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The role of TaABF1 in abscisic acid-mediated suppression of ⍰-amylase gene expression in cereal grains

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Abstract

The phytohormones gibberellin (GA) and abscisic acid (ABA) regulate important developmental events in germinating seeds. Specifically, GA induces the expression of hyrolase genes, like the α-amylase gene *Amy32b*, which mobilize starch reserves to be used by the embryo, and ABA suppresses this induction. Recent advancements identified ABA and GA receptors and key components in the signaling pathways, however, the mechanism of crosstalk between the hormones remains largely unknown. To further elucidate the mechanism of ABA suppression of GA-induced genes, we focused on the transcription factor TaABF1, a member of the ABA response element binding factor family. TaABF1 has been shown to physically interact with the SnRK2 kinase PKABA1 and overexpression of TaABF1 or PKABA1 can suppress *Amy32b*. We carried out particle bombardment experiments to investigate how TaABF1 suppresses *Amy32b* and how TaABF1 is activated by ABA. The role of TaABF1 in ABA-mediated suppression of *Amy32b* is more complicated than hypothesized. Unlike PKABA1, overexpression of TaABF1 did not cause a decrease of *GAMyb* expression and in fact resulted in an increase of *GAMyb* expression. When *TaABF1* and *GAMyb* were simultaneously overexpressed in aleurone, the *GAMyb* induction of *Amy32b* was unaffected, indicating that the target of TaABF1 action must be upstream of GAMyb. Furthermore, TaABF1 and ABA demonstrated an additive effect on the suppression of *Amy32b*. Based on our findings, we propose a model in which PKABA1 activates two separate targets, one being TaABF1 which then modifies an unknown target upstream of GAMyb and the other being an unknown transcription factor that suppresses *GAMyb* transcription.

Introduction

In order to survive and grow in rapidly changing environmental conditions, plants use complex hormone interactions to regulate growth and reproduction. Two of the phytohormones necessary for developmental regulation are gibberellin (GA) and abscisic acid (ABA). GA and ABA stimulate complex signaling cascades that culminate in the induction or suppression of genes specific for seed development and germination. Because of the potential advancements in crop control and regulation, the molecular mechanisms involved in GA and ABA signaling have been a focus of research for many years.

Gibberellins (GA) regulate a wide range of developmental events in plants. In addition to seed development and germination, GA has been shown to be involved in root and stem growth, and flowering and fertility. GA-deficient mutants demonstrated increased seed abortions and this failure to develop normally was attributed to reduced levels of bioactive GA in very young seeds. Treatment with exogenous GA, however, could not restore normal seed development because the exogenous GA was unable to enter the seeds. Nevertheless, the effect of GA deficiency on seed abortion could be negated by simultaneous expression of mutations that give a constitutive GA response (Swain and Singh 2005), demonstrating the role of GA and the GA stimulated response in early stages of seed development. Similarly, extreme dwarf mutants of pea and *Arabidopsis* in which GA biosynthesis was blocked exhibited shorter roots and stems than wild-type plants. Treatment of the dwarf plants with GA enhanced the shoot and root elongation (Yaxley et al. 2001; Fu and Harberd 2003), thereby providing evidence for GA's involvement in root and stem growth. Early studies found that fruit

development in tomato and earlier flowering in several plants was also induced by treatment with GA (Wittwer et al. 1957).

There are 136 naturally occurring gibberellins (MacMillan 2002) which all share similar chemical structure (Figure 1), yet relatively few demonstrate intrinsic biological activity. This specificity has facilitated the identification of features crucial for bioactivity. Key structural aspects needed for activity include a hydroxyl group on C3 and a carboxyl group on C6. Furthermore, hydroxylation on C2 causes inactivation, which is an important mechanism for growth regulation in angiosperms (Yamaguchi 2008). The slight structural differences between bioactive and inactive gibberellins are indicative of the tight fit of GA in a specific pocket of a GA receptor (Harberd et al. 2009).

Another plant hormone vital to the regulation of plant development, abscisic acid (ABA) (Figure 1), has opposing effects to GA. Under conditions of stress, seed maturation, and dormancy, ABA regulates growth and stomatal aperture. Elucidation of ABA's roles in freezing, salt, and water stress led to its characterization as a stress hormone. In fact, ABA concentrations in leaves can increase up to 50 times under draught conditions, which is the most dramatic change in concentration reported for any hormone in response to an environmental signal (Schurr et al. 1992). Under water stress conditions, ABA stimulates stomatal closing to prevent excess water loss to transpiration, promotes root growth, and inhibits shoot growth.

Seed dormancy is controlled by the ratio of ABA to GA and the importance of this balance was demonstrated clearly in an early experiment by Koornneef and colleagues (Koorneef et al. 1982). Seeds of a GA-deficient mutant that was unable to

germinate in the absence of exogenous GA were mutagenized and then screened for revertants. The revertants, or seeds that had regained their ability to germinate, were mutants of ABA synthesis. This result showed that GA synthesis is not required in the absence of ABA, since seeds lacking both GA and ABA can still germinate. Consequently, in wild-type seeds the finely tuned ratio of ABA to GA is what regulates seed germination, not simply GA synthesis.

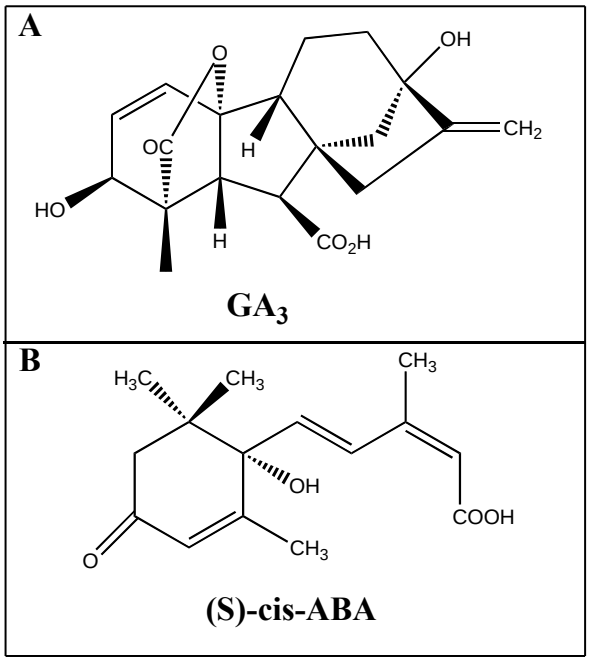

Figure 1. Chemical structures of bioactive (**A**) gibberellin (GA_3) and (**B**) abscisic acid ((S)-cis-ABA)

ABA suppresses GA stimulated developmental events such as germination and storage reserve breakdown by repressing many GA-induced genes whose expression is required for these events. For example, during germination of cereal grains, the embryo secretes GA to the aleurone layer. The aleurone cells that surround the starchy endosperm have thick primary cell walls and large numbers of protein-storing vacuoles called protein bodies (Bethke et al. 1997). The primary function of these cells is the synthesis and

release of hydrolytic enzymes into the starchy endosperm during and after germination, allowing the starch reserves to be broken down and used by the growing embryo. The enzymes α-amylase and β-amylase are responsible for the starch degradation. α-Amylase specifically hydrolyzes starch chains to produce oligosaccharides with α-1,4-linked glucose residues while β-amylase degrades the oligosaccharides from the ends to form maltose. These energy-rich products can then be used by the growing embryo for early developmental events.

In the aleurone layer, GA promotes the expression of several genes encoding these hydrolytic enzymes (Ritchie and Gilroy 1998; Lovegrove and Hooley 2000). The expression of these genes is suppressed by ABA during dormancy, seed development, and in seeds under unfavorable germination conditions. Consequently, these interactions between GA and ABA in cereal aleurone layers make aleurone cells an excellent system to investigate the molecular mechanisms involved in hormonally regulated gene expression (Lovegrove and Hooley 2000).

Important insights into participants involved in the molecular mechanism of GA signaling came from "slender" mutants of rice (*slr1*) and barley (*sln1*) (Ikeda et al. 2001; Chandler et al. 2002). When homozygous for the recessive *slender1* mutation the plants were abnormally tall, similar to plants that have been treated with high levels of GA. Since the mutants were not treated with GA, the *slender1* must have resulted in the GA response being constitutively turned on. In fact, these mutants lacked an important negative regulator of the GA response.

The *SLENDER* gene in cereal grains is orthologous to the *Arabidopsis GAI (GIBBERELLIN-INSENSITVE*) and *RGA* (*REPRESSOR OF ga1-3*) genes, which encode

a class of transcriptional regulators, the DELLA-domain proteins (Silverstone et al. 1997). DELLA-domain proteins belong to the GRAS family of transcription factors, which gets its name from the first three members of the group : GAI, RGA, and SCR (SCARECROW). The DELLA-domain proteins have a regulatory domain at the N-terminal end in which the first five amino acid residues are aspartic acid (D), glutamic acid (E), leucine (L), leucine (L), and alanine (A). Mutation in the DELLA domain of the slender protein yields a GA-insensitive dwarf phenotype, while mutation in the GRAS repressor domain results in the slender phenotype (Ikeda et al. 2001). This result elucidated the specific roles of the DELLA and GRAS domains. The DELLA domain is required for degradation of the repressor protein and therefore a nonfunctional DELLA domain yields an overactive repressor protein and little to no GA signaling, evidenced by the dwarf phenotype. Conversely, the GRAS domain is required for the repressor function, and a mutation in this domain results in an inactive repressor protein, an overactive GA response, and thus a slender plant.

Identification of the SLR1 DELLA repressor protein spurred a hunt for the GA receptor. A rice gene (*GID1*), originally identified by the loss of function *gid1* mutation that results in GA-insensitive dwarfism, was shown to encode a nuclear GA receptor protein (Ueguchi-Tanaka et al. 2005). GID1 is localized predominantly in the nucleus and acts as a soluble GA receptor. GID1 has high affinity for bioactive GA and low to nonexistent affinity for inactive GA. Furthermore, GID1 binds specifically with the rice DELLA SLR1 when both proteins are in yeast and in the presence of bioactive GA (Figure 2).

Recent crystal structure studies have elucidated the molecular spatial arrangements directing the interactions between GID1, GA, and a DELLA protein. The GID1 protein has a central pocket in which bioactive GA can bind. Polar groups in the bioactive GA interact directly with GID1 and hydrogen bond with water molecules, causing the tight specificity of fit of GA into its receptor. The binding of GA causes a conformational change in GID1 resulting in the N terminus forming a lid to the GA binding pocket (Murase et al. 2008). Afterwards, the upper surface of the lid binds with the N-terminal region of the DELLA protein. The binding of the DELLA protein is believed to cause a conformational change in the C-terminal domain of the DELLA protein, which induces substrate recognition by the enzyme $SCF^{SLY1/GID2}$ E3 ubiquitin ligase. This leads to ubiquitination, which then results in the DELLA protein being broken down by the proteasome, allowing the GA signaling pathway to be activated (Shimada et al. 2008).

Several studies, however, have found evidence to suggest that the site of GA perception in aleurone cells may be on the plasma membrane. GA derivatives that were impermeable to the plasma membrane still stimulated the expression of GA-inducible genes (Beale et al. 1992). Moreover, GA-inducible genes were not up-regulated when GA was injected into the cytoplasm of barley aleurone protoplasts (Gilroy and Jones 1994). Because GID1 is a soluble receptor, it is feasible that cereal aleurone layers utilize both soluble and plasma-membrane-bound GA receptors. Evidently, further research is needed to clarify the number and identity of GA receptors.

Early responses to GA include the activation of G-proteins (Jones et al. 1998), which cause an increase in cGMP (Penson et al. 1996) and cytoplasmic Ca^{2+}

concentrations (Gilroy 1996), culminating in the induction of a transcription factor, GAMYB (Gubler et al. 1995; Gubler et al. 1999). cGMP levels increase in barley aleurone layers two hours after GA treatment (Bethke et al. 1997) and specific inhibitors that prevent the transient increase in cGMP reduced the accumulation of *GAMyb* and *α-amylase* mRNA. These results identify cGMP as a component of the GA signal transduction pathway.

GAMYB is a member of the MYB superfamily of transcription factors that play regulatory roles in developmental processes and defense responses in plants. GAMYB promotes the expression of genes, like low- and high-pI α-amylases, proteinases, and β-glucanases by specifically binding to the GA-responsive element (GARE: 5′-TAACAAA-3′) present in these promoters. Mutations in the GARE inhibited GAMYB's binding to and transactivation of these target promoters (Zentella et al. 2002). An inhibitor of translation, cycloheximide, had no effect on the production of *GAMyb* mRNA in response to GA, however, cycloheximide did inhibit *α-amylase* transcription. These results identify *GAMyb* as a primary or early response gene and *α-amylase* as a secondary or late response gene (Taiz and Zeiger 2006).

The ABA signal transduction pathway in aleurone layers has been a target of research for many years. Many ABA signaling components have been identified, however, the way they work together to form a complete ABA signaling network has not been well established. Recently, however, several advances have led to the identification of the ABA receptors and their three-dimensional structures, as well as an understanding of how key regulatory phosphatases and kinases are controlled by ABA. A new model has been proposed in which soluble PYR/PYL/RCAR ABA receptors directly regulate

PP2C phosphatases, which then directly regulate SnRK2 kinases (Park et al. 2009; Ma et al. 2009) (Figure 2).

Identifying the receptors for ABA had eluded researchers for many years, however, recently *PYRABACTIN RESISTANCE1 (PYR1)* was found in a chemical genetic screen using pyrabactin, a selective ABA agonist that inhibits only some of the pathways regulated by ABA. *PYR1* encodes one of the 14 members of the START family of proteins, which share a conserved hydrophobic ligand-binding pocket. *Pyr1* and *pyr1-like (pyl)* mutants were insensitive to ABA *in vivo*, however, expression of PYR1 or PYL4 restored ABA sensitivity. These results demonstrated that PYR1 and PYLs are functionally redundant and mediate multiple ABA responses. Furthermore, ABA was shown to promote the interaction of PYR1 with group A PP2Cs, which led to the inhibition of the enzymatic activity of the PP2Cs. Based on these results, researchers concluded that PYR1 and PYLs combined with different PP2Cs form a large family of ABA receptors (Park et al. 2009). Another research team independently identified this receptor family, however the PYR1 and PYL were named RCAR, for regulatory component of ABA receptor (Ma et al. 2009).

In the absence of ABA, the phosphatase PP2C acts as a constitutive negative regulator of a family of kinases (SnRK2) whose autophosphorylation is required for kinase activity on downstream targets. When ABA binds the receptor, it facilitates the PYR/PYL/RCAR receptor to then bind and repress PP2P. Once PP2P is inactivated, the SnRK2 kinase can autoactivate and phosphorylate downstream transcription factors which then induce transcription of a promoter containing the ABA response complex

(Sheard and Zheng 2009). This response complex consists of the ABA-responsive *cis*-acting promoter elements (ABREs) together with a coupling element.

Members of the ABA response element binding factor (ABF) family of basic Leu zipper (bZIP) transcription factors have been shown to function in ABA signaling. bZIP transcription factors have a highly conserved basic region responsible for sequence-specific DNA binding and a less conserved amphipathic sequence in the form of a coiled-coil (Nijhawan et al. 2007). As transcription factors, the ABFs interact with specific ABRE and trans-activate downstream gene expression. Several members of the ABF family, AtAB15, AtABF1, AtABF2 (Fujii et al. 2007; et al. Furihata 2006), OsTRAB1 (Kobayashi et al. 2005), and OREB1 (Chae et al. 2007) have been shown to be phosphorylated by SnRK2 kinases and play a clear role in stimulating the expression of ABA-induced genes (Casaretto and Ho 2005; Oh et al. 2005).

While many aspects of ABA and GA induction have been elucidated, the mechanisms involved in the crosstalk between the hormones remain largely unknown. The GA-induced, ABA-suppressed transcription of the α-amylase promoter, *Amy32b,* in the aleurone layer of cereal grains has been a classical experimental system to study the interaction between ABA and GA. Using this system, two major mechanisms of GA-suppression have been identified: ABA-induced WRKY transcriptional regulators, and the ABA-induced Ser/Thr protein kinase PKABA1 (Figure 2).

WRKY genes belong to a gene superfamily encoding transcription factors involved in the regulation of a variety of biological processes. Four of the six *WRKY* genes expressed in rice aleurone cells are ABA-inducible (Xie et al. 2005). Overexpression of rice *WRKY* genes *OsWRKY51* and *OsWRKY71* specifically and synergistically repressed

the GA-induction of *Amy32b* (Xie et al. 2006). These transcription factors, however, do not influence ABA-induced gene expression (Xie et al. 2005). Bimolecular fluorescence complementation assays revealed that OsWRKY51 does not bind to the *Amy32b* promoter *in vitro*, but rather interacts with OsWRKY71 and enhances the binding affinity of OsWRKY71 to W boxes in the *Amy32b* promoter (Xie et al. 2006). The expression of *Amy32b* then was shown to be dependent on the ratio of GAMYB activator and OsWRKY51/OsWRKY71 repressors. A barley ortholog to OsWRKY1, HvWRKY38, was also characterized to block the inductive effects of SAD, a DOF protein, and HvGAMYB whenever these proteins were present individually. When SAD and HvGAMYB were simultaneously acting on *Amy32b*, HvWRKY38 could not repress the induction, further supporting the conclusion that *Amy32b* expression is regulated by the ratio of activators and repressors (Zou et al. 2008).

The alternative mechanism of ABA-GA crosstalk involves the SnRK2 Ser/Thr protein kinase PKABA1 (Gómez-Cadenas et al. 1999). *PKABA1* transcript levels increase in response to ABA in aleurone cells as well as in scutellar, root, and shoot tissues (Holappa and Walker-Simmons 1995). Conversely, *PKABA1* levels decrease below detectable levels in GA-treated aleurone (Gómez-Cadenas et al. 2001). PKABA1 has also been shown, using two-hybrid assays, to bind specifically to TaABF1, a member of the ABF family of bZIP transcription factors from wheat. PKABA1 produced in transformed cell lines was able to phosphorylate synthetic peptides representing three specific regions of TaABF1. Taken together, these findings indicate that TaABF1 may serve as a physiological substrate for PKABA1 in the ABA signal transduction pathway during ABA-suppressed gene expression (Johnson et al. 2002). TaABF1 is currently the

only ABF found to play any role in the ABA-mediated inhibition of gene expression and is the only transcription factor known to act in both ABA induction and ABA suppression pathways.

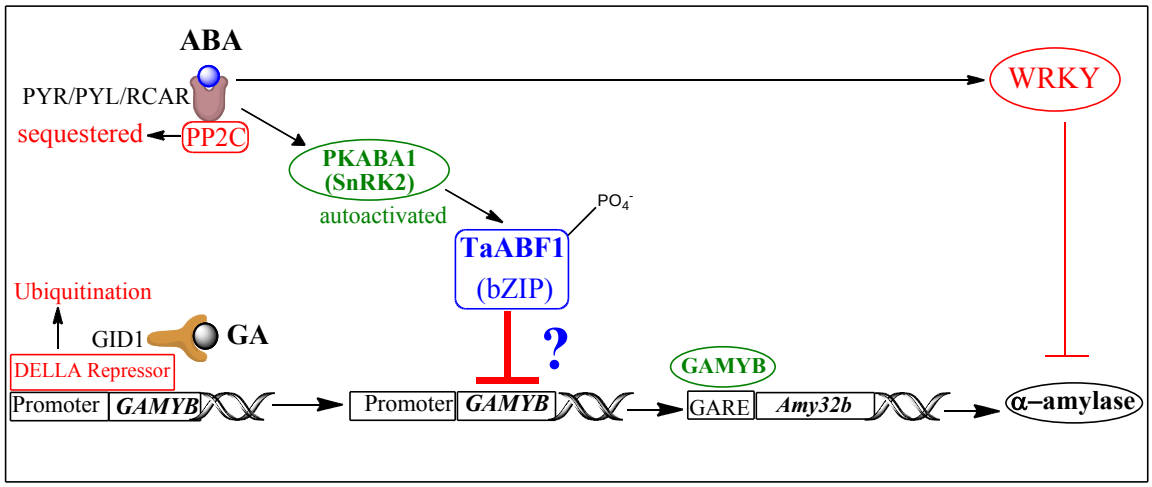

Figure 2. Current predicted model of GA and ABA perception and crosstalk in aleurone.

Both PKABA1 and TaABF1 have been shown to act as intermediates in ABA antagonism of GA-induced gene expression. PKABA1 was able to fully substitute for ABA in inhibiting the expression of *Amy32b* and Cys proteinase genes in GA-treated barley aleurone layers (Gómez-Cadenas et al. 1999). Similarly, TaABF1 has been shown to completely eliminate the GA-induced expression of the *Amy32b* α-amylase promoter in the absence of ABA (Johnson et al. 2008). RNA inhibition of *TaABF1* did not prevent either ABA-mediated or PKABA1-mediated suppression of the *Amy32b* promoter, suggesting that another protein may act redundantly with TaABF1 during cereal imbibition (Johnson et al. 2008).

The target of TaABF1 along the GA-induction pathway has yet to be definitively established. Both ABA and PKABA1 repress the GA-induction of *GAMyb*. In a *slender* mutant in the absence of GA, *GAMyb* and *α-amylase* were highly expressed, but this

constitutive expression was still inhibited by ABA, PKABA1, or an inhibitor of cGMP synthesis (Gómez-Cadenas et al. 2001). Based on these observations, it has been hypothesized that PKABA1, and therefore TaABF1, act upstream from the formation of functional GAMyb but downstream of the site of action of the *Slender* gene product. Furthermore, because PKABA1 inhibits GA induction of the *GAMyb* promoter, it has been postulated that at least part of the action of PKABA1 is to downregulate GAMyb at the transcriptional level (Gómez-Cadenas et al. 2001). Involvement of TaABF1 in this downregulation and its molecular target, however, has yet to be determined.

Materials and Methods

Seed Preparation

The embryos were removed from Himalaya barley seeds with a sterilized razor blade. The embryo-less seeds were then subjected to a wash with E-pure water, 10% bleach, and then 5 consecutive washes with sterilized water. The seeds were placed on a sterilized vermiculite plate, soaked in imbibing solution (20 mM sodium succinate, 20 mM calcium chloride, pH=5.0) containing 10 μg/mL chloramphenicol, and incubated at 24° for 48 hours.

After 48 hours, the pericarp/testa layers were peeled off of each seed using sterilized fine point tweezers in order to expose the aleurone layer. Once the pericarp/testa layers were removed, the seeds were returned to the vermiculite plate and incubated at 24° for 16-20 hours (modified from Lanahan et al. 1992)

DNA Preparation

The DNA for each bombardment was prepared to contain 2.5 μg *UBI:Luciferase* internal control (pAHC18 plasmid), 2.5 μg of the reporter construct, and the desired concentration of *UBI:Effector.* Effector substitute (pAHC17) and sterile water were added to each DNA preparation to make each sample have the same amount of DNA and a total volume of 5 μL. A control was also prepared to contain only 5 μL H_2O (modified from Lanahan et al. 1992).

Microcarrier Preparations

To prepare the microcarriers, 30 mg of 1.6 μm gold microparticles were suspended in 1 mL of 70% ethanol. The particles were then allowed to settle for 15 minutes and pelleted using a microcentrifuge. The particles were resuspended in 1 mL sterile water, allowed to settle for 1 minute and then pelleted again. This was repeated three times. The microcarriers were finally stored in 0.5 mL sterile 50% glycerol at 4°C (modified from Lanahan et al. 1992).

Macrocarrier Preparations

In order to bind the prepared DNA to 1.6 μm gold microcarriers, the microcarriers were first resuspended vigorously and 50 μL were added to each microcentrifuge tube containing the prepared DNA. Immediately after, 50 μL of 2.5M $CaCl_2$ was added and the microcentrifuge tubes were quickly vortexed. 20 μL of 0.1M spermidine was then added and the samples were vortexed for 2-3 minutes. The particles were allowed to settle for 1 minute and then centrifuged for 2 seconds to pellet the DNA-bound gold. The microcarriers were then washed with 70% ethanol, 100% ethanol, and resuspened in 48 μL 100% ethanol. Eight microliters of the resuspended microcarriers were spread evenly

on each macrocarrier (1 macrocarrier for control, 4 macrocarriers per experimental treatment) and allowed to air dry (Figure 3) (modified from Lanahan et al. 1992).

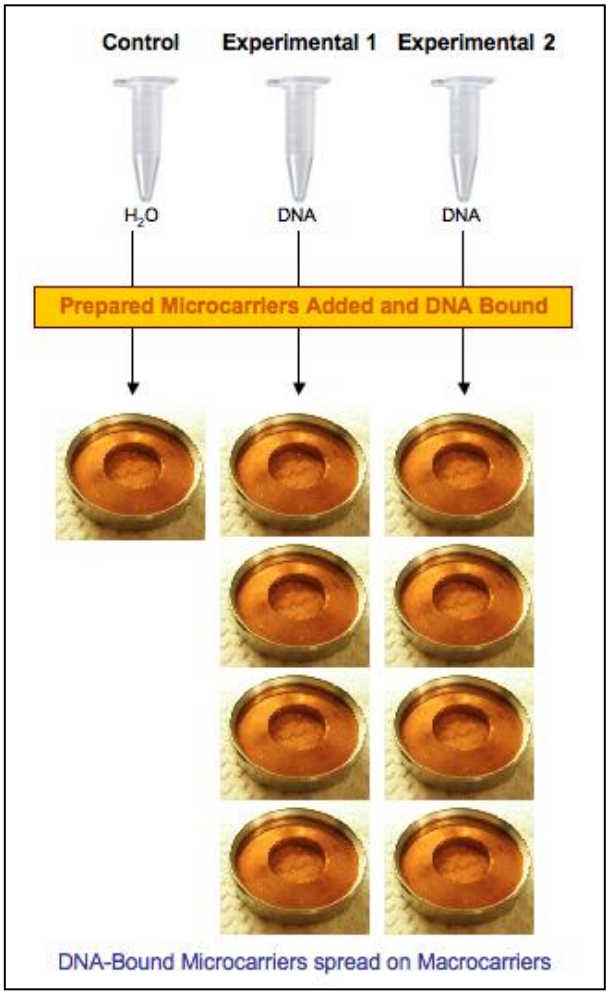

Figure 3. Diagram of macrocarrier preparation for control and experimental groups.

Particle Bombardment

A 1350 psi rupture disk, stopping screen, and macrocarrier, were loaded into a PSD-1000/He particle delivery system. Eight embryoless seeds were arranged in a small circle (~2.5 cm in diameter) at the center of a piece of filter paper on a Petri dish and then loaded into the particle delivery system. A vacuum was applied (28 in Hg) and then held

while the fire switch was applied until the rupture disk burst. The target seeds were then removed and placed in Petri dishes containing 4 mL of imbibing solution, 10 mg/mL chloramphenicol, and the appropriate concentration of gibberellin (GA_3) and/or absicic acid (Figure 4). Four groups of eight seeds were bombarded for each treatment. The seeds were then shaken in the Petri dishes at 24° for 24 hours (modified from Lanahan et al. 1992).

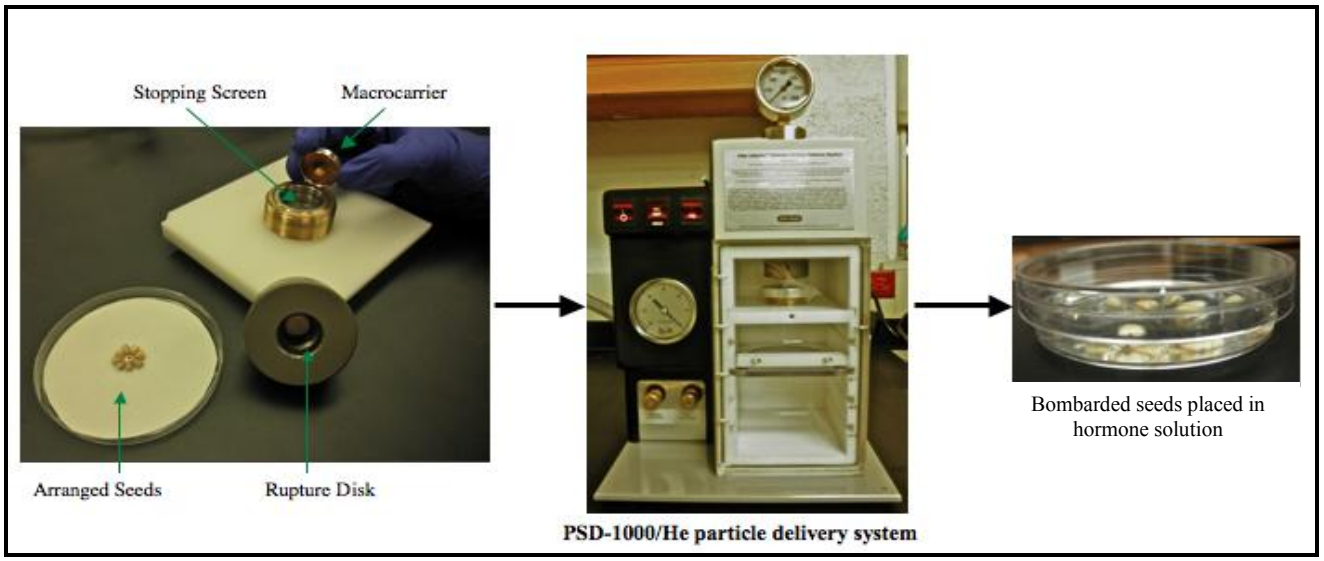

Figure 4. Diagram of one particle bombardment and subsequent incubation in hormone solution.

Enzyme Assays

Groups of four bombarded seeds (eight groups for each treatment) were ground in chilled mortars and pestles containing 800 µL grinding buffer (20% glycerol containing 100 mM $NaPO_4$ pH 7.2, 5 mM DTT, 20 µg/mL leupeptin) until completely liquefied and then poured into a microcentrifuge tube. The microcentrifuge tubes containing the seed extract were then centrifuged at maximum speed at 4° for ten minutes. Immediately after centrifugation, the clear supernatants were poured into a new set of microcentrifuge tubes

and stored on ice (Figure 5). Luciferase assay mixture (200 μL), composed of luciferase assay buffer (60 mM Tris sulfate pH 7.7, 20 mM $MgCl_2$, 20 mM DTT, 2 mM EDTA) with 1 mM luciferin and 1 mM ATP was aliquoted into 12 x 75 mm glass test tubes. Seed extract (100 μL) was added to the assay tube and vortexed quickly. The tube was immediately placed into a luminometer (Berthold Detection Systems, *Sirius*) and a measurement of light output was taken. This process was repeated for each sample of the bombardment.

Seed extract (50 μL of each sample) was then added to 200 μL GUS (β-glucuronidase) assay buffer (2.5 mM MUG (4-methylumbelliferyl β-D-glucuronide), 50 mM Na phosphate pH 7.2, 2 mM EDTA, 10 mM DTT, 10 μg/mL leupeptin, 0.2 M methanol, 0.02% sodium azide). These solutions were incubated at 37° in the dark for 20 hours. After 20 hours, the samples were centrifuged for 5 minutes and placed on ice. 250 μL of 0.2 M sodium carbonate was added to each well of a 96 well plate. 6.25 μL of each assay mixture was added into the corresponding well of the 96 well plate and the MU **(**methylumbelliferone**)** fluorescence was read under the following conditions: excitation=360 nm, emission=460 nm, sensitivity=42, by a FLx800 microplate fluorescence reader (BIO-TEK Instruments, Inc) (modified from Shen et al. 1996).

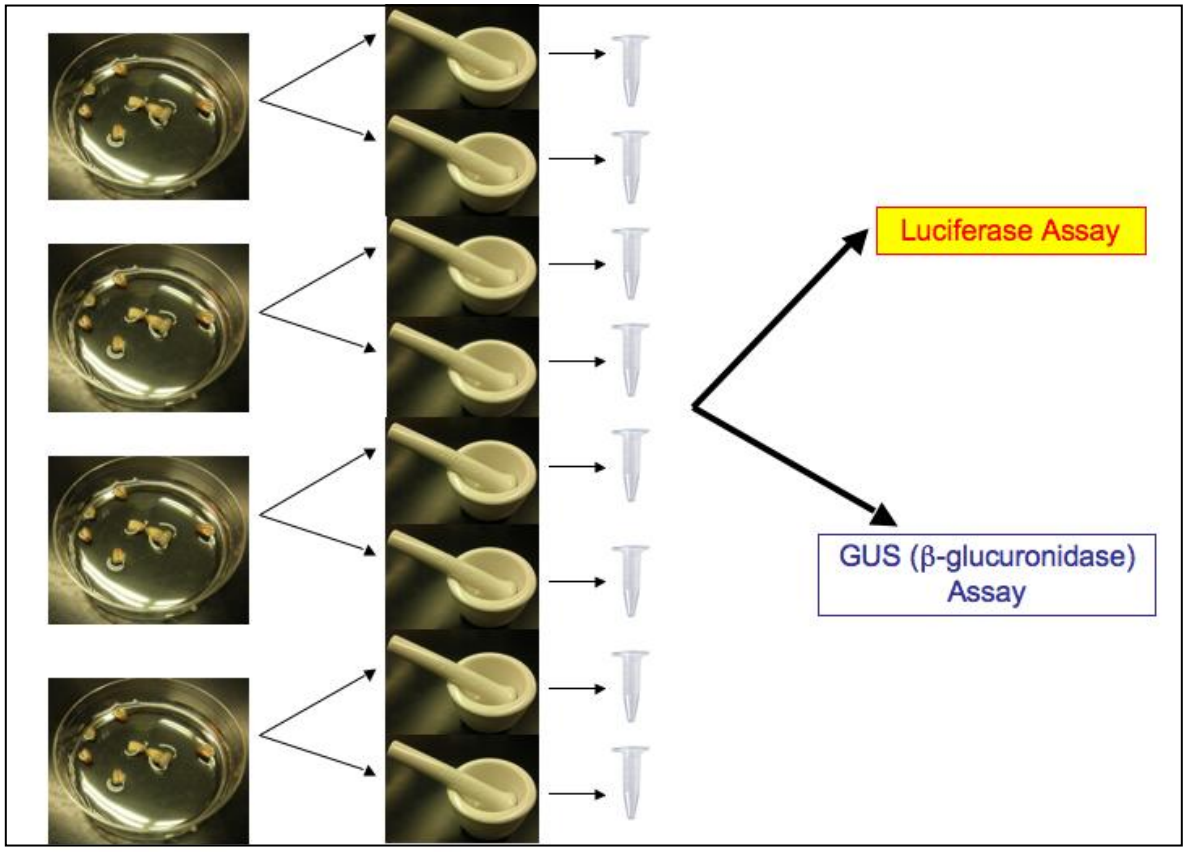

Figure 5. Schematic of the grinding of bombarded seeds and enzyme assays of seed extracts for one experimental treatment (eight samples per treatment).

Data Analysis

To normalize the data for GUS activity, the MU fluorescence value for the control (treatment prepared with only water rather than DNA) was subtracted from the MU fluorescence value for each sample. This was then divided by the luciferase activity for the sample, which also had the control luciferase activity value subtracted from it. This fraction was then multiplied by 2×10^6 to obtain the final normalized value. Only samples with luciferase activity above 15×10^3 RLU/s were used in order to ensure reliable results. The mean and standard error of each treatment group was then calculated (modified from Shen et al. 1996).

Results

Sensitivity of Amy32b to ABA and TaABF1

The GA-induced gene *Amy32b* has been shown to be suppressed by both ABA and TaABF1 (Johnson et al. 2008). The relative sensitivity to these two suppressors was investigated by introducing the *Amy32b:GUS* reporter construct into aleurone cells using particle bombardment and then exposing the bombarded cells to varying concentrations of ABA (Figure 6). Low concentrations of ABA (0.2 μM) resulted in almost full suppression of *Amy32b*, indicating a high degree of sensitivity of *Amy32b* to ABA. The relative expression of *Amy32b* had an indirect relationship to the concentration of ABA, in that higher concentrations of ABA yielded lower levels of *Amy32b:GUS* activity until *Amy32b* was completely suppressed. To compare the sensitivity of *Amy32b* to ABA and TaABF1, *Amy32b:GUS* reporter construct along with different amounts of *UBI: TaABF1:*effector construct were cobombarded into aleurone cells (Figure 6c). *Amy32b* also demonstrated a high degree of sensitivity to TaABF1, illustrated by low effector:reporter ratios of TaABF1:Amy32b (10%) resulting in almost full suppression of *Amy32b.* Furthermore, like ABA, TaABF1 had an indirect relationship with the relative amount of *Amy32b* expression.

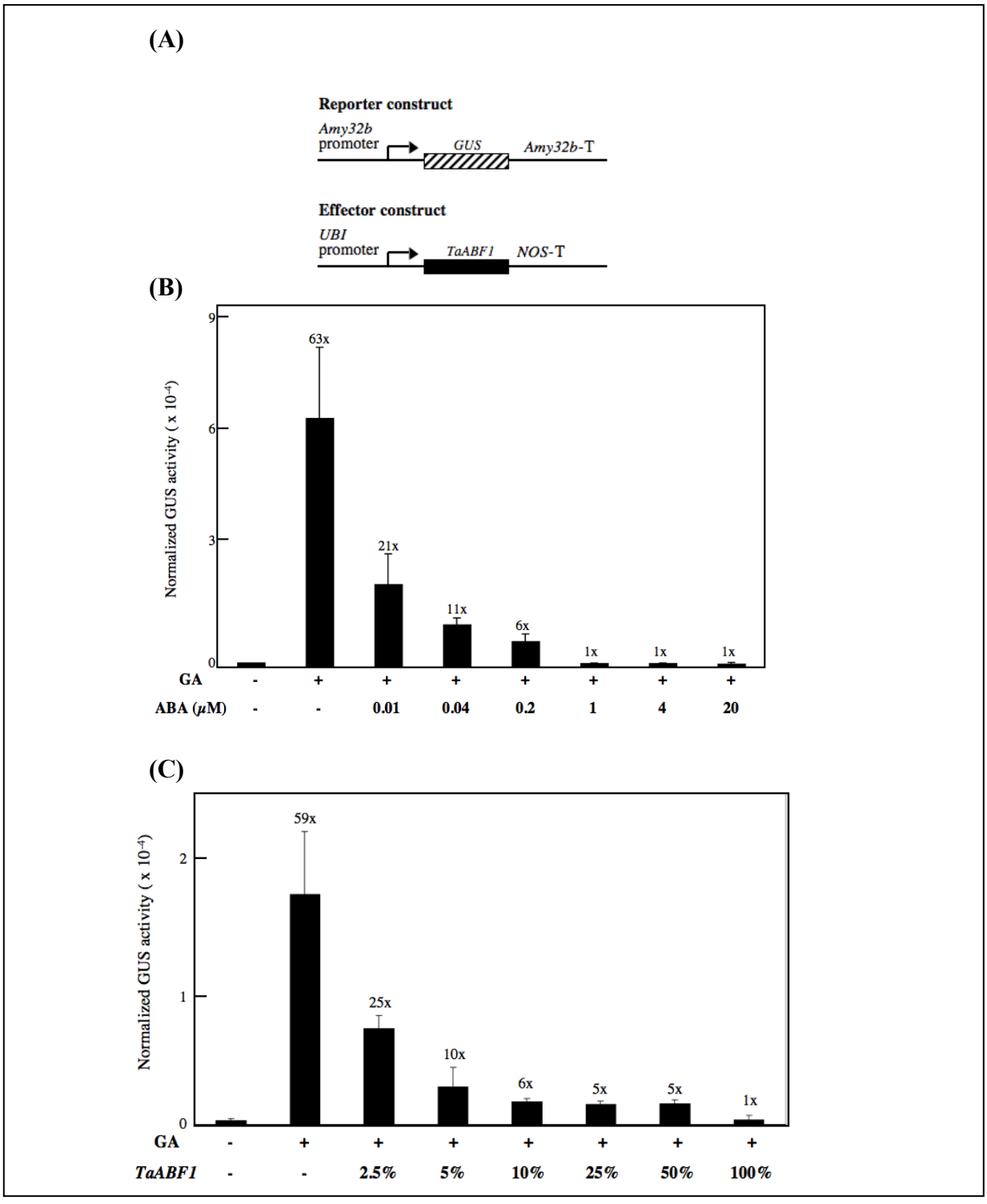

Figure 6. Dose Response Curves of ABA and TaABF1 suppression of the *Amy32b* promoter. **(A)** Diagram of the reporter and effector constructs used in the experiment. **(B)** The reporter construct *Amy32b:GUS* and the internal control construct *UBI:luciferase* were cobombarded into barley aluerone cells, and then seeds were incubated in different concentrations of ABA **(C)** The amount of reporter (*Amy32b:GUS*) and internal control plasmid (*UBI:luciferase*) DNA were held constant, while that of the *UBI:TaABF1* effector varied with respect to the reporter (0%, 2.5%, 5%, 10%, 25%, 50%, 100%). GUS activity was normalized relative to luciferase activity. Bars indicate GUS activities after 24 h of incubation with (+) or without (-) 1 μM GA_3. Data are means ± SE.

Additive Effect of ABA and TaABF1

Previous studies have found puzzling results regarding the activation of TaABF1 by ABA in bombarded aleurone cells. *TaABF1* mRNA was not induced by exogenous ABA (Johnson et al 2002), suggesting that *TaABF1* is not activated at a transcriptional level, and overexpression of *TaABF1* has been shown to fully suppress the GA-induction of *Amy32b* in the absence of ABA (Johnson et al 2008), suggesting that TaABF1 is not post-transcriptionally activated by ABA. To further elucidate the activation of TaABF1 by ABA in the ABA-mediated suppression of the *Amy32b* promoter we investigated the combined effect of ABA and TaABF1 on the expression of *Amy32b*. Based on the dose response curves (Figure 6), we determined the levels of ABA (0.0005 μM or 0.005 μM) and *TaABF1* (2.5% Effector:Reporter) that yielded a mid-level of *Amy32b* suppression. Aleurone cells were bombarded with the *Amy32b:GUS* reporter construct as well as amounts of *UBI:TaABF1* that yielded mid-levels of *Amy32b* suppression. The bombarded aleurone cells were then treated with 1 μM GA, 0.0005 μM or 0.005 μM ABA, or a combination of both. Simultaneous treatment with both ABA and TaABF1 resulted in a greater amount of *Amy32b* suppression than either treatment individually (Figure 7). This result suggests that ABA and TaABF1 work additively in the suppression of *Amy32b*.

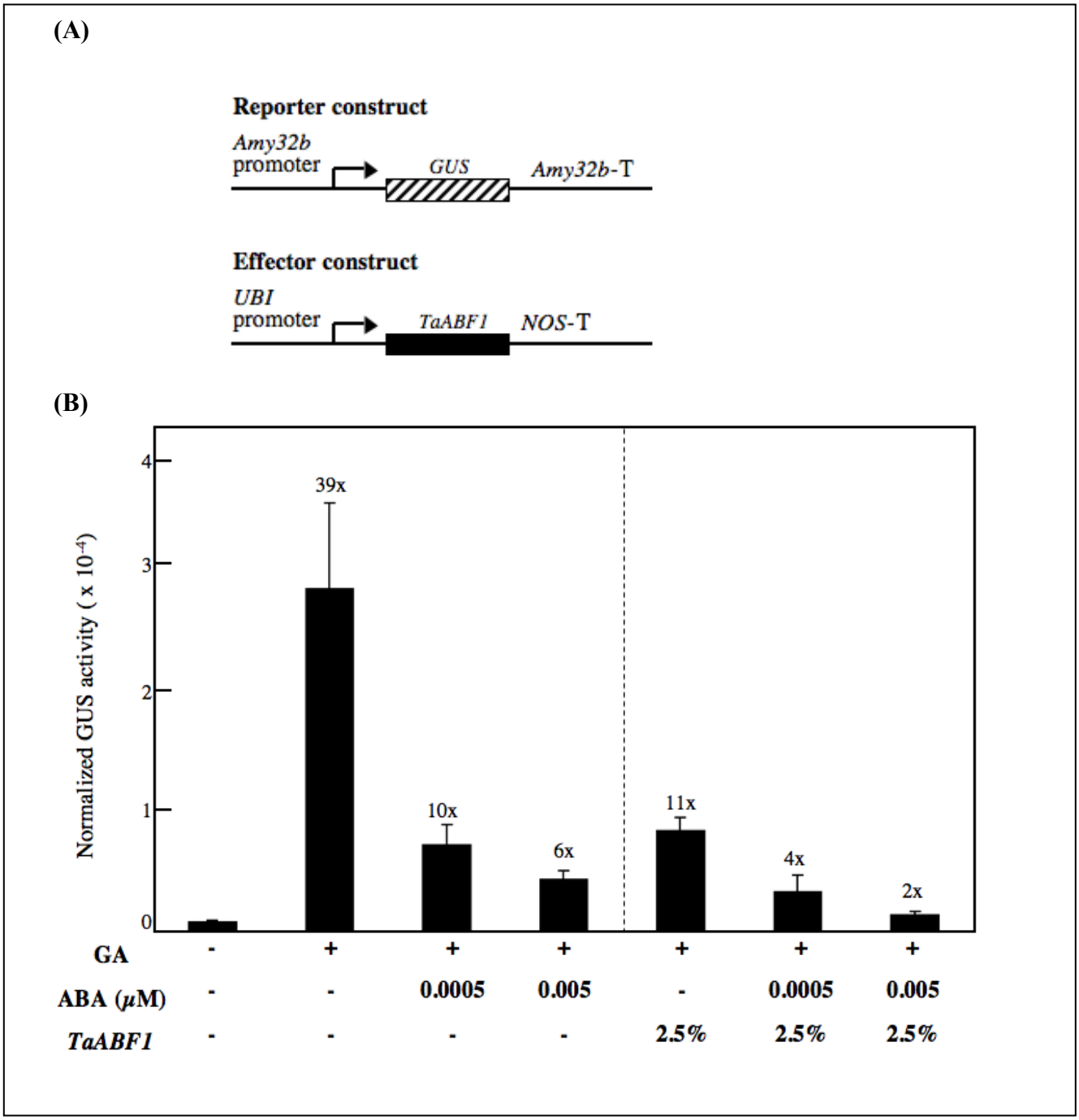

Figure 7. Additive Effect of ABA and *TaABF1*. (A) Diagram of the reporter and effector constructs used in the experiment. **(B)** The *Amy32b:GUS* reporter construct and *UBI:luciferase* internal control with (+) or without (-) 2.5% of the effector construct, *UBI:*TaABF1, relative to the reporter construct were cobombarded into barley aleurone cells. The seeds were incubated (24h) with (+) or without (-) GA_3 and with different concentrations of ABA (0.0005 μM or 0.005 μM) or without (-) ABA. GUS activity was normalized relative to luciferase activity. Bars indicate GUS activity means ± SE.

Location of TaABF1 action

Because TaABF1 has been hypothesized to be a transcriptional repressor of *GAMyb* (Johnson et al. 2008), we sought to determine how TaABF1 affects *GAMyb* transcription. Barley aleurone cells were cobombarded with *UBI:TaABF1* effector construct, *GAMyb:GUS* reporter construct, and *UBI:luciferase* internal control and then treated with 1 μM GA, or a combination of 1 μM GA and 20 μM ABA. Previous experiments (Gómez-Cadenas et al. 2001) demonstrated that responses of the *GAMyb* promoter are highly complex and dependent on the timing of analysis. These previous experiments demonstrated that the GAMyb promoter was most responsive to hormone treatments at 18 hours after bombardment. Because the expression from the *GAMyb* promoter is time sensitive, seeds were tested at 18 hours (Figure 8b) or 24 hours (Figure 8c) after bombardment. TaABF1 increased transcription of the *GAMyb* promoter in samples incubated for both time periods. Subtle differences in the magnitude of response to TaABF1 as well as the effect of ABA were observed at the different times. After 18 hours, the TaABF1-mediated induction of *GAMyb* was slightly greater than that observed after 24 hours. ABA-induced suppression of the TaABF1-mediated induction of *GAMyb* was not observed after 18 hours, but was observed after 24 hours. The effects of overexpression of *TaABF1* on *GAMyb* gene expression were the opposite of what had been predicted, indicating the role of TaABF1 in ABA-mediated suppression of *Amy32b* is more complicated than previously predicted.

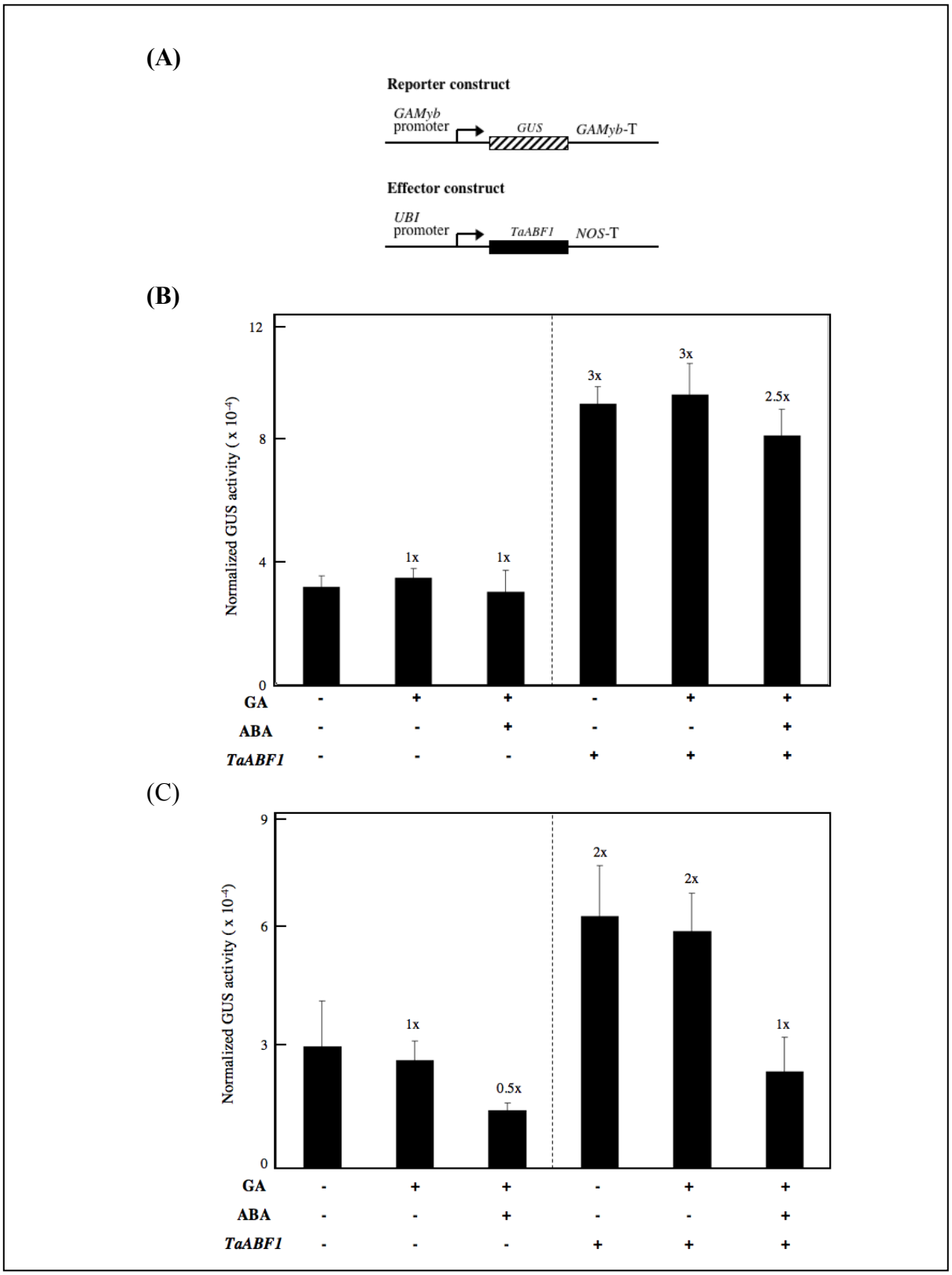

Figure 8. Effect of *TaABF1* on *GAMyb* gene expression. (A) Diagram of the reporter and effector constructs used in the experiment. **(B)** The reporter construct (*GAMyb:GUS)* and an internal control construct (*UBI:luciferase)* were co-bombarded into barley aleurone cells either with (+) or without (-) the effector construct (*UBI:TaABF1*). The bombarded aleurone cells were incubated with (+) or without (-) 1 μM GA_3 and 20 μM ABA for 18 hours or **(C)** 24 hours. GUS activity was normalized relative to luciferase activity. Data are means ± SE.

The forgoing results show that TaABF1 does not suppress *GAMyb* expression as might have been expected. However, they do clearly show that TaABF1 regulates the expression of GAMyb, consistent with the hypothesis that TaABF1 acts upstream of GAMyb in ABA signaling. To clarify the location of TaABF1 action in the signaling pathway, the *Amy32b:GUS* reporter construct along with the *UBI:TaABF1* effector construct, *UB1:GAMyb* effector construct, or a combination of both effectors were co-bombarded into barley aleurone. Either GA or GAMyb were able to induce *Amy32b*. While TaABF1 could suppress GA-induction of *Amy32b*, TaABF1 could not suppress GAMyb-induction of Amy32b. (Figure 9). This result indicates that TaABF1 acts prior to GAMyb along the GA-signaling pathway because GAMyb still induced *Amy32b* despite the presence of TaABF1.

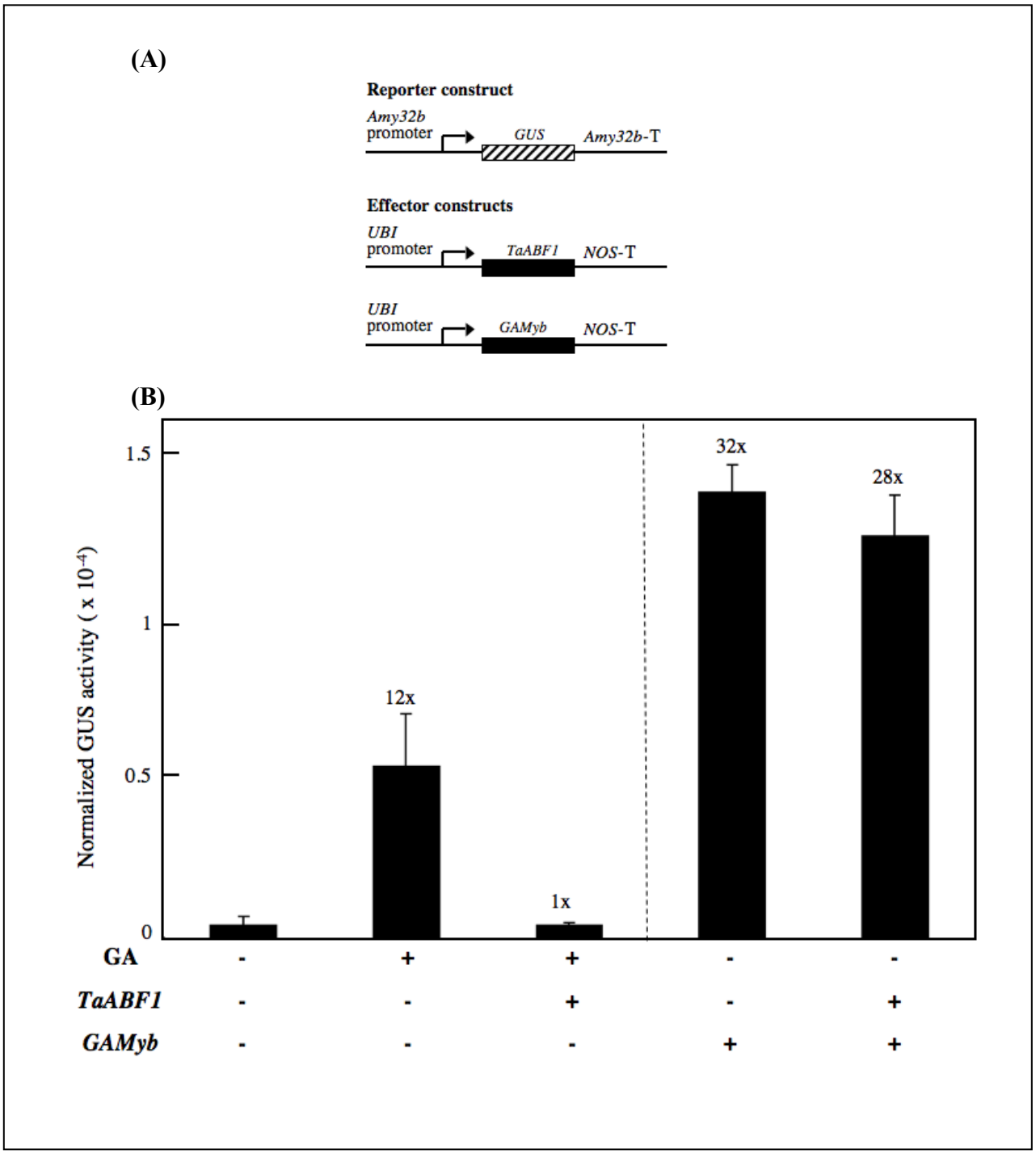

Figure 9. TaABF1 does not counteract GAMyb induction of *Amy32b*. (a) Diagram of the reporter and effector constructs used in the experiment. **(b)** The reporter construct (*Amy32b:GUS)* with (+) or without (-) the effector constructs *UBI:TaABF1* and *UBI:GAMyb*, and an internal control (*UBI:luciferase)* were co-bombarded into barley aleurone cells. The bombarded aleurone cells were incubated with (+) or without (-) 1 μM GA_3 for 24 hours. GUS activity was normalized relative to luciferase activity. Data are means ± SE.

Discussion

The expression of hydrolase genes during and after seed germination is regulated by the hormones GA and ABA. GA induces the expression of these hydrolases while ABA suppresses this induction. Some key components involved in the mechanism of ABA-mediated repression of hydrolase genes, including PKABA1 (Gómez-Cadenas et al. 1999; Holappa and Walker-Simmons 1995), TaABF1 (Johnson et al. 2002), HvDOF19 (Moreno-Risueño et al. 2007), and WRKY proteins (Xie et al. 2005; Zou et al. 2008), have been identified, however, the way they work together has yet to be well established. The work reported in this thesis provides evidence that the role of TaABF1 in ABA-mediated suppression of the *Amy32b* gene is more complicated than previously postulated (Johnson et al. 2008). Based on these results, we have proposed a model for the role of TaABF1 in ABA-mediated suppression of *Amy32b* (Figure 10). In this model, TaABF1, after being phosphorylated by PKABA1, acts on an unknown target (X), located upstream of *GAMyb*. Modification of target X results in increased *GAMyb* expression, which is then inhibited by a second target of PKABA1 phosphorylation, (Y).

Because PKABA1 has been shown to physically interact with TaABF1 (Johnson et al. 2002), and PKABA1 represses the GA-induction of *GAMyb* at a transcriptional level (Gómez-Cadenas et al. 2001), it was hypothesized that TaABF1 also downregulates *GAMyb* expression. On the contrary, overexpression of TaABF1 actually resulted in an increase in *GAMyb* transcription. This surprising result could be explained by TaABF1 modifying GAMyb at a protein level rather than a transcriptional level. Because the *GAMyb* promoter (Gubler et al. 1999) does not contain a binding site (ABRE) for ABF's, it is logical that TaABF1 does not directly bind *GAMyb* and regulate transcription. If

TaABF1 rendered GAMyb unable to bind to the target promoter, a feedback loop could induce *GAMyb* transcription. Another transcription factor, HvDOF19, has been shown to have such an inhibiting effect on GAMyb. HvDOF19 binds to GAMyb and interferes with the DNA binding of GAMyb to its target promoter (Moreno-Risueño et al. 2007). The effect of HvDOF19 on the transcription of *GAMyb*, however, has not been studied.

Unlike *HvDOF19*, which when cobombarded with *GAMyb* resulted in an inhibition of GAMyb induction of *Amy32b* (Moreno-Risueño et al. 2007), bombardment with *TaABF1* did not prevent the GAMyb induction of *Amy32b.* This result indicates that TaABF1 does not posttranslationally modify GAMyb and suggests that instead TaABF1 acts on a target upstream of GAMyb in the GA signaling pathway. The finding that TaABF1 alters *GAMyb* transcription, even if in the opposite way than expected, also supports the conclusion that TaABF1 acts prior to GAMyb.

The puzzling compilation of results that (1) TaABF1 effectively suppresses *Amy32b* in the absence of ABA, (2) TaABF1 causes the upregulation of *GAMyb* transcription, and (3) TaABF1 does not inhibit the GAMyb induction of *Amy32b* demonstrate that the role of TaABF1 in ABA signaling is more complex than previously hypothesized and likely involves multiple branching pathways. It may be possible that TaABF1 inhibits a target upstream of GAMyb and the inhibition of this target results in upregulation of the transcription of *GAMyb*. The upregulation of *GAMyb* may then be counteracted by other factors induced by ABA. HvDOF19 (Moreno-Risueño et al. 2007), shown to inactivate GAMyb binding of target DNA, would render GAMyb inactive at a protein level. Additionally, PKABA1, shown to downregulate *GAMyb* transcription

(Gómez-Cadenas et al. 2001), may phosphorylate a transcription factor other than TaABF1 that directly binds the *GAMyb* promoter and represses transcription.

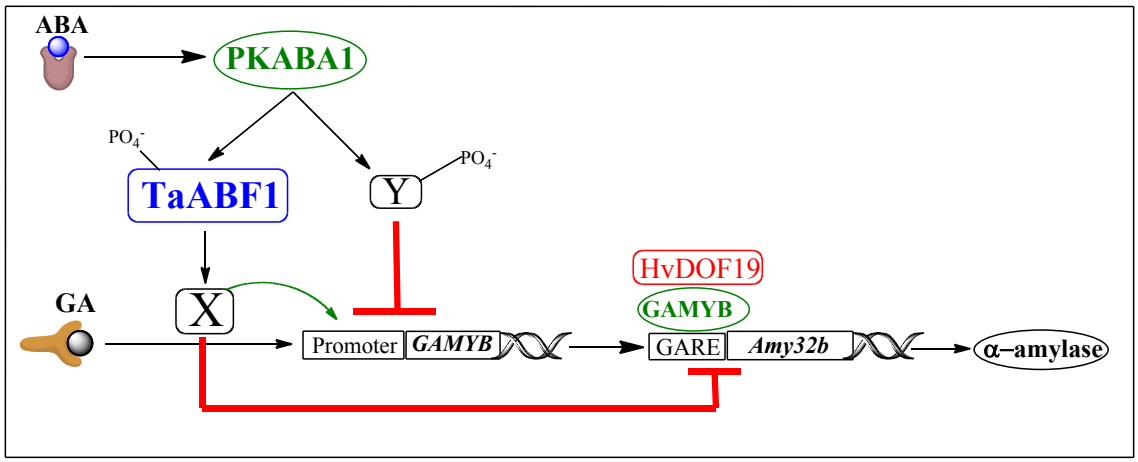

Figure 10. Proposed model for the role of TaABF1 in ABA signaling

The observed repression of TaABF1 induction of *GAMyb* when aleurone cells were treated with exogenous ABA (Figure 8C.) supports this hypothesis. Furthermore, ABA has been shown to only partially counteract GA induced expression of *GAMyb* (Gubler et al. 2002), implying that other pathways besides *GAMyb* suppression are involved in regulating *Amy32b*. Similarly, RNAi experiments with PKABA1, TaABF1 (Johnson et al. 2008), or HvDOF19 (Moreno-Risueño et al. 2007) resulted in unaffected ABA suppression of *Amy32b* demonstrating none of these factors work independently, and redundant pathways must also exist. Nevertheless, these results still raise unanswered questions: if TaABF1 does not inhibit *GAMyb* at a transcriptional or protein level, how does TaABF1 inhibit *Amy32b,* and how is this pathway still functional in the absence of ABA? Further work is needed to answer these questions.

The mechanism by which TaABF1 is activated in imbibing grains remains unknown. Genes encoding other members of the ABF family have been found to have a variety of responses to exogenous ABA. The Arabidposis genes *AtABF1, AtABF2,*

AtABF3, AtABF4 (Choi et al. 2000; Uno et al. 2000) and *AtDPBF1* (Kim et al. 2002) are all induced by ABA in whole plants. In *Arabidopsis* seeds, *AtABI5* and *AtABF1* transcripts are strongly induced, while *AtABF3* is weakly induced by ABA (Finkelstein et al. 2005; Lopez-Molina et al. 2001). Both protein and mRNA levels of the barley gene *HvAB15* increased modestly in response to ABA (Hobo et al. 1999). TaABF1 mRNA levels, however, do not change with the addition of exogenous ABA or GA (Johnson et al. 2008). Because TaABF1 does not appear to be regulated transcriptionally, it must be activated in some other manner.

Although the ability of TaABF1 to completely suppress *Amy32b* in the absence of ABA in bombarded aleurone cells suggests that it does not require ABA-induced posttranslational activation, TaABF1 may be activated in this manner in normal imbibing grains. It was previously postulated (Johnson et al. 2008) that during ABA signaling, the primary effect of TaABF1 phosphorylation is stabilization, which would result in more available TaABF1 to suppress *Amy32b*. Conversely, when TaABF1 is overexpressed in bombarded aleurone cells, the amount of TaABF1 protein may be sufficiently high that stabilization by phosphorylation is no longer needed. This hypothesis was based on previous studies that found ABA induces both phosphorylation and stabilization of AtABI5 (Lopez-Molina et al. 2001; Lopez-Molina et al. 2003). These studies, however, did not demonstrate that the phosphorylation itself is required for the increased stability. Alternatively, in the results reported here we found that very low concentrations of TaABF1 and ABA resulted in nearly full suppression of the *Amy32b* promoter. Because *Amy32b* is so sensitive to TaABF1 it may also be possible that low levels of endogenous

ABA are capable of modifying and activating enough TaABF1 to elicit a full response in the bombarded cell.

The hypothesis that TaABF1 is posttranslationally modified, possibly via phosphorylation, is supported by our finding that ABA and TaABF1 had an additive effect on *Amy32b* suppression. When exogenous ABA was added to aleurone cells overexpressing *TaABF1*, this may have resulted in modification of the TaABF1 protein, consequently resulting in greater suppression than either exogenous ABA or overexpression of *TaABF1* alone. It is feasible that during the ABA response, PKABA1 phosphorylates and thus stabilizes or activates TaABF1 because it is already know that ABA induces *PKABA1* (Johnson et al. 2008) and PKABA1 can bind to and phosphorylate TaABF1 (Johnson et al. 2002).

While the observed additive effect could be explained by posttranslational modification of TaABF1, this is not the sole possibility. The apparent multiple branches of ABA signaling could also explain the additive effect of TaABF1 and ABA. As observed by the effects of TaABF1 on *GAMyb*, it is likely that multiple factors and pathways are involved in the suppression of *Amy32b*. These other factors may include a PKABA1-activated suppressor of *GAMyb* as well as a TaABF1-mediated factor upstream of GAMyb. Furthermore, rice WRKY proteins OsWRKY51, OsWRKY71 (Xie et al. 2005), and barley ortholog HvWRKY38 (Zou et al. 2008) have been identified as ABA-inducible repressors of *Amy32b* (Xie et al. 2006). When these separate branches of ABA signaling are active and the TaABF1 pathway is overexpressed, the level of *Amy32b* expression would be lower than that caused by either the overexpressed TaABF1 pathway or ABA signaling pathway alone.

The role of TaABF1 in ABA signaling is further complicated by differences in the way TaABF1 acts in ABA-suppressed genes and ABA-induced genes. While the ABA suppressed gene (*Amy32b*) promoter exhibited a high degree of sensitivity to ABA and TaABF1, the ABA induced gene (*HVA1*) promoter did not demonstrate the same level of sensitivity (Harris et al. in preparation). Furthermore, while ABA and TaABF1 clearly demonstrated an additive effect on the *Amy32b* promoter, ABA and TaABF1 did not have an additive effect on the *HVA1* promoter (Keyser 2010). These results suggest that the mechanism of TaABF1 action in ABA-induced and ABA-suppressed genes is different. Moreover, because TaABF1 and ABA did not appear to have an additive effect on the *HVA1* promoter, the mechanism by which TaABF1 is itself activated may differ between these pathways. Further investigation of the effects of ABA, GA, and a combination of ABA and GA on the phosphorylation, stability, and protein abundance of TaABF1 may provide insight into the difference of TaABF1 action and activation in ABA suppressed and ABA induced pathways.

Additional studies are also needed to determine downstream targets of TaABF1 in the ABA-mediated suppression of *Amy32b*. ABFs have been shown to physically associate with other classes of transcription factors (Nakamura et al. 2001) and with 14-3-3 proteins (Shoonheim et al. 2007), a family of regulators whose function nor action mechanism in plant hormonal signaling has been fully established. It is possible that TaABF1 binds to other transcription factors, a regulatory molecule like a 14-3-3 protein, or directly suppresses a gene that is located upstream of *GAMyb*.

Further research may also focus on regulation of the *GAMyb* promoter. The effects of other factors involved in ABA signaling, like HvDOF19, on *GAMyb*

transcription may provide insight into the possible existence of a feedback loop. Similarly, identification and characterization of the transcription factor activated by PKABA1 to bind and suppress *GAMyb* may further explain the observed increase of *GAMyb* transcription in response to overexpression of *TaABF1*.

The activation and action of TaABF1 in the crosstalk between ABA and GA is more complex than previously proposed. In this study we demonstrate that TaABF1, unlike PKABA1, does not regulate *Amy32b* by suppressing *GAMyb* expression. This result indicates that more unidentified factors are involved in ABA signaling including a transcription factor that is activated by PKABA1 and directly suppresses *GAMyb* as well as a target of TaABF1 action. Furthermore, we provide evidence that TaABF1 may be activated by ABA via posttranslational modification, however further work must be performed before this can be concluded with confidence.

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