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Benjamin R. Keyser
Colby College

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Benjamin R. Keyser

Waterville, ME

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Advisor: Russell Johnson _____

Reader: Judy Stone _____

Reader: Lynn Hannum _____

ABSTRACT:

The plant hormone Abscisic acid (ABA) plays a central role in maturation and germination in seeds, as well as mediating adaptive responses to abiotic environmental stresses. ABA induces the expression of many genes, including late-embryogenesis-abundant genes such as *HVA1*. To elucidate the ABA signaling pathway leading to *HVA1* expression, we focus on the bZIP factor TaABF1. Analysis of the interplay between ABA and TaABF1 in the aleurone cells of imbibing cereal grains indicated that the two are not additive in their induction of the *HVA1* promoter. A synthetic ABA analog, PBI-51, did not specifically inhibit the effect of exogenous ABA on *HVA1* expression while 1-butanol (which inhibits phospholipase D, an early step in ABA perception) did. Furthermore, inhibition of endogenous ABA perception using 1-butanol reduced *HVA1* induction by the overexpression of *TaABF1*. This result suggests that TaABF1 may undergo an ABA-induced posttranslational modification. However, the lack of synergism between ABA and TaABF1 overexpression in *HVA1* induction does not support this conclusion. Therefore, our findings indicate that the branch of ABA signaling leading to *HVA1* is more complex than previously believed. We propose a model of ABA signaling involving TaABF1 and other putative components that result in the stimulation of ABA-induced genes.

INTRODUCTION

During plant development, the phytohormone abscisic acid (ABA) plays a critical role in mediating plant stress responses as well as regulating various events during seed development. Structurally, ABA is a 15-carbon isoprenoid compound and is synthesized within the chloroplast or other plastids via cleavage of a 40-carbon carotenoid intermediate produced from isopentenyl diphosphate. Its naturally occurring form is the *cis* isomer with an S configuration at the chiral 1' ring position (Figure 1).

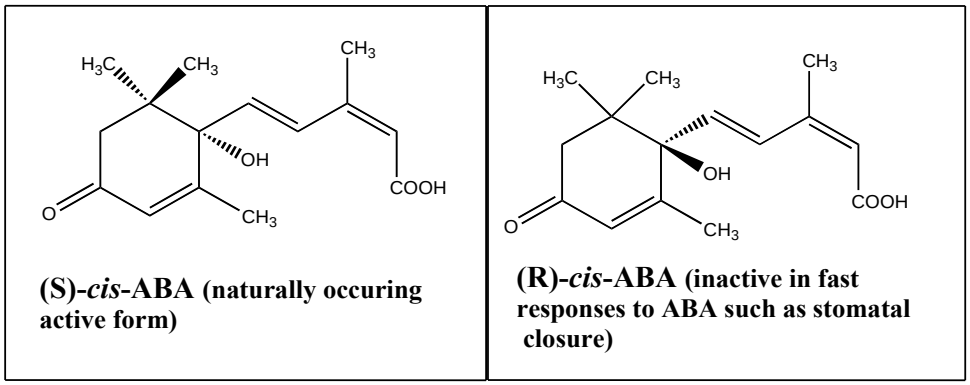


Figure 1. Chemical structure of the *cis*-ABA enantiomers.

During the last two thirds of seed development endogenous ABA levels increase, while at the very end of seed development levels return to a lower level within the dry seed. ABA is known to regulate important processes that occur during seed development; specifically, the acquisition of seed dormancy, seed desiccation tolerance, and accumulation of nutritive reserves (Busk and Pages, 1998).

The investigation of ABA's role in seed development and plant stress responses has been facilitated by the generation of mutant plants expressing ABA insensitive or ABA hypersensitive phenotypes. Specifically, ABA insensitive and ABA hypersensitive mutants have allowed for the determination of genes involved in ABA signaling. For example, a mutation in the maize

VIVIPARIOUS 1 (*VP1)* gene, which encodes a seed-specific transcription factor, leads to vivipary (McCarty et al., 1989). This demonstrates the mutant's inability to arrest precocious germination while still attached to the mother plant. While this mutant does not have reduced ABA content, vivipary occurs due to the mutant's insensitivity to ABA (McCarty et al., 1989). Another mutation in the *RED EMBRYONIC AXIS (REA)* gene produces an occasionally viviparous mutant which is indicative of its defective ABA perception (Sturaro et al., 1996).

Another class of mutants named ABA-insensitive *abi1* through *abi5* was discovered by screening for seeds able to germinate in the presence of ABA concentrations inhibitory to wild-type seed germination in *Arabidopsis thaliana* (Finkelstein et al., 1994). The *abi1, abi2, and abi3* mutants also have a significant reduction in seed dormancy, loss of stomatal aperture regulation (leading to wilting), and decreased expression of ABA-inducible genes (Koornneef et al., 1989). Therefore *ABI* loci are believed to be involved in the inhibition of seed germination via endogenous ABA. The inability to modify stomatal aperture in *abi* mutants arises from ABA insensitivity of S-type anion channels, K^+ channels, and actin reorganization. The application of high external calcium concentrations can induce closure of the mutant stomata, indicating that they also lack the ability to initiate Ca^{2+} signaling (Himmelbach et al., 2003)

As implied by their name, the *abi* mutants are insensitive to ABA. However, it is now known that the *abi1* and *abi2* mutations are dominant since the mutant allele is able to block the gene product of the remaining wild-type allele, causing the inhibition of the ABA response. The *ABI1* and *ABI2* genes encode homologous protein serine/threonine phosphatase 2Cs (PP2Cs) that inhibit the ABA response presumably through dephosphorylation of target proteins such as protein kinases, Ca^{2+}-binding proteins, and transcription factors (Leung et al., 1997; Himmelbach et al., 2003). Recessive (null) mutants of *ABI1*and *ABI2* actually have enhanced sensitivity to

ABA, while the overexpression of *ABI1* and *ABI2* genes causes decreased ABA sensitivity (Gosti et al., 1999). The *ABI3* gene is orthologous to the *VP1* gene in maize, encoding a similar transcription factor (Giraudat et al., 1992), and the *ABI5* gene encodes a basic leucine zipper transcription factor (Finkelstein et al., 2000). Therefore, the ABI loci all play a role in ABA signaling within the seed.

Like the *abi1* and *abi2* recessive mutations, *ERA* mutations discovered in *Arabidopsis thaliana* confer enhanced response to ABA. Specifically, mutations in the *ERA1* and *ERA3* genes cause deficient seed germination in the presence of low ABA (0.3 μM) concentrations that are normally unable to inhibit germination in the wild-type seed (Cutler et al., 1996). While the relationship between ABI1 and ERA1 proteins remains unclear, the *ERA1* gene has been determined to encode the β subunit of a protein farnesyl transferase. This class of enzymes has been show to modify various signal transduction proteins necessary for membrane localization (Cutler et al., 1996).

Generation of another ABA-hypersensitive mutant occurs via a point mutation in the *SUPERSENSITIVE TO ABA AND DROUGHT 1 (SAD1)* locus. The *SAD1* gene encodes a member of a small nuclear ribonucleoprotein complex that is capable of splicing, exporting, and degrading RNA. ABA-hypersensitivity can also occur due to a mutation in *HYPONASTIC LEAVES 1 (HYL1)* gene. The *HYL1* gene encodes a double-stranded RNA-binding protein that uses microRNA production to regulate the stability of specific transcripts (Vazquez et al., 2004). The ABA hypersensitivity in this mutation occurs due to an accumulation of the ABI5 transcription factor.

Along with the discovery of many ABA-hypersensitive and ABA-insensitive mutants, various mutations of components involved in the ABA pathway cause altered seed responses to environmental stress. In arabidopsis, the calcineurin B-like proteins (CBLs) have been shown to be differentially regulated by environmental stress and by ABA, suggesting the presence of multiple isoforms and their involvement in stress-related ABA signaling. The putative role of the CBLs is as a Ca^{2+} sensor that can regulate downstream CBL-interacting protein kinases (CIPKs), allowing for the transduction of calcium signals into a physiological response (Luan et al., 2002). Knockout of the *CBL9* gene or the *CIPK3* gene was able to suppress ABA-induced and stress-induced inhibition of seed germination. Involved upstream of calcium signaling is the *OPEN STOMATA 1 (OST1)* gene that encodes an autophosphorylating protein kinase (Assmann, 2004). In Arabidopsis, a mutation in *OST1* causes stomata to be left open since the guard cells become insensitive to ABA.

As described above, genetic screens for mutants with altered ABA perception have led to the identification of genes and proteins involved in the ABA signaling pathway. However, determining the genes whose expression is altered by ABA is also vital in understanding ABA signaling, especially since many physiological responses to ABA are dependent upon ABA-mediated gene expression (Johnson et al., 2002). From transcriptome analyses, ABA is known to alter genomic expression (Seki et al., 2002). In a random massive sequencing of arabidopsis transcripts more than 600 transcripts were found to be upregulated by ABA (Hoth et al., 2002). Approximately 600 other transcripts were found to be downregulated by ABA.

Within vegetative tissues, ABA-regulated genes respond primarily to abiotic stress that can lead to cellular dehydration (Xiong et al., 2002). During seed development or in the presence of environmental stress, ABA levels increase in the seed causing expression of ABA-induced

genes. In maturing seeds, *LATE EMBRYOGENESIS ABUNDANT (LEA)* genes are expressed during the acquisition of dessication tolerance (Ried and Walker-Simmons, 1993; Rock, 2000) and the putative role of LEA proteins is to protect the seed and plant under water-or salt-stress conditions (Xu et al., 1996). Another gene expressed in maturing seeds is *KIN1* which encodes an antifreeze-like protein that protects the seed from very low temperatures **(**Cutler et al., 1995).

The use of particle bombardment followed by transient gene expression analysis has enabled the functional dissection of many ABA-responsive promoters associated with physiological ABA responses. Specifically, various cis-acting promoter elements have been identified and shown to be involved in ABA-induced gene expression. One category of cis-acting elements contains a 8-10 base pair G-box ACGT core motif within its sequence and has been named ABRE (ABA Response Element). Analysis of expression in the ABA-inducible genes *RAB16A* from rice and *EM* from wheat alongside *in vitro* binding assays determined ABREs to be vital for transcription (Mundy et al., 1990). Only a subset of ABRE-like motifs within a given promoter is required for ABA regulation and the sequence flanking the ACGT core plays a role in determining function *in vivo* and protein binding *in vitro* (Izawa et al., 1993). Another category of cis-acting elements was discovered in the maize *C1* gene. It contains an ABRE-like motif not essential to ABA-regulation of the gene and an Sph element, which is essential for ABA induction of the gene (Kao et al., 1996).

Detailed analyses using deletion and linker-scanning studies of a subset of "strong" ABREs allowed for the identification of the minimal ABA-responsive complexes (ABRCs) which is the promoter sequence containing the ABRE and coupling elements such as CE1, CE3. These coupling elements are only active in combination with the ABRE. A single ABRE and single CE, or multiple ABREs, have been show to be necessary and sufficient in allowing a

synthetic promoter to respond to ABA (Shen and Ho, 1995). Further research on ABRCs has demonstrated that in the *HVA1* promoter, the ABRE has the consensus 'ACGTGGC' sequence and the CE3 element has the consensus sequence 'GCGTGTC' (Shen et al., 2004). Spacing between the ABRE and CE is constrained to less than 20 bp since induction of a promoter by ABA is greatly reduced when the two units have greater separation. However, the relative orientation of the ABRE and CE in functional promoters was flexible (Shen et al., 2004).

Many transcriptional regulators of ABA-induced and ABA-suppressed genes have been identified, including Viviparious 1/ABI3, the APELATA2 (AP2)-type transcription factors, the basic Leucine zipper domain (bZIPs), and the MYB- and MYC-class transcription factors. (Finklestein et al., 2002). The MYB and MYC transcription factor families are expressed in response to abiotic stress and function in a slower response system than the bZIP-ABRE system. Moreover, the ABA-inducible gene *RD22* contains a MYB binding sequence, which is involved in induction by ABA (Busk and Pages, 1998). Another family of transcription factors, the homeodomain Leu zipper (HD-Zip) family, also increased its expression in the presence of ABA or abiotic stress, although its function is unclear (Finkelstein et al., 2002).

Further investigation of ABRCs using yeast one-hybrid screens has show that only a subset of the bZIP family of transcription factors recognizes and binds to either the ABRE sequence or CE in an ABA-regulated promoter region. This bZIP subfamily, which includes ABI5 in arabidopsis, TaABF1 in wheat, and TRAB1 in rice and their other homologs, has been classified as ABRE binding factors (ABFs) (Choi et al., 2000). All bZIP proteins harbor a bZIP domain composed of a DNA-binding basic region and the Leu zipper dimerization region (Khurana et al., 2008). Moreover, the ABF transcription factors also contain three unique N-terminal conserved motifs with putative phosphorylation sites and a basic region slightly varied

from that of other bZIPs (Ho and Casaretto, 2002). A specific group of ABFs known as ABI5-like bZIPs has been shown to bind as a dimer to the ACGT-box of the ABREs or to CE3 to activate the ABRC containing promoters (Ross and Shen, 2006). The ability of these ABFs to do so indicates that these transcription factors are directly involved in the ABA signaling cascade leading to ABA-altered gene expression.

The ABI5-like bZIP, TRAB1, has been shown to interact specifically with the VP1 protein, which does not directly bind an ABRE. In doing so, TRAB1 likely works to regulate VP1-dependent ABA induced transcription (Hobo, 1999). The *TRAB1*gene and its barley homolog *HvABI5* have been shown to increase expression in drought and ABA-treated seedlings. Furthermore, a wheat ortholog of the *HvABI5* gene, *Wabi5*, increases its expression in response to low temperature, drought and exogenous ABA treatment (Takumi et al., 2008). The WABI5 protein functions as a transcription factor that induces LEA genes in response to abiotic stress.

HvABI5 was able to bind to ABRCs and was able to transactive *HVA1* and *HVA22* ABRC-β-glucuronidase (GUS) reporter genes when introduced into barley aleurone cells using particle bombardment. This transactivation only occurred in the presence of the HvVP1 transcription factor indicating that both the HvVP1 and HvABI5 are necessary for the ABA induction of gene expression (Ho and Casaretto, 2002). The *HVA1* gene encodes a group 3 LEA protein that confers drought resistance in transgenic plants (Xu et al., 1996) while *HVA22* has been proposed to play a role intracellular vesicular trafficking (Brands and Ho, 2002).

Another ABF transcription factor able to induce *HVA1* gene expression via particle bombardment is TaABF1. TaABF1 was identified in wheat via a yeast two hybrid screen where TaABF1 bound specifically to PKABA1, an ABA-induced Ser/Thr protein kinase (Johnson et

al., 2002). This binding only occurred when a functioning nucleotide binding domain was present in PKABA1, indicating binding of ATP must occur for subsequent binding of TaABF1. Furthermore, TaABF1 contains peptide sequences that are phosphorylated by PKABA1 while TaABF1 and PKABA1 gene expression patterns in wheat are very similar. Therefore, PKABA1 likely phosphorylates TaABF1 during late seed development and imbibition in response to ABA (Hirayama and Shinozaki, 2007). Along with TaABF1 phosphorylation, the prolonged presence of TaABF1 mRNA in dormant cereal grains and not in afterippened grains during imbibition suggests that TaABF1 may function in regulating dormancy and germination (Johnson et al., 2002). This putative role of TaABF1 is further supported by the mapping of TmABF, a *Triticum monococcum* ortholog of TaABF1, to a quantitative trait locus for dormancy on chromosome 3 (Nakamura et al., 2007).

Multiple other ABFs have also been shown to undergo post-translational modification. One method of modification is phosphorylation via a protein kinase. Phosphorylation leads to altered transcriptional activation activity or altered stability of the ABF. The presence of ABA causes phosphorylation of ABI5 in young seedings, which is required to preserve the seedlings in a state of developmental arrest (Lopez-Molina et al., 2001). TRAB1 is rapidly phosphorylated in response to ABA and hyper-osmolarity, demonstrating that this ABF is involved in the stress induced ABA pathway (Kagaya et al., 2002). ABA and abiotic stress also causes phosphorylation of arabidopsis TRAB1 homologs AREB1 and AREB2 at multiple sites and the addition of the phosphate group is required for their transcriptional activation activity (Uno et al., 2000). A family of rice SnRK2 protein kinases, which are related to PKABA1, were determined to be responsible for this modification of TRAB1 as well as for the phosphorylation of another bZIP factor, OSRK1 (Chae et al., 2007).

Instances of ABA-induced stabilization of ABFs via phosphorylation were identified in *Arabidopsis thaliana* ABI5 (AtABI5). Phosphorylation of AtABI5 yields reduced degradation which may also occur in its barley homolog HvABI5 (Casaretto and Ho, 2005). Conversely, a novel ABI5-binding protein, AFP, is believed to mark AtABI5 for ubiquitin-mediated degradation (Lopez-Molina et al., 2003). The mechanism by which AFP acts is similar to that of the bZIP HY5. HY5 is more stable in darkness due to a weaker association between HY5 and the E3 ubiquitin ligase COP1, which targets HY5 for proteasome-dependent degradation (Hardtke et al., 2000). Furthermore, small ubiquitin-related modifier conjugation of ABI5 via the E3 ligase SIZ1 is able to attenuate ABA signaling. The double mutant *siz1-2 afp-1* has even greater ABA sensitivity than the single mutant *siz-1*, indicating that SIZ1 independently suppresses ABI5 function in ABA signaling (Miura et al., 2009).

Much research has focused on identifying components of the ABA pathway that function upstream of the transcription factors, such as ABA receptors. To date, only members of the START domain protein family known as PYR/PYLs (pyrabactin resistance 11/PYR1-like) have been definitively identified as ABA receptors (Fujii et al., 2009). Several PYR/PYLs were shown to interact with and inhibit clade-A PP2Cs. These PP2Cs, such as ABI1, ABI2, and HAB1, negatively regulate ABA responses while ABA-activated SnRK2 kinases positively regulate ABA signaling (Santiago et al., 2009). Through an unidentified mechanism, inhibition of PP2Cs allows for activation of a subfamily of SnRK2 kinases (SnRK2.2, SnRK 2.3, and SnRK 2.6 in arabidopsis) and these kinases phosphorylate ABFs, likely modifying their ability to bind to ABRCs and regulate ABA-responsive gene expression (Schütze et al., 2008).

While ABA signaling in plants has been considered to be very complex, a group of researchers have proposed a simple model composed of the essential components necessary to

cause altered gene expression. In the presence of ABA, the PYR/PYL soluble receptor proteins can interrupt the interaction between PP2Cs and SnRK2s, stopping PP2C from dephosphorylating SnRK2s and allowing SnRK2 to return to its autophosphorylated default state (Figure 2).

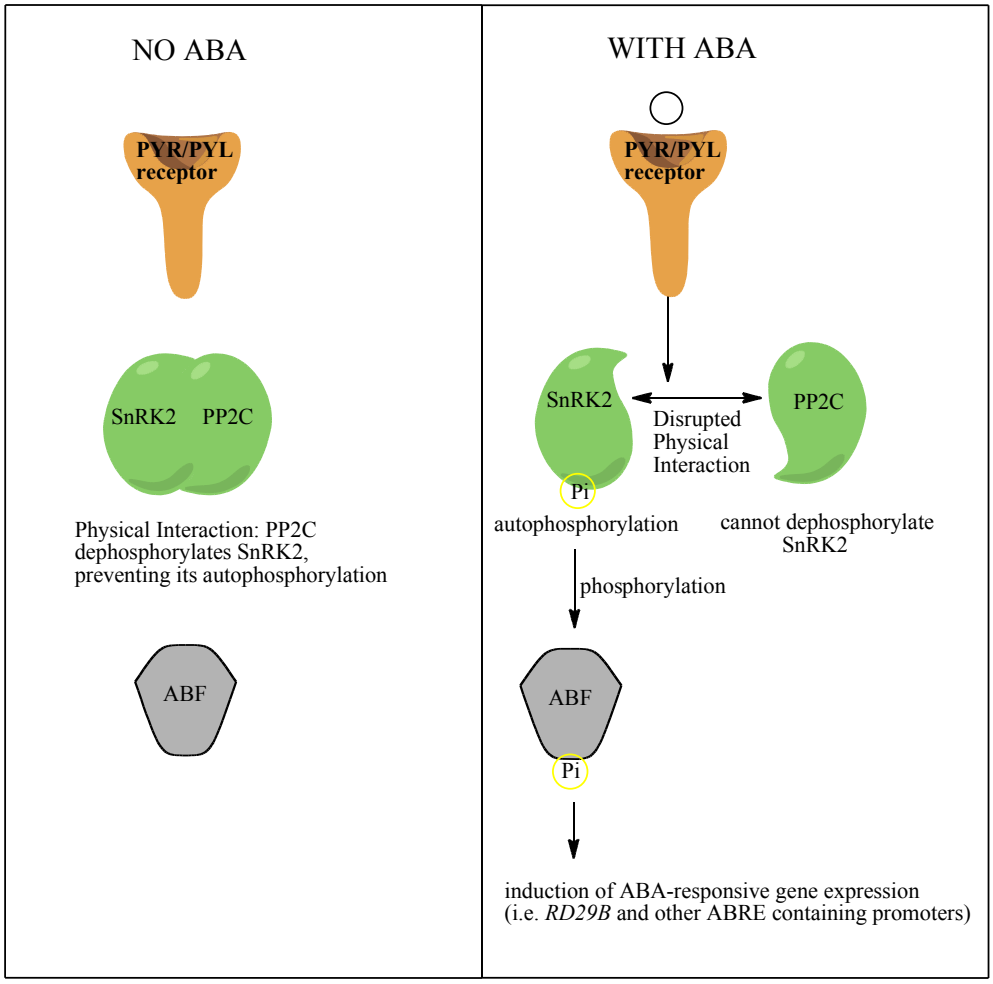


Figure 2. A model of the ABA signaling pathway including the PYR/PYL receptor, SnRK2, PP2C, ABF, and an ABA-responsive promoter.

The model of ABA signaling (Figure 2) beginning with the PYR/PYL receptor involves the posttranslational modification of transcription factors, which leads to altered gene expression.

However, ABA signaling in guard cells has been proposed to involve second messengers such as Ca^{2+} and phosphatidic acid (Hirayama and Shinozaki, 2007).

As mentioned above, transient particle bombardment experiments have been instructive in understanding the role and location of various components within the ABA signaling pathway, as well as crosstalk with other phytohormone signaling pathways such as gibberellin (GA). Upon bombardment of *HVA1:GUS* and *HVA22*:*GUS* reporter constructs in barley aleurone cells, basal expression was low in the absence of ABA and was greatly increased by addition of ABA. Co-bombardment of TaABF1 driven by a strong constitutive promoter with the *HVA1:GUS* reporter construct greatly increased reporter expression and was able to substitute for the presence of ABA. These results demonstrate that TaABF1 is involved in ABA-induced gene expression from ABRC-containing promoters. The addition of ABA to aleurone cells transiently overexpressing TaABF1 did not increase *HVA1:GUS* reporter expression since it was already at maximal levels (Johnson et al., 2008). Therefore, further investigation must be done to determine the combinatorial effects of exogenous ABA and overexpression of TaABF1. Moreover, bombardment of TaABF1 has been shown to inhibit ABA-suppressed gene expression in the *Amy32b* promoter indicating that TaABF1 is involved in mediating both ABA-induced and ABA-suppressed gene expression (Johnson et al., 2008).

Within particle bombardment experiments, the use of double stranded RNA interference (RNAi) technology has become a powerful tool in determining the role of various molecules with the biological systems (Zentella et al., 2002). The technique was first implemented using PKABA1 RNAi constructs controlled by the maize constitutive *UBI1* promoter, which was able to cause a sequence-specific suppression of the target *PKABA1* transcript.

Particle bombardment experiments with TaABF1 RNAi in barley aleurone cells determined that reducing levels of TaABF1 did not alter basal levels of *HVA1* induction or ABA-induced expression of the *HVA1* promoter. These findings suggest that TaABF1 is not absolutely required for ABA induction of the *HVA1* promoter or other ABRC containing genes during imbibition and there may be redundant proteins that can function in place of TaABF1 (Johnson et al., 2008). Using qRT-PCR, it was determined that modification of TaABF1 mRNA levels does not occur in response to ABA, suggesting that TaABF1's function in ABA signaling is not linked to up- or down-regulation of *TaABF1* transcription.

Another particle bombardment experiment in barley aleurone cells utilized 14-3-3 RNAi constructs to assess their role in ABA induction of an ABRC3 containing reporter. Proteins of the 14-3-3 family have well-defined functions as regulators of plant primary metabolism and ion homeostasis but their connection with ABA signaling had been unknown (Schoonheim et al., 2007). Transient transformation of cells with 14-3-3 RNAi led to decreased ABA induction of an ABRC-containing reporter construct. This suggests that 14-3-3 adaptor proteins are involved in ABA signaling. Furthermore, using yeast two-hybrid assays, ABFs were shown to bind various 14-3-3 proteins. Specifically, the ABF HvABI5 interacts with three different 14-3-3 proteins via two specific 14-3-3 binding motifs that are essential for 14-3-3 binding and proper *in vivo* trans-activation activity of ABRC promoters (Schoonheim et al., 2007).

While RNAi experiments have allowed for specific inhibition of various gene products, researchers have also sought to identify specific inhibitors of ABA perception. ABA perception was found to be specifically inhibited by 0.1 % 1-butanol since its presence lead to an inhibition of ABA-regulated processes (Ritchie et al., 1998). 1-butanol is believed to inhibit ABA perception by inhibiting phospholipase D activity which causes reduced production of

phosphatidic acid (PPA). PPA is involved in triggering subsequent ABA responses of the aleurone cell (Ritchie et al., 1998). Another known inhibitor of ABA perception is PBI-51, an enantiomeric synthetic acetylenic abscisic acid analog that displayed a reversible antagonistic effect with ABA. PBI-51 was proposed to be a competitive inhibitor of ABA for receptor binding (Wilen et al., 1993). PBI-51 has also been reported to act as an ABA antagonist in the ABA-inducible expression of the genes for napin and oleosin in *Brassica napus,* and in ABA-induced stomatal closure (Wilen et al., 1993; Yamazai et al., 2003). However, PBI-51 was observed to reduce the efficiency of germination and of post-germination growth in Arabidopsis, indicating that it may act as an ABA agonist as well as an ABA antagonist depending on the experiment (Hirayama et al., 2004).

To further elucidate TaABF1's role in ABA signaling, experiments combining the inhibition of ABA perception with transient transformation of cereal grains via particle bombardment were carried out and are reported in this thesis. In doing so, the effect of any endogenous ABA was inhibited and the effects of ABA on the overexpression of TaABF1 or other components of the ABA pathway could be more clearly assessed. Specifically, the change in ABRC-containing promoter induction by TaABF1 in the "absence" of endogenous ABA was determined. This method of inhibiting ABA and observing the change in ABA-regulated gene expression by various transcription factors, protein kinases, or other components of the ABA pathway will be instrumental in understanding the interactions and modifications occurring within the ABA signaling network.

MATERIALS AND METHODS

PARTICLE BOMBARDMENT

Seed Preparation:

Embryos of Himalaya barley (*Hordeum vulgare)* seeds were removed using a sterilized razor blade. Seed lacking their embryos were washed while shaking in E-pure water and 10% bleach. They were then washed while shaking five consecutive times in sterile water for five minutes. These seeds were placed in imbibing solution (20 mM $CaCl_2$, 20 mM Na succinate, pH 5.0) containing 10 μg/mL chloramphenicol and transferred to a vermiculite plate to incubate at 24˚C for 48 hours. The pericarp/testa layers were subsequently removed using sterilized fine point tweezers, exposing the aleurone layer. The seeds were returned to the vermiculite plate to incubate at 24˚C for 16-20 hours (modified from Lanahan et al., 1992).

DNA preparation:

Effector, reporter, and internal control construct DNA were all bound to 1.6 μm gold microcarriers. For each bombardment group, 2.5 μg *UBI:Luciferase* and 2.5 μg of the *HVA1:GUS* reporter construct was used. Appropriate effector substitute (*pAHC17*) was added to allow each sample to have equal amounts of DNA and dH_2O was added to reach a total volume of 5 μL. A sample containing 5 μL of dH_2O served as the control (modified from Lanahan et al., 1992).

Microcarrier preparation:

To prepare the microcarriers, 30 mg of 1.6 μm gold microparticles were weighed out and suspended in 1 mL of 70% ethanol. The particles were allowed to settle for 15 minutes. The particles were pelleted via microcentrifugation. The pellet was resuspended in 1 mL of sterile water, the particles were allowed to settle for 1 minute, and then re-pelleted using a microcentrifuge. This procedure was performed three times and the prepared microcarriers were stored in 0.5 mL of sterile 50% glycerol at 4˚C (modified from Lanahan et al., 1992).

Macrocarrier preparation:

The prepared microcarriers were vortexed until they were fully suspended and 50 μL of the microcarriers were subsequently added to the 5 μL DNA mixture. Following the addition of the microcarriers, 50 μL 2.5 M $CaCl_2$, followed by 20 μL 0.1 M spermidine were added. This allowed the DNA to bind to the gold microparticles. The mixture was vortexed, settled and then spun in a microcentrifuge for 2 seconds to pellet the DNA-bound gold. The supernatant was removed and the DNA bound-gold pellet was washed in 70% ethanol, 100% ethanol, and then resuspended in 48 μL of 100% ethanol. DNA-bound gold (8 μL) was spread onto each macrocarrier (each DNA treatment had four macrocarriers) and allowed to air dry (Figure 3) (modified from Lanahan et al., 1992).

Biolistic Gene Gun Protocol:

A sterilized 1350 psi rupture disk, a stopping screen, and the macrocarrier containing DNA-bound gold were loaded into the PSD-1000/He particle delivery system (BIO-RAD) (Figure 3). Eight prepared embryoless seeds were arranged with their thinner ends pointing inward in a circle (~2.5 cm in diameter) around the center mark of a petri dish on filter paper.

The petri dish was placed into the gene gun at a 6 cm target distance. Vacuum was applied to the shooting chamber until 28 in Hg were reached. Pressure was then applied to the rupture disk until it burst. The bombarded seeds were removed from the chamber and placed in their respective treatment solutions containing 4 mL imbibing solution, 10 mg/mL chloramphenicol, and ABA if required. The seeds were subsequently shaken while incubating in petri dishes at 24˚C for 24 hours (modified from Lanahan et al., 1992).

Luciferase and GUS assays:

The bombarded seeds were ground (eight groups of four seeds for each treatment) in 800 μL of grinding buffer (100 mM $NaPO_4$, pH 7.2, 5 mM DTT, and 20 μg/mL leupeptin**)** in a mortar and pestle. All ground seed extracts were centrifuged (14,000 x g) at 4˚C for 10 minutes. The supernatant was decanted into a new tube and 100 μL of the supernatant from each sample was added to 200 μL of luciferase assay mixture (45 mM Tris sulfate pH 7.7, 15 mM $MgCl_2$ 15 mM DTT, 1.5 mM EDTA, 1 mM luciferin, 1 mM ATP) in a 12 x 75 mm test tube. Each tube was vortexed quickly and the light emitted was immediately measured using a luminometer.

For GUS assays, each seed extract (50 μL) was added to 200 μL of GUS (β-glucuronidase) assay buffer (GAB) which contained 2.5 mM 4-Methylumbelliferyl β-D-glucuronide (MUG), 50 mM Na phosphate pH 7.2, 2 mM EDTA, 10 mM DTT, 10 μg/mL leupeptin, 20% methanol, 0.02% sodium azide. The mixture of each sample was incubated at 37˚C in the dark for 20 hours. After incubation, each sample was centrifuged at maximum speed for 5 minutes. From each sample 6.25 uL was added to one well of a 96 well plate along with 250 μL of 0.2 M Na_2CO_3. The methylumbelliferone (MU) fluorescence of the plate was read

using an FLx800 (Biotek Instruments Inc.) microplate fluorescence reader (excitation=360 nm, emission= 460 nm, sensitivity=42) (modified from Shen et al., 1996).

Data Analysis:

To normalize the GUS activity across all samples, the MU fluorescence value from the control sample, which was prepared with water instead of DNA, was subtracted from the MU fluorescence value of each sample. This value was then divided by the luciferase control value subtracted from the luciferase value of that sample (Ho and Casaretto, 2002) and then multiplied by 2 million to obtain the normalized GUS value [(GUS value-GUS control)/(LUC value-LUC control)] x 2,000,000. Only samples with luciferase values of 18,000 RLU/s were used to ensure that sufficient bombardment of DNA occurred. The mean normalized GUS activity for each treatment group was then calculated along with the standard error. A t-test (Excel) was used when necessary to determine whether differences in the normalized GUS activity between treatment groups within a bombardment were significant ($p<0.05$) (modified from Shen et al., 1996).

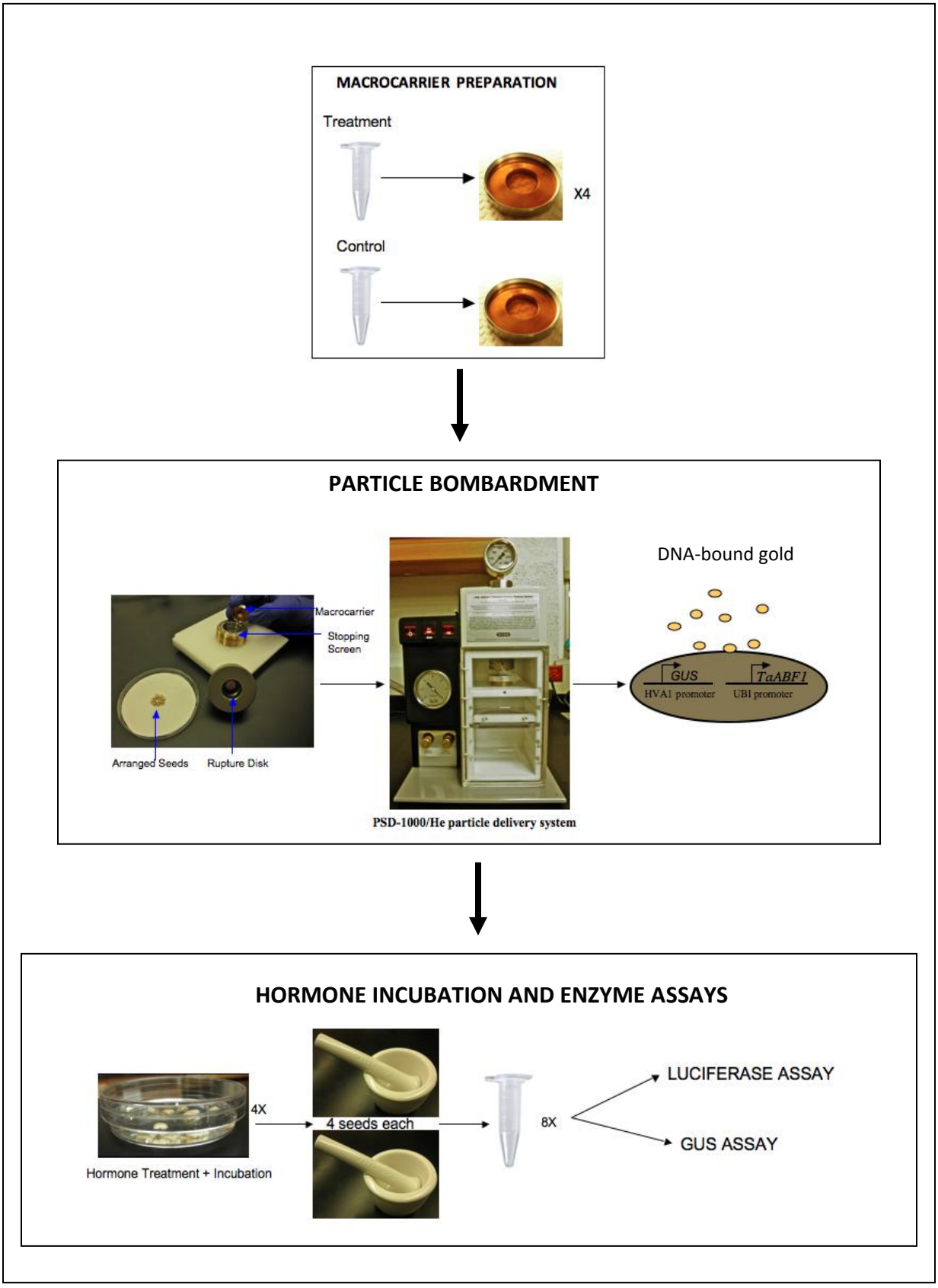


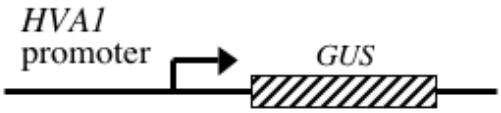
Figure 3. Bombardment protocol scheme for one experimental treatment group.

RESULTS

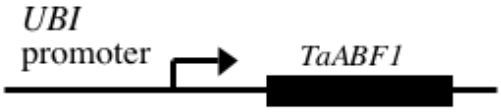
TaABF1 and ABA have no combinatorial effect:

Since *TaABF1* is a component of the ABA signaling pathway involved in ABA-inducible gene expression, we wished to determine the combinatorial effect of submaximal levels of *TaABF1* and ABA on the induction of ABA-inducible genes. First, a reporter construct (*HVA1:GUS)* containing the *GUS* reporter gene driven by the ABA-inducible promoter *HVA1* was introduced into barley aleurone cells via particle bombardment (Figure 4a). A modest 3.5-fold induction was observed in the *HVA1* promoter in the presence of 0.02 μM ABA and a 9-fold induction occurred in the presence of 0.2 μM ABA (Figure 4b). As expected, *HVA1:GUS* expression was greater with an increased concentration of ABA since both concentrations were below the maximal induction concentration of 20 μM (Johnson et al., in preparation). Introduction of the effector construct *UBI:TaABF1* (Figure 4a) into the aleurone cells resulted in a 4-fold induction of the *HVA1* promoter. *HVA1:GUS* expression was not increased upon overexpression of *TaABF1* in the presence of 0.02 μM ABA in comparison to the 0.02 μM ABA treatment alone. Furthermore, in the presence of 0.2 μM ABA the addition of *TaABF1* actually reduced the *HVA1* expression. These results indicate that there is no additive effect between ABA and TaABF1 for ABA-inducible gene expression since increasing levels of *TaABF1* did not cause greater induction of the *HVA1* promoter by ABA.

(A) **Reporter construct**



Effector construct



(B)

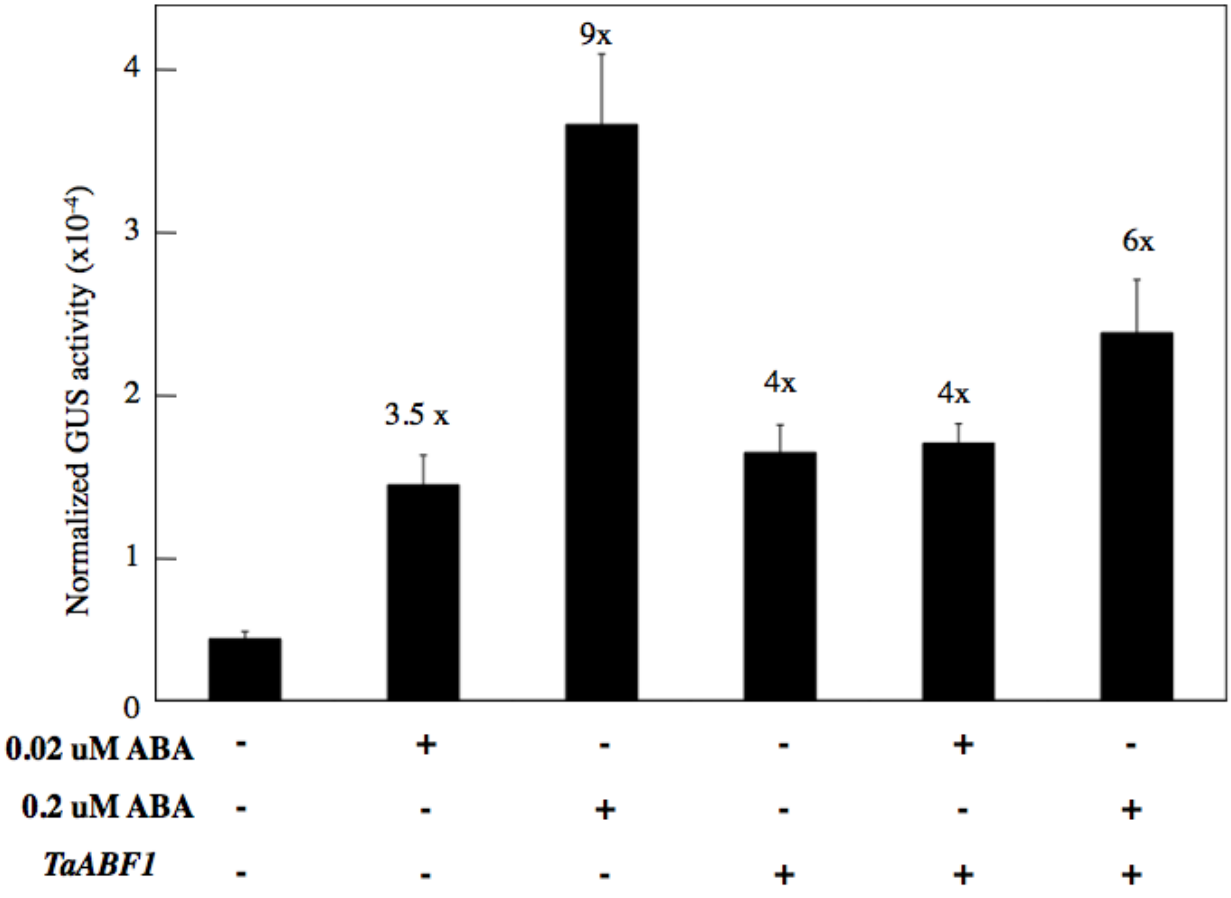


Figure 4. Effect of *TaABF1* on ABA induction of *HVA1:GUS.* **(a)** Diagram of the reporter and effector constructs used in the experiment. **(b)** The reporter construct, *HVA1:GUS,* and the internal control construct, *UBI:luciferase,* were co-bombarded into barley aleurone cells either with (+) or without (-) the effector construct [*UBI:TaABF1*], using a 1:20 molar ratio of effector and reporter constructs. Bars indicate normalized GUS activities +/- SE after 24 h of incubation with (+) or without (-) 0.02 μM or 0.2 μM ABA.

PBI51 does not inhibit ABA perception in aleurone cells:

In search of a specific inhibitor of ABA perception, we wished to determine whether PBI51 was able to inhibit ABA-induced gene expression. First, a reporter construct (*HVA1:GUS)* containing the *GUS* reporter gene driven by the ABA-inducible promoter *HVA1* was introduced into barley aleurone cells via particle bombardment (Figure 5a). In the presence of PBI51, *HVA1:GUS* induction was unchanged in comparison to the control level of expression caused by endogenous ABA (Figure 5b). Incubation in 10 μM ABA yielded 53-fold induction of the *HVA1* promoter. Incubation with 10 μM ABA and 50 μM PBI51 resulted in unchanged *HVA1:GUS* induction (+/- SE). Therefore, PBI51 was not able to inhibit ABA-induced gene expression.

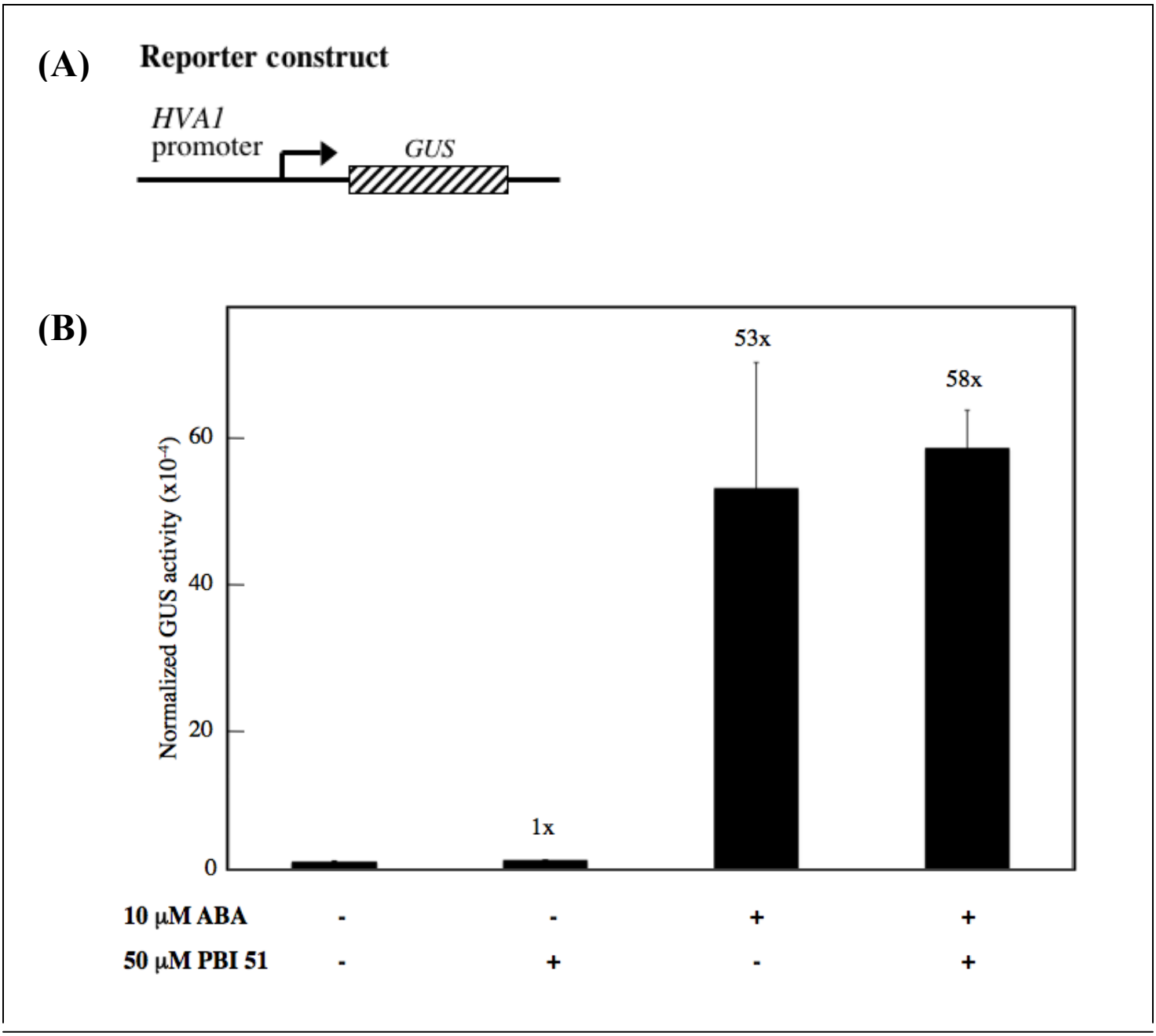


Figure 5. PBI51 does not inhibit ABA-induced gene expression. . **(a)** Diagram of the reporter construct used in the experiment. **(b)** The reporter construct, *HVA1:GUS*, and the internal control construct, *UBI:luciferase*, were co-bombarded into barley aleurone cells. Bars indicate normalized GUS activities +/- SE after 24 h of incubation with (+) or without (-) 10 μM ABA or 50 μM PBI51.

1-butanol inhibits ABA-induced gene expression:

To identify a specific inhibitor of ABA perception, we sought to determine whether 1-butanol was able to inhibit ABA-induced gene expression. To do so, a reporter construct (*HVA1:GUS)* containing the *GUS* reporter gene driven by the ABA-inducible promoter *HVA1* was introduced into barley aleurone cells via particle bombardment (Figure 6a). Incubation with 20 μM ABA resulted in 49-fold induction of *HVA1:GUS* (Figure 6b). The ability of 1-butanol and 2-butanol to inhibit ABA induction *HVA1:GUS* was assessed since 1-butanol has been shown to specifically inhibit ABA perception in barley aleurone cells while 2-butanol does not (Ritchie et al., 1998). The presence of neither 0.1% 1-butanol ($p=0.34$) nor 0.1% 2-butanol ($p=0.30$) was able to significantly inhibit induction of the *HVA1* promoter by ABA. Also, the *HVA1:GUS* expression was not significantly different between the 0.1% 1-butanol and 0.1% 2-butanol treatments ($p=0.99$). However, incubation with 0.4% 1-butanol was able to strongly inhibit induction of the *HVA1* promoter by ABA ($p=0.00026$) while 0.4% 2-butanol had much less of an effect, although it was still a significant one ($p=0.013$). However, 1-butanol was able to specifically inhibit *HVA1:GUS* expression at 0.4% since the inhibition observed from 0.4% 1-butanol was significantly greater than that of 0.4% 2-butanol ($p=2.98 \times 10^{-5}$). This suggests that levels of 1-butanol in a 0.1% solution were too low to inhibit ABA while the 0.4% solution had an optimal level of 1-butanol to inhibit ABA. Levels of 1-butanol greater than that present in a 0.4% solution (i.e. 1% 1-butanol) caused non-specific inhibition of ABA-induced gene expression. Specifically, 1 % 1-butanol reduced ABA induction of the *HVA1* promoter to levels of endogenous *HVA1* expression and also inhibited other horomone (GA) signaling (data not shown).

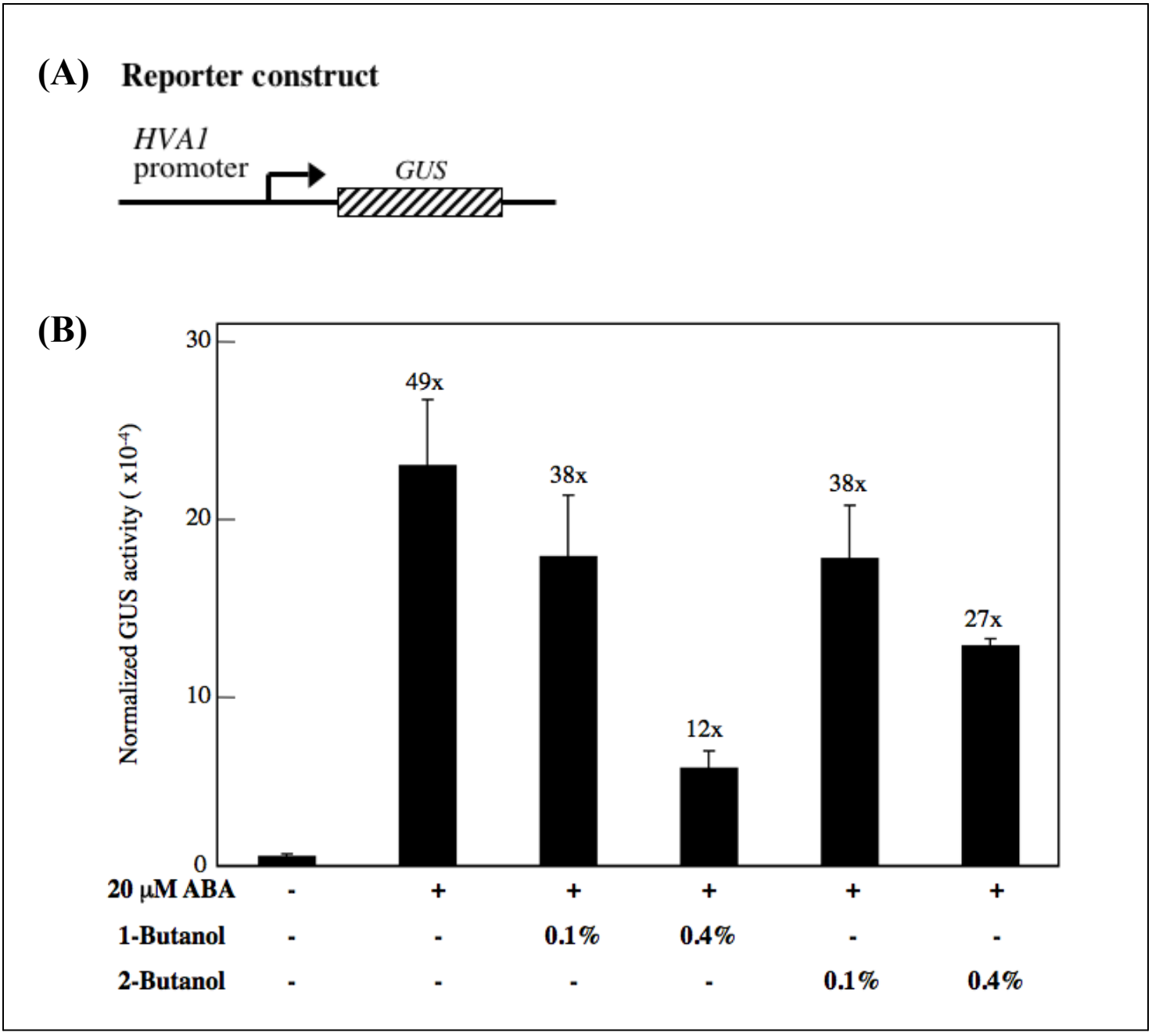


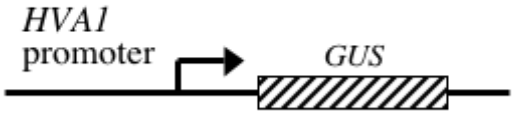
Figure 6. Inhibition of ABA induction of *HVA1:GUS* with 1-butanol. **(a)** Diagram of the reporter construct used in the experiment. **(b)** The reporter construct, *HVA1:GUS*, and the internal control construct, *UBI:luciferase*, were co-bombarded into barley aleurone cells incubated for 24 h with (+) or without (-) 20 μM ABA, 0.1 % 1-butanol, 0.4% 1-butanol, 0.1 % 2-butanol, or 0.4 % 2-butanol. Bars indicate normalized GUS activities +/- SE.

1-butanol inhibits TaABF1 induction of ABA-inducible genes:

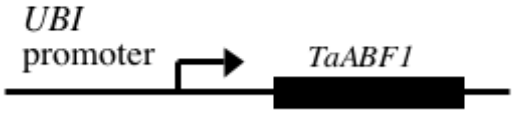
Since 0.4% 1-butanol was shown to specifically inhibit ABA perception, we wished to determine whether 0.4% 1-butanol was able to alter the effect of TaABF1 overexpression on ABA-inducible gene expression. First, a reporter construct (*HVA1:GUS)* containing the *GUS* reporter gene driven by the ABA-inducible promoter *HVA1* was introduced into barley aleurone cells via particle bombardment (Figure 7a). Incubation with 0.4% 1-butanol inhibited induction of *HVA1:GUS* by endogenous ABA (Figure 7b). As previously shown (Figure 6), the presence of 0.4 % 1-butanol was able to inhibit the effect of 20 μM ABA on *HVA1* expression. Introduction of *UBI:TaABF1* into the aleurone cells yielded a 62-fold induction of the *HVA1* promoter while bombardment of *UBI:TaABF1* in the presence of 0.4% 1-butanol inhibited the effects of TaABF1 on *HVA1* expression (11-fold). Therefore, 0.4 % 1-butanol was able to inhibit the effect of TaABF1 overexpression on ABA-inducible gene expression. This suggests that ABA is required for post-translational activation of TaABF1 since inhibition of endogenous ABA perception caused a decrease in the induction of the *HVA1* promoter by TaABF1.

(A)

Reporter construct



Effector construct



(B)

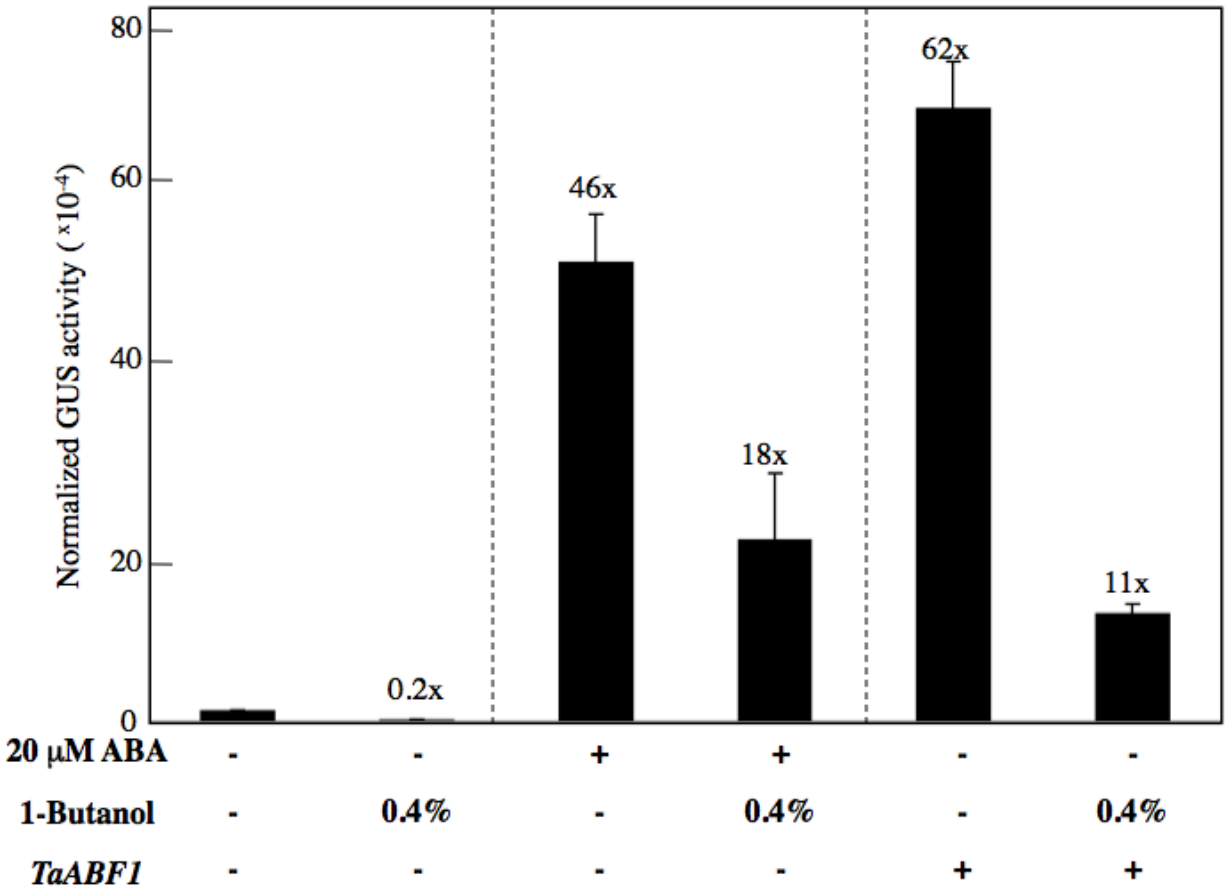


Figure 7. (a) Diagram of the reporter and effector constructs used in the experiment. **(b)** The reporter construct, *HVA1:GUS*, and the internal control construct, *UBI:luciferase*, were co-bombarded into barley aleurone cells either with (+) or without (-) the effector construct [*UBI:TaABF1*], using a 1:1 molar ratio of effector and reporter constructs. Bars indicate normalized GUS activities +/- SE after 24 h of incubation with (+) or without (-) 20 μM or 0.4% 1-butanol.

DISCUSSION

These experiments further investigate the role of TaABF1 in the ABA signaling pathway. Our data provide insight into the potential for post-translational activation of TaABF1 and the effects of this putative TaABF1 modification on ABA-induced gene expression. We also demonstrate an effective method to specifically inhibit ABA perception. However, our results indicate that the role of TaABF1 in the branch of ABA signaling leading to *HVA1* is more complex than previously proposed models show (Johnson et al., 2008).

TaABF1 was previously shown to induce ABRC-containing promoters (i.e. *HVA1*), which likely occurs via direct binding to the ABRC and with the help of other factors, activates transcription of the promoter. This hypothesis is substantiated by the fact that TaABF1 contains a conserved DNA-binding domain present in other ABFs that is known to bind to ABRC (Choi et al., 2000; Hobo et al., 1999). Future DNA-protein binding experiments must be performed to validate this predicted action of TaABF1 on ABRC-containing promoters (Johnson et al., 2008).

Upstream of TaABF1 binding to ABRC, the mechanism of TaABF1 activation by ABA remains unclear. TaABF1 has been proposed to undergo an ABA-dependent phosphorylation

that activates the protein. Other ABFs such as ABI5 (Lopez-Molina et al., 2001), TRAB1 (Kagaya et al., 2002), AREB1, and AREB2 (Uno et al., 2000) have been implicated in ABA-induced post-translational activation via phosphorylation. Primarily, the SnRK2 class of protein kinases has been found responsible for the phosphorylation of ABFs (Chae et al., 2007). The protein kinase PKABA1, a SnRK2, has been shown to phosphorylate peptide sequences from TaABF1 (Johnson et al., 2002). However, PKABA1 is not involved in ABA-induced gene expression since its overexpression barely induces the *HVA1* promoter and does not increase ABA induction of the *HVA1* promoter upon its bombardment (Gomez-Cadenas, 1999). Furthermore, bombardment of PKABA1 RNAi into barley aleurone cells does not inhibit the effects of ABA on *HVA1* expression, demonstrating that ABA induction of ABRC-containing promoters occurs independently of PKABA1 (Zentella et al., 2002). If post-translational modification (i.e. phosphorylation) of TaABF1 leading to *HVA1* induction occurs, it is likely through a different SnRK2 protein kinase and does not involve PKABA1.

Assuming TaABF1 undergoes ABA-induced post-translational modification to become activated, it is logical to predict that in the absence of exogenous ABA, overexpression of *TaABF1* would not induce the *HVA1* promoter. Furthermore, exogenous ABA and the bombardment of *TaABF1* should have an additive effect on increasing *HVA1* expression. However, previous findings show that the effect of *TaABF1* overexpression on ABRC-containing promoters was observed in the absence of exogenous ABA (Johnson et al., 2008). This is consistent with action of other ABFs such as OsTRAB1 (Hobo et al., 1999) and HvABI5 (Casaretto and Ho, 2005).

Contrary to predictions based on an ABA-induced post-translational event, earlier experiments showed that the effects of TaABF1 overexpression and exogenous ABA on ABA-

induced gene expression (*HVA1*) were not additive (Johnson et al., 2008). However, these conclusions were drawn from the addition of ABA at a concentration (20 μM) able to maximally induce the *HVA1* promoter (Harris et al., in preparation). Therefore, the absence of additive effects seen with TaABF1 and ABA in ABA-induced gene expression could not be definitively determined.

We re-examined the potential for additive induction of the *HVA1* promoter by overexpression of *TaABF1* and exogenous ABA at lower concentrations (0.02 μM and 0.2 μM) that do not maximally induce the promoter (Figure 4). Again, the effects of *TaABF1* overexpression and exogenous ABA on ABA-induced gene expression (*HVA1*) were not additive**.** This suggests that ABA-induced posttranslational activation of TaABF1 does not occur under these conditions.

Overexpression of another ABF in rice seedlings, *AtABF3*, slightly upregulated ABA-induced gene expression without exogenous ABA. Upon treatment with ABA, greater upregulation occurred in the seedlings (Oh et al., 2005), indicating that this ABF functions in a different manner than TaABF1. In ABA-suppressed gene expression (i.e. *Amy32b),* the effects of exogenous ABA and overexpression of *TaABF1* are additive. This suggests that posttranslational modification of TaABF1 may occur (potentially through PKABA1) in the ABA signaling branch leading to *Amy32b* (Harris, 2010).

To further explore the potential for ABA to initiate the posttranslational modification of TaABF1 in ABA-induced gene expression, it was necessary to identify a specific inhibitor of ABA. Previous findings showed that PBI51 reversibly inhibits the perception of ABA and acts as an ABA antagonist of ABA-induced gene expression in microspore-derived embryos of *Brassica*

napus (Wilen et al., 1993) and of ABA-induced stomatal closure in *Vicia faba* **(**Yamazaki et al., 2003). This suggests that PBI-51 directly competes with ABA by binding to the ABA receptor (Wilen et al., 1993). In contrast to these findings, we found that PBI-51 was unable to inhibit ABA perception in barley aleurone cells (Figure 5). Interestingly, another study (Hirayama et al., 1999) has shown that PBI-51 exhibits agonistic effects on the action of ABA in germination and post-germination growth of Arabidopsis. This result implies that PBI-51 may act differently depending on the activity of the ABA-recognizing components found in a particular species or cell type. This could explain the lack of ABA-specific inhibition by PBI51 since PBI51 may have had no effect on ABA signaling in our barley aleurone cells.

Previous work demonstrated that 0.1% 1-butanol caused specific inhibition of ABA perception in barley aleurone cells (Ritchie et al., 1998). While our findings showed that 0.1% 1-butanol slightly inhibited ABA-induced gene expression, we found this inhibition statistically insignificant as well as non-specific since 0.1% 2-butanol exhibited nearly identical inhibition (Figure 6). However, we determined that 0.4% 1-butanol was able to strongly and specifically inhibit ABA from inducing an ABRC-containing promoter (*HVA1)*. Therefore, using 0.4 % 1-butanol to inhibit ABA perception will be a useful tool in elucidating the role of various components within the ABA signaling pathway. Specifically, it will enable the determination of whether ABA perception is required for the activity of a given component.

Using 1-butanol to specifically inhibit ABA perception, we examined the effects of inhibiting the perception of endogenous ABA on the ability of TaABF1 to induce *HVA1* expression. We found that treatment with 0.4% 1-butanol decreased induction of the *HVA1* promoter by the overexpression of *TaABF1* (Figure 7). Therefore, perception of endogenous ABA must be required for the activation of TaABF1. These results further suggest that TaABF1

undergoes ABA-induced posttranslational activation. If TaABF1 posttranslational activation occurs, it is likely through a phosphorylation event catalyzed by an SnRK2 protein kinase. The hypothesis that TaABF1 is modified at the protein level is supported by previous findings that *TaABF1* mRNA abundance is not increased by ABA (Johnson et al., 2008). Therefore, upregulation of *TaABF1* at the mRNA level is not required for its function in ABA-induced gene expression.

Multiple functions of TaABF1 phosphorylation have been proposed including the classic model of activation by addition of a phosphate group. Another role of TaABF1 phosphorylation may be to stabilize the protein. This stabilization could lead to an increase in available TaABF1 and cause the stimulation of ABA-induced genes (Johnson et al., 2008). However, overexpression of *TaABF1* yields such high levels of TaABF1 protein that stabilization via phosphorylation may not be required to increase ABA-induced gene expression. It has already been shown that ABA induces both phosphorylation and stabilization of AtABI5 (Lopez-Molina et al., 2001; Lopez-Molina et al., 2003). However, our finding that suggests a posttranslational modification of TaABF1 occurs (Figure 7) may contradict the stabilization hypothesis since inhibiting endogenous ABA causes a decrease in the induction of *HVA1* when *TaABF1* is overexpressed. According to the stabilization hypothesis, the overexpression of *TaABF1* should cause increased *HVA1* expression.

The conclusion that ABA induces the posttranslational modification of TaABF1 does not agree with our finding that exogenous ABA and the overexpression of *TaABF1* were not additive in their induction of *HVA1* (Figure 4). This discrepancy between our results and previously proposed models of the ABA signaling pathway indicate that ABA induction of *HVA1* through TaABF1 is more complex than previously hypothesized (Johnson et al., 2008).

Various aspects of the ABA signaling pathway may have caused the data we obtained. One possible factor is the redundancy of TaABF1 in the induction of the *HVA1* promoter. Bombardment of *TaABF1* RNAi into aleurone cells was shown to not inhibit ABA induction of *HVA1*, suggesting that other ABFs act redundantly with TaABF1 (Johnson et al., 2008). Future work will include the generation of a *taabf1* null mutant and assess the level of *HVA1* expression in the mutant versus the wild-type plant. If *HVA1* expression is not reduced in the mutant plant then other ABFs must act redundantly with TaABF1.

Other groups have reported functional redundancy between members of the ABF family. The knockout of ABFs known to elicit an ABA response can often show no altered effect due to redundancy. For example, decrease in expression of the ABF genes *EEL, EREB3,* and *AtbZIP67* in transgenic arabidopsis caused no observable phenotypic change from wild-type (Bensmihen et al., 2005). Likewise, Arabidopsis *abf1* and *abf3* mutants did not exhibit phenotypic differences from wild-type (Finkelstein et al., 2005). Conversely, bombardment of an *HvABI5* RNAi construct into aleurone cells greatly reduced induction of ABRC-containing promoters (Casaretto and Ho, 2003), indicating that no ABFs function redundantly with HvABI5.

Another factor that may complicate the ABA signaling branch leading to *HVA1* is the possible role of a 14-3-3 adaptor protein. The involvement of 14-3-3 proteins in increasing ABA induced *HVA1* expression has already been shown using 14-3-3 RNAi bombardment experiments (Schoonheim et al., 2007). Moreover, ABFs including HvABI5 (a TaABF1 ortholog) are known to interact with 14-3-3 proteins (Schoonheim et al., 2007). Therefore, it is likely that a 14-3-3 protein is able to bind to TaABF1 and facilitate a phosphorylation event (via a SnRK2), or the stabilization of TaABF1. The presence of a 14-3-3 protein in the pathway could have caused the results we observed. To investigate whether a 14-3-3 protein binds to TaABF1, a

yeast two-hybrid screen and assay could be performed with various known 14-3-3 proteins and TaABF1.

Future work will also address whether phosphorylation of TaABF1 occurs in the branch of the ABA signaling leading to *HVA1*. A yeast two-hybrid screen involving many SnRK2 protein kinases and TaABF1 will determine whether an SnRK2 kinase besides PKABA1 is able to bind to TaABF1. If one is found, a phosphorylation analysis of the SnRK2 kinase and TaABF1 peptide sequences will be performed *in vitro.* If bombardment of this SnRK2 protein into aleurone cells induces the *HVA1* promoter, it would show that TaABF1 is phosphorylated and thereby activated by an SnRK2 kinase in the branch of ABA signaling leading to *HVA1*.

In summary, our results suggest the potential for posttranslational modification of TaABF1 within the ABA signaling branch leading to ABRC-containing promoters. However a subset of our results contradicted this hypothesis, indicating that the ABA signaling pathway involved with ABA-induced gene expression is more complex than previously proposed. A new hypothetical model of the branch of ABA signaling leading to *HVA1* is presented with the inclusion of potentially involved components (Figure 8). It is possible that a more complex version of the model proposed for ABA signaling through the PYR/PYL ABA receptor (Fujii et al., 2009) occurs for *HVA1* induction. In our proposed model (Figure 8b) binding of ABA to the PYR/PYL receptor disrupts the physical interaction between an SnRK2 and a PP2C. This enables SnRK2 to be autophosphorylated and activated. SnRK2 subsequently phosphorylates TaABF1, activating it. In its activated form, TaABF1 can bind to the ABRC of *HVA1* and induce transcription of the gene. Other ABFs that function redundantly with TaABF1 and a 14-3-3 protein are also shown (Figure 8b).

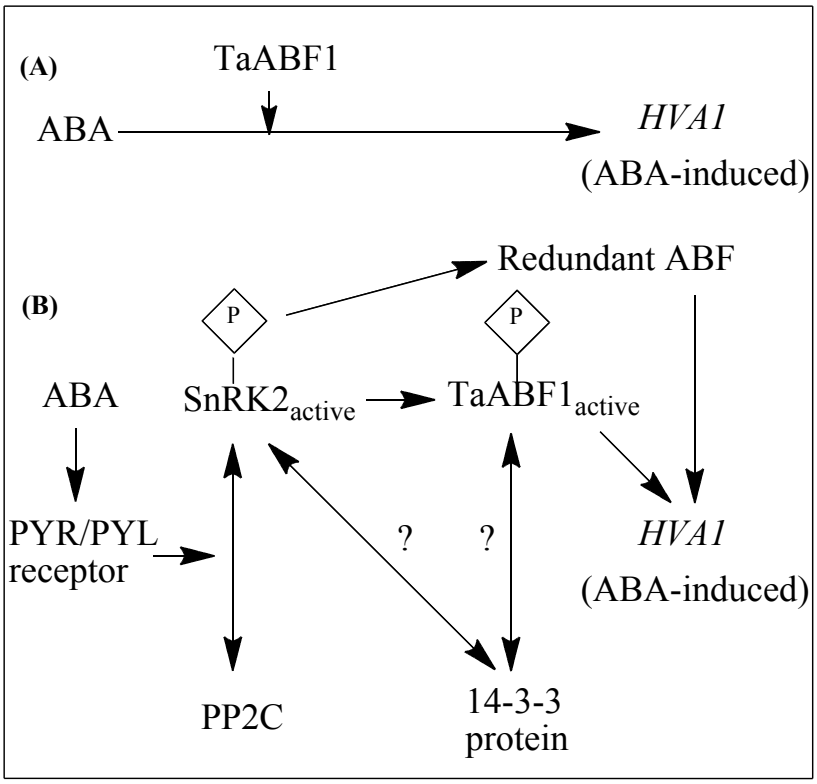


Figure 8. The proposed model ABA signaling for the branch leading to *HVA1*. (a) The previously proposed model. (b) A hypothetical model of ABA signaling incorporating potentially involved components.

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References

Anderberg RJ, Walker-Simmons MK (1992) Isolation of a wheat cDNA clone for an abscisic acid-inducible transcript with homology to protein kinases. Proc Natl Acad Sci U S A 89 (21):10183-10187

Bensmihen S, Giraudat J, Parcy F (2005) Characterization of three homologous basic leucine zipper transcription factors (bZIP) of the ABI5 family during *Arabidopsis thaliana* embryo maturation. J Exp Bot 56(412):597-603

Brands A, Ho TH (2002) Function of a plant stress-induced gene, *HVA22*. Synthetic enhancement screen with its yeast homolog reveals its role in vesicular traffic. Plant Physiol 130(3):1121-1131

Busk PK, Pages M (1998) Regulation of abscisic acid-induced transcription. Plant Mol Biol 37(3):425-435

Casaretto J, Ho TH (2003) The transcription factors HvABI5 and HvVP1 are required for the abscisic acid induction of gene expression in barley aleurone cells. Plant Cell 15(1):271-284

Casaretto JA, Ho TH (2005) Transcriptional regulation by abscisic acid in barley (*Hordeum vulgare L.)* seeds involves autoregulation of the transcription factor HvABI5. Plant Mol Biol 57(1):21-34

Choi H, Hong J, Ha J, Kang J, Kim SY (2000) ABFs, a family of ABA-responsive element binding factors. J Biol Chem 275(3):1723-1730

Cutler S, Ghassemaian M, Bonetta D, Cooney S, McCourt P (1996) A protein farnesyl transferase involved in abscisic acid signal transduction in Arabidopsis. Science 273(5279):1239-1241

Finkelstein R, Gampala SS, Lynch TJ, Thomas TL, Rock CD (2005) Redundant and distinct functions of the ABA response loci ABA-INSENSITIVE(ABI)5 and ABRE-BINDING FACTOR (ABF)3. Plant Mol Biol 59(2):253-267

Finkelstein RR, Gampala SS, Rock CD(2002) Abscisic acid signaling in seeds and seedlings. Plant Cell 14 Suppl:S15-45

Finkelstein RR, Lynch TJ (2000) The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. Plant Cell 12(4):599-609

Fujii H, Chinnusamy V, Rodrigues A et al (2009) In vitro reconstitution of an abscisic acid signalling pathway. Nature 462(7273):660-664

Giraudat J, Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM (1992) Isolation of the Arabidopsis *ABI3* Gene by Positional Cloning. Plant Cell 4(10):1251-1261

Gomez-Cadenas A, Verhey SD, Holappa LD, Shen Q, Ho TH, Walker-Simmons MK (1999) An abscisic acid-induced protein kinase, PKABA1, mediates abscisic acid-suppressed gene expression in barley aleurone layers. Proc Natl Acad Sci U S A 96(4):1767-1772

Gosti F, Beaudoin N, Serizet C, Webb AA, Vartanian N, Giraudat J (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. Plant Cell 11(10):1897-1910

Hardtke CS, Gohda K, Osterlund MT, Oyama T, Okada K, Deng XW (2000) HY5 stability and activity in Arabidopsis is regulated by phosphorylation in its COP1 binding domain. EMBO J 19(18):4997-5006

Harris LJ (2010) The role of TaABF1 in abscisic acid-mediated suppression of α-amylase gene expression in barley (*Hordeum vulgare)* aleurone. Honors Thesis, Colby College.

Harris LJ, Keyser BK, Martinez SA, Dryer WE, Johnson RR (in preparation) Functional Analysis of TaABF1 during abscisic acid signaling in cereal grains.

Himmelbach A, Yang Y, Grill E (2003) Relay and control of abscisic acid signaling. Curr Opin Plant Biol 6(5):470-479

Hirayama T, Kieber JJ, Hirayama N et al (1999) RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell 97(3):383-393

Hirayama T, Shinozaki K (2007) Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. Trends Plant Sci 12(8):343-351

Hobo T, Kowyama Y, Hattori T (1999) A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. Proc Natl Acad Sci U S A 96(26):15348-15353

Hoth S, Morgante M, Sanchez JP, Hanafey MK, Tingey SV, Chua NH (2002) Genome-wide gene expression profiling in *Arabidopsis thaliana* reveals new targets of abscisic acid and largely impaired gene regulation in the *abi1-1* mutant. J Cell Sci 115(24):4891-4900

Izawa T, Foster R, Chua NH (1993) Plant bZIP protein DNA binding specificity. J Mol Biol 230(4):1131-1144

Johnson RR, Shin M, Shen JQ (2008) The wheat PKABA1-interacting factor TaABF1 mediates both abscisic acid-suppressed and abscisic acid-induced gene expression in bombarded aleurone cells. Plant Mol Biol 68(1-2):93-103

Johnson RR, Wagner RL, Verhey SD, Walker-Simmons MK (2002) The abscisic acid-responsive kinase PKABA1 interacts with a seed-specific abscisic acid response element-binding factor, TaABF, and phosphorylates TaABF peptide sequences. Plant Physiol 130(2):837-846

Kagaya Y, Hobo T, Murata M, Ban A, Hattori T (2002) Abscisic acid-induced transcription is mediated by phosphorylation of an abscisic acid response element binding factor, TRAB1. Plant Cell 14(12):3177-3189

Kao CY, Cocciolone SM, Vasil IK, McCarty DR (1996) Localization and interaction of the cis-acting elements for abscisic acid, VIVIPAROUS1, and light activation of the *C1* gene of maize. Plant Cell 8(7):1171-1179

Kobayashi F, Maeta E, Terashima A, Takumi S (2008) Positive role of a wheat HvABI5 ortholog in abiotic stress response of seedlings. Physiol Plant 134(1):74-86

Koornneef M, Hanhart CJ, Hilhorst HWM, Karssen CM (1989) *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. Plant Physiol 90(2):463-469

Lanahan MB, Ho TH, Rogers SW, Rogers JC (1992) A gibberellin response complex in cereal alpha-amylase gene promoters. Plant Cell 4(2):203-211

Leung J, Merlot S, Giraudat J (1997) The arabidopsis *ABSCISIC ACID-INSENSITIVE2 (ABI2*) and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell 9(5):759-771

Lopez-Molina L, Mongrand S, Chua NH (2001) A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proc Natl Acad Sci U S A 98(8):4782-4787

Lopez-Molina L, Mongrand S, Kinoshita N, Chua NH (2003) AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. Genes Dev 17(3):410-418

McCarty DR, Carson CB, Stinard PS, Robertson DS (1989) Molecular analysis of *viviparous-1*: an abscisic acid-insensitive mutant of maize. Plant Cell 1(5):523-532

Miura K, Lee J, Jin JB, Yoo CY, Miura T, Hasegawa PM (2009) Sumoylation of ABI5 by the Arabidopsis SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. Proc Natl Acad Sci U S A 106(13):5418-5423

Mundy J, Yamaguchi-Shinozaki K, Chua NH (1990) Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice *RAB* gene. Proc Natl Acad Sci U S A 87(4):1406-1410

Nakamura S, Komatsuda T, Miura H (2007) Mapping diploid wheat homologues of Arabidopsis seed ABA signaling genes and QTLs for seed dormancy. Theor Appl Genet 114(7):1129-1139

Nijhawan A, Jain M, Tyagi AK, Khurana JP (2008) Genomic survey and gene expression analysis of the basic leucine zipper transcription factor family in rice. Plant Physiol 146(2):333-350

Oh SJ, Song SI, Kim YS et al (2005) Arabidopsis CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. Plant Physiol 138(1):341-351

Ried JL, Walker-Simmons MK(1993) Group 3 Late Embryogenesis Abundant Proteins in Desiccation-Tolerant Seedlings of Wheat (*Triticum aestivum L.*). Plant Physiol 102(1):125-131

Ritchie S, Gilroy S (1998) Abscisic acid signal transduction in the barley aleurone is mediated by phospholipase D activity. Proc Natl Acad Sci U S A 95(5):2697-2702

Schoonheim PJ, Sinnige MP, Casaretto JA et al (2007) 14-3-3 adaptor proteins are intermediates in ABA signal transduction during barley seed germination. Plant J 49(2):289-301

Schütze K, Harter K, Chaban C (2008) Post-translational regulation of plant bZIP factors. Trends Plant Sci 13(5):247-255

Seki M, Ishida J, Narusaka M et al (2002) Monitoring the expression pattern of around 7,000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. Funct Integr Genomics 2(6):282-291

Shen Q, Ho TH (1995) Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel cis-acting element. Plant Cell 7(3):295-307

Shen Q, Zhang P, Ho TH (1996) Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. Plant Cell 8(7):1107-1119

Shen Q, Casaretto J, Zhang P, Ho T-HD (2004) Functional definition of ABA-response complexes: the promoter units necessary and sufficient for ABA induction of gene expression in barley (*Hordeum vulgare L.*) Plant Mol Biol 54:111-124

Sturaro M, Vernieri P, Castiglioni P, Binelli G and Gavazzi G (1996) The *rea* (*red embryonic axis*) phenotype describes a new mutation affecting the response of maize embryos to abscisic acid and osmotic stress. Journal of Experimental Botany 47(6):755--762

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc Natl Acad Sci U S A 97(21):11632-11637

Wilen RW, Hays DB, Mandel RM, Abrams SR, Moloney MM (1993) Competitive inhibition of abscisic acid-regulated gene expression by stereoisomeric acetylenic analogs of abscisic acid. Plant Physiol 101(2):469-476

Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. Plant Cell 14 Suppl:S165-83

Xu D, Duan X, Wang B, Hong B, Ho T, Wu R (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol 110(1):249-257

Yamazaki D, Yoshida S, Asami T, Kuchitsu K (2003) Visualization of abscisic acid-perception sites on the plasma membrane of stomatal guard cells. Plant J 35(1):129-139

Zentella R, Yamauchi D, Ho TH (2002) Molecular dissection of the gibberellin/abscisic acid signaling pathways by transiently expressed RNA interference in barley aleurone cells. Plant Cell 14(9):2289-2301