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

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PHYSIOLOGICAL AND MOLECULAR FUNCTIONS

OF HAP3B IN FLOWERING TIME REGULATION

AND COLD STRESS RESPONSE

by

Mingxiang Liang

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ABSTRACT

Physiological and Molecular Function of HAP3b in Flowering Time Regulation and

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by

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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 *At5g63470* and *At1g56170*.

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. 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*. *TC191694* overexpression plants were early flowering compared to *HAP3b*-overexpression and wild-type plants while overexpression of *TC176294* plants were not.

(143 pages)

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Mingxiang Liang

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ABBREVIATIONS

35S	CaMV 35S promoter
ABA	abscisic acid
CBF	CCAAT binding factor
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
cDNA	complimentary DNA
CO	CONSTANS
Col	Columbia
COP1	CONSTITUTIVE PHOTOMORPHOGENESIS 1
CYC1	ISO-1-CYTOCHROME C
ELF 3/4	EARLY FLOWERING 3 and 4
ESD1	EARLY IN SHORT DAYS
FKF1	FLAVIN-BINDING, KELCH REPEAT, F-BOX 1
FLC	FLOWERING LOCUS C
FLD	FLOWERING LOCUS D
FRI	FRIGIDA
FRL1	FRIGIDA LIKE 1
FT	FLOWERING LOCUS T
GA	gibberellic acid
gDNA	genomic DNA
GI	GIGANTEA
GID 1	GIBBERELLIN INSENSITIVE DWARF1
HAP	HEME ACTIVATOR PROTEIN
Hd3a	FT ortholog in rice
HOS1	HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE
HvCO	CO ortholog in barley
ICE1	INDUCER OF CBF EXPRESSION 1
LD	LUMINIDEPENDENS
LEC1	LEAFY COTYLEDON1
Ler	Landsberg erecta
LFY	LEAFY
LHY	LATE ELONGATED HYPOCOTYL
LHP1	LIKE HETEROCHROMATIN PROTEIN 1
MS	Murashige and Skoog
n	number of plants
NF-Y	NUCLEAR FACTOR-Y
OsSOC1	SOC1 ortholog in rice
PIE1	PHOTOPERIOD INDEPENDENT EARLY FLOWERING
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
SPA	SUPPRESSOR OF PHYA
TaGI1	GI ortholog in wheat
TOC1	TIMING OF CAB1

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 CCGNNCCC 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 *FT* and *FLC* (*FLOWERING LOCUS C*) to activate expression of *LFY* (*LEAFY*) and *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*) (Blazquez et al., 1998; Moon et al., 2003). The activation of *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 *GAMYB*-like transcripts (Achard et al., 2004).

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 FLC. Any upstream gene that induces *FLC* could result in late flowering. Dominant *FRIGIDA* (*FRI*) is such a case. FRI promotes *FLC* expression by enhancing *FLC* transcription and splicing, resulting in delayed flowering (Geraldo et al., 2009). Other genes including *FRIGIDA LIKE 1* (*FRL1*) (Michaels et al., 2004), *EARLY IN SHORT DAYS* (*ESD1*) (Gomez-Mena et al., 2001), *PHOTOPERIOD INDEPENDENT EARLY FLOWERING* (*PIE1*) (Noh and Amasino, 2003) and *VERNALIZATION INDEPENDENCE GENES* (*VIP*) (Zhang and Van Nocker, 2002) can also repress flowering by promoting *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 transcriptioncally 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 *fve* plants, the expression level of *CBF*s 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 *CBF*s were not changed in *hos9* and *hos10* mutant plants (Zhu et al., 2004). In addition, *CBF*s 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 *fve* 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*

caused late flowering under long day conditions (Hayama et al., 2003). OsGI

functions to upregulate *Hd1* (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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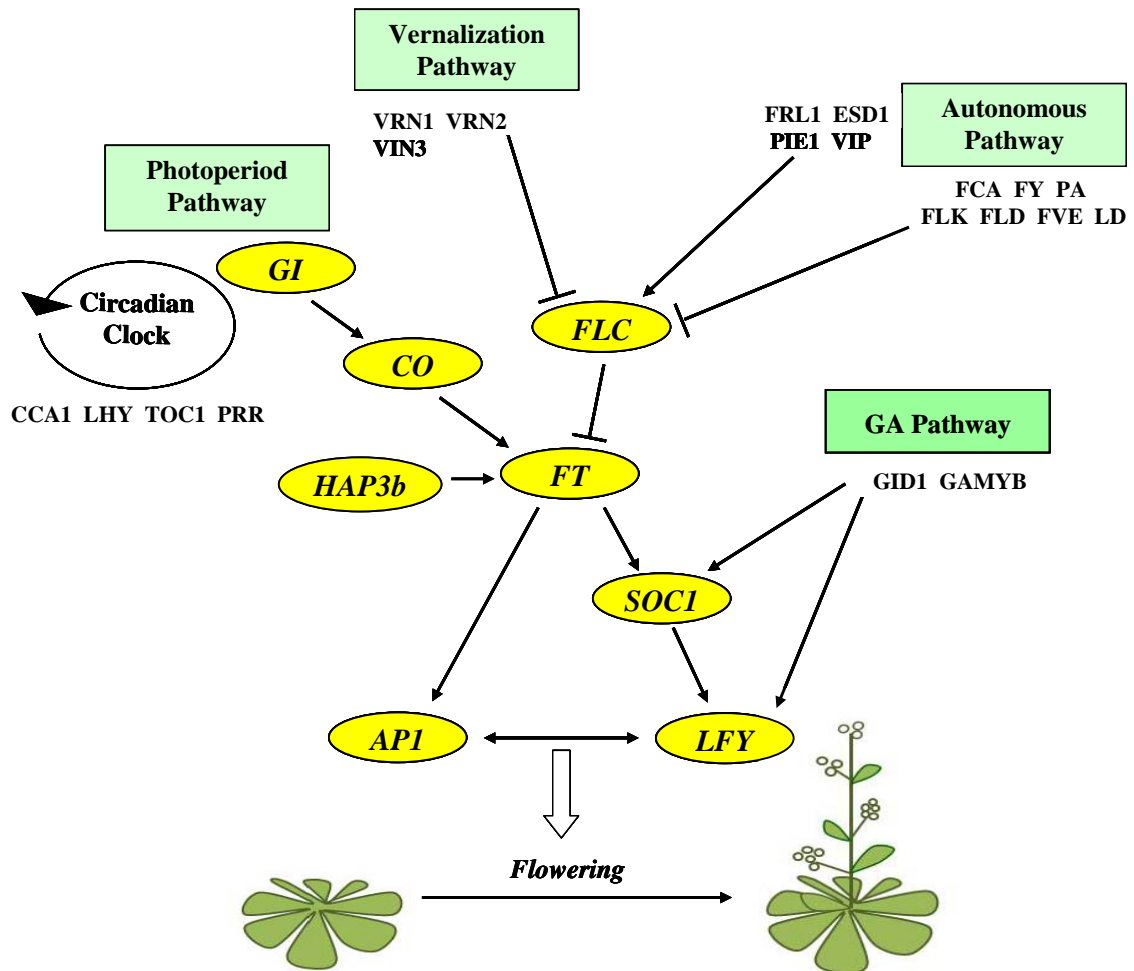
Figure 1: Illustration of the main flowering pathways in *Arabidopsis*. Adapted

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. *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 (Inducer of CBF Expression 1) is so far the most upstream transcription factor. ICE1 positively regulates *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 *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 *CBF1*, *CBF2* and *CBF3* to negatively control expression of *CBF* genes. It was also shown that the *Arabidopsis* 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 *fve* plants, the expression level of *CBF*s 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 *CBF*s were not changed in *hos9* and *hos10* mutant plants (Zhu et al., 2004). In addition, *CBF*s 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 (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 at 23ºC. 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 (4ºC) 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 (P_{actin}:HAP3b). RNA used for the chip

experiment was from five independent biological samples from two independent

experiments. Each sample represented a collection of leaves from 12 plants.

To confirm expression of selected genes from the microarray experiments, a

quantitative PCR was used. Seeds of wild-type, *hap3b* mutant, *HAP3b*-overexpression

transgenic plants, and overexpression control plants (C1= P_{actin}:GUS) were

germinated in a single Murashige and Skoog-Phytagel 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 4ºC. 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 0ºC 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 -12ºC (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 -20ºC 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-reguated
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 *CBF*s 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*-overexpresion 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.

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**Table 2.1. Genes Downregulated in Overexpression Plants But Upregulated in
hap3b Mutant on Affymetrix Genechip**

Affy ID	Mean signal for wt	Mean signal for *hap3b*	Fold change (*hap3b* / wt)	Signal for ox	Fold change (ox / wt)	Gene ID	Annotation
265480_at	3593.88	4489.58	1.25	2384.76	0.66	At2g15970	Cold-acclimation protein putative (FL3-5A3)
265471_at	341.30	436.25	1.28	206.03	0.60	At2g37130	Peroxidase 21 (PER21) (P21) (PRXR5)
265260_at	42.09	54.41	1.29	28.34	0.67	At2g43000	No apical meristem (NAM) family protein
263948_at	199.85	260.75	1.30	129.90	0.65	At2g35980	Harpin-induced family protein (YLS9)
264005_at	140.27	186.28	1.33	81.06	0.58	At2g22470	Arabinogalactan-protein (AGP2)
245463_at	147.55	197.10	1.34	78.30	0.53	At4g17030	Expansin-related
259384_at	222.17	299.35	1.35	145.97	0.66	At3g16450	Jacalin lectin family protein:
249867_at	104.20	142.75	1.37	65.89	0.63	At5g23020	2-Isopropylmalate synthase 2 (IMS2)
254066_at	99.63	137.98	1.38	48.15	0.48	At4g25480	CBF3
248337_at	640.51	889.43	1.39	426.60	0.67	At5g52310	Low-temperature-responsive protein 78 (LTI78) / Desiccation-responsive protein 29A (RD29A)
266168_at	2233.35	3163.16	1.42	1168.17	0.52	At2g38870	Protease inhibitor putative
264314_at	107.43	152.40	1.42	78.34	0.73	At1g70420	Expressed protein
246481_s_at	15308.85	21845.70	1.43	9741.40	0.64	At5g15960	Stress-responsive protein (KIN1)
263497_at	636.51	928.26	1.46	423.50	0.67	At2g42540	Cold-responsive protein / cold-regulated protein (cor15a)

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

Gene or Annotation	Affy ID	Mean signal for wt	Mean signal for *hap3b*	Fold change (*hap3b* / wt)	Signal for ox	Fold change (ox / wt)	Gene ID
RD29A or lit78	248337_at	640.51	889.43	1.39	426.60	0.67	At5g52310
KIN1	246481_s_at	15308.85	21845.70	1.43	9741.40	0.64	At5g15960
WCOR413	265480_at	3593.88	4489.58	1.25	2384.76	0.66	At2g15970
COR47, RD17	259570_at	699.07	862.50	1.23	561.28	0.80	At1g20440
COR15b	263495_at	1833.77	2229.57	1.22	1585.84	0.86	At2g42530
COR15a	263497_at	636.51	928.26	1.46	423.50	0.67	At2g42540
erd7	264787_at	857.82	1018.73	1.19	709.52	0.83	At2g17840
erd10, dehydrin	259516_at	859.50	945.45	1.10	712.96	0.83	At1g20450
Protease Inhibitor	254818_at	1513.42	1842.48	1.22	863.44	0.57	At4g12470
Protease Inhibitor	254805_at	2568.51	3154.43	1.23	2312.17	0.90	At4g12480
LEA	259426_at	1576.13	1782.25	1.13	1336.96	0.85	At1g01470
RD29B	248352_at	12.86	13.14	-	12.86	-	At5g52300
Putative c2h2 zinc finger transcription factor	245711_at	71.29	90.06	1.26	152.77	2.14	At5g04340
Salt-tolerance zinc finger protein	261648_at	141.44	266.94	1.89	385.20	2.72	At1g27730
AP2 domain containing protein	245807_at	10.43	9.83	-	9.55	-	At1g46768
Phosphoinositide specific phospholipase C	247794_at	134.25	122.73	0.91	114.64	0.85	At5g58670
Glycine-rich RNA binding protein 7	263548_at	29793.20	28891.45	0.97	31633.00	1.06	At2g21660
Putative sugar transporter protein sugar transporter	253188_at	284.07	295.34	1.04	295.34	1.04	At4g35300
Delta 9 desaturase	263249_at	2221.89	2279.86	1.03	2381.17	1.07	At2g31360
Putative galactinol synthase	264511_at	27.12	25.66	-	21.81	-	At1g09350
Tyrosine transaminase like protein	254232_at	789.88	838.60	1.06	801.40	1.01	At4g23600
Pyruvate decarboxylase-1 (Pdc1)	253416_at	17.81	17.82	-	13.89	-	At4g33070
Protease inhibitor II	266119_at	7745.94	7065.00	0.91	10656.10	1.38	At2g02100
Putative trypsin inhibitor	260551_at	1634.43	1617.51	0.99	1251.13	0.77	At2g43510
Xero2/dehydrin	252102_at	149.77	143.32	0.96	158.36	1.06	At3g50970
Drought-induced protein like	245523_at	239.62	208.28	0.87	238.79	1.00	At4g15910
Unknown protein	259768_at	764.71	773.35	1.01	898.29	1.17	At1g29390
Proline-rich protein	245749_at	146.45	148.75	1.02	131.65	0.90	At1g51090
Water stress-induced protein	262958_at	4154.84	3911.21	0.94	4078.57	0.98	At1g54410
Unknown protein	267261_at	517.08	535.60	1.04	378.03	0.73	At2g23120
Unknown protein	266225_at	2325.51	2463.62	1.06	2092.14	0.90	At2g28900
Putative auxin-regulated protein	267461_at	8045.57	8867.78	1.10	8501.27	1.06	At2g33830
Hypothetic protein	245316_at	394.08	346.11	0.88	372.74	0.95	At4g14000
Abscisic acid-induced- like protein	254085_at	360.97	343.39	0.95	379.24	1.05	At4g24960
Low temperature and salt responsive protein homolog	253627_at	5242.70	6334.21	1.21	6398.68	1.22	At4g30650
Farnesylated protein (ATFP6)	252956_at	1039.51	996.09	0.96	998.80	0.96	At4g38580
Hypothetic protein	246435_at	8.28	8.33	-	10.09	-	At5g17460
Ripening-related protein- like	247450_at	7243.70	7585.09	1.05	5298.06	0.73	At5g62350

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   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

Gene	Affy ID	Mean signal for wt	Mean signal for *hap3b*	Fold change (*hap3b* / wt)	Signal for ox	Fold change (ox / wt)	Gene ID
ICE1	258310_at	325.89	320.95	0.985	325.578	1.001	At3g26744
CBF1/DREB1b	254074_at	5.68	7.44	-	5.73	-	At4g25490
CBF2/DREB1c	254075_at	42.85	51.97	-	41.26	-	At4g25470
CBF4	248389_at	10.86	10.82	-	9.54	-	At5g51990
DREB2B	256430_at	48.50	44.43	0.92	47.59	0.98	At3g11020
DREB2A	250781_at	92.77	95.32	1.03	69.31	0.75	At5g05410
DREB-like	267026_at	16.68	16.31	-	11.86	-	At2g38340
RD22	246908_at	5232.40	5119.61	0.98	4790.04	0.92	At5g25610
RD21	249187_at	434.13	496.90	1.14	492.29	1.13	At5g43060
RD21A	245803_at	4102.00	4249.24	1.04	3717.99	0.91	At1g47128
RD19A	252927_at	9372.48	8745.45	0.93	10567.90	1.13	At4g39090
ERD1	248487_at	413.17	390.30	0.94	365.35	0.88	At5g51070
ERD15	267104_at	2123.90	2292.99	1.08	2660.13	1.25	At2g41430
ERD11/13	267154_at	5847.45	6128.94	1.05	5457.51	0.93	At2g30870

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. -, 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

AGI ID	Annotation	Promoter length	0~200	200~400	400~600	600~800	800~1000	1000~1600
At2g37130	Peroxidase 21 (PER21) (PRXR5)	2000		242(+) 314(+)	559(−)		821(+)	1040(−), 1390(+), 1451(+), 1486(+), 1605(+), 1742(−), 1830(−)
At2g43000	No apical meristem (NAM) family protein	1990	56(−) 176(+)		497(−)			1100(−), 1311(−), 1632(−), 1893(+)
At2g35980	Harpin-induced family protein (YLS9)	813	155(+)	303(+)	418(−)			
At2g22470	Arabinogalactan-protein (AGP2)	2000	66(−)	312(−)			990(+)	1382(+), 1611(+), 1949(+)
At4g17030	Expansin-related	2000	183(+)	377(+)			833(−) 859(−) 997(+)	1239(+)
At3g16450	Jacalin lectin family protein	643	118(−)	273(+)	506(+) 514(+)			
At5g23020	2-isopropylmalate synthase 2 (IMS2)	2000	32(+)			639(−)		1661(+), 1913(−)
At4g25480	CBF3	1980			442(−)	762(−)		1576(+), 1915(+)
At2g38870	Protease inhibitor putative	1142			414(−) 455(−) 589(+)	781(+)		
At1g70420	Expressed protein	2000	54(+)	396(−) 434(+) 499(+) 522(+)			851(+) 971(−)	1452(−), 1974(−)
At4g25490	CBF1	1766	20(−) 32(−)	379(−)		601(+) 631(+)	851(−)	1098(−), 1329(+), 1686(+)
At4g25470	CBF2	1978	37(−)				804(−)	1531(+), 1775(−), 1802(+), 1876(−)
At5g51990	CBF4	1235	40(+)		495(+) 511(+) 559(+)		895(+)	

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 predicated 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

Primer Sequences (5'-3')	Descriptions
GGCCACGAGTTGTCCGAAGAA	5'-upstream, CBF1, gene expression
TATTAGTAACTCCAAAGCGACACG	3'-downstream, CBF1, gene expression
ACGACGTCGCCGCCATAGC	5'-upstream, CBF2, gene expression
ACCATTTACATTCGTTTCTCACAA	3'-downstream, CBF2, gene expression
TTCAGCAAACCATACCAACAAAAA	5'-upstream, CBF3, gene expression
GCATCTCAA ACATCGCCTCATC	3'-downstream, CBF3, gene expression
GAATCACCAGCGACGACAACA	5'-upstream, COR47, gene expression
CTCCACCACACTCTCCGACACT	3'-downstream, COR47, gene expression
ATGAGAATGGTGCGACTAAGATG	5'-upstream, RD29A, gene expression
TGACAATTCGGACAGAGGATGAT	3'-downstream, RD29A, gene expression
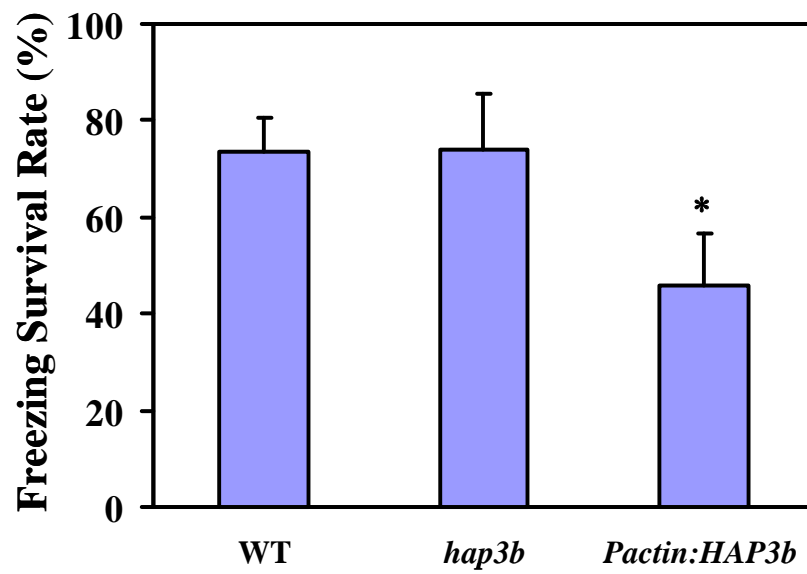
Figure 2.1 Decreased survival rate of *HAP3b*-overexpression (*Pactin:HAP3b*) plants under freezing stress compared to wild-type plants (WT) and *hap3b* mutant. Data are means ± SE from five independent experiments. *, indicates P<0.05 compared with wild type.
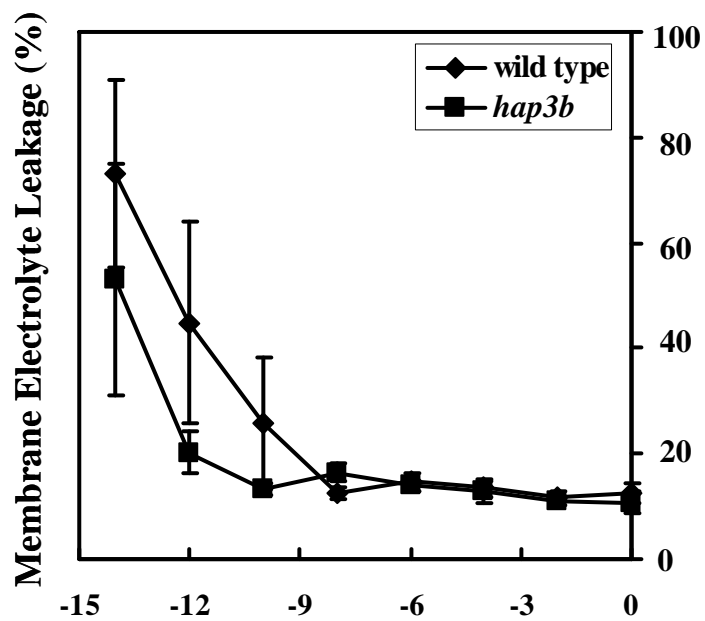
Figure 2.2 Difference in membrane leakage of wild-type plants and mutant *hap3b* under freezing temperatures. Data are means ± SE of three independent experiments, and each experiment consisted of measurements of three samples (individual leaves) for both wild-type and *hap3b* plants at each freezing temperature.
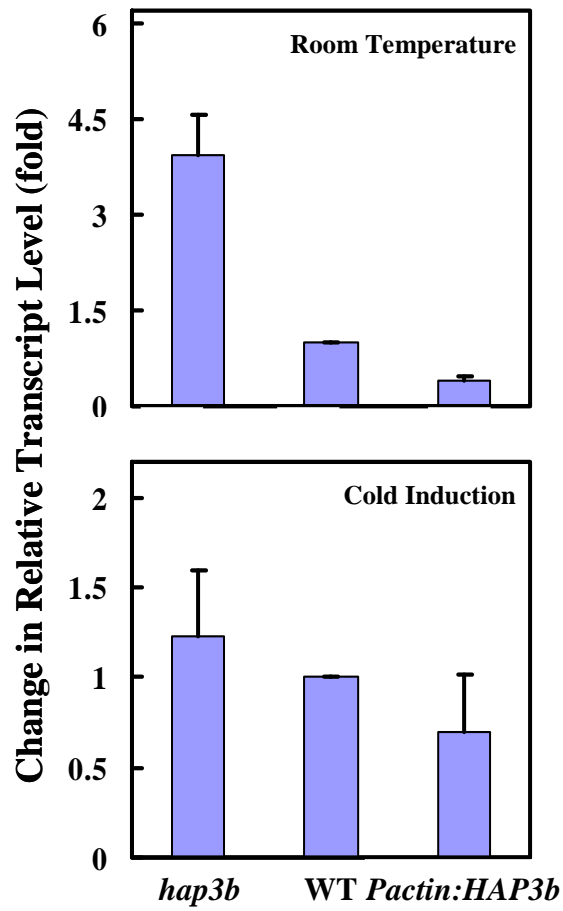
Figure 2.3 Relative *CBF3* transcript levels in *hap3b* mutant and
HAP3b-overexpression plants grown at room temperature or treated with low
temperature compared to wild-type plants. Data are means ±SE of three independent
experiments.
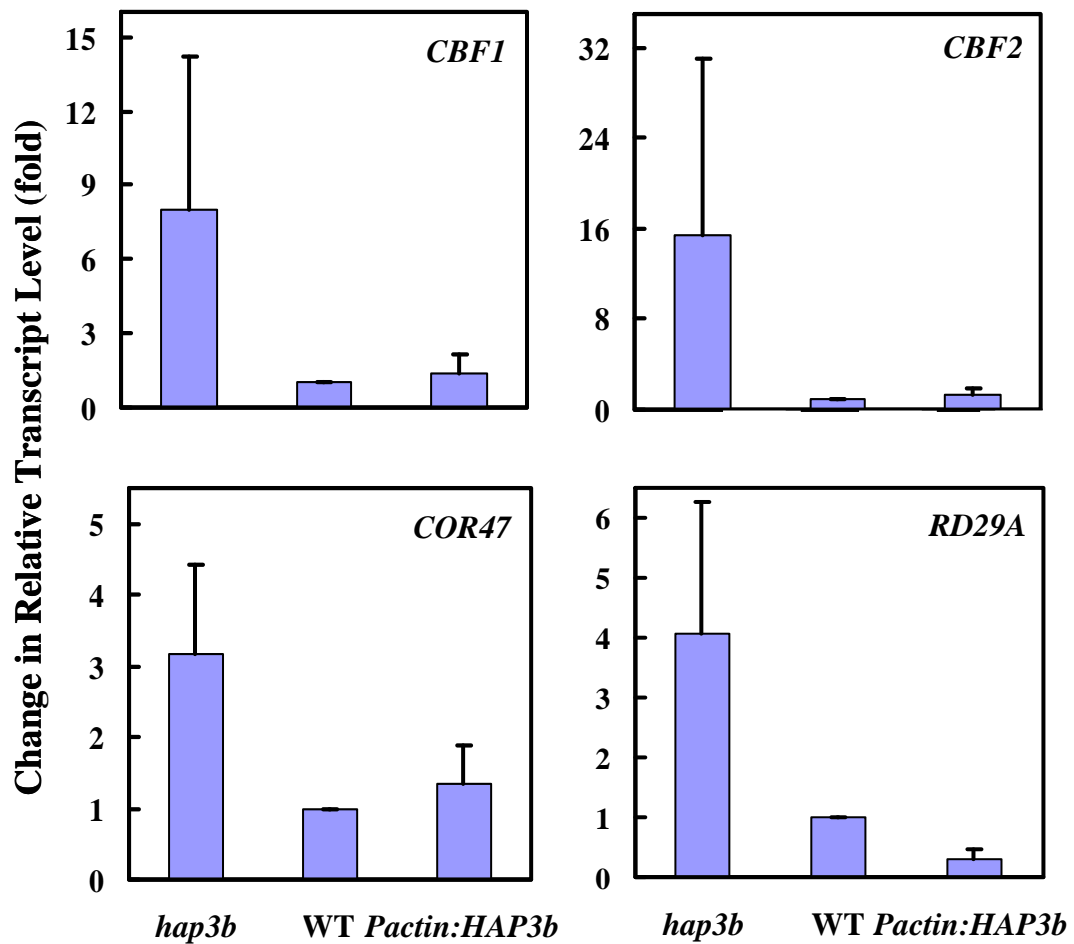
Figure 2.4 Relative transcript levels of *CBF* genes and their regulated genes in *hap3b* mutant and *HAP3b*-overexpression plants compared with wild-type plants grown at room temperature. Data are means ±SE of three independent experiments.
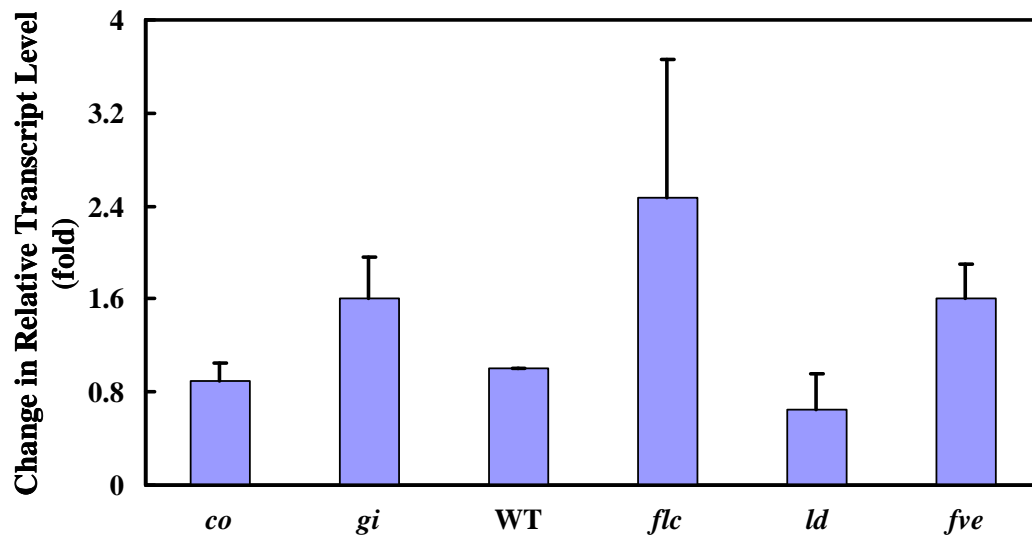
Figure 2.5 Relative transcript levels of *CBF3* in various flowering time mutants compared to wild-type plants grown at room temperature. *CBF3* transcript level in each genotype was first normalized using an actin gene and the normalized transcript level was then compared with the level in wild-type plants. Data are means ±SE of three independent experiments.
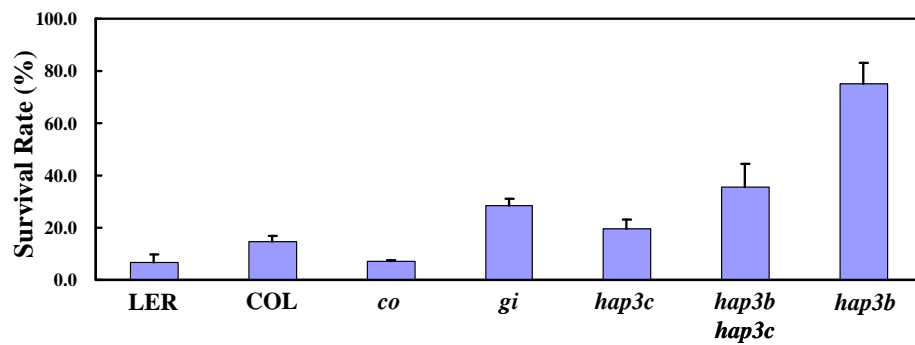
Figure 2.6 Survival rate of wild-type plants and various flowering time mutants after freezing treatment. Two-week-old plants grown on solid medium in Petri dishes were incubated at -20°C for 50 min. Survival plants were scored 10 d after treatment. Data are means ± SE of three independent experiments.

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; 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 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 ^{TM} 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 H$_2$O. PCR was run with the following

parameters: 1. 95ºC, 5 min; 2. 95ºC, 30 sec; 3. 50-55ºC, 30 sec; 4. 72ºC, 1 min kb^{-1};

for 35 cycles from step 2 to 4; 5. 72ºC, 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 *Agrobacterium tumefaciens* 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.

3.3.6 Protein extraction and *in vitro* coimmunoprecipitations

Seedlings (15 g, fresh weight), wild-type or *HAP3b*-overexpression line

(overexpression of *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 ℃ for 30

min , filtered through four layers of Miracloth (Calbiochem, #475855, Gibbstown, NJ),

and centrifuged at 12, 000 g for 10 min at 4 ℃. 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 μg of

anti-green fluorescent protein multiclonal antibody (Invitrogen, A11122, Carlsbad,

CA). Extracts containing similar amounts of total protein were incubated with 100 μL

IgG beads overnight at 4 ℃ with gentle rotation. The IgG beads were collected after

centrifugation at 3000 g for 1 min at 4 ºC. 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 H_2O in one

minute at 15 mL min^{-1}. The peptides were eluted from the Trapping column over a

100 μm ×100 mm BEH 130 C_{18} 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% H_2O, 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 F_1 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 (4ºC) 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 *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 Prn1 *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.

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

HAP2 AGI ID	other name		HAP5 AGI ID	other name	
At3g14020		+	At1g08970	AtHAP5c	++
At1g17590		-	At1g54830		++
At1g72830	AtHAP2c	-	At1g56170	AtHAP5b	++
At3g05690	AtHAP2b	-	At5g63470		++
At5g06510		-	At5g50480		++
At1g54160		-	At5g50470		++
At1g30500		-	At5g38140		++
At2g34720		-	At3g48590	AtHAP5a	++
At3g20910		-	At5g50490		++
At5g12840	AtHAP2a	-	At5g27910		++
At5g15840	CO	-			

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

HAP5 AGI ID	Domain 1-66bp	Domain 1-369bp	Domain 67-573bp	Domain 370-573bp
At1g08970	-	++	++	-
At1g54830	-	++	++	-
At1g56170	-	++	++	-
At5g63470	-	++	++	-
At5g50480	-	++	++	-
At5g50470	-	++	++	-
At5g38140	-	++	++	-
At3g48590	-	++	++	-
At5g50490	-	++	++	-
At5g27910	-	++	++	-

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

HAP5 AGI ID	At3g14020
At1g08970	-
At1g54830	-
At1g56170	++
At5g63470	++
At5g50480	-
At5g50470	-
At5g38140	-
At3g48590	-
At5g50490	-
At5g27910	-

Note: ++, positive interactions; -, negative interactions. AGI, Arabidopsis Genome Initiative.

Table 3.4   Identification of Proteins That Interact with HAP3b in a CoImmunoprecipitation Assay

ID	Description	Accession	mW (Da)	pI (pH)	Peptides	Coverage (%)
1.	Glucoside glucohydrolase 2 hydrolase hydrolyzing *o* glycosyl compounds (TGG2)	At5g25980	53388	6.4	16	39.2
2.	Peroxisomal NAD malate dehydrogenase 2 (PMDH2)	At5g09660	37345	8.0	8	38.1
3.	Galacturan 1,4, alpha galacturonidase pectinase (exopolygalacturonase)	At1g02790 (At3g07850) (At3g14040)	45571	8.3	7	17.6
4.	Epithiospecifier modifier 1 carboxylesterase (ESM1)	At3g14210	44032	7.6	5	26.8
5.	Ribosomal protein l12, a structural constituent of ribosome (RPL12-A)	At3g27830	20062	5.4	1	7.3

Table 3.5 Prediction of Nuclear Location of *Arabidopsis* HAPs Using PredictNLS Software.

HAP2		HAP3		HAP5	
AGI ID	PredictNLS	AGI ID	PredictNLS	AGI ID	PredictNLS
At5g12840	+	At2g38880	-	At3g48590	+
At3g05690	-	At5g47640	-	At1g56170	+
At1g72830	-	At4g14540	-	At1g54830	+
At2g34720	-	At1g09030	-	At5g63470	+
At1g54160	-	At2g47810	-	At5g50490	-
At3g14020	-	At5g47670	-	At5g50480	-
At1g30500	-	At2g13570	-	At5g50470	-
At1g17590	-	At2g37060	-	At5g27910	-
At3g20910	-	At1g21970	-	At1g08970	+
At5g06510	-	At3g53340	-	At1g07980	-
CONSTANS	-	At2g27470	-	At3g12480	+
AnHAPB	+	At5g08190	-	At5g38140	-
AoHAPB	+	At5g23090	-	At5g43250	-
ScHAP2	+				
NF-YA	+				

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*, *HAP2*s and *HAP5*s in the Yeast Two-Hybrid Analysis and Overexpression in *Arabidopsis*

Primer Sequences (5'-3')	Descriptions
TGAATTCATGCAAGTGTTTCAAAGGAA	5'-upstream, to clone At1g54160 in YTH
TAGATCTTCAAGTCCCTGACATGAGAG	3'-downstream, to clone At1g54160 in YTH
TGAATTCATGACTTCTTCAATCCATGA	5'-upstream, to clone At1g30500 in YTH
TAGATCTTCAAGATGTACCACTAGAAGCA	3'-downstream, to clone At1g30500 in YTH
TGAATTCATGACTTCTTCAGTACATGAG	5'-upstream, to clone At2g34720 in YTH
TGTCGACTCAAGATCTACCATTAGGAC	3'-downstream, to clone AT2g34720 in YTH
TGAATTCATGGGAATTGAAGACATGCA	5'-upstream, to clone At3g20910 in YTH
TAGATCTTCATTTAATGGCTAGACGAGCTT	3'-downstream, to clone At3g20910 in YTH
TGTCGACCTATGCAATCAAAACCGGGAA	5'-upstream, to clone At5g12840 in YTH
TAGATCTTTATGGTGCACCAGAAGAATTCAGG	3'-downstream, to clone At5g12840 in YTH
TGAATTCATGGGGGATTCCGACAGGG	5'-upstream, to clone At5g47640 in YTH
TGTCGACTTAAGTCCTTGTCCTACCGGAGG	3'-downstream, to clone At5g47640 in YTH
TGAATTCATGGAACGTGGAGCTCCCT	5'-upstream, to clone At1g21970 in YTH
TAGATCTTCACTTATACTGACCATAATGGTCAAAA	3'-downstream, to clone At1g21970 in YTH
TGAATTCATGGATCAACAAGACCATGG	5'-upstream, to clone At1g08970 in YTH
TAGATCTCTAATTTTCCTGGTCAGGTTGGT	3'-downstream, to clone At1g08970 in YTH
TGAATTCATGGATCAACAAGGACAATC	5'-upstream, to clone At1g54830 in YTH
TAGATCTCTAATTGTCAGGATCCTGCT	3'-downstream, to clone At1g54830 in YTH
TGAATTCATGGAGCAGTCAGAAGAGGG	5'-upstream, to clone At1g56170 in YTH
TAGATCTTTAAGACTCATCAGGGTGTTGCT	3'-downstream, to clone At1g56170 in YTH
TGAATTCAGAGAAGCCAGGGTCCTGAGAT	5'-upstream, to clone CO in YTH
TAGATCTTCTCTTTGCGAACCGGCCA	3'-downstream, to clone CO in YTH
TGAATTCATGGATAAGAAAGTTTCATT	5'-upstream, to clone At1g17590 in YTH
TAGATCTTCAGATATGGACAGAGAAATG	3'-downstream, to clone At1g17590 in YTH
TGTCGACCTATGATGCATCAGATGTTGAA	5'-upstream, to clone At1g72830 in YTH
TAGATCTTCAGATATGGACAGAGAAATGGTGCA	3'-downstream, to clone At1g72830 in YTH
TGAATTCATGCAAGAGTTCCATAGTAG	5'-upstream, to clone At3g14020 in YTH
TAGATCTTCACATGAGGACTGAGACAT	3'-downstream, to clone At3g14020 in YTH
TGTCGACCTATGGCTATGCAAACTGTGAG	5'-upstream, to clone At3g05690 in YTH
TAGATCTTCAGGTTTTGAAATTGCAGCAGC	3'-downstream, to clone At3g05690 in YTH
TGAATTCATGCAAACTGAGGAGCTTTT	5'-upstream, to clone At5g06510 in YTH
TAGATCTTCATATATTAAGTTTGCAGCAGCC	3'-downstream, to clone At5g06510 in YTH
TCCCGGGAATGGACAATAACAACAACA	5'-upstream, to clone At5g63470 in YTH
TCTGCAGTCAACCTTGGCTATCGAGATT	3'-downstream, to clone At5g63470 in YTH
TGAATTCATGGCTGAGAACAACAACAACAAC	5'-upstream, to clone At5g50480 in YTH
TAGATCTTCAATTTCCGCCGCCGTTTC	3'-downstream, to clone At5g50480 in YTH
TGAATTCATGGAAGAGAACAACGGCAA	5'-upstream, to clone At5g50470 in YTH
TAGATCTTCAATTACCGCCGCTGCTTC	3'-downstream, to clone At5g50470 in YTH
TGAATTCATGAGGAGGCCAAAGTCATC	5'-upstream, to clone At5g38140 in YTH

TAGATCTTCACTGGAGATCACAGTTGAGGTC	3'-downstream, to clone At5g38140 in YTH
TGAATTCATGGATACCAACAACCAGCAA	5'-upstream, to clone At3g48590 in YTH
TGTCGACTTAACCTTGGCCGTCGAGA	3'-downstream, to clone At3g48590 in YTH
TGAATTCATGGAGAACAACAACAACAACC	5'-upstream, to clone At5g50490 in YTH
TGTCGACTTAATTCCCACCGTTTCCT	3'-downstream, to clone At5g50490 in YTH
TGAATTCATGGAGAACAACAACGGCA	5'-upstream, to clone At5g27910 in YTH
TAGATCTTTAGTTTCCGTCGTCACCTCCT	3'-downstream, to clone At5g27910 in YTH
TGTCGACTCCAGTCCTCTCCCCTTCGATC	3'-downstream, to clone partial 47640 in YTH
TGTCGACGGAGGACTGTCCGTTCTGG	3'-downstream, to clone partial 47640 in YTH
TGAATTCCTAGGGAGGCCACAGACTGG	5'-upstream, to clone partial 47640 in YTH
TGAATTCTTGTCTCCAAGAGAGCAAGACAGG	5'-upstream, to clone partial 47640 in YTH
TCAGCTACATCGCTGTCTACC	5'-upstream, to screen the At5g63470 mutant
ACACAGAGCAGCAAATTCCTC	3'-downstream, to screen the At5g63470 mutant
ACCATCAGAGAGAACTGCCAC	5'-upstream, to screen the At1g54160 mutant
ACAGTATCATGCGATTCTCCG	3'-downstream, to screen the At1g54160 mutant
GAAATCCGACAACTACACCAATC	5'-upstream, to screen the At1g56170 mutant
CCTCTTCTGACTGCTCCATTG	3'-downstream, to screen the At1g56170 mutant
ATCCCAAACAAACCAGACTTAG	5'-upstream, to screen the At3g48590 mutant
TCGGATGCATATGAACTTTGG	3'-downstream, to screen the At3g48590 mutant
TACCACTAGAAGCAGCCATGG	5'-upstream, to screen the At1g30500 mutant
TCTTTTGGTGGTGCTCTCTTG	3'-downstream, to screen the At1g30500 mutant
TGGTTCACGTAGTGGGCCATCG	LBa1 primer
GCGTGGACCGCTTGCTGCAACT	LBb1 primer
TTTAATTAAATGGATACCAACAACCAGCAA	5'-upstream, to overexpress At3g48590
GGCCGGCCTTAACCTTGGCCGTCGAGA	3'-downstream, to overexpress At3g48590
TTTAATTAAATGACTTCTTCAATCCATGA	5'-upstream, to overexpress At1g30500
GGCCGGCCTCAAGATGTACCACTAGAAGCA	3'-downstream, to overexpress At1g30500
TTTAATTAAATGCAAGAGTTCCATAGTAG	5'-upstream, to overexpress At3g14020
GGCCGGCCTCACATGAGGACTGAGACAT	3'-downstream, to overexpress At3g14020
TTTAATTAAATGGACAATAACAACAACA	5'-upstream, to overexpress At5g63470
GGCCGGCCTCAACCTTGGCTATCGAGATT	3'-downstream, to overexpress At5g63470

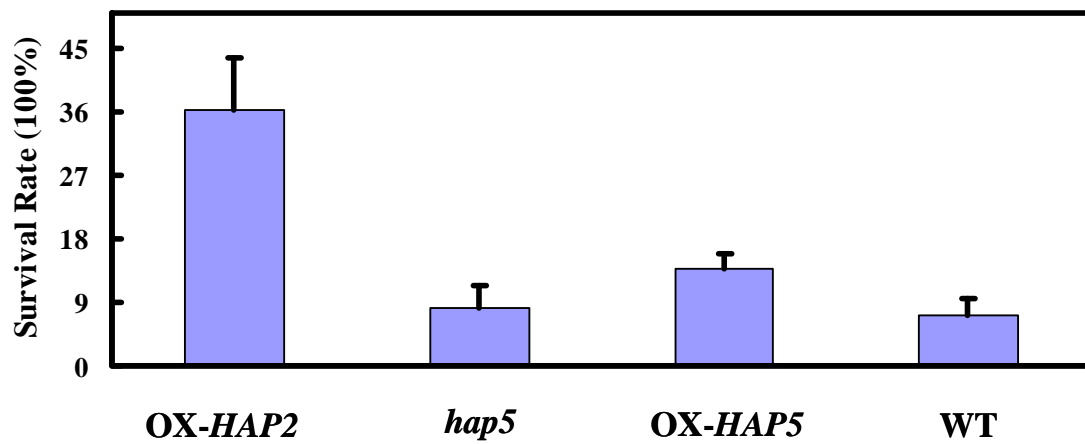**Figure 3.1** Survival rate of wild-type plants, two overexpression plants of identified HAP2 (At3g14020) and HAP5 (At1g56170) and one *hap5* (SALK_111422c for At3g14020) mutant plants after freezing treatment. Two-week-old plants grown on solid medium in Petri dishes were incubated at -20°C for 50 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/tgi/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 ($60 \times 20 \times 6$ cm^3) 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 ± 1ºC). 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 30ºC and 8h dark at 25ºC 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):

RWC (%) = [(FW1 − dry weight) ÷ (FW2 − dry weight)] × 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 *HAP3*s 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 *HAP3*s 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-ovexpression 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.

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Table 4.1   Protein Sequences That Are Most Similar to Barley HAP3 in NCBI Database

	Top 3 hits	e-value	Annotation	Full length sequence
TC171559	NP_001152278	5E-74	Nuclear transcription factor Y subunit B-3 [*Zea mays*]	NP_001152278
	BAC76331	9E-71	HAP3 [*Oryza sativa* Japonica Group]	
	P25209	2E-70	Nuclear transcription factor Y subunit B in *Zea mays*	
TC176294	NP_001050358	1E-66	Os03g0413000 [*Oryza sativa* (japonica cultivar-group)]	NP_001050358
	XP_002467695	3E-63	Hypothetical protein SORBIDRAFT_01g032590 [*Sorghum bicolor*]	
	NP_001147638	6E-63	Nuclear transcription factor Y subunit B-3 [*Zea mays*]	
TC191694	XP_002463163	1E-70	Hypothetical protein SORBIDRAFT_02g038870 [*Sorghum bicolor*]	XP_002463163
	NP_001147727	2E-68	Nuclear transcription factor Y subunit B-3 [*Zea mays*]	
	NP_001060230	3E-60	Os07g0606600 [*Oryza sativa* (japonica cultivar-group)]	
TC164749	NP_001056383	4E-55	Os05g0573500 [*Oryza sativa* (japonica cultivar-group)]	NP_001056383
	XP_002440289	2E-52	Hypothetical protein SORBIDRAFT_09g029140 [*Sorghum bicolor*]	
	EEE64770	3E-52	Hypothetical protein OsJ_19626 [*Oryza sativa* Japonica Group]	
TC166526	P25209	5E-71	Nuclear transcription factor Y subunit B in *Zea mays*	P25209
	NP_001105435	6E-71	CAAT-box DNA binding protein subunit B (NF-YB) [*Zea mays*]	
	NP_001141333	2E-70	Hypothetical protein LOC100273424 [*Zea mays*]	

Note: The deduced protein sequences of five HvTCs were used in BlastP analysis.

Table 4.2   Expression of Barley HAPs in Different Tissues

Gene	Developmental stage								
	5-day etiolated seedlings			15-day soil-grown plants	30-day soil-grown plants		55-day soil-grown plants		
	Root	Coleoptile	Shoot	Seedling	Leaf	Stem	Leaf	Stem	Awn
TC191694	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
TC176294	0/3	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
TC164749	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
TC171559	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
TC161801	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3

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)

Gene	Database Information – cDNA Libraries
TC191694	3-week-old root and top three leaves of heading stage
TC176294	Germination shoots; 7-day-old green seedlings infected with *Blumeria graminis* f. sp. *hordei*, and leaves were harvested 24, 48 and 72 hr post-inoculation
TC164749	Embryos dissected from developing grains (21 days post anthesis)
TC171559	Developing endosperm tissue in 10, 12, 15 DPA (days post anthesis); 7-day-old green leaves; pericarp in 0-7 DAP
TC161801	Rachis, embryo, endosperm tissues in 10, 12, and 15 DPA; whole spikes with awns collected at 20 DAP; 3-week-old root, callus

Table 4.4   Primers for Gene Expression and Overexpression of Barley *HAPs* in *Arabidopsis*

| Primer Sequences (5'-3')	Descriptions	
GACGCGCTTCCCTCCTGTTCTTG	5'-upstream, TC191694,	gene expression
TCACCGCCTCCGCCATTGTTCCTT	3'-downstream, TC191694,	gene expression
CCGCCGCGTCGATGCTTGAT	5'-upstream, TC176294,	gene expression
TCGCGGAACTTGTGGAGGTAG	3'-downstream, TC176294,	gene expression
ATCGCCAAGGACGCCAAGGAGACC	5'-upstream, TC171559,	gene expression
ACCTAATGTTGGGGCGGCTTCTT	3'-downstream, TC171559,	gene expression
GGCGACGTCTCTGTTAAAAAGGAT	5'-upstream, TC161801,	gene expression
GAACCGAATGACAGCCACGACGAG	3'-downstream, TC161801,	gene expression
CCGGGAGATGGAGGGTGAC	5'-upstream, TC164749,	gene expression
CGACGCCGTAAGCAGCAGATA	3'-downstream, TC164749,	gene expression
GCACCGCCGCCCGCTCTCCAC	5'-upstream, TC168497,	gene expression
CGGGCTTGTCCTGCGATTCTTCTT	3'-upstream, TC168497,	gene expression
GGCGCGCCATGGCCGACGACGACAGCGGC	5'-upstream, TC171559,	overexpression
GGCCGGCCTCAGGTATCCCCATTATGGTAC	3'-downstream, TC171559,	overexpression
GGCGCGCCATGCCGGACTCCGACAACGAC	5'-upstream, TC191694,	overexpression
GGCCGGCCCAGATACGCCAATAACGAAGAAGC	3'-downstream, TC191694,	overexpression
GGCGCGCCATGCCGGACTCGGACAACGAC	5'-upstream, TC176294,	overexpression
GGCCGGCCTTACAGCTGGCGGCGTGCCGTGGG	3'-downstream, TC176294,	overexpression
CGCCCTCCTCCCGCACCAA	5'-upstream, TC164749,	full-length CDS sequencing
CAGCCGCTTTATCTTCTTCAGTCA	3'-upstream, TC164749,	full-length CDS sequencing
ATGTCGGACGAGCCGGCGAGC	5'-upstream, TC166526,	full-length CDS sequencing
CAGACGGCAACAACAACAACAACC	3'-upstream, TC166526,	full-length CDS sequencing
GCTCAATCAGGCAAAGCCCT	5'-upstream, TC168497,	full-length CDS sequencing
CGTAGATGCCCAGCTGCATCT	3'-upstream, TC168497,	full-length CDS sequencing
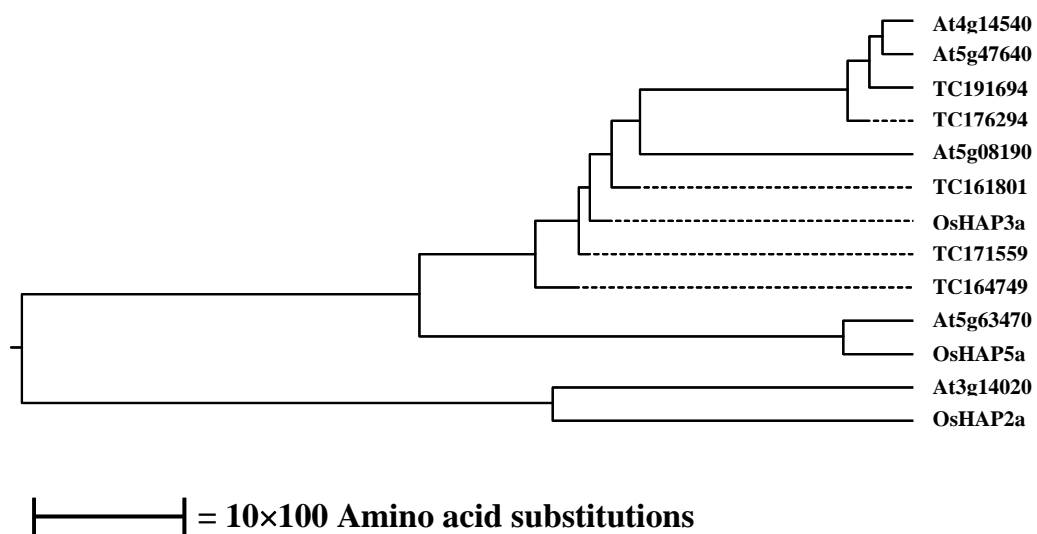
Figure 4.1 Phylogenetic analysis of putative barley HAP3s with selected rice and *Arabidopsis* HAP3s and other HAPs. Sequence alignment analysis was performed using a ClustalW method in the MegAlign program. The following are the corresponding accession numbers for each full-length protein in NCBI database: 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*).
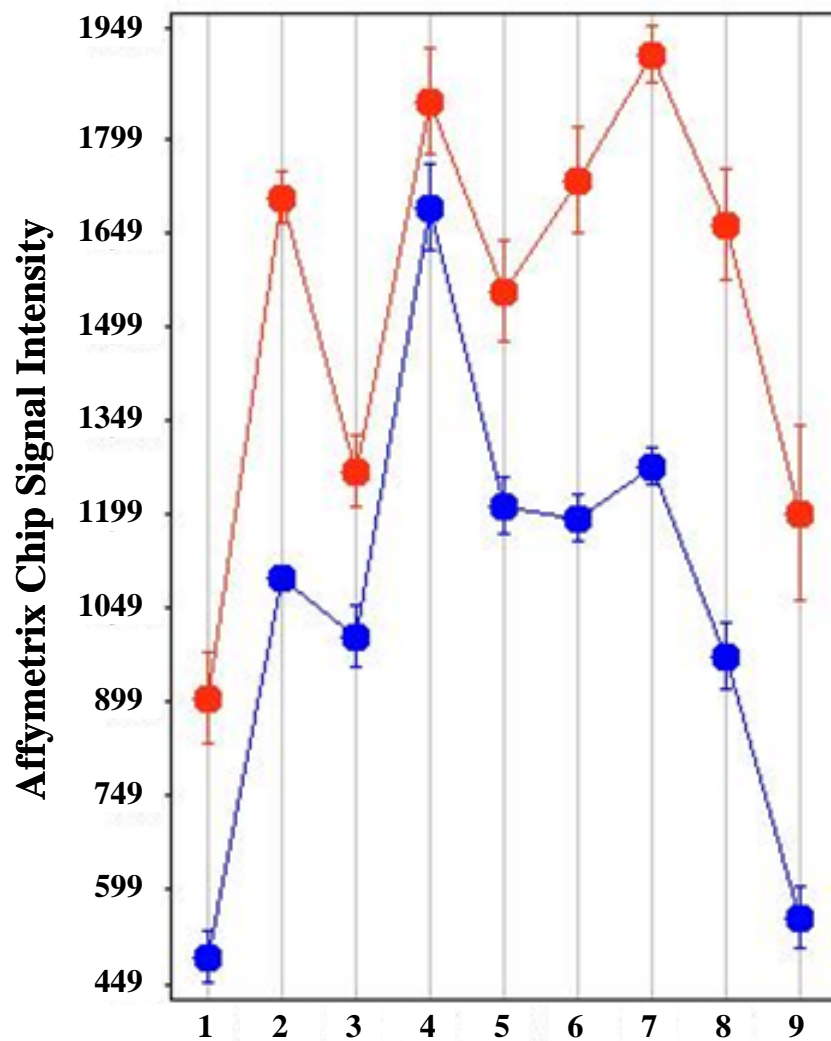
1. Germinated seeds   **6. Young flower**
2. Seedling   **7. Developed flower**
3. Young rosette   **8. Flowers and siliques**
4. Developed rosette   **9. Mature siliques**
5. Bolting

Red: HAP3b   **Blue: HAP3c**

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.
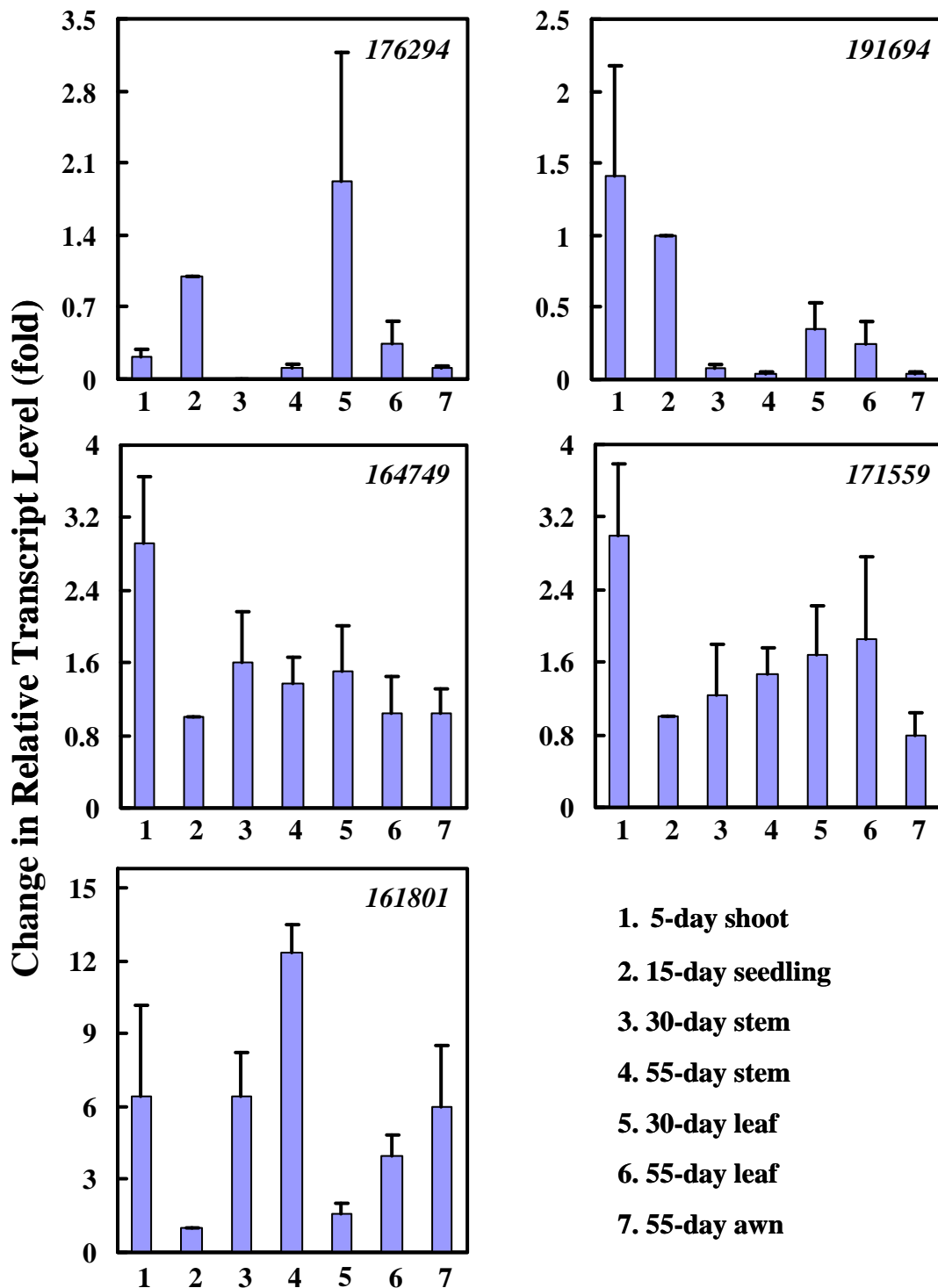
Figure 4.3 Relative transcript levels of five putative barley *HAP3*s in different tissues at different developmental stages. Transcript levels were examined using semi-quantitative PCR, then normalized to an 18s gene and compared with the level of 15-d old seedlings. Data are means ± SE of three independent experiments.
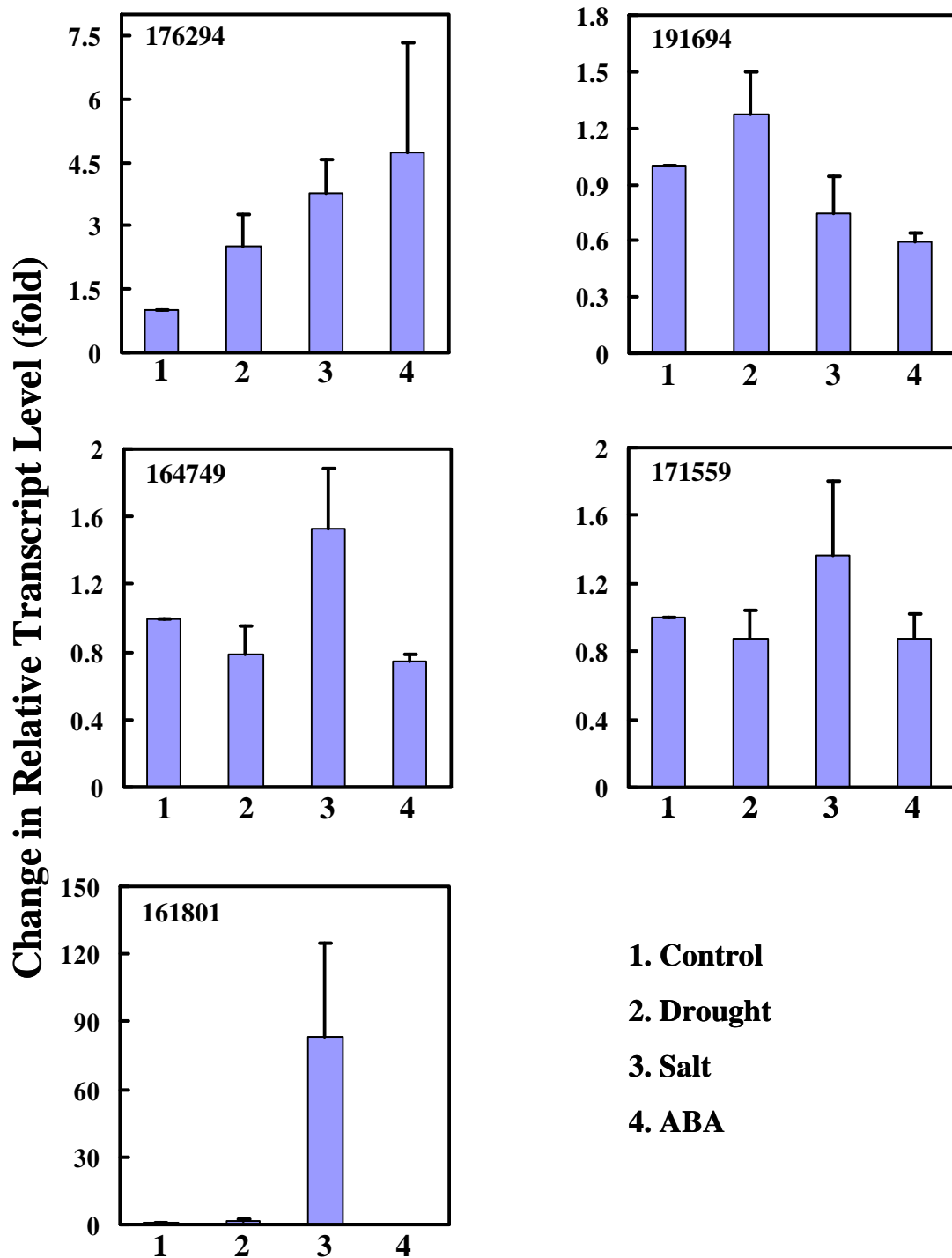
Figure 4.4 Change in relative transcript levels of putative *HAP3* genes in barley seedlings subjected to salinity (150 mM NaCl), drought or ABA (10 µM) treatments. Transcript levels were examined using semi-quantitative PCR, then normalized to an 18s gene and compared with control plants. Data are means ± SE of three independent experiments.
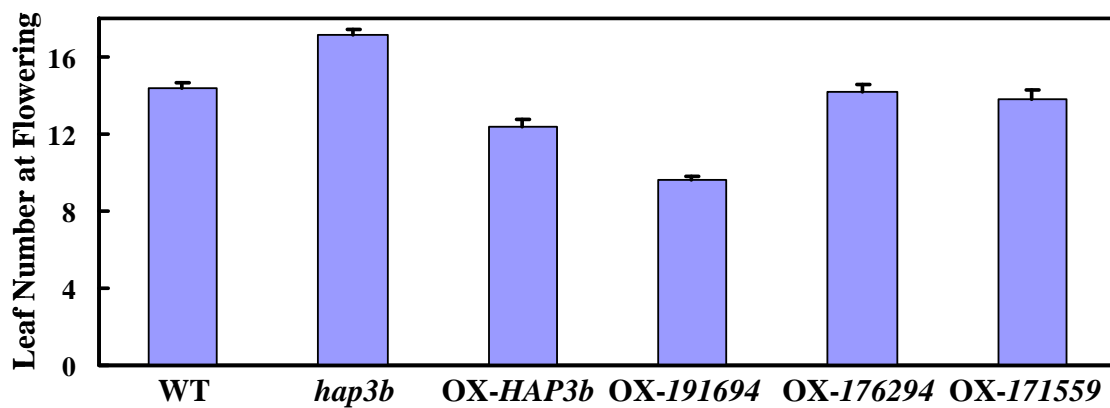
Figure 4.5 Early flowering in TC191694-overexpression plants grown under a 16-h/8-h light/dark photoperiod. OX-191694 plants (CaMV35S:TC191694 in wild-type background) developed fewer leaves compared with control WT plants and *HAP3b*-overexpression plants before flowering. OX-176294 and OX-171559 plants had the same number of leaves compared to WT plants. The data are means±SE (n = 22?-30) from three independent experiments.
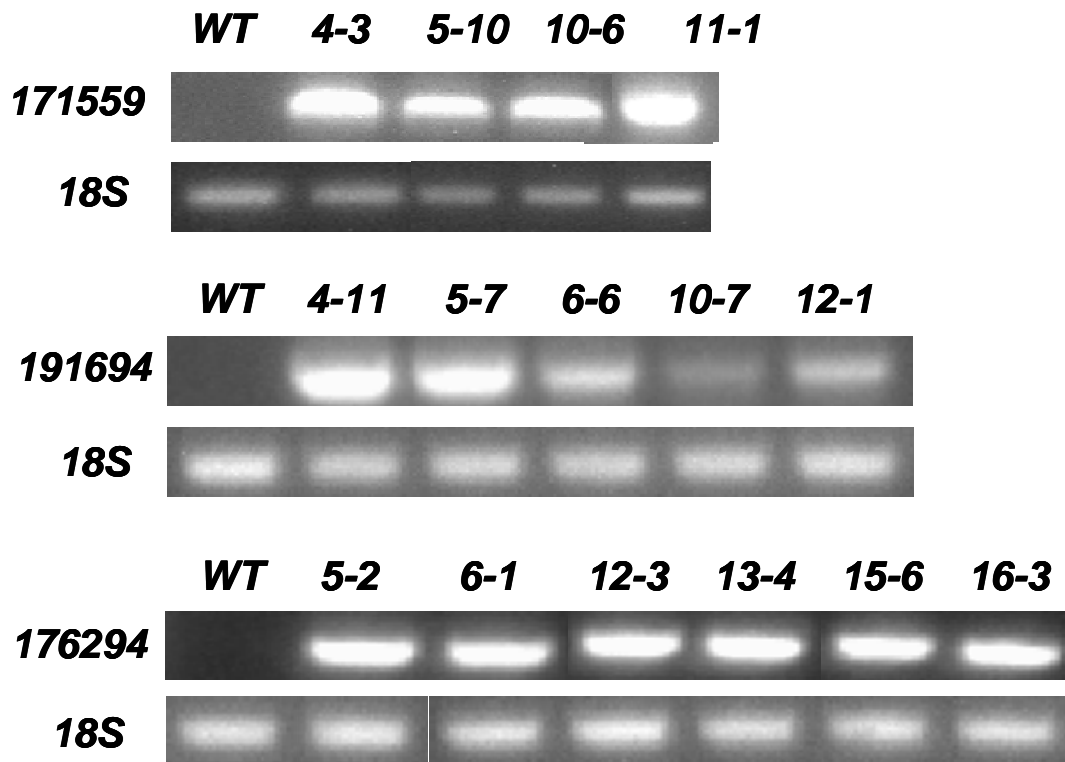
Figure 4.6   All transgenetic lines were true overexpression lines. Total RNA were extracted from each overexpression line and WT *Arabidopsis*. Semi-RT-qPCR method was used to determine transcript level and 18S gene was used as a control.

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 *HAP3*s 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 *HAP3*s 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.

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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

Cai X, Ballif J, Endo S, Davis E, **Liang M**, Chen D, DeWald D, Kreps J, Zhu T, Wu Y. 2007. A putative CCAAT-binding transcription factor is a regulator of flowering timing in *Arabidopsis*. *Plant Physiology* 145: 98–105

Liang M, Haroldsen V, Cai X, Wu Y. 2006. Expression of a putative laccase gene, *ZmLAC1*, in maize primary roots under stress. *Plant, Cell & Environment* 29:746-753

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Liang M, Yuan S et al. 2002. Report on the screening of cyanopyridine hydroxylation. *Journal of Nanjing Normal University* (Natural sciences), 2002(4):122-124

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