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Functional analysis of TaABF1 during abscisic acid and gibberellin signalling in aleurone cells of cereal grains

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Abstract

The wheat transcription factor TaABF1 physically interacts with the protein kinase PKABA1 and mediates both abscisic acid (ABA)-induced and ABA-suppressed gene expression. In bombarded aleurone cells of imbibing grains, the effect of TaABF1 in down-regulating the gibberellin (GA)-induced *Amy32b* promoter was stronger in the presence of exogenous ABA. As these grains contained low levels of endogenous ABA, the effect of TaABF1 may also be mediated by ABA-induced activation even in the absence of exogenous ABA. Levels of TaABF1 protein decreased slightly during imbibition of afterripened grains. However, TaABF1 levels (especially in aleurone layers) were not substantially affected by exogenous ABA or GA, indicating that changes in TaABF1 protein level are not an important part of regulating its role in hormone signalling. We found that TaABF1 was phosphorylated *in vivo* in aleurone cells, suggesting a role for post-translational modification in regulating TaABF1 activity. Induction of *Amy32b* by overexpression of the transcription factor GAMyb could not be prevented by TaABF1, indicating that TaABF1 acts upstream of *GAMyb* transcription in the signalling pathway. Supporting this view, knockdown of TaABF1 by RNA interference resulted in increased expression from the *GAMyb* promoter. These results are consistent with a model in which TaABF1 is constitutively present in aleurone cells, while its ability to down-regulate *GAMyb* is regulated in response to ABA.

Keywords: abscisic acid, germination, gibberellin, imbibition, wheat

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Introduction

In imbibing cereal grains, gibberellin (GA) stimulates important developmental events such as germination and storage reserve breakdown, and these events are inhibited by abscisic acid (ABA). The underlying mechanism regulating these developmental events includes a number of GA-induced genes (including *Amy32b*) whose expression is also repressed by ABA (Lovegrove and Hooley, 2000; Johnson, 2003). ABA is perceived by receptors (Ma *et al.*, 2009; Park *et al.*, 2009) that, upon ABA binding, inhibit the activity of protein phosphatase 2C. In some cases this can lead directly to changes in gene expression by allowing the phosphorylation/activation of SnRK2 (SNF1-related kinase 2) protein kinases, which in turn phosphorylate and activate transcription factors from the ABF (ABRE binding factor) family (Fujii and Zhu, 2009). In other cases, the pathway to ABA-regulated gene expression appears to be more complex, as signalling molecules such as WRKY proteins (Xie *et al.*, 2006; Zhang *et al.*, 2009), transcription factor binding proteins (Garcia *et al.*, 2008; Sirichandra *et al.*, 2010) as well as ubiquitin (Stone *et al.*, 2006; Zhang *et al.*, 2007) and SUMO E3 ligases (Miura *et al.*, 2009) are involved. In addition, hydrogen peroxide, nitric oxide (Takemiya and Shimazaki, 2010) and phosphatidic acid (Ritchie and Gilroy, 1998; Zou *et al.*, 2004) serve as intermediates in some responses to ABA.

The signal transduction pathway leading to ABA-suppressed gene expression in cereal grains involves the ABA-induced SnRK2 kinase PKABA1 (Gómez-Cadenas *et al.*, 2001) and the transcription factor TaABF1 (*Triticum aestivum* ABF1). While the expression of TaABF1 itself is grain-specific (Johnson *et al.*, 2002), other related wheat ABFs (called TaABI5s) are expressed in roots and leaves (Ohnishi *et al.*, 2008). Proteins similar to TaABF1 (Johnson *et al.*, 2008) are also present in barley (HvABF1, for *Hordeum vulgare* ABF1) (Schoonheim *et al.*, 2007), rice (OsABI5/OREB1) (Zou *et al.*, 2008), and Arabidopsis

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(ABI5) (Lopez-Molina *et al.*, 2001). Overexpression of either PKABA1 or TaABF1 can substitute for exogenous ABA in inhibiting the expression of GA-induced genes in imbibing cereal grains, while TaABF1 (but not PKABA1) can substitute for ABA in stimulating the expression of ABA-induced genes (Gómez-Cadenas *et al.*, 1999; Johnson *et al.*, 2008). Among the extensive ABF family (group A bZip proteins) (Jakoby *et al.*, 2002), only TaABF1, HvABF1 and HvABF2 have been found to have a role in GA-regulated signalling. These proteins are therefore likely to play a critical role in the intersection of ABA and GA response pathways. As wheat cultivars with greater seed dormancy contain higher *TaABF1* transcript levels (Rikiishi *et al.*, 2010) and expression of both PKABA1 and HvABI5 is higher in barley grains under conditions that prevent germination (Mendiondo *et al.*, 2010), TaABF1 and related ABF proteins may play an important role in regulating cereal grain dormancy.

As the level of *PKABA1* mRNA increases in response to ABA (Gómez-Cadenas *et al.*, 1999), signalling through PKABA1 is likely to be mediated (in part) by ABA-induced increases in *PKABA1* transcription. In contrast, *TaABF1* mRNA levels are not regulated by ABA (Johnson *et al.*, 2008), suggesting that regulation of TaABF1 at the protein level must be an important component of the signalling pathway. This is indeed the case for some other members of the ABF family, as TRAB1 (Kagaya *et al.*, 2002), AtABI5 (Piskurewicz *et al.*, 2008), and OREB1/OsABI5 (Hong *et al.*, 2011) are phosphorylated in response to ABA. The fact that PKABA1 can phosphorylate peptide sequences from TaABF1 *in vitro* (Johnson *et al.*, 2002) suggests that TaABF1 may also be regulated in this manner, but the specific phosphorylation sites that are important *in vivo* and their roles in different aspects of ABA and GA signalling are still unknown.

Although TaABF1 suppresses GA-induced expression of *Amy32b* in aleurone cells, the mechanism for this suppression is also unknown. *Amy32b* is directly up-regulated by the transcription factor GAMyb (Gubler *et al.*, 1999; Hong *et al.*, 2012), which binds to a consensus GA-response element present in the promoter of *Amy32b* and other GA-inducible genes. It is therefore conceivable that TaABF1 could act by down-regulating GAMyb, either directly or indirectly, to inhibit *Amy32b* expression.

To further define the role of TaABF1 in hormone signalling, we investigated the role of ABA and GA in the upstream regulation of TaABF1 and the relationship of TaABF1 with downstream signalling molecules. We report that ABA works in concert with TaABF1 to down-regulate *Amy32b*, that TaABF1 is phosphorylated *in vivo*, and that TaABF1 acts upstream of *GAMyb* transcription.

Materials and methods

Bombardment

The *UBI::Luciferase* internal control plasmid pAHC18 (Christensen and Quail, 1996), reporter constructs *Amy32b::GUS* (Lanahan *et al.*, 1992), *HVA1::GUS* (Shen *et al.*, 1993), *GAMyb::GUS* (Gómez-Cadenas *et al.*, 2001), and effector constructs *UBI::TaABF1*, *UBI::PKABA1*, *UBI::TaABF1Ri* (Johnson *et al.*, 2008), and *UBI::GAMyb* (Gómez-Cadenas *et al.*, 2001) have been described previously.

De-embryonated grains of Himalaya barley were imbibed for 2 d, and the pericarp and testa were removed. After one more day of imbibition, the DNA mixture (in 1:1 ratio) of *Amy32b::GUS* (or *HVA1::GUS* or *GAMyb::GUS*) and *UBI::Luciferase*, with or without an effector construct, was bombarded into embryoless barley grains (at least four replicates per test construct), using a Bio-Rad PDS-1000/He system (Bio-Rad, Hercules, California, USA). After incubation for 24 h with various treatments, GUS assays and luciferase assays were performed as described previously (Shen *et al.*, 1996). Detailed methods for seed preparation and particle bombardment are included in the Supplementary Materials (available online).

ABA assays

ABA was extracted from embryoless grains based on a previously published method (Gubler *et al.*, 2008) and the ABA concentration of extracts was measured using a Phytodetek competitive enzyme-linked immunosorbent assay (ELISA) kit (Agdia, Elkhart, Indiana, USA). Five embryoless grains were ground in a mortar and pestle in 80% methanol, and the homogenate was mixed overnight and centrifuged to pellet plant debris. The pellet was extracted twice with 80% methanol and the supernatants were combined and concentrated in a vacuum concentrator. The aqueous extract (approximately 100 μl) was diluted to 1 ml by addition of Tris-buffered saline (25 mM Tris, 100 mM NaCl, 1 mM $MgCl_2$, 3 mM sodium azide, pH 7.5) and the ABA content was measured using a competitive ELISA as described by the Phytodetek protocol.

Protein analysis

For each treatment, eight whole wheat grains (cultivar NuWest) were placed on to filter paper saturated with 5 mM 2-(*N*-morpholino) ethanesulphonic acid (MES) pH 5.7 at 24°C for 0–48 h. Each sample was then ground with a mortar and pestle in 2 ml of seed-grinding buffer (SGB; 65 mM potassium phosphate, 0.4% 2-mercaptoethanol, 0.1% polyvinylpolypyrrolidone, 1% Sigma proteinase inhibitor cocktail, pH 6.7).

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After centrifugation, the supernatant was retained for protein analysis. De-embryonated grains (eight per treatment) were sterilized with 10% bleach before placement on filter paper saturated with imbibing solution (IS; 20 mM sodium succinate, 20 mM calcium chloride, pH 5.0) for 48 h. They were then transferred to IS containing 1 μM GA or 20 μM ABA and allowed to incubate for 0–48 h. Each sample was ground in 1.5 ml SGB and centrifuged to obtain protein extracts. Isolated aleurone layers were obtained by incubating sterilized embryoless grains on filter paper saturated with IS for 4 d at 24°C. Aleurone layers (still attached to the seed coat) were then dissected away from the starchy endosperm. For each treatment, aleurone layers from 12 grains were placed into a 60-mm Petri dish containing 4 ml IS for 48 h. Each sample was then ground in 0.8 ml of SGB and centrifuged to obtain protein extracts.

Proteins were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (NuPage 10% Bis-Tris; Life Technologies, Carlsbad, California, USA) and transferred to polyvinylidene fluoride (PVDF) membranes for Western blotting. TaABF1 was detected using an antibody made to a synthetic peptide containing amino acids 121–140 (Open Biosystems/Fisher Scientific custom antibody service, Pittsburgh, Pennsylvania, USA). In Western blots carried out for validation, the antibody detected a 42 kDa band (the correct size for TaABF1) in plant tissues consistent with the known distribution of *TaABF1* mRNA (Johnson *et al.*, 2002, 2008) and a band could be detected using 50 pg of purified GST::TaABF1 recombinant protein. Detection of α-amylase was carried out using a commercial antibody (AgriSera, Vännäs, Sweden). Bands were visualized using the ECL Plus detection system (Life Technologies). Blot images were generated and quantified using a BioRad ChemiDoc XRS + Imaging system and the accompanying QuantityOne software.

Aleurone protein extracts used for isoelectric focusing (IEF) and gel filtration analysis were made as described above except that each sample was ground in 0.8 ml of seed-grinding phosphate-buffered saline (137 mM NaCl, 27 mM KCl, 43 mM sodium phosphate, 14 mM potassium phosphate, 0.5% Triton X-100, 0.1% polyvinylpolypyrrolidone, 1% Sigma proteinase inhibitor cocktail, pH 7.3). For phosphatase treatments, 60 μl of the protein extract (3 μg protein per μl) was combined with 8 μl of 10 × phosphatase buffer [500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM dithiothreitol (DTT), pH 7.9], 4 μl of water and 8 μl of calf intestinal phosphatase (CIP; 10 units μl^{-1}, New England Biolabs, Ipswich, Massachusetts, USA) and incubated at 37°C. For mock treatments, proteins were incubated as above but without the inclusion of CIP. The phosphatase-treated protein samples were then transferred into 1 × IEF pH 3–10 sample buffer (Life Technologies) using Amicon spin filters, separated on Life Technologies pH 3–10 IEF gels, and transferred to PVDF membranes for Western blotting.

Results

Regulation of Amy32b and HVA1 promoters by ABA and TaABF1

Previous work has shown that the *Amy32b* (amylase) promoter is activated by GA and this induction can be prevented by either ABA or by overexpression of TaABF1. Expression from the *HVA1* (*Hordeum vulgare* aleurone 1) promoter can be induced either by ABA or by overexpression of TaABF1 (Johnson *et al.*, 2008). Here, we have analysed in more detail the response of these promoters to different concentrations of ABA and different amounts of TaABF1.

The expression of an *Amy32b::GUS* reporter construct (Fig. 1A) introduced into aleurone cells by particle bombardment was strongly induced (>50-fold) by 1 μM GA, and this induction was reduced or eliminated by the presence of exogenous ABA (Fig. 1B). A concentration of only 0.01 μM ABA provided a more than 50% reduction in GA inducibility, while complete inhibition of the induction of *Amy32b* by GA was observed with concentrations of 1 μM ABA or higher. In contrast, much higher concentrations of ABA were required for induction of the *HVA1* promoter (Fig. 1C). A concentration of 1 μM was required to reach 50% of maximal induction, and induction continued to increase even above 4 μM ABA.

We also conducted dose/response experiments to determine the amount of *UBI::TaABF1* effector required to regulate the *Amy32b* and *HVA1* promoters. As previously determined (Johnson *et al.*, 2008), effector:reporter ratios as low as 0.05 were sufficient to strongly reduce (by (> 50%) the GA induction of *Amy32b*, and higher ratios of effector:reporter had an even greater effect (Fig. 1D). In contrast, effector: reporter ratios of at least 0.2 were required to strongly regulate *HVA1* expression (Fig. 1E). These results clearly show that *Amy32b* expression is much more sensitive to low levels of ABA and TaABF1 than is *HVA1* expression, and suggest that TaABF1 regulates these two genes via a different mechanism.

The role of ABA in TaABF1-mediated signalling

The fact that TaABF1 can regulate the *Amy32b* and *HVA1* promoters in the absence of exogenous ABA (Fig. 1D and E; and Johnson *et al.*, 2008) suggests that overexpressed TaABF1 protein might not require ABA-induced modification to be effective. However, the possibility exists that the bombarded aleurone cells contain endogenous ABA, and this ABA might be able

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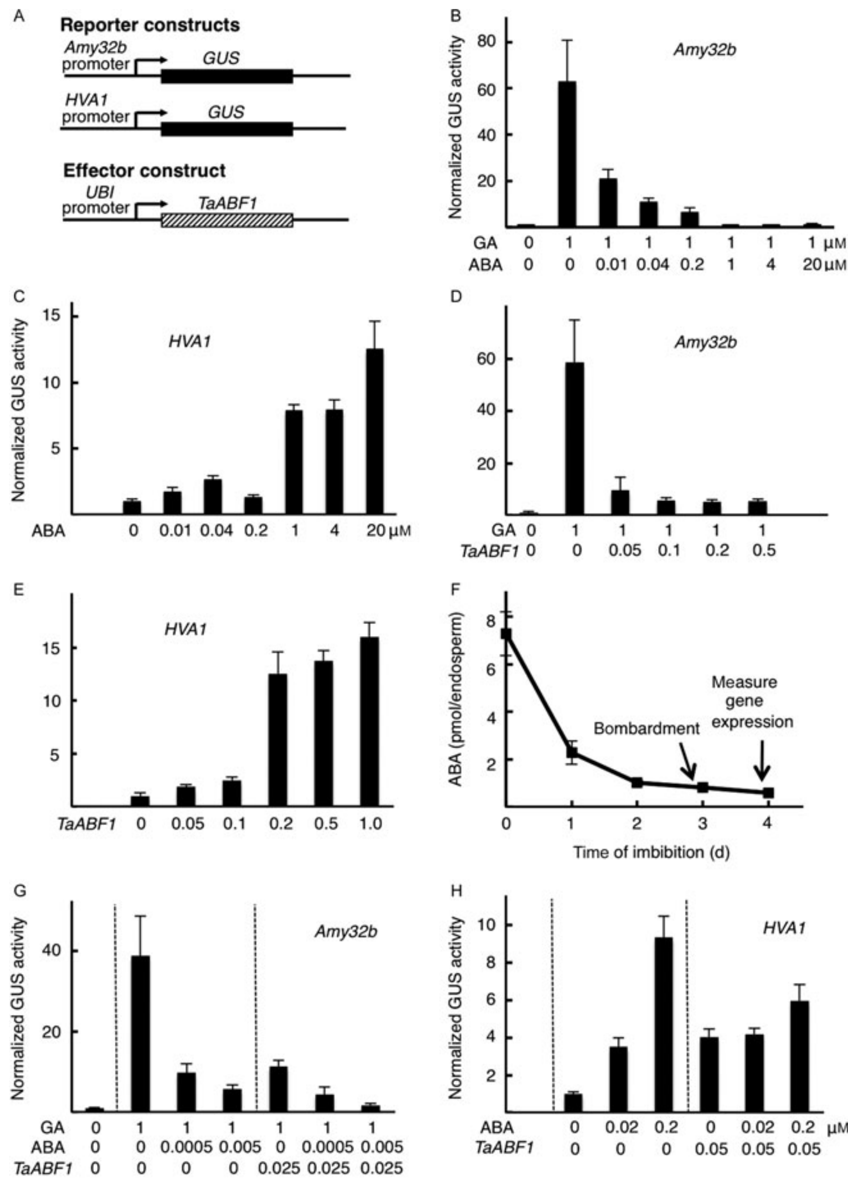

Figure 1. Regulation of *Amy32b* and *HVA1* promoters by ABA and TaABF1. (A) Reporter and effector constructs used in the experiments. (B) The *Amy32b::GUS* reporter and the internal control construct, *UBI::luciferase*, were co-bombarded into barley aleurone cells. Bars indicate GUS activities after 24 h of incubation with or without 1 μM GA and with concentrations of ABA ranging from 0 to 20 μM. GUS activity was normalized in every independent transformation relative to the luciferase activity. Data are means ± SE of at least four replicates. (C) The *HVA1::GUS* reporter and the internal control construct, *UBI::luciferase*, were co-bombarded into barley aleurone cells. Bars indicate GUS activities after 24 h of incubation with concentrations of ABA ranging from 0 to 20 μM. GUS activity was normalized and reported as in (B). (D) The effector construct, *UBI::TaABF1*, was co-bombarded into barley aleurone cells along with the reporter construct, *Amy32b::GUS*, and the internal control construct, *UBI::luciferase*. The amount of reporter and internal control plasmid DNA was always constant, while the amount of *TaABF1* effector (relative to the reporter) varied from 0 to 0.5. GUS activities were measured after 24 h of incubation with or without 1 μM GA. (E) The effector construct, *UBI::TaABF1*, was co-bombarded into barley aleurone cells along with the reporter construct, *HVA1::GUS*, and the internal control construct, *UBI::luciferase*. The amount of reporter and internal control plasmid DNA was always constant, while the amount of *TaABF1* effector (relative to the reporter) varied from 0 to 1.0. (F) ABA content of imbibed embryoless barley grains. Grains were prepared and imbibed as if for bombardment, as described in the Materials and methods section. Data are means ± SE of three biological replicates. After 2 d of imbibition the error bars are smaller than the symbols. (G) The reporter construct *Amy32b::GUS*, and the internal control construct *UBI::luciferase* were co-bombarded into barley aleurone cells either with or without the effector construct, *UBI::TaABF1* (effector/reporter ratio of 0.025). Bars indicate GUS activities after 24 h of incubation with or without GA (1 μM) and ABA (0.0005 or 0.005 μM). (H) 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* (effector/reporter ratio of 0.05). Bars indicate GUS activities after 24 h of incubation with or without ABA (0.02 or 0.2 μM).

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to act on TaABF1. By measuring the amount of ABA in imbibing embryoless grains, throughout the normal process of preparing them for bombardment and subsequent incubation, we found that the amount of ABA decreased substantially during the 4-day imbibition period (Fig. 1F). Nevertheless, grains used for bombardment (after 3 d of imbibition) still retained a physiologically relevant level of endogenous ABA (about 0.5 pmol per endosperm).

If ABA-induced post-translational modification of TaABF1 is required for its full activity, we would expect that ABA-regulated genes stimulated or repressed by TaABF1 would show increased stimulation or repression, respectively, upon addition of exogenous ABA. When we introduced the *UBI::TaABF1* effector construct into aleurone layers (at a effector:reporter ratio of 0.025), we found that TaABF1 caused a significant (but incomplete) repression of the GA-induced expression of *Amy32b* (Fig. 1G). The addition of exogenous ABA resulted in still further repression of *Amy32b*. The combined repressive effect for ABA and TaABF1 together was greater than for either ABA or TaABF1 alone. We also tested the combined effects of ABA and TaABF1 on *HVA1* induction. In this case, we found that the effects of the two inducers were not additive (Fig. 1H). While higher levels (0.2 μM) of ABA caused a modest increase in *HVA1* induction from cells already induced with TaABF1, the level of *HVA1* expression in these cells was actually less that in cells treated with 0.2 μM ABA alone.

The fact that ABA and TaABF1 had an additive effect when repressing *Amy32b* expression is consistent with TaABF1 requiring a modifying activation by ABA in the pathway leading to ABA-suppressed gene expression. However, the fact that added ABA did not potentiate TaABF1 to activate *HVA1* expression is further evidence that TaABF1 acts via a different mechanism in the pathway leading to *HVA1* expression.

Effects of GA and ABA on TaABF1 protein levels

One way in which ABA might act to facilitate TaABF1-mediated repression of *Amy32b* would be to increase the amount of TaABF1 present. While previous work (Johnson *et al.*, 2008) has shown that the amount of *TaABF1* mRNA does not increase in response to ABA, protein levels have not been reported. When whole grains were imbibed in buffer alone, we found that TaABF1 levels decreased slightly over 48 h. In the presence of 20 μM ABA, however, TaABF1 showed a slight increase during imbibition. We also observed that the presence of 1 μM GA during imbibition caused a slight increase in TaABF1 (Fig. 2A). Since the effects of exogenous GA and ABA may be masked in whole grains by endogenous hormones secreted by the embryo, we also measured the effects

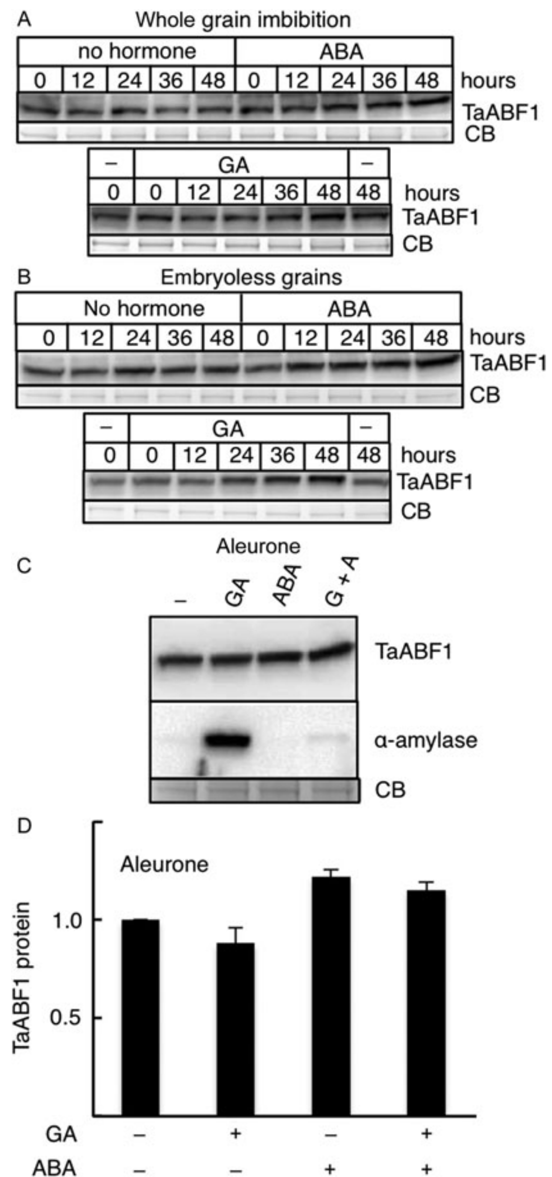

Figure 2. Effect of GA and ABA on TaABF1 protein levels. (A) Whole wheat grains were placed on moist filter paper at 24°C with or without 20 μM ABA or 1 μM GA and allowed to imbibe for 0–48 h. (B) Embryoless grains were sterilized and allowed to imbibe as in (A). (C) Isolated aleurone layers were allowed to imbibe for 48 h. Proteins were then isolated from whole grains, embryoless grains or aleurone layers, and 20 μg of total soluble protein was analysed by SDS-PAGE and Western blotting using anti-TaABF1 and anti-α-amylase antibodies. An unidentified protein band stained with Coomassie Blue (CB) is shown as a loading control. (D) Western blotting was carried out on three separate biological replicates treated as in (C). TaABF1 bands from the blots were quantified as described in the Materials and methods section. Data are means ± SE.

of GA and ABA on embryoless grains. In this case we observed that grains maintained a constant level of TaABF1 in the absence of exogenous hormones, and increased modestly in the presence of ABA or GA

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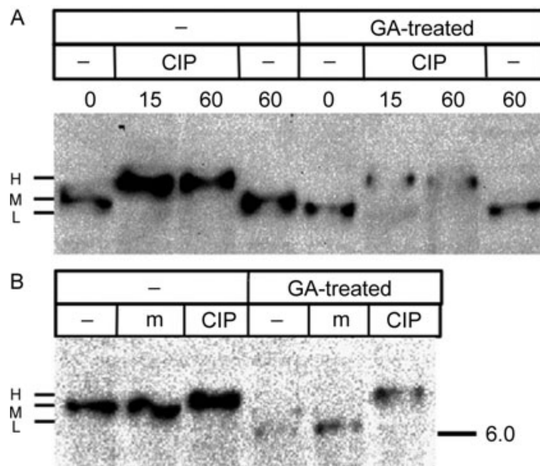

Figure 3. Phosphorylation of TaABF1. (A) Isolated aleurone layers were allowed to imbibe at 24°C with or without 1 μM GA for 48 h. Proteins were then isolated and incubated with or without calf intestinal phosphatase (CIP) for 15 or 60 min. (B) Aleurone layers were incubated as in (A) and isolated proteins were either treated with CIP for 60 min or given a mock (m) treatment. Twenty micrograms of each protein was then analysed by isoelectric focusing followed by Western blotting. The position of TaABF1 bands with high (H), medium (M) or low (L) isoelectric point (pI) are indicated. Bovine carbonic anhydrase was used as a pI 6.0 standard.

(Fig. 2B). These measurements of TaABF1 (as a fraction of total protein) in whole endosperms must be interpreted carefully, considering the fact that degradation of storage proteins in the starchy endosperm is taking place during imbibition, and the level of this degradation increases in the presence of GA. To address this complication, we also measured the level of TaABF1 protein in isolated aleurone layers. In this case, 48-h treatments with GA, ABA or both, all resulted in small changes in TaABF1 levels that are unlikely to be biologically significant (Fig. 2C, D). The strong response of α-amylase protein to GA and ABA confirms that the aleurone cells were healthy and responding normally to hormones (Fig. 2C). Considering all of these results together, we conclude that neither GA nor ABA causes a substantial change in the level of TaABF1 protein in the aleurone cells of imbibing grains. Thus, the effect of ABA in potentiating TaABF1-mediated gene regulation in these cells is apparently not caused by an ABA-induced increase in the amount of TaABF1 protein.

Phosphorylation of TaABF1

We next examined the possibility that TaABF1 might be regulated by post-translational modification. While SDS-PAGE analysis (Fig. 2C) indicated that neither GA nor ABA induced a change in the apparent size (42 kDa) of TaABF1, the principal TaABF1 band on an IEF gel shifted to a lower isoelectric point (pI) (from band M to band L) after GA treatment (Fig. 3A). Both the untreated and GA-treated TaABF1 shifted to a higher pI (band H) after phosphatase treatment, while no shift was observed after a mock phosphatase treatment (Fig. 3A, B). These results indicate that TaABF1 was phosphorylated *in vivo* both before and after the GA treatment, and that the GA-induced change in mobility may be associated with phosphorylation. Inclusion of pI standards (Fig. 3B) indicated that the principal band of GA-treated TaABF1 (band L) had a pI of about 6.0, while that of untreated TaABF1 (band M) was slightly higher.

TaABF1 does not prevent induction of Amy32b by GAMyb

In addition to the upstream activation of TaABF1, we also investigated its downstream connection to later steps in hormone signalling, such as the GA-induced expression of *Amy32b*. To more precisely determine the location of TaABF1 within the GA/ABA signalling pathway, we tested its ability to interfere with GA and GAMyb-induced gene expression. As expected, the

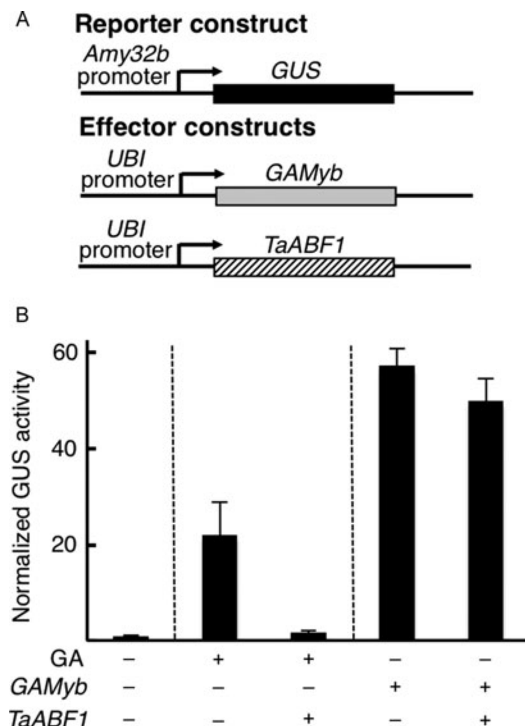

Figure 4. Induction of *Amy32b* by GA and GAMyb. (A) Reporter and effector constructs used in the experiment. (B) The reporter construct, *Amy32b::GUS*, and the internal control construct, *UBI::luciferase*, were co-bombarded into barley aleurone cells with (+) or without (−) the effector constructs, *UBI::GAMyb* and *UBI::TaABF1*. The effector constructs were used at an effector/reporter ratio of 1.0. GUS activities were measured after 24 h of incubation with or without 1 μM GA. GUS activity was normalized and reported as in Fig. 1.

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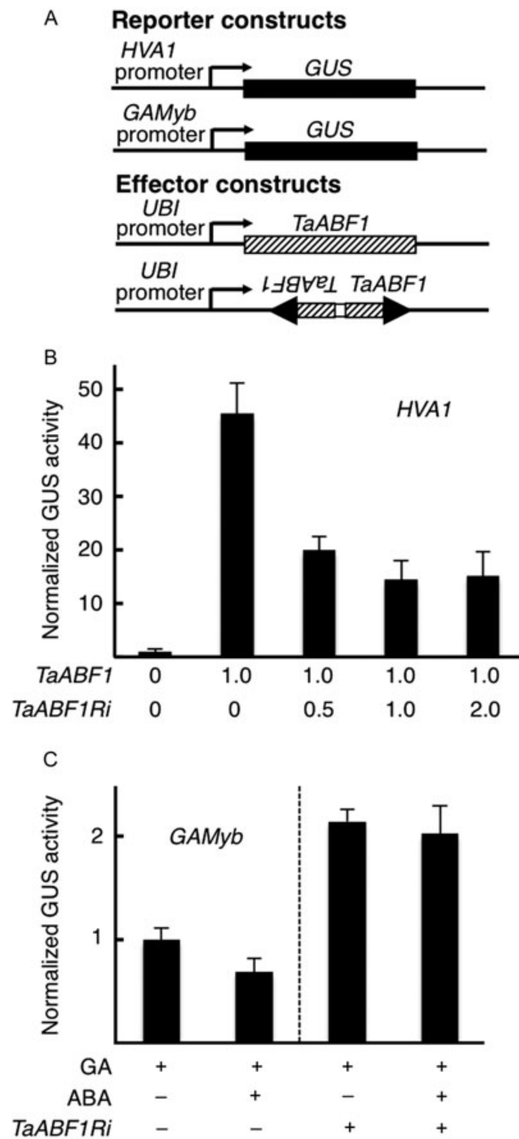

Figure 5. Regulation of *GAMyb* by ABA and TaABF1. (A) Reporter and effector constructs used in the experiment. (B) The reporter construct, *HVA1::GUS*, and the internal control construct, *UBI::luciferase*, were co-bombarded into barley aleurone cells with the effector constructs *UBI::TaABF1* and *UBI::TaABF1Ri* in the proportions indicated. GUS activities were measured after 72 h of incubation. (C) The reporter construct, *GAMyb::GUS*, and the internal control construct, *UBI::luciferase*, were co-bombarded into barley aleurone cells with (+) or without (−) the effector construct *UBI::TaABF1Ri* (effector/reporter ratio of 1.0). GUS activities were measured after 72 h of incubation with (+) or without (−) 1 μM GA and 20 μM ABA. GUS activity was normalized and reported as in Fig. 1.

induction of *Amy32b* by GA was prevented by the TaABF1 effector (Fig. 4), as TaABF1 acts downstream of GA. However, TaABF1 could not prevent induction of *Amy32b* by the *GAMyb* effector construct, indicating that TaABF1 must act upstream of GAMyb in the signalling pathway.

Knockdown of TaABF1 increases GAMyb expression

If TaABF1 acts upstream of *GAMyb*, then it would be expected that altered levels of TaABF1 would in turn disrupt expression of *GAMyb*. In preliminary experiments we determined that a *TaABF1* RNAi construct could partially knock down the effects of TaABF1 in bombarded aleurone cells (Fig. 5A, B). We then utilized the RNAi construct to reduce *TaABF1* expression in cells expressing a *GAMyb::GUS* reporter construct. To allow time for endogenous TaABF1 protein already present at the time of the bombardment to decay, assays of *GAMyb::GUS* expression were carried out at 72 h post-bombardment. In the absence of any effector construct, exogenous ABA resulted in a modest decrease in the level of *GAMyb* expression (Fig. 5C). In the presence of the *TaABF1* RNAi construct, however, ABA did not repress *GAMyb* expression. *GAMyb* expression was in fact increased both in the absence and presence of exogenous ABA, strongly suggesting that TaABF1 is normally required to suppress *GAMyb*.

Discussion

Here we have provided functional analysis to better understand the action of TaABF1 during ABA and GA signalling in aleurone cells. We have determined that TaABF1 activity is not strongly regulated at the level of protein abundance, and our results suggest the importance of post-translational modification, such as phosphorylation. We have determined that TaABF1 regulates the expression of *Amy32b* at a point upstream of *GAMyb* transcription. Our results support the existing model that TaABF1 is involved in both ABA-induced and ABA-suppressed gene expression. For the activation of *HVA1*, it is most likely that TaABF1 acts by directly binding to the ABA response complex (ABRC) in the promoter of that gene, based on the established activity of related proteins (Casaretto and Ho, 2005; Zou *et al.*, 2008). For the inhibition of *Amy32b*, the higher sensitivity to both TaABF1 and ABA and the additive effect of TaABF1 and ABA suggest that a different mechanism is involved. As discussed below, it appears that one role of TaABF1 is to down-regulate the *Amy32b*-activating transcription factor GAMyb. As there is no consensus ABRC present in the *GAMyb* promoter, TaABF1 may bind to other DNA sequences or may inhibit *GAMyb* indirectly.

As overexpression of TaABF1 can regulate gene expression in bombarded embryoless barley grains in the absence of exogenous ABA (Johnson *et al.*, 2008), it was important to determine if these grains contained a physiologically relevant level of endogenous ABA. Previous work has shown that the ABA content of afterripened barley grains (Jacobsen *et al.*, 2002) and

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wheat embryos (Reid and Walker-Simmons, 1990) decreases during the first day of imbibition, but this was not determined at later times, and has not been measured in embryoless grains. Our results clearly show that in embryoless barley grains, the decline in ABA level continued, although at a slower pace, after the first day (Fig. 1F). At 3–4 d of imbibition, the ABA level was about 0.5 pmol ABA per endosperm (equivalent to a concentration in the low nM range). This level of endogenous ABA could itself be sufficient to allow activation of overexpressed TaABF1, but still allow for further TaABF1 activation if exogenous ABA is added.

Previous work (Johnson *et al.*, 2008) has shown that very high levels of either TaABF1 alone or ABA alone can maximally suppress *Amy32b* expression. Thus, under those conditions the effects of TaABF1 and ABA are not additive. However, we have shown here (Fig. 1) that under more physiologically realistic conditions of lower TaABF1 and ABA levels, they do in fact suppress *Amy32b* in an additive manner. The fact that overexpressed TaABF1 suppressed *Amy32b* expression more strongly in the presence of exogenous ABA is consistent with the requirement for an ABA-induced activating modification in this pathway. The lack of a similar additive response for the induction of *HVA1* expression most likely indicates that ABA does not act on TaABF1 in the same manner for the pathway leading to ABA-induced gene expression.

Some ABF proteins such as AtABI5 (Lopez-Molina *et al.*, 2001), HvABI5 (Casaretto and Ho, 2003) and OsABI5 (Zou *et al.*, 2008) are upregulated at the mRNA level by exogenous ABA, and this may be an important part of their overall response to ABA. However, this is not the case for TaABF1, whose mRNA levels do not change in response to either ABA or GA (Johnson *et al.*, 2008). TaABF1 is in fact unusual among ABFs that have been tested, in that its transcript levels are insensitive to ABA. To further investigate the mechanism through which ABA (together with GA) acts on TaABF1, we carried out a series of experiments measuring the level and status of TaABF1 protein under a variety of conditions.

We have now determined (Fig. 2) that ABA and GA have little effect on the level of TaABF1 protein in imbibing wheat grains. This conclusion is based on a thorough analysis of TaABF1 protein in whole grains, embryoless grains and isolated aleurone layers. It is interesting to note that while *TaABF1* mRNA decreased substantially during 48 h of imbibition (Johnson *et al.*, 2008), the amount of TaABF1 protein stayed almost constant during this period, suggesting a high degree of stability. While slight increases in TaABF1 protein were observed in hormone-treated whole grains and embryoless grains, we doubt that these differences are biologically significant, especially when considering that very little difference was observed in the aleurone, which is the tissue in which TaABF1 has been observed to mediate ABA responses (Johnson *et al.*, 2008). The increased levels of TaABF1 (as a fraction of total protein) in embryoless grains treated with GA are more likely the result of GA-induced degradation of storage proteins than an actual increase in TaABF1. This idea is supported by the fact that embryoless grains treated for 36 and 48 h with GA contained lower total protein than untreated grains (data not shown) and by the fact that GA treatment of aleurone layers did not change the level of TaABF1 protein. The fact that TaABF1 protein levels are not hormone-regulated contrasts with the case for some other ABF family members. For example, exogenous ABA caused a threefold increase in HvABI5 protein in whole barley grains (Casaretto and Ho, 2005), while AtABI5 protein in imbibing Arabidopsis seeds increased more than tenfold in response to exogenous ABA (Lopez-Molina *et al.*, 2001).

While TaABF1 protein levels did not appear to be under regulation, we observed that TaABF1 was phosphorylated in aleurone cells. Through a combination of IEF analysis and phosphatase treatment, we determined that TaABF1 in aleurone layers is phosphorylated in the absence of exogenous hormone treatment. This phosphorylation might be the result of PKABA1 that was activated by endogenous ABA, a scenario that needs to be tested in future studies. Treatment with exogenous GA resulted in a shift of pI of TaABF1 in IEF gels, suggesting the possibility of hormone-induced post-translational modification.

Work by several groups has shown that ABFs are regulated via reversible phosphorylation in a number of ways (Schutze *et al.*, 2008). The ability of the rice ABF TRAB1 to activate transcription of downstream genes is dependent on ABA-induced phosphorylation by members of the SnRK2 kinase family (Kagaya *et al.*, 2002). Another rice ABF, OREB1/OsABI5, can be phosphorylated by several types of kinases, including SnRK2, SnRK3 and casein kinase II. Phosphorylation at some sites in OREB1/OsABI5 increases its activity as a transcription factor while phosphorylation at other sites decreases its activity (Hong *et al.*, 2011). A 14-3-3 binding domain within HvABF2 negatively regulates that protein's transcriptional activation activity (Schoonheim *et al.*, 2009). As binding of 14-3-3 proteins is often phosphorylation dependent, this suggests that phosphorylation in the 14-3-3 binding domain might inhibit HvABF2. The precise relationship of the specific phosphorylation events in OREB1 and HvABF2 to signalling by ABA and GA is not yet clear. Interestingly, at least one phosphorylation event in Arabidopsis ABI5 is regulated by both GA and ABA (Piskurewicz *et al.*, 2008), and an ABA-induced phosphorylation event on AtABF3 increases its stability (Sirichandra *et al.*, 2010). Whether phosphorylation at specific sites regulates the ability of TaABF1 to mediate ABA-induced and ABA-suppressed gene expression, and whether those phosphorylation events are

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regulated by hormones, are important questions that will require attention in the future.

Active TaABF1 protein is assumed to modulate downstream actors in the pathway leading to suppression of *Amy32b* expression. The fact that PKABA1 acts upstream of GAMyb in this pathway and results in *GAMyb* down-regulation (Gómez-Cadenas *et al.*, 2001) led us to test whether TaABF1 would have a similar effect. Based on the fact that overexpressed TaABF1 was able to prevent GA-induced *Amy32b* expression but not GAMyb-induced *Amy32b* expression (Fig. 4), we concluded that TaABF1 acts downstream of GA and upstream of *GAMyb* transcription. This conclusion is supported by the fact that reducing the expression of TaABF1 by RNAi resulted in higher *GAMyb* expression, and prevented the ABA-induced reduction in *GAMyb* (Fig. 5). We conclude from these results that an important role of TaABF1 is to reduce the level of *GAMyb* transcription in aleurone cells, leading to reduced *Amy32b* expression. The fact that bombardment of aleurone cells with a *TaABF1* RNAi construct could only partially reduce the activity of a co-bombarded *UBI::TaABF1* construct (Fig. 5B) suggests that the endogenous *TaABF1* gene (in Fig. 5C) may also have been only partially knocked down. Nevertheless, the effect was sufficient to cause an increase in *GAMyb* expression.

In summary, this report provides evidence that TaABF1's activity in down-regulating the GA-induced *Amy32b* promoter is potentiated by ABA, that TaABF1 is phosphorylated *in vivo*, and that it down-regulates *GAMyb* transcription which in turn leads to down-regulation of *Amy32b*.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0960258513000081

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