romier et al., 2003; Tuncher et al., 2005).
A different situation was found in plants. Even though CO and HAP3b were detected in nuclei in plants (Wenkel et al., 2006), according to the predication from PredictNLS software, HAP3b and CO do not carry a nuclear localization signal (NLS) while some of the HAP5s have a NLS (http://cubic.bioc.columbia.edu/services/predictNLS/). Most of HAP2s do not carry an NLS either, except for At5g12840 (Table 3.5). This information implies that some of the HAP5s or HAP2s might determine the specificity of the trimer complex just like HAPB in A. nidulans. Our finding that HAP2 only interacts with two HAP5s and HAP3b seems to support this scenario.

Why two HAP5s could interact with this particular HAP2 is not clear. Based on protein sequence alignment, these two identified HAP5s are not closely related and are located in different tissues (Siefers et al., 2009). In addition, the two HAP5s had different gene expression patterns. At5g63470 was mostly expressed in root, seedling, and flower tissue while At1g56170 showed no expression in these tissues (Siefers et al., 2009). Thus, it is possible that two HAP5s might function differently by forming different heterotrimer complexes but sharing the HAP2 (At3g14020) in their complexes.

3.5.3 HAP3b interacts with other proteins in vivo

The LEC1 gene was the first HAP3 gene cloned and studied in plants. A recent study showed LEC1 could recruit bZIP67, an ABA-response element binding factor to form a complex to activate the promoter of CRUCIFERIN C to control seed embryo development (Yamamoto et al., 2009). In another study, LEC1 or LEC1-like
protein was found to function with NF-YA5, NF-YC4, Gcr1, Gpa1 and Pn1 in vitro to mediate blue light or ABA response (Warpeha et al., 2007). Thus these studies indicated that HAP forms a complex that may involve many other non-HAP proteins for specific transcription activation. The results from CO-IP analysis in this study also showed that HAP3b might interact with other proteins. Among them, beta-thioglucoside glucohydrolase 2 (TGG2) was a hydrolase involved in glucosinolate breakdown and insect defense (Barth and Jander, 2006) while peroxisomal NAD-malate dehydrogenase 2 (PMDH2) played a role in fatty acid beta-oxidation (Pracharoenwattana et al., 2007). Ribosomal protein L12-A (RPL12-A) is a component of the 60s ribosome in Arabidopsis. All other proteins identified in this study seem to be enzymes in secondary metabolites production. In fact, all these proteins appeared to be cytoplasmic proteins. Since HAP3b may be present in cytoplasm (without an NLS), it is possible for HAP3b to interact with these proteins before moving into nuclei.

It is also possible, however, that proteins identified from CoIP were the result of non-specific binding. A GFP-tagged HAP3b protein was used in this CoIP analysis. Despite some success in CoIP analysis (Mandel and Gozes, 2007; Umezawa et al., 2009), GFP tag is not often used in the CoIP test owing to its weak binding activity to its antibody and therefore low specificity (personal communications with other researchers). In addition, ectopic over-expression of HAP3b-GFP may also induce an artifact in protein-protein interaction. Additional examination is thus needed to verify the proteins identified from the CoIP studies.
3.5.4 HAP3b and CO control flowering through independent pathways

CO interacts with HAP5a and HAP3a to delay flowering (Wenkel et al., 2006). HAP5a protein in tomato was also proven to recruit CO-like proteins to promote flowering (Ben-Naim et al., 2006). All these results suggested that in the long-day photoperiod pathway, CO may form a complex with HAPs. However, how different HAPs can promote or delay flowering and whether they all act through CO proteins was not clear (Cai et al., 2007). The results from yeast two-hybrid analysis in this study showed that HAP3b did not interact with CO, suggesting that CO and HAP3b may work independently. The notion was further supported by the results from co hap3b double mutants which showed significant delay in flowering compared to either of the single mutants. Thus, HAP3b may even work in a pathway independent from CO.

3.5.5 An HAP2 that interacts with HAP3b is involved in freezing tolerance

Functional analysis of identified HAP2 and HAP5 that interact with HAP3b in the yeast two-hybrid analysis revealed unexpected results. Mutants and overexpression plants of these genes did not show altered flowering time. Instead, HAP2-overexpression plants showed improved freezing tolerance, opposite to the phenotype of HAP3b-overexpression plants. The results could suggest that HAP3b plays a key role in the HAP2/3/5 complex in flowering time regulation, and a HAP3b and HAP2 interaction may determine the final outcome of cold stress response in plants. Future studies are needed to address how HAP2-overexpression can improve
freezing tolerance and how HAP3b and HAP2 in a single complex could achieve
opposite effects on freezing tolerance.

3.6 References

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Table 3.1 Interactions of HAP3b with HAP2 and HAP5 Detected Using the Yeast Two-Hybrid System

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<td>AtHAP5c</td>
</tr>
<tr>
<td>At1g17590</td>
<td>-</td>
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<td>++</td>
</tr>
<tr>
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<td>AtHAP2c</td>
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<td>AtHAP5b</td>
</tr>
<tr>
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<td>AtHAP2b</td>
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<td>++</td>
</tr>
<tr>
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<td>-</td>
<td>At5g50480</td>
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<tr>
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<td>-</td>
<td>At3g48590</td>
<td>AtHAP5a</td>
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<td>At5g50490</td>
<td>++</td>
</tr>
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<td>AtHAP2a</td>
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<tr>
<td>At5g15840</td>
<td>CO</td>
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Note: No autoactivation of single vectors was detected (data not shown). HAP3b and another HAP3, At1g21970, were also tested and an interaction was detected between these two HAP3s. At5g15840 (CCT domain of CO), HAP2 like. AGI, Arabidopsis Genome Initiative. +, positive interactions; -, negative interactions; ++, strong positive interactions.
Table 3.2 Interactions of HAP3b Domains with HAP5 Detected Using the Yeast Two-Hybrid System

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Note: AGI, Arabidopsis Genome Initiative. No autoactivation of single domains was detected (data not shown). ++, positive interactions; -, negative interactions.
Table 3.3 Interactions of At3g14020 with HAP5 Detected Using the Yeast Two-Hybrid System

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Note: ++, positive interactions; -, negative interactions. AGI, Arabidopsis Genome Initiative.
Table 3.4  Identification of Proteins That Interact with HAP3b in a CoImmunoprecipitation Assay

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<th>Description</th>
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<th>mW (Da)</th>
<th>pI (pH)</th>
<th>Peptides</th>
<th>Coverage (%)</th>
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<td>1.</td>
<td>Glucoside glucohydrolase 2 hydrolase hydrolyzing o glycosyl compounds (TGG2)</td>
<td>At5g25980</td>
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<td>6.4</td>
<td>16</td>
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<td>2.</td>
<td>Peroxisomal NAD malate dehydrogenase 2 (PMDH2)</td>
<td>At5g09660</td>
<td>37345</td>
<td>8.0</td>
<td>8</td>
<td>38.1</td>
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<td>3.</td>
<td>Galacturan 1,4, alpha galacturonidase pectinase (exopolygalacturonase)</td>
<td>At1g02790 (At3g07850) (At3g14040)</td>
<td>45571</td>
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<td>4.</td>
<td>Epithiospecifier modifier 1 carboxylesterase (ESM1)</td>
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<td>5.</td>
<td>Ribosomal protein 112, a structural constituent of ribosome (RPL12-A)</td>
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<td>20062</td>
<td>5.4</td>
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<td>7.3</td>
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Table 3.5 Prediction of Nuclear Location of *Arabidopsis* HAPs Using PredictNLS Software.

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Note: +, in nucleus; -, not in nucleus; AnHAPB, *Aspergillus nidulans* HAPB (Accession number, AACD01000129); AoHAPB, *Aspergillus oryzae* (Accession number, AB010430); ScHAP2, *Saccharomyces cerevisiae* HAP2 (Accession number, P06774); NF-YA, Human NF-YA (Accession number, NM_021705). The above four non-*Arabidopsis* proteins were used as positive control since their locations were already confirmed (Steidl et al., 2004).
Table 3.6 Primers Used for Cloning *HAP3b, HAP2s and HAP5s* in the Yeast Two-Hybrid Analysis and Overexpression in *Arabidopsis*

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5'-upstream, to screen the At5g63470 mutant
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3'-downstream, to screen the At5g63470 mutant
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5'-upstream, to screen the At1g54160 mutant
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3'-downstream, to screen the At1g54160 mutant
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5'-upstream, to screen the At1g54160 mutant
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3'-downstream, to screen the At1g56170 mutant
ATCCAAACAAACACAGACTTATG
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3'-downstream, to screen the At3g48590 mutant
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5'-upstream, to screen the At1g30500 mutant
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3'-downstream, to screen the At1g30500 mutant
TGTTTCACGTAGTGGGACCATCG
LBa1 primer
GCCGACCGGCTTGGACTGCACT
LBb1 primer
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5'-upstream, to overexpress At3g48590
GGCCGACTTAAACTGGCCGTCGAGA
3'-downstream, to overexpress At3g48590
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5'-upstream, to overexpress At1g30500
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3'-downstream, to overexpress At1g30500
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5'-upstream, to overexpress At3g14020
GGCCGACTTAAACTGGCCGTCGAGA
3'-downstream, to overexpress At3g14020
TTAATTTAAATGGATACCAACACCAACCA
5'-upstream, to overexpress At5g63470
GGCCGACCGGCTTGGACTGCACT
3'-downstream, to overexpress At5g63470
Figure 3.1 Survival rate of wild-type plants, two overexpression plants of identified HAP2 (At3g14020) and HAP5 (At1g56170) and one hap5 (SALK_111422c for At3g14020) mutant plants after freezing treatment. Two-week-old plants grown on solid medium in Petri dishes were incubated at -20°C for 50 min. Survival plants were scored 7 d after treatment. Data are means ± SE of three independent experiments.
CHAPTER 4
IDENTIFICATION OF HAP3b AND HAP3c HOMOLOGS IN BARLEY AND THEIR FUNCTION

4.1 Abstract

HAP3 proteins are a group of transcription factors that play important roles in plant growth/development and response to environmental stress, such as embryogenesis, flowering time control, and drought tolerance. The objectives of this study were to identify HAP3 members in barley (*Hordeum vulgare*) and study the functions of a subset of barley HAP3s. From database sequence analysis, cloning, and sequencing, we confirmed that barley plants have at least six full-length members in the HAP3 family. Phylogenetic analysis showed that each barley HAP3 was different, forming its own cluster with the corresponding HAP3s from other plant species. The results indicated that the HAP3 family evolved before the divergence of monocots and dicots. Each barley HAP3 also showed its own expression pattern in different tissues, at different developmental stages and under various environmental stresses. In particular, TC176294 showed the highest sequence similarity to HAP3b in *Arabidopsis*. Its high expression was associated with flowering. In addition, TC176294 was upregulated by various abiotic stresses and by exogenous ABA application. Thus, TC176294 might be a barley ortholog of HAP3b. TC191694 showed the highest sequence similarity to HAP3c and might be a barley ortholog of HAP3c. To test this hypothesis, we over-expressed three barley *HAP3* s including TC176294 and TC191694 in *Arabidopsis*. TC191694-overexpression plants were
early flowering compared to $HAP3b$-overexpression and wild-type plants while
TC176294-overexpression plants were not. These results suggest that barley and
Arabidopsis have conserved mechanism in flowering time control using HAP3c.

4.2 Introduction

The developmental transition from vegetative to reproductive growth is essential
for successful reproduction and requires the proper integration of external stimuli such
as day length and temperature. In the floral transition of the dicot Arabidopsis,
environmental control is partially modulated through the photoperiod pathway
(Simpson and Dean, 2002). GIGANTEA ($GI$) and CONSTANS ($CO$) genes are two
major players in the photoperiod pathway. GI forms a complex with
FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) to bind the promoter of
$CO$ to regulate its expression (Sawa et al., 2007). GI also affects the CIRCADIAN
CLOCK ASSOCIATED 1 ($CCA1$) and LATE ELONGATED HYPOCOTYL ($LHY$)
genes, which are components of the circadian clock in Arabidopsis to regulate $CO$
expression (Fowler et al., 1999). CO activates $SOC1$, an integrator of four major
known flowering pathways (Moon et al., 2003), through $FT$ (Yoo et al., 2005). FT
protein was proved to be a long distance “florigen” in Arabidopsis flowering (Jaeger
and Wigge, 2007).

Several other genes, such as the recently identified stress-responsive $HAP3b$,
also function in the photoperiod pathway but the position of $HAP3b$ in this hierarchy
is still not clear (Cai et al., 2007). Interestingly, $HAP3c$, a gene showing the greatest
similarity to $HAP3b$ in the Arabidopsis HAP3 gene family, had an additive effect with
HAP3b in promoting flowering; Flowering in the hap3b hap3c double mutant was much more delayed. Thus, HAP3b and HAP3c play important but independent roles in controlling flowering time in the long-day photoperiod pathway (Kumimoto et al., 2008). HAP3b and HAP3c in Arabidopsis promoted flowering by increasing the transcript levels of SOC1 but the mechanism behind this was elusive. HAP3b and HAP3c are two of the thirteen HAP3 members in Arabidopsis (Siefers et al., 2009).

Similar flowering time-regulating components have been identified in rice, the model plant for the short-day photoperiod in monocots. OsGI, an ortholog of the Arabidopsis GI had a similar expression pattern (Hayama et al., 2002), and aberrant expression of OsGI caused late flowering under long day conditions in rice (Hayama et al., 2003). OsGI functions to upregulate Hd1 (the CO ortholog). Hd1 has a dual role in regulating Hd3a (the FT ortholog) depending on the photoperiod. Under long day conditions, Hd1 represses Hd3a expression while under the short days Hd1 enhances Hd3a expression. OsSOC1 is expressed in similar tissue and at similar developmental stages as SOC1 in Arabidopsis (Tadege et al., 2003). HAP genes have been characterized in rice but their role in flowering control has not been examined (Thirumurugan et al., 2008).

Some major cereal crops, such as wheat and barley, are long-day flowering monocot plants. Wheat TaHd1-1 was identified to have the same role as CO in Arabidopsis (Nemoto et al., 2003). Wheat TaGI1 (the GI ortholog) functioned in flowering time control just like GI in Arabidopsis (Zhao et al., 2005). Barley HvCO and HvGI were recently identified (Griffiths et al., 2003; Dunford et al., 2005). The
flowering integrators wheat TaFT and barley HvFT also were found to function similarly to FT in *Arabidopsis* (Li and Dubcovsky, 2008; Kikuchi et al., 2009). The other integrator wheat WSOC1 was found to play roles in both photoperiod and vernalization pathways (Shitsukawa et al., 2007). Thus, it appears that these long-day photoperiod cereal plants have similar regulatory machineries of flowering time control as *Arabidopsis*. Again, whether HAP is involved in flowering time control in these plants has not been studied.

The objectives of this study were to identify HAP3b and HAP3c counterparts in barley (*Hordeum vulgare*) and determine whether they were also involved in flowering time control in long-day cereal plants.

4.3 Materials and Methods

4.3.1 Bioinformatics analysis

To identify all the HAP3 family members in barley, two strategies were employed: First, the key word ‘HAP or NF-Y or CCAAT’ was used to search the barley EST database (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gireport.pl?gudb=barley). All the hits were then analyzed based on the sequence similarity to *Arabidopsis* HAP3b (NF-YB1 or At5g47640) and HAP3c (NF-YB3 or At4g14540). Second, the protein and nucleotide sequences of *Arabidopsis* At5g47640 (HAP3b) and At4g14540 (HAP3c) were used to blast the barley EST database. The sequences with high similarity (low e-values) were retrieved from the database.

These candidate genes were then cloned and sequenced for further analysis. For sequence alignment analysis, HAP sequences from other plants which showed high
similarity to *HvHAP3* were retrieved from GenBank based on a BLASTP analysis (http://www.ncbi.nih.gov). The protein sequences, starting from the first methionine, were used in the alignment analysis using a ClustalW method in the MegAlign program (DNASTAR, Inc., WI, USA). A phylogenetic tree was generated based on the ClustalW protein sequence alignment analysis.

### 4.3.2 Plant materials and growth conditions

To harvest very young barley roots and coleoptiles, seeds of barley (UT1960-483, OR741209//ID633019/Woodvale/3/Short2//ID633019/Woodvale/4/Brigham) were arranged on two layers of brown germination paper (Seedburo Equipment Co., IL, USA) which were pre-soaked with 90 mL ddH2O in a glass tray (60×20×6 cm³) and were then covered with another two layers of germination paper. The tray was wrapped with a layer of Saran wrap (The Glad Product Company, CA, USA) and kept vertical in the dark for 5 d at room temperature (23±1°C). Twelve holes were made in the Saran wrap using a dissecting needle to allow air exchange while minimizing water loss. Five-day-old coleoptiles, young shoots, and roots were harvested. All experiments had three biological replications.

Soil grown barley plants were used for gene expression in other tissues or at different developmental stages. Three barley seeds were germinated in a 10.5 L pot in a growth chamber with a light intensity of 300 μmol m⁻² s⁻¹ during a 16h light at 30°C and 8h dark at 25°C cycle. Leaf and stem tissues were harvested 15, 30, and 55 d after germination. Peat moss was used as potting mixture (Canadian Sphagnum Peat Moss, Sunshine, Alberta, Canada) and 600 mL nutrition solution (Peter’s Peat-Lite Special
20-10-20, 100 ppm N, OH, USA) for each pot was applied daily after 10-d germination.

4.3.3 Stress treatment

To study expression of *HvHAP3*s under stress, young seedlings were subjected to various stress treatments. Seeds were germinated between wet germination papers in vertically arranged glass trays covered with Saran wrap as described above. 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. The plants were grown under a 16-h/8-h light/dark cycle. After germination, coleoptiles and later leaves and stems were grown outside the germination paper while roots continued to grow downward between the germination papers. At the 7th d, water at the bottom of the glass trays was removed and replaced with 50 mL solution containing 0.5 mM KCl and 1 mM CaCl$_2$ (control) or 50 mL of the same solution plus 300 mM NaCl (salt stress). Leaves were harvested 4 h after treatment. For drought treatment, barley seedlings were exposed to air by lifting Saran wrap and removing the water at the bottom of the tray (drought stress) for 4 h. Normal transpiration of photosynthesizing plants quickly used up water from the germination paper, and plants started to wilt during the 4-h treatment. For ABA treatment, barley roots were treated with 50 mL 10 μM ABA methanol solution or with 50 mL methanol-containing ddH$_2$O without ABA (control) for 4 h. The trays containing seedlings were tilted so that the roots were partially submerged in a thin layer of solution. Treatment and sample harvesting of three replicates were conducted at the same time of the day on different days. Samples were immediately dipped in
liquid nitrogen and stored at -80ºC until use.

4.3.4 Water content measurement

Five to six young seedlings were collected from each treatment (well-watered control and drought stress) at harvest for measurement of relative water content (RWC). The seedlings were weighed immediately after harvesting for fresh weight (FW1). The tissues were then submerged in distilled water and kept in the dark at 4ºC overnight. The tissues were weighed again on the second day after being blotted dry (FW2). Dry weight (DW) was obtained after tissues were dried in an oven at 80ºC for 48 h. RWC was calculated using the following equation (http://www.plantstress.com/files/RWC.htm):

\[
\text{RWC} (%) = \frac{\text{FW1} - \text{dry weight}}{\text{FW2} - \text{dry weight}} \times 100
\]

4.3.5 Genomic DNA extraction

Leaf tissues were harvested from soil-grown seedlings either individually or in bulk depending on the experiments. Genomic DNA was extracted using a quick cetyl-trimethyl-ammoniumbromide method (Rogers and Bendich, 1988) and used for PCR reactions.

4.3.6 RNA extraction and reverse transcription

Total RNA was extracted using RNAwiz (Ambion, #9736, TX, USA). DNase treatment of RNA samples were applied using RQ1 RNase-Free DNase I (Promega, #M6101, WI, USA). DNase-treated RNA was first tested in a PCR reaction to ensure no genomic DNA contamination and then reverse transcribed into cDNA using
SuperScript III Reverse Transcriptase (Invitrogen, #18080-093, CA, USA) by following the manufacturers’ protocols.

4.3.7 Quantitative PCR

The individual gene (TC191694, TC176294, TC171559, TC161801, or TC164749) fragment was amplified using barley cDNA as templates in regular PCR. Primers are listed in Table 4.4. For the genes with introns, primers were designed flanking an intron so that genomic DNA contamination was readily detected in RNA samples based on the size difference in PCR products. PCR products were extracted from the gel and sequenced. These PCR products were then diluted into different concentrations and used as templates to construct the standard curves. PCR reactions for the standard curve and all the samples of the same gene were run at the same time and analyzed on the same agarose gel. The barley 18S housekeeping gene was used for normalization of the amount of cDNA used in each PCR reaction.

4.3.8 Plasmid construction and plant transformation

The barley candidate genes that showed high similarity to Arabidopsis HAP3b and HAP3c were cloned into a Zero-Blunt Clone vector (Invitrogen, CA, USA), sequenced, and then subcloned into a PBI121 vector (driven by a CaMV 35S promoter). All PCR amplifications were carried out with high-fidelity DNA polymerase (Stratagene PfuUltra DNA polymerase, CA, USA). The genes were transformed into Arabidopsis wild-type plants with Agrobacterium tumefaciens (ABA4404) using the floral dipping method (Clough and Bent, 1998). The transformants were selected on agar plates containing 50 μg mL\(^{-1}\) carbinicillin and
kanamycin and verified using PCR with construct-specific primers. All the
overexpression plants were selected for two more generations and homozygous
transgenic plants (T3) were used for further characterization. Expression levels of the
transgenes in Arabidopsis were examined using semi-qRT-PCR as described above.

4.3.9 Flowering time

Seeds of Arabidopsis wild-type and overexpression transgenic plants were
germinated in the same flat containing well-watered potting mix. After a 2-day cold
treatment, seeds were germinated and plants were grown under long-day 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.

4.4 Results

4.4.1 Identification of HAP3 candidates in barley

More than 40 annotated entries, TCs (tentative contigs) and ESTs were found by
searching the barley EST database using the keywords “CCAAT”, “NF-Y”, “HAP” or
“Nuclear transcription factor Y”. Through careful sequence similarity analysis, 12
TCs/ESTs were chosen as HAP3 candidates by aligning with Arabidopsis HAP3b,
HAP3c, HAP2 or HAP5. Using Arabidopsis HAP3b or HAP3c protein sequences in a
TBLASTP search analysis of barley database, 9 hits showed different degrees of
similarity and were retrieved. Eight of these TCs/ESTs are common to the gene list
from the key word search. In combination, a total of 13 TC/EST sequences were
preliminarily considered barley HAP3 candidates. They are: AJ461344, AJ485376,
DN155102, TC189179, TC175935, TC158430, TC168497, TC164749, TC171559, TC176294, TC191694, TC166526 and TC161801. Among them, AJ461344, AJ485376, DN155102, TC189179, TC175935 and TC158430 were short sequences, had low scores (Probability < e-11) in protein sequence blast analysis using HAP3b or HAP3c, and were not even present in nucleotide blast analysis. Thus, these sequences were not included for further analysis.

To ensure sequence accuracy of the remaining TCs, these seven TC sequences were PCR amplified from either genomic DNA (gDNA) or cDNA templates, cloned and sequenced. TC191694 and TC176294 only showed small nucleotide sequence discrepancies compared to the ones in the database. The TC171559 Coding Sequences (CDS) sequence was identical to the original in the database. The barley database indicated that TC168497 had a full length CDS. Sequencing of PCR products, however, showed that there were some sequence insertions and deletions in this contig and this TC was not a full-length sequence. New primer pairs were designed according to the new predicted CDS sequence but failed to produce PCR products from either gDNA or cDNA samples. Since TC166526 and TC164749 only had partial CDS sequences, effort was made to clone the missing parts of the genes. Clones containing ESTs of these TCs were first ordered from B-Bridge International, Inc. (CA, USA). However, the resulting sequences from these commercial clones were totally different from the ones deposited in the database. A different approach was then taken. Using the partial barley TC sequences (TC166526 and TC164749) to blast against other related cereal crop genome databases such as rice, wheat, and corn,
it was found that these TCs showed high identity with two full-length wheat HAP3s, with 77.8% and 90.4% nucleotide sequence identity. By aligning the identified wheat sequences with barley TCs, primer pairs that flanked the region between the first ATG and the first stop codon were designed and used in PCR amplification. One primer matched the wheat sequence and the other corresponded to the barley TC. The primer pairs were designed and used in PCR amplifications. Either barley cDNA or gDNA were used as template. As a result, both barley TCs were successfully cloned. After sequencing, these clones appeared to be the full length sequence and contained the original partial barely TC sequences. It turned out that TC166526 only differed by 6 nucleotides from TC161801 in the CDS region. Thus, these two TCs are potentially allelic and encoded by the same gene. Of the two, TC161801 was chosen for further analysis. The full-length barley CDS was then blasted back against the wheat EST database. The top hit is the same wheat TC, suggesting this cloning strategy may work well for cloning genes across the barley and wheat genomes.

4.4.2 Phylogeny and alignment analysis

The deduced protein sequences of TC191694, TC171559, TC176294, TC164749 and TC161801 were further blasted against the NCBI database to confirm if they are indeed HAP3s. The top three hits from the blast result were all annotated HAP3s from various plants. This is also true when only the full-length hits were considered (Table 4.1).

The phylogenetic relationship among *Arabidopsis*, barley, and rice HAP3s was investigated by constructing a phylogenetic tree using ClustalW in the DNAstar
MegAlign program. The alignment included five barley TCs and selected HAP3s, HAP5s and HAP2s from Arabidopsis and rice. Protein sequence homology was measured as percentage of identical amino acid residues. All five barley full-length genes were clustered with Arabidopsis and rice HAP3 and separated from HAP2 and HAP5 (Fig. 4.1). Clustering of monocot HAP3 with dicot HAP3 was observed in the tree, implying that duplication of these genes occurred before the divergence of monocots and dicots. TC176294 was highly homologous to HAP3b (66.1% identity). TC191694 was most similar to HAP3c (71.7% identity). TC161801, TC171559 and TC164749 were closely grouped with rice OsHAP3a.

4.4.3 Diverse gene expression patterns of barley HAP3s at different growth stages

To further understand the relationship between identified barley HAP3 genes with Arabidopsis HAP3b and HAP3c, expression patterns of barley HAP3 genes were compared with those of Arabidopsis HAP3b and HAP3c. Information from the Genevestigator database showed that the transcript level of Arabidopsis HAP3b or HAP3c in leaves was gradually increased from young seedlings, reaching the highest levels at flowering stage or in mature leaves. The transcript levels then decreased rapidly in the late stages of flowering and seed formation (Fig. 4.2). As shown in Table 4.2, five barley HAP3 genes were all expressed in leaves of all stages. Most barley HAP3s were also expressed in roots, coleoptiles, stems, and flower organs (including awns) at various stages. One exception is TC176294, whose transcripts were not detected in young roots. These expression results were supported by the
information in the database as these gene sequences are present in various EST libraries (Table 4.3).

Relative transcript levels of barley HAPs in different tissues at different developmental stages were examined using semi-quantitative PCR and compared with the level of 15-d old young seedlings. TC176294 was expressed at relatively high levels in young seedlings and expressed at the highest level in developing leaves (30-d old), but the transcript level was reduced to very low levels in maturing leaves and awns. The transcript levels were low in stems regardless of development stage. TC191694 was expressed at the highest level in young seedlings but the transcript levels declined drastically in all the tissues in developing and mature organs (Fig. 4-3). TC164749 and TC171559 were also expressed at the highest level in very young seedling (5-d old) and their transcript levels were expressed at lower but relatively similar levels in all the tissues of different stages (Fig. 4-3). TC161801 was expressed at the highest level in stems and the levels appeared to increase with plant maturation, reaching the highest level in 55-d-old stems. Change in the transcript levels in leaves showed a similar pattern to those in stems, although the relative expression was lower than that in stems. Interestingly, the transcript level in awns was even greater than in maturing leaves (Fig. 4-3).

4.4.4 Change in barley HAP3 transcript levels under different stress conditions

Previous studies showed that the transcript level of HAP3b of Arabidopsis was enhanced by treatments of low temperature (4°C), 100 mM NaCl, or 200 mM mannitol (Kreps et al., 2002). Data from the microarray database
(www.arabidopsis.org) showed that HAP3b transcript levels also responded to ABA treatment and heat stress in addition to cold, osmotic, and drought stress, showing the greatest upregulation in response to salinity stress, drought and ABA. To investigate whether any of the barley HAP3 genes were regulated in a similar way, transcript levels of these barley HAP3s were examined after barley plants were subject to salt, drought and ABA treatments. Relative water content of drought-stressed plants was 79% compared to 92.8% of control plants. The results showed that TC176294 transcript levels were upregulated by all the stress treatments, with the greatest induction by ABA treatment. TC191694 showed a slight decrease in transcript levels after ABA treatment. The transcript levels of TC171559 and TC164749 were not affected by these treatments. TC161801, however, showed only one response: a drastic increase in transcript level in response to high salt treatment.

4.4.5 TC191694-overexpression plants are early flowering compared to HAP3-overexpression and WT plants

To determine whether TC176294 and TC191694 are orthologs of HAP3b and HAP3c in Arabidopsis, we overexpressed these two barley genes in Arabidopsis since Arabidopsis transformation is easy and quick. We included TC171559 as a control. Only TC191694-overexpression plants were early flowering among three barley genes compared to WT. In fact, TC191694-overexpression plants even flowered earlier compared to HAP3b-overexpression plant (Fig. 4.5). All overexpression lines were true overexpression plants since their transcript levels were increased greatly compared to that in WT plants (Fig. 4.6).
4.5 Discussion

Previous studies showed that stress-responsive *HAP3b* in *Arabidopsis* regulates flowering time through the long day photoperiod pathway. Our recent work indicates that HAP3b also suppresses freezing tolerance in *Arabidopsis* (Chapter 2) and promotes root elongation (Ballif, 2007). The main objective of this study was to identify *HAP3b* and *HAP3c* ortholog genes in barley through sequence similarity comparison, gene expression pattern, and gene function analysis.

4.5.1 TC176294 in barley is a possible ortholog of HAP3b

Based on sequence similarity analysis, six *HAP3*-like genes were identified from the barley EST database. The data indicated that barley, like other plant species, has a multigene family of *HAP3* genes (Edwards et al., 1998; Ito et al., 2005). Compared to *Arabidopsis* which has 13 *HAP3* genes, barley had seven members in the gene family which had full-length sequences. There were six members only having partial gene sequences. Since the barley genome is not completely sequenced, it is very possible that other HAP3 members exist in the genome but are currently missing in the EST database. There are 11 annotated HAP3 members in the rice genome. Thus, monocot plants appear to have a similar number of HAP3 members as dicot plants.

The barley *HAP3*-like genes could be classified into two groups based on the phylogenetic study using deduced amino acid sequences (Fig 4.4). TC176294 and TC191694 share high sequence similarity and are clustered with HAP3b and HAP3c which are two closely-related members in the *Arabidopsis* HAP3 family. Thus, TC176294 and TC191694 could potentially be the orthologs of HAP3b and HAP3c.
and these two genes likely evolved before the divergence of monocots and dicots. Other barley HAP3-like genes are grouped with rice OsHAP3a which was involved in rice chloroplast biogenesis (Miyoshi et al., 2003).

TC176294 showed the highest similarity to HAP3b in Arabidopsis while TC191694 showed the highest similarity to HAP3c. The high similarity is clearly demonstrated from protein sequence alignment. All HAP3 proteins have a conserved central domain and diverse N-terminal and C-terminal domains. The deduced protein sequence of TC176294 showed nearly identical central domain to HAP3b. In addition, they shared 59.1% and 20% sequence identity in N-terminal and C-terminal domains, respectively, while TC191694 shared 50% and 57.1% with HAP3c in N-terminal and C-terminal domains, respectively.

Gene expression patterns provide additional supporting evidence for TC176294 and TC191694 to be potential HAP3b and HAP3c orthologs. HAP3b in Arabidopsis was expressed in leaves and various other tissues. Its transcript level was increased rapidly at the stage of phase transition from vegetative to reproductive growth under long-day (LD) conditions which was consistent with its role in flowering (Cai et al., 2007). The expression pattern of barley TC176294 in leaves showed a similar trend, reaching the highest level on the 30th day (before pollination) and decreased on the 55th day (after pollination) under LD conditions. In contrast to Arabidopsis HAP3b, which is also expressed in roots and plays a role in root elongation (Ballif, 2007), transcripts of TC176294 were not detected in barley roots. HAP3b in Arabidopsis is expressed at very low levels in the very tip of the root. TC176294 transcripts may
escape detection in barley roots due to low and localized expression. Alternatively, TC176294 may have lost its function in root elongation in barley during evolution, if TC176294 is indeed an ortholog of HAP3b. TC191694 expression also showed a similar pattern, showing the highest expression in young leaves (15th day). Transcripts of other HAP3-like genes in barley showed relatively constant expression in leaves and were not correlated with flowering process. Thus, both sequence similarity analysis and gene expression patterns, together with other evidence discussed below, suggest that TC176294 may be an ortholog of HAP3b and TC191694 may correspond to HAP3c in Arabidopsis.

4.5.2 TC176294 transcript levels respond to multiple stresses

More and more evidence shows that HAPs play important roles in stress response. Overexpression of HAP3a (At2g38880) in Arabidopsis and orthologous maize ZmNF-YB2 conferred improved performance under drought stress (Nelson et al., 2007). Transcript levels of NF-YA (At1g54160), a HAP2 member in Arabidopsis, were highly up-regulated by drought. Transgenic HAP2-overexpression plants were more resistant to drought stress (Li et al., 2008). HAP3b transcript levels were initially observed to be highly induced by salt, osmotic stress, and ABA in Arabidopsis (Kreps et al., 2002; Cai et al., 2007). Instead of improving stress tolerance directly, overexpression of HAP3b promotes early flowering (Cai et al., 2007) and at the same time suppresses freezing tolerance (Chapter 2). Interestingly, TC176294 appears to be the only HAP3 among the five barley HAP3s studied whose transcript levels were increased under multiple stresses and ABA treatment. Thus, the results again suggest
that TC176294 may indeed be an ortholog of *HAP3b* and may play similar roles in barley (see discussion below). TC161801 transcript levels were only increased under salt treatment, and the other barley *HAP3* genes showed no or only moderate changes in response to stress treatments, suggesting TC161801 may play a specific role in salinity tolerance and different barley *HAP3*s may have different functions.

### 4.5.3 TC191694 is an ortholog of *Arabidopsis* HAP3c

TC191694 showed the highest sequence similarity to *Arabidopsis* HAP3c, and also caused plants early flowering when it was overexpressed in *Arabidopsis*, indicated the TC191694 is an ortholog of HAP3c. Our results demonstrated the conserved function of HAP3c protein. However, whether TC191694 will also performed similar role in barley in flowering time control needs to be demonstrated.

Overexpression of TC176294 did not show an effect on flowering, even though TC176294 showed the highest sequence similarity and had similar expression patterns to *HAP3b* among these five barley *HAP3*s. The reasons why TC176294 overexpression plants were not early flowering are not clear at this time. The simplest possibility is that TC176294 is not the true *HAP3b* ortholog, i.e. a true *HAP3b* ortholog is not in the database yet. In *Arabidopsis*, there are at least 10 members in each HAP family, and in rice at least 11 HAP3s have been identified. Thus, it is very possible there are many other HAP3s in the barley genome to be discovered. Though TC176294 showed an overall high similarity to HAP3b, the highest similarity in amino acid sequence was identified in the central conserved domain. For C-terminus, TC176294 only shares 20% identity with HAP3b while TC191694 showed 57%
identity with HAP3c. It is also possible that TC176294 is indeed originally a HAP3b but its function may have been lost in or evolved so that it may need additional components in barley for function in flowering. Thus, a direct study of TC176294 and TC191694’s function in barley in flowering time control is needed in the future.

4.6 References


Zhao XY, Liu MS, Li JR, Guan CM, Zhang XS (2005) The wheat TaGI1, involved in photoperiodic flowering, encodes an Arabidopsis GI ortholog. Plant Molecular Biology 58: 53-64
Table 4.1  Protein Sequences That Are Most Similar to Barley HAP3 in NCBI Database

<table>
<thead>
<tr>
<th>Top 3 hits</th>
<th>e-value</th>
<th>Annotation</th>
<th>Full length sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC171559</td>
<td>5E-74</td>
<td>Nuclear transcription factor Y subunit B-3 [Zea mays]</td>
<td>NP_001152278</td>
</tr>
<tr>
<td>BAC76331</td>
<td>9E-71</td>
<td>HAP3 [Oryza sativa Japonica Group]</td>
<td></td>
</tr>
<tr>
<td>P25209</td>
<td>2E-70</td>
<td>Nuclear transcription factor Y subunit B in Zea mays</td>
<td></td>
</tr>
<tr>
<td>TC176294</td>
<td>1E-66</td>
<td>Os03g0413000 [Oryza sativa (japonica cultivar-group)]</td>
<td>NP_001050358</td>
</tr>
<tr>
<td>XP_002467695</td>
<td>3E-63</td>
<td>Hypothetical protein SORBIDRAFT_01g022590 [Sorghum bicolor]</td>
<td></td>
</tr>
<tr>
<td>NP_001147638</td>
<td>6E-63</td>
<td>Nuclear transcription factor Y subunit B-3 [Zea mays]</td>
<td></td>
</tr>
<tr>
<td>TC191694</td>
<td>1E-70</td>
<td>Hypothetical protein SORBIDRAFT_02g038870 [Sorghum bicolor]</td>
<td>XP_002463163</td>
</tr>
<tr>
<td>NP_001147727</td>
<td>2E-68</td>
<td>Nuclear transcription factor Y subunit B-3 [Zea mays]</td>
<td></td>
</tr>
<tr>
<td>NP_001060230</td>
<td>3E-60</td>
<td>Os07g0606600 [Oryza sativa (japonica cultivar-group)]</td>
<td></td>
</tr>
<tr>
<td>TC164749</td>
<td>4E-55</td>
<td>Os05g0573500 [Oryza sativa (japonica cultivar-group)]</td>
<td>NP_001056383</td>
</tr>
<tr>
<td>XP_002440289</td>
<td>2E-52</td>
<td>Hypothetical protein SORBIDRAFT_09g029140 [Sorghum bicolor]</td>
<td></td>
</tr>
<tr>
<td>EEE64770</td>
<td>3E-52</td>
<td>Hypothetical protein Os_19626 [Oryza sativa Japonica Group]</td>
<td></td>
</tr>
<tr>
<td>TC166526</td>
<td>5E-71</td>
<td>Nuclear transcription factor Y subunit B in Zea mays</td>
<td>P25209</td>
</tr>
<tr>
<td>P25209</td>
<td>6E-71</td>
<td>CAAT-box DNA binding protein subunit B (NF-YB) [Zea mays]</td>
<td></td>
</tr>
<tr>
<td>NP_001141333</td>
<td>2E-70</td>
<td>Hypothetical protein LOC100273424 [Zea mays]</td>
<td></td>
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</tbody>
</table>

Note: The deduced protein sequences of five HvTCs were used in BlastP analysis.
Table 4.2  Expression of Barley HAPs in Different Tissues

<table>
<thead>
<tr>
<th>Gene</th>
<th>Developmental stage</th>
<th>5-day etiolated seedlings</th>
<th>15-day soil-grown plants</th>
<th>30-day soil-grown plants</th>
<th>55-day soil-grown plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Root</td>
<td>Coleoptile</td>
<td>Shoot</td>
<td>Seedling</td>
</tr>
<tr>
<td>TC176294</td>
<td>0/3</td>
<td>0/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

Note: Gene expression was examined using RT-PCR. The ratio in the table indicates the times that transcripts were detected from samples of three independent biological experiments.
### Table 4.3  Expression of Barley HAPs Based on EST in cDNA Libraries (NCBI or DFCI Barley Gene Index Database)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Database Information – cDNA Libraries</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC191694</td>
<td>3-week-old root and top three leaves of heading stage</td>
</tr>
<tr>
<td>TC176294</td>
<td>Germination shoots; 7-day-old green seedlings infected with <em>Blumeria graminis</em> f. sp. <em>hordei</em>, and leaves were harvested 24, 48 and 72 hr post-inoculation</td>
</tr>
<tr>
<td>TC164749</td>
<td>Embryos dissected from developing grains (21 days post anthesis)</td>
</tr>
<tr>
<td>TC171559</td>
<td>Developing endosperm tissue in 10, 12, 15 DPA (days post anthesis); 7-day-old green leaves; pericarp in 0-7 DAP</td>
</tr>
<tr>
<td>TC161801</td>
<td>Rachis, embryo, endosperm tissues in 10, 12, and 15 DPA; whole spikes with awns collected at 20 DAP; 3-week-old root, callus</td>
</tr>
</tbody>
</table>
### Table 4.4  Primers for Gene Expression and Overexpression of Barley HAPs in Arabidopsis

<table>
<thead>
<tr>
<th>Primer Sequences (5'-3')</th>
<th>Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>GACGCCTTCCTCCCTGTCTTTTG</td>
<td>5'-upstream, TC191694, gene expression</td>
</tr>
<tr>
<td>TCACCGCTCCGCCATGGTTCTTT</td>
<td>3'-downstream, TC191694, gene expression</td>
</tr>
<tr>
<td>CCGCGGCTGATGCTTTGAT</td>
<td>5'-upstream, TC176294, gene expression</td>
</tr>
<tr>
<td>TCGCCGAGCTGTGGAGGTAG</td>
<td>3'-downstream, TC176294, gene expression</td>
</tr>
<tr>
<td>ATCCAGACGGACAGCAAGGAC</td>
<td>5'-upstream, TC171559, gene expression</td>
</tr>
<tr>
<td>GCCGCTCTCTGTTAAAAGGAT</td>
<td>3'-downstream, TC171559, gene expression</td>
</tr>
<tr>
<td>GAACCGAATGAGCAGACAGGAG</td>
<td>5'-upstream, TC1761801, gene expression</td>
</tr>
<tr>
<td>CCGGGAGATGGAGGTGAC</td>
<td>3'-downstream, TC1761801, gene expression</td>
</tr>
<tr>
<td>CGACCGCTGTAAGCAAGGAT</td>
<td>5'-upstream, TC164749, gene expression</td>
</tr>
<tr>
<td>GCCGCCGGCCTTCCTCACAGGAG</td>
<td>3'-downstream, TC164749, gene expression</td>
</tr>
<tr>
<td>CGGGCTCTCGGATACAGCGGAAGGC</td>
<td>5'-upstream, TC168497, gene expression</td>
</tr>
<tr>
<td>GGCGCGCCATGCCGGACTCCGACA</td>
<td>3'-downstream, TC168497, gene expression</td>
</tr>
<tr>
<td>GGCGACGTCTCTCTTCTTTCT</td>
<td>5'-upstream, TC168497, gene expression</td>
</tr>
<tr>
<td>GGGCTCTCCCGCAACCAA</td>
<td>3'-downstream, TC168497, gene expression</td>
</tr>
<tr>
<td>CAGCCGCTTTATCTCTCAGTCA</td>
<td>5'-upstream, TC164749, full-length CDS sequencing</td>
</tr>
<tr>
<td>ATGTCCGAGAGCCGGTAGAGC</td>
<td>3'-upstream, TC166526, full-length CDS sequencing</td>
</tr>
<tr>
<td>CAGACGGCAACAACAAACAAAGGCA</td>
<td>5'-upstream, TC166526, full-length CDS sequencing</td>
</tr>
<tr>
<td>GCTCAATCGGAAAGGCCCT</td>
<td>5'-upstream, TC168497, full-length CDS sequencing</td>
</tr>
<tr>
<td>CGTAGATGCCCAGCTGAT</td>
<td>3'-upstream, TC168497, full-length CDS sequencing</td>
</tr>
</tbody>
</table>
Figure 4.1 Phylogenetic analysis of putative barley HAP3s with selected rice and Arabidopsis HAP3s and other HAPs. Sequence alignment analysis was performed using a ClustalW method in the MegAlign program. The following are the corresponding accession numbers for each full-length protein in NCBI database: AAO39912 for At1g14540, NP_199575 for At5g47640, NP_001078545 for At5g08190, BAH19731 for At5g63470 and NP_188018 for At3g14020 in Arabidopsis; BAC76331 for OsHAP3a, BAF64449 for OsHAP5a and BAF64435 for OsHAP2a in rice (Oryza sativa).
Figure 4.2 Relative transcript levels of *Arabidopsis HAP3b* (At5g47640) and *HAP3c* (At4g14540) in different tissues at different developmental stages. The signals on Affymetrix chips representing the relative transcript levels of each gene were retrieved from the Genevestigator database.
Figure 4.3 Relative transcript levels of five putative barley HAP3s in different tissues at different developmental stages. Transcript levels were examined using semi-quantitative PCR, then normalized to an 18s gene and compared with the level of 15-d old seedlings. Data are means ± SE of three independent experiments.
Figure 4.4 Change in relative transcript levels of putative *HAP3* genes in barley seedlings subjected to salinity (150 mM NaCl), drought or ABA (10 µM) treatments. Transcript levels were examined using semi-quantitative PCR, then normalized to an 18s gene and compared with control plants. Data are means ± SE of three independent experiments.
Figure 4.5 Early flowering in TC191694-overexpression plants grown under a 16-h/8-h light/dark photoperiod. OX-191694 plants (CaMV35S:TC191694 in wild-type background) developed fewer leaves compared with control WT plants and HAP3b-overexpression plants before flowering. OX-176294 and OX-171559 plants had the same number of leaves compared to WT plants. The data are means±SE (n = 22?-30) from three independent experiments.
Figure 4.6 All transgenic lines were true overexpression lines. Total RNA were extracted from each overexpression line and WT *Arabidopsis*. Semi-RT-qPCR method was used to determine transcript level and 18S gene was used as a control.
CHAPTER 5

SUMMARY

Heme-activated proteins (HAPs), also known as nuclear factor Y proteins (NF-Ys) or CCAAT-binding Factors (CBFs), are transcription factors that have multiple roles in plant growth and development, such as embryogenesis, flowering time control, and drought tolerance.

In Arabidopsis (Arabidopsis thaliana) HAP3b has been shown to regulate flowering time. In the present study I found that HAP3b was also involved in controlling response to cold stress. Transcript profiling and gene expression analyses indicated that HAP3b repressed the CBF3 regulon under normal growth conditions. As a result, plants with HAP3b-overexpressed showed decreased survival rates while plants homozygous for the null allele hap3b showed an improved freezing tolerance compared to wild-type plants. Since HAP3b is upregulated by multiple abiotic stresses and promotes flowering, HAP3b could be an important link between flowering time control and low temperature response pathways, an evolutionary advantage for Arabidopsis to complete reproductive growth under stress by efficiently using energy and resources.

In yeast and mammalian systems, HAP genes activate transcription by forming a heterotrimer consisting of HAP2, HAP3, and HAP5. To understand the mechanism of HAP3b in Arabidopsis, i.e. whether it also acts through forming a heterotrimer, the yeast two-hybrid system and the protein coimmunoprecipitation method were used to identify the proteins that could interact with HAP3b. From yeast two-hybrid analyses,
it was found that HAP3b could interact with one (At3g14020) of ten HAP2s and all ten HAP5s tested. Further analyses showed that the newly identified HAP2 protein could only interact with two HAP5 proteins, those encoded by At5g63470 and At1g56170. Thus, HAP3b in Arabidopsis may also form a heterotrimer and HAP2 might determine the specificity of the interaction. However, protein coimmunoprecipitation analyses revealed a different set of proteins that interacted with HAP3b. To provide supporting evidence for the protein-protein interaction data, a genetic approach was used to examine the functions of some of the identified proteins in flowering time control and freezing tolerance.

To address whether HAPs also play important roles in major crop plants, HAP3 genes in barley (Hordeum vulgare L.) were identified and characterized. From database sequence analyses, cloning, and sequencing, it was found that barley plants have at least six full-length members in the HAP3 family. Phylogenetic analyses showed that each barley HAP3 was different, forming its own cluster with the HAP3s from other plant species. The results indicated that the HAP3 family evolved before the divergence of monocots and dicots. Each barley HAP3 also showed its own expression pattern in different tissues, at different developmental stages and under various environmental stresses. In particular, TC176294 showed the highest sequence similarity to HAP3b in Arabidopsis and its high expression was associated with flowering. In addition, TC176294 was upregulated by various abiotic stresses and by abscisic acid (ABA). Thus, TC176294 might be a barley ortholog of HAP3b. TC191694 showed the highest sequence similarity to HAP3c and might be a barley
ortholog of HAP3c. To test this hypothesis, we overexpressed three barley HAP3s including TC176294 and TC191694 in Arabidopsis. TC191694 overexpression plants were early flowering compared to HAP3b-overexpression and wild-type plants while overexpression of TC176294 plants were not.

These results have provided new insights into the physiological functions and molecular mechanism of HAP3b in Arabidopsis. Identification of HAP3s and their expression patterns in barley sheds light on the evolutionary history of the gene family and potential conserved gene functions. Further studies are needed to determine whether the knowledge generated in this study will be useful for crop improvement.
CURRICULUM VITAE

Mingxiang Liang, Ph.D. Candidate
Department of Plants, Soils, and Climate
Utah State University
Tel: 1-435-213-5910
Email: a.L@aggiemail.usu.edu

Education and Training
Ph.D. student in Plant Science, since Jan 2007, Utah State University
M.S. in Microbiology, June 2003, Nanjing Normal University (CHINA)
B.S. in Biology, June 2000, Nanjing Normal University (CHINA)

Employment History
Graduate Research Assistant  Jan. 2007–present
Department of Plants, Soils, and Climate, Utah State University
Center of Integrated BioSystems, Utah State University
Visiting Scholar  March 2004–Sept. 2005
Department of Plants, Soils, and Biometeorology, Utah State University
Research Scientist  June 2003–March 2004
Nanjing Biotech Inc., China

Honors and Awards
1. Graduate Fellowship, Plants, Soils, and Climate Department, 2008-2009
2. Graduate Student Research Grant (CIBR Student), USU, 2007-2008
3. Graduate Fellowship, Plants, Soils, and Climate Department, 2007-2008
4. Graduate student poster competition finalist, Intermountain Systems Biology
   Symposium, USU, 2007
5. Graduate student research symposium, 3rd prize, USU, 2007

Major Research Interests
Stress physiology: Plant response to salt, drought, and cold stress
Plant flowering: gene function in controlling flowering time

Publications and Patent
Chen D*, Liang M*, DeWald D, Weimer, B, Davis E, Peel M, Bugbee B, Wu Y
(* Co-first author) 2008. Identification of drought response genes from two alfalfa
cultivars using Medicago truncatula microarrays. Acta Physiologiae Plantarum
30:183-199


**Manuscripts in Preparation**

**Liang M** et al., HAP3b suppresses freezing stress response in *Arabidopsis*

**Liang M** et al., Molecular mechanism of HAP3b in regulating flowering time in *Arabidopsis*

**Liang M** et al., HAP3 genes in barley: their expression and functions

**Presentation**

- **Oral talk at national/regional conferences**
  1. A putative CCAAT-binding transcription factor is a regulator of flowering timing in *Arabidopsis*. Feb 2008 Western Section American Society of Plant Biologists Annual winter Meeting, Orem, UT, USA
  2. Understanding the mechanism of HAP3b regulating the flower timing in *Arabidopsis*. Jan 2008 Graduate Student Support Program Center for Integrated BioSystems Research, Logan, UT, USA
  3. The first genetic evidence: laccase in lignin synthesis. April 2007 USU Graduate Student Senate Project, Logan, UT, USA
-Poster Presentations at national/regional conferences
Liang M, Davis E, Gardner D, Cai X, Wu Y. Involvement of AtLAC15 in lignin synthesis of *Arabidopsis* seeds. June 2007 Intermountain System Biology Meeting, Logan, UT, USA

-Departmental graduate seminar presentations (PSB7970)
1. How does HAP3b regulate the flower time in *Arabidopsis*? Oct 2008
2. Laccase in lignin synthesis. Feb 2008