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Protein Kinase Activity Toward TaABF1 in Imbibing Grains

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By

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Protein Kinase Activity Toward TaABF1 in Imbibing Grains

Abstract

The hormones gibberellin and abscisic acid are essential for plant responses to changing environmental conditions, and can send opposing signals. In wheat, the transcription factor TaABF1 plays an important role at the intersection of a gibberellin-induced/abscisic acid-suppressed pathway. When gibberellin dominates, the GA-induced gene, *Amy32b,* is transcribed. When abscisic acid is dominant, TaABF1 is active and it downregulates GA-induction of *Amy32b*, while promoting ABA-induced transcription of the gene *HVA1*. The activity of TaABF1 is thought to be regulated by post-translational phosphorylation at key serine residues. In this study, to determine TaABF1 phosphorylation by wheat kinases, we purified recombinant histidine tagged TaABF1 protein, incubated it with kinases extracted from the aleurone layer of wheat grains, and assessed phosphorylation status using liquid chromatography-mass spectrometry. We have designed a successful purification scheme, and are able to identify synthetic test peptides and peptides that result from tryptic digestion of BSA and purified un-phosphorylated TaABF1. However, we are still working to optimize protein recovery after the phosphorylation assay for analysis by liquid chromatography-mass spectrometry.

Introduction

In cereal grains, including wheat, the phytohormones gibberellin (GA) and abscisic acid (ABA) are important signaling molecules that allow plants to adapt to environmental stimuli. When conditions favor germination, GA is produced, which signals for the transcription of genes involved in germination and the hydrolysis of starch reserves (Bethke et al., 1997; Lovegrove and Hooley, 2000). Contrarily, when conditions do not favor germination, as in high salinity

environments, during drought, or in low temperatures, grains produce ABA. The ABA signals for the inhibition of the GA-induced signaling pathways and promotes transcription of genes involved in the prolongation of seed dormancy (Lovegrove and Hooley 2000). Because understanding the mechanisms of GA and ABA crosstalk and antagonism could prove important for designing new advancements in agricultural crop control, such molecular pathways have long been a focus of cereal grain research.

In addition to regulating germination and seed development, GA has been shown to control plant root growth, stem growth, and flowering. Plants that are deficient in GA experience an increase in number of seed abortions as well as abnormal seed development; such events can be detrimental to plant reproduction and crop yield. Although these phenotypes cannot be reversed through introduction of exogenous GA, which cannot easily enter the seed, they can be negated with mutations mimicking a constitutive GA response (Swain and Singh 2005).

ABA, which has opposing effects to GA, is an equally essential plant hormone. During times of environmental stress, ABA is responsible for ceasing stem/shoot growth, promoting root growth, closing stomata, and inducing transcription of genes for drought, salinity, and cold tolerance. Such genes code for various proteins including: water channel and transport proteins, detoxification enzymes, protection factors of macromolecules, enzymes for osmolyte biosynthesis, proteases, transcription factors, protein kinases and phosphatases, and proteins involved in phospholipid metabolism (Shinozaki and Yamaguchi-Shinozaki 2007). Thus, both GA and ABA play important roles in numerous processes that are critical for plant survival.

The ratio of GA to ABA, not either hormone alone, is responsible for the control of seed dormancy and germination in plants. The roles of the hormones were demonstrated through an experiment that utilized *Arabidopsis thaliana* seeds that lacked functional GA synthesis, and

therefore could not germinate. When these seeds were further mutagenized, only the new mutants that also lacked functional ABA synthesis were able to germinate. Thus, a seed is able to germinate if it is lacking both ABA and GA or if it is able to synthesize GA (Koorneef et al. 1982), but it cannot germinate if it produces ABA alone. Such effects of GA and ABA are mediated through signal transduction. However, GA and ABA signaling pathways are complex; in general, regulation of phosphorylation via kinase and phosphatase activity (Lovegrove and Hooley 2000) as well as calcium/calmodulin signaling appears to be important in pathways involving both hormones (Ritchie and Gilroy 1998).

In GA signaling pathways, signal transduction occurs through calcium/calmodulin upregulation, cGMP messaging, and protein phosphatase activity (Kuo et al. 1996; Jones et al. 1998; Penson et al. 1996; Lovegrove and Hooley 2000). To characterize GA signal transduction, researchers utilized cereal grain mutants with defects in GA signaling. *Slender*, a barley mutant defective in the *Sln1* gene, exhibits constitutive production of GA-induced α-amylase in aleurone cells, which form a distinct layer surrounding the starchy endosperm. Further, the SLN1 protein is thought to be involved in early repression of GA signaling upstream of cGMP and kinase activation (Gomez-Cadenas et al. 2001). Though *Sln1* mRNA levels do not change in response to GA, SLN1 protein levels decrease, indicating that GA can signal for SLN1 degradation (Gomez-Cadenas et al. 2001). The mechanism by which GA signals for SLN1 degradation relies on SLN1's DELLA domain. DELLA repressors, the family of proteins that contain DELLA domains, can be marked for degradation in response to GA via ubiquitination (Silverstone et al. 2001; Willige et al. 2007). In addition to the barley *Slender* mutant, other cereal crops with mutations in protein DELLA domains, including wheat *Reduced height1* (RH1 protein) mutants and maize *dwarf8* (D8 protein) mutants also exhibit insensitivity to GA-dependent degradation

(Peng et al. 1999). Another barley mutant, *Spindly (Spy)*, also exhibits impaired GA signaling (Robertson et al. 1998). The SPY protein, like SLN1, blocks the GA-induced expression of α-amylase; however, it may also be involved in ABA-induced gene expression (Robertson et al. 1998), giving it a role in the complex networks of GA/ABA crosstalk.

Another major breakthrough in understanding GA signaling was the identification of GA INSENSITIVE DWARF (GID1) proteins. The soluble GID1 proteins are GA receptors that were first identified in the *gid1* rice mutant (Ueguchi-Tanaka et al. 2005), and were later identified in *Arabidopsis* (Nakajima et al. 2006). The GID1 receptor is considered a nuclear receptor, but is also thought to exist on the plasma membrane (Ueguchi-Tanaka et al. 2005). In both rice and *Arabidopsis,* GID1 proteins have been shown to interact with DELLA repressors (Ueguchi-Tanaka et al. 2005; Nakajima et al. 2006). A study involving an *Arabidopsis* mutant that lost its three GID1 receptors found that these mutants exhibit GA-insensitivity, and that the N-terminal DELLA domain was required for GID1 interactions (Griffiths et al. 2006). Thus, the degradation of DELLA proteins is caused by GA perception, as the DELLA domain serves as a receiver domain for activated GID1 GA receptors (Willige et al. 2007).

To initiate the GA signaling pathway, GA binds to one of its receptors, such as GID1. From there, signal transduction eventually results in the downstream binding of transcription factors to the promoters of GA-inducible genes, such as those for α-amylase production. These promoters contain distinct sequences that have been extensively studied: the amylase box, the GA response element (GARE), and the pyrimidine box (Skriver et al. 1991; Gubler and Jacobsen 1992; Lanahan et al. 1992). It is at the GARE that positive and negative regulators are able to bind and control transcription. For example, a zinc-finger protein in barley is able to bind at the GARE box and act as a transcriptional repressor (Raventos et al. 1998).

In ABA signaling pathways, ABA can bind to receptors known by three different names: PYROBACTIN RESISTANCE 1 (PYRs), PYR1-like family (PYLs) (Park et al. 2009), and regulatory component of ABA family receptors (RCARs) (Ma et al. 2009). ABA binding to PYR/PYL/RCAR receptors activates these receptors and promotes their interaction with type 2C protein phosphatases (PP2Cs), thus inactivating these PP2Cs. PP2Cs, when active, are negative regulators of ABA signaling, but are not involved in ABA-mediated suppression of GA-induced genes (Allen et al. 1999; Shen et al. 2001). Further, PP2Cs have been shown to interact with SNF1-related kinases (SnRKs) by dephosphorylating them. SnRKs are serine/threonine kinases of which there are three subfamilies: SnRK1, SnRK2, and SnRK3 (Kulik et al. 2011). SnRK2 kinases are a subfamily whose members are important regulators of stress responses in plants. Specifically, they serve as positive regulators of ABA signaling when phosphorylated (Allen et al. 1999). The activity of many SnRK kinases is controlled by (de-)phosphorylation of the kinase activation loop (Kulik et al. 2011). Thus, the interaction between PYR/PYL/RCAR and PP2Cs inactivates the PP2Cs, blocks PP2C inhibition of SnRK2s, and propagates the ABA signal (Park et al. 2009). Transcription factors, such as VP1, can simultaneously promote ABA-induced gene expression and inhibit the expression of some GA-induced genes. Protein kinases such as PKABA1 and some WRKY transcription factors can also inhibit GA-induced genes in response to ABA presence (Shen et al. 2001; Xie et al. 2006).

Within the cereal grain, aleurone cells make up an important system for the study of GA and ABA signaling and antagonism. These cells have GA and ABA receptors localized to their plasma membranes (Bethke et al. 1997; Hooley et al. 1991; Gilroy and Jones 1994; Gilroy 1996). It is within the aleurone cells, therefore, where the GA-induced/ABA-suppressed and ABA-induced signaling pathways occur. The embryo secretes GA to the aleurone cells in order to

initiate germination, and in dormant wheat cultivars, ABA concentration at aleurone cells is high compared to non-dormant cultivars (Bethke et al. 1997). Because the pathways of interest take place in the aleurone layer and because the aleurone cells are readily separated from other cell types in the wheat grain, they can be used as a model for the study of GA and ABA pathways.

In aleurone of dormant wheat grains, when ABA concentrations are low, GA production and the binding of GA to its receptor results in a series of signaling events that lead to the downstream activation of the GA-induced transcription factor GAMyb. GAMyb is a Myb-related protein that contains two transcriptional activation domains, and GAMyb can activate transcription by binding to GARE boxes (Gubler et al. 1995 and 1999). GAMyb transactivates the expression of genes such as *Amy32b. Amy32b* codes for a low pI α-amylase that can hydrolyze and break down starch reserves during germination (Gomez-Cadenas et al. 2001; Gubler et al. 1999; Lanahan et al. 1992). When barley aleurone was experimentally exposed to GA, GAMyb protein levels rapidly increased (Grubler et al. 2002), resulting in up-regulation of α-amylase production (Gomez-Cadenas et al. 2001). This observation suggests that GAMyb concentration is important for the regulation of *Amy32b* transcription.

In contrast, ABA signals in the wheat aleurone layer suppress this GA-induced pathway, while simultaneously up-regulating the expression of ABA-induced genes (Figure 1). When aleurone cells are exposed to GA, they increase production of GAMyb protein; however, the introduction of ABA partially blocks this GA induced expression (Gomez-Cadenas et al. 2001). The ABA-suppressed pathway is regulated by both PKABA1, a SnRK2 kinase (Gomez-Cadenas et al. 2001), and TaABF1 (*Triticum aestivum* ABF1), a basic leucine zipper (bZIP) transcription factor found in wheat (Johnson et al. 2002). PKABA1 has been shown to specifically disrupt α-amylase production (Gomez Cadenas et al. 1999), and when ABA is present, *Amy32b* expression

is significantly reduced (Harris et al. 2013). Active SnRK2s, including PKABA1, are thought to affect expression at promoters that contain ABA-response elements (ABREs) via phosphorylation of ABRE binding factors (ABFs) at their RXXS/T consensus sequence. ABFs exist as dimers, and their bZIP domain can bind to ABREs, modulating gene expression (Choi et al. 2000). The phosphorylation of ABFs by SnRK2s has been shown to occur in the presence of ABA (Fujii et al. 2007). Thus, ABA-mediated inactivation of PP2Cs allows for PKABA1 (or other SnRK2) activation, which allows ABF activation, ABA signal transduction, and inhibition of the GA-induced pathway.

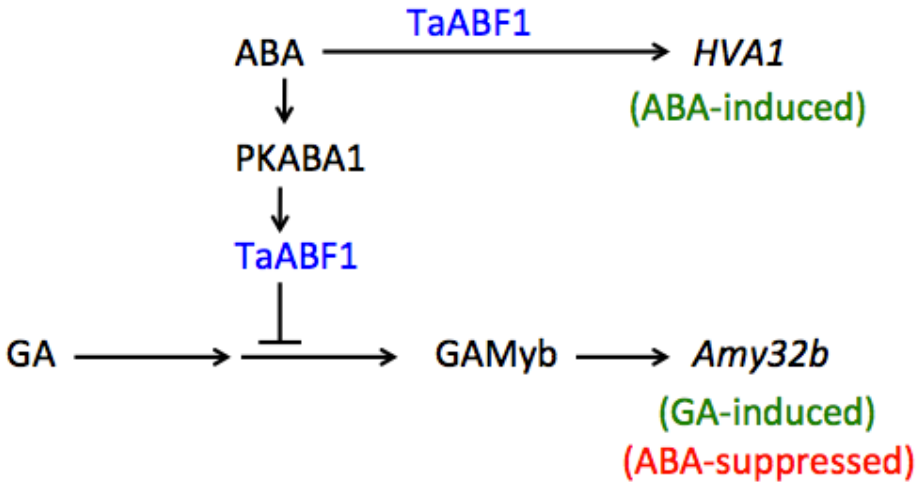

Figure 1. Current model for the GA and ABA signaling pathways in aleurone (Adapted from Johnson et al. 2008).

Recent genomic analyses reveal that 187 bZIP transcription factors, including ABFs, have been identified in the wheat genome (Li et al. 2015). The bZIP proteins that are involved in ABA mediated signaling belong to Subgroup-A. Bioinformatics analysis of 41 Subgroup-A

Triticum aestivum bZIP (TabZIP) transcription factors in wheat revealed that many of these family members had highly conserved motifs and similar exon-intron composition, but had variable intron positions within the leucine zipper regions (Li et al. 2016), suggesting that their regulation may be similar, but that they probably bind different DNA sequences. Many of these family members, such as TabZIP174, were localized to the nucleus; however, TabZIP9 appears to be simultaneously present in the nucleus, cytoplasm, and plasma membrane (Li et al. 2016). Additionally, overexpression of TabZIP174 resulted in the induction of ABA-induced stress responsive genes (Li et al. 2016). Such structural similarities suggest that the function and control of activation may be similar between ABFs and other TabZIP family members.

It has previously been shown that PKABA1 expression in barley grains increases in conditions that prevent germination (Rikiishi et al. 2010) and that levels of PKABA1 mRNA increase in response to ABA (Gomez-Cadenas et al. 1999), indicating that ABA-induced *PKABA1* transcription likely mediates the ABA signaling pathway. *TaABF1* mRNA levels (Johnson et al. 2008) and protein abundance (Harris et al. 2013), however, are not regulated by ABA, suggesting that post-translational modifications to the TaABF1 protein might be key for its regulation. Many ABF transcription factors are controlled by post-translational modifications, including phosphorylation, so this method of regulation would not be unusual for TaABF1. For example, ABA induces phosphorylation of TRAB1, a rice ABF, by a SnRK2 kinase (Kobayashi et al. 2005). The activity of HvABI5, a barley ABF that is involved in induction of the ABA-regulated gene *HVA1*, is also controlled by phosphorylation (Casaretto and Ho 2005). And, phosphorylation occurs at all four conserved regions of OREB1, another rice ABF (Chae et al. 2007). Such post-translational modifications impact stability, interactions with other proteins, (de-) activation, and localization within the cell (Shutz et al. 2008). In plant ABFs, there are three

N-terminal conserved regions—C1, C2, and C3—and a conserved C-terminal bZIP binding domain (Figure 2A). Potential phosphorylation sites may exist within these conserved regions.

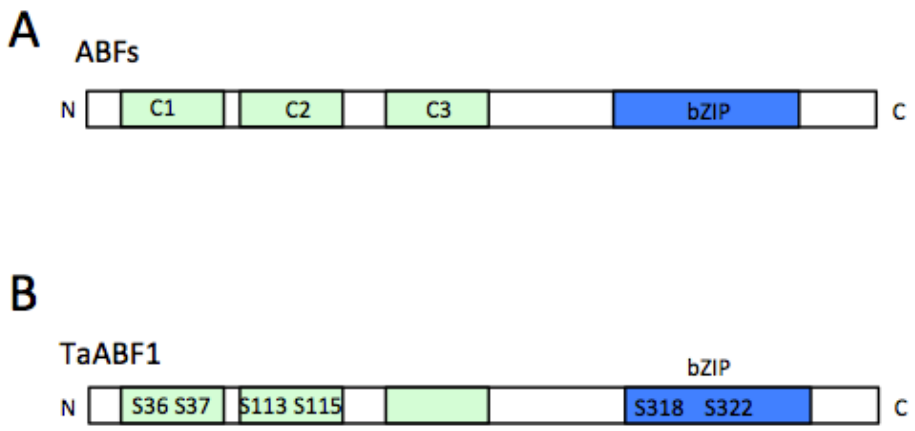

Figure 2. (A) Diagram of conserved regions of a general ABF transcription factor. **(B)** Diagram of TaABF1 with the serine residues that are potential phosphorylation sites identified. Conserved regions 1-3 (C1, C2, C3) are highlighted in green and the conserved bZIP domain is highlighted in blue.

TaABF1, a wheat member of the ABF family, contains the characteristic ABF conserved regions and bZIP domain. It is involved in the intersection of GA and ABA signaling in aleurone cells (Johnson et al. 2002, 2008; Harris et al. 2013). TaABF1 is important for the up-regulation of ABA-induced genes like *HVA1*, and for the down-regulation of GA-induced/ABA-suppressed genes, like *Amy32b*. In the absence of hormones, constitutive TaABF1 expression can eliminate GA-induced *Amy32b* expression and stimulate the ABA-inducible *HVA1* and *HVA22* genes (Johnson et al. 2008). This observation suggests that TaABF1 acts downstream of ABA. Because *HVA1* and *HVA22* contain ABRE regions in their promoters, it is likely that TaABF1 is able to bind to them directly to promote transcription, while TaABF1 likely cannot bind directly to the GARE region of the *Amy32b* promoter.

The role of TaABF1 in ABA suppression of GA-induced genes, therefore, is slightly more complex. Harris and colleagues demonstrated that TaABF1 could not inhibit *Amy32b* expression that was induced through overexpression of GAMyb, and that the knockdown of TaABF1 by RNAi results in increased expression from the *GAMyb* promoter (2013). Thus, TaABF1 must act upstream of *GAMyb* transcription in aleurone cells. However, when RNAi was used to inhibit *TaABF1* transcription, it did not prevent the ABA-induced suppression of *Amy32b* or the induction of *HVA1*, suggesting that another protein may act redundantly with TaABF1 in aleurone cells (Johnson et al. 2008). Current models reflect the findings that active TaABF1 must somehow inhibit *GAMyb* transcription, although the mechanism(s) through which this occurs are unknown (Figure 1).

The method by which TaABF1 becomes activated and suppressed in GA signaling may involve phosphorylation by PKABA1 (and/or other kinases). A previous study utilized a two-hybrid assay to demonstrate that TaABF1 binds to the PKABA1 kinase, and that PKABA1 is able to phosphorylate synthetic peptides that represent regions of TaABF1. Further, PKABA1 specifically interacts with TaABF1, while mutated versions of PKABA1 do not appear to phosphorylate TaABF1 (Johnson et al. 2002). These findings suggest that TaABF1 is a substrate for PKABA1.

To investigate the potential role of phosphorylation in regulating TaABF1 activity, critical serine residues in the conserved regions of TaABF1 (Figure 2B) were mutated to aspartic acid to mimic phosphorylation or to alanine to prevent phosphorylation. These mutants were introduced to barley aleurone via particle bombardment. Mutagenesis studies revealed that mimicking phosphorylation at the serine residue 113 (S113), inhibited the activity of TaABF1, while simultaneous mimicked phosphorylation of S36, S37, S113, and S115 increased its activity

(Johnson, unpublished data). Thus, these may be key residues for controlling the activity of TaABF1 if their phosphorylation and de-phosphorylation occurs *in vivo.*

Understanding TaABF1 regulation is critical to understanding GA and ABA crosstalk in cereal grain aleurone. To better understand phosphorylation of TaABF1 *in vivo*, we sought to determine whether phosphorylation occurs at specific residues of TaABF1 by wheat kinases, and to determine whether GA and ABA control the phosphorylation patterns. To do so we purified a recombinant, histidine tagged TaABF1 (His_6::TaABF1) from *Escherichia coli* cells. We then assessed the ability of wheat protein kinases extracted from aleurone cells to phosphorylate TaABF1 at specific locations—specifically, those key serine residues—and tested whether the TaABF1 phosphorylation was regulated by GA and ABA.

Materials and Methods

Protein Construct Preparation and transformation

A TaABF1 cDNA (Johnson et al. 2002) was cloned into the pDEST17 vector using the Gateway cloning system (Invitrogen, product number 12535-019), and the resulting plasmid (p17/TaABF1) was transformed into BL21 (DE3) pLysE *E. coli* cells.

Protein Expression and Purification

A single colony of *E. coli* BL21(DE3)pLysE containing the p17/TaABF1 expression plasmid was grown overnight at 37°C in 50 mL of LB medium containing 34 µg/mL chloramphenicol and 100 µg/mL ampicillin in a shaking incubator. Ten mL of the overnight culture were used to inoculate 500 mL LB containing 100 µg/mL ampicillin, and grown at 37°C

in a shaking incubator until OD_{600} was 0.3. The cells were then induced with 1 mM IPTG, and incubated for 3 hours at 37°C in a shaking incubator. Cells were harvested by centrifugation (6000 x g, 4°C, 10 min) and stored at -80°C. Cell pellets were re-suspended in native purification buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0), lysed by sonication for 3X 15 seconds (Virisonic 100, level 12), and centrifuged (8000 x g, 4°C, 8 min). The supernatant was discarded and the pellet, which contained the His_6::TaABF1, was re-suspended in guanidinium lysis buffer (6 M Guanidine HCl, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8) to denature and solubilize proteins, and mixed. The re-suspension was sonicated on ice for 3X 15 seconds (Virisonic 100, level 12), centrifuged (3000 x g, 4°C, 15 minutes), and supernatant was collected. To transition back to native conditions, a buffer exchange into native conditions (20mM Tris, 100 mM NaCl, pH 7.4) was completed using 10,000 molecular weight cutoff centrifugal filter tubes. The final solution was concentrated to about 1 mL using centrifugal filter tubes, and protein concentration was measured with a Bradford assay (reagent: Bio Rad., product number 500-0006).

Western Blotting

Thirty µg of purified His_6::TaABF1 was separated on a NuPAGE 10% Bis-Tris Protein gel (ThermoFisher Scientific, product number NP0301BOX) with MOPS running buffer. A western blot was carried out on a polyvinylidene difluoride (PVDF) membrane using the XCell II Blot Module (ThermoFisher Scientific, product number EI9051) as previously described (Penna and Cahalan 2007). TaABF1 detection with anti-TaABF1 antibodies was carried out as previously described (Harris et al. 2013). His tag detection was carried out in the same way as TaABF1 detection except that THE™ His Tag Antibody, mAb, Mouse (GenScript, product

number A00186-100) was used as the primary antibody and goat anti-mouse IgG (GenScript, product number A00160) was used as the secondary antibody. Bands were visualized using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, product number RPN2232) with a ChemiDoc imager.

Grain Imbibition and Kinase Extraction

Embryos were cut off of wheat grains (NuWest, Bozeman Harvest, 2003) and discarded. Embryo-less grains were washed with sterile water for 10 minutes, 10% bleach for 20 minutes, and then sterile water for five times 5 minutes. Grains were added to imbibing solution (20 mM sodium succinate, 20 mM calcium chloride, pH 5.0) with 10 mg/mL chloramphenicol, poured onto a vermiculite plate (petri dish with layer of vermiculite covered with filter paper), and allowed to imbibe at 24°C for 4 days. After imbibition, forceps were used to separate the aleurone layer from the starchy endosperm. Starchy endosperms were discarded and aleurone layers were washed in imbibing solution with 10 mg/mL chloramphenicol, 24°C, shaking, overnight. Aleurone layers were then ground using a chilled mortar and pestle in grinding buffer (50mM Tris pH 7.6, 1 mM DTT, 1mM PMSF, 0.1% PVPP, 0.1% protease inhibitor cocktail, 0.1% protease inhibitor cocktail 2, and 0.1% protease inhibitor cocktail 3) (Sigmafast protease inhibitor cocktail tablets: Sigma, product numbers S8830-20TAB, S8820-20TAB). The solution was centrifuged at 10,000 x g, and supernatant that contained kinases was collected as fresh aleurone extract.

Phosphorylation Assay and Protein re-purification

The fresh aleurone extract (described above) was combined with 50 mM Tris buffer, pH 7.6 and/or purified His_6::TaABF1 (Table 1). Reaction mixtures were incubated for 30 minutes at 30°C. One experimental mixture was not nickel column re-purified, in the hope that a distinct His_6::TaABF1 band would be visible after SDS-PAGE separation without needing the extra re-purification step. The remaining control and experimental mixtures were re-purified using nickel affinity chromatography to recover the His-tagged protein. For the re-purification, the ProBond Purification System with denaturing buffer conditions was used as described in the user manual (Invitrogen, product number K85001).

Table 1. Reaction mixtures for phosphorylation assay (reporting characteristic protein concentrations)

	Nickel column re-purified		**Not re-purified**
	Control	Experimental	Experimental
Tris, pH 7.6	50 mM	50 mM	50 mM
Fresh Aleurone Extract (total protein)	1.15 mg/mL	1.15 mg/mL	1.15 mg/mL
His_6::TaABF1	--	0.25 mg/mL	0.25 mg/mL
Total Reaction Volume	2.00 mL	2.00 mL	0.20 mL

Tryptic Digestion and Liquid Chromatography-Mass Spectrometry

After re-purification, protein samples were separated on a NuPAGE 10% Bis-Tris Protein gel (ThermoFisher Scientific, product number NP0301BOX) with MOPS running buffer. TaABF1 bands were excised with a razor blade and subject to tryptic digestion according to procedures from the In-Gel Tryptic Digestion Kit (ThermoFisher Scientific, product number 89871X). Tryptic digested protein samples were filtered with a 0.2 µm filter. Liquid Chromatography-Mass Spectrometry was carried out using an Agilent 6230 TOF LC-MS

instrument and an Agilent Poroshell 120 EC-C18 [5 cm x 3mm, 2.7 μm] column. The following mass spectrometry-grade buffers were used: A1 (5% acetonitrile, 0.1% formic acid), B1 (90% acetonitrile, 0.1% formic acid), A2 (10% methanol), and B2 (90% methanol). Samples were eluted from the column at 0.4 mL/min using the following buffer gradient: 0-80% B1 over 10 min (Table 2). Buffers A2 and B2 were used for the column wash so that the column would be stored in a final solution of 70% methanol (Table 3).

Table 2. LC-MS run method

Time (min, cumulative)	A1	B1
0	100%	0%
0.5	100%	0%
10.5	20%	80%
15.5	20%	80%
16.5	100%	0%

6 min post-run

Table 3. LC-MS wash method for column storage

Time (min, cumulative)	A2	B2
0	100%	0%
5	0%	100%
7	100%	0%
11	0%	100%
13	100%	0%
17	0%	100%
18	22%	78%
20	22%	78%

Results

Recombinant His-tagged TaABF1 can be purified from E. coli cells

Before we could determine whether kinases extracted from wheat aleurone are able to phosphorylate TaABF1, we needed to produce a recombinant histidine tagged form of the protein (His_6::TaABF1). A vector containing the protein construct was transformed into *E. coli* so that the *E. coli* could produce the His_6::TaABF1 protein for purification. After initial *E. coli* cell lysis, His_6::TaABF1 was not soluble in native buffer, a buffer in which the TaABF1 protein should be folded into its native conformation, and was located in the insoluble pellet after centrifugation (Figure 3A). Little to no His_6::TaABF1 remained soluble in native buffer after initial cell lysis.

To solubilize the His_6::TaABF1, we denatured the proteins in the pellet with a guanidinium buffer. Then, we attempted to refold the protein by several modes of buffer exchange back into native conditions to determine which method would optimize soluble protein yield. We attempted to use the ProBond nickel purification system (Invitrogen) under denaturing conditions and hybrid conditions. Hybrid conditions require the use of both denaturing and native buffers. We attempted dialysis for a gradual transition from the guanidinium solution to native conditions, and we attempted to use centrifugal filter tubes (spin columns) for quick buffer exchange from guanidinium to native conditions. The ProBond nickel purification system under hybrid conditions and the dialysis yielded almost no His_6::TaABF1. The spin columns appeared to give the highest soluble His_6::TaABF1 protein yield (though some protein became insoluble again) and this yield was approximately equivalent to using the ProBond nickel column (Invitrogen) under denaturing conditions (Figure 3A). Strangely, the His_6::TaABF1 did not always travel the same distance during the SDS-PAGE separation. For example, the

His_6::TaABF1 traveled further in the post-buffer exchange samples than in the induced *E. coli* or post-lysis pellet samples (Figure 3A).

We chose to continue to use the spin columns for buffer exchange back to native conditions, as this was the simplest method that produced the highest protein yield. However, this method was not always consistent. For example, in native buffer (20 mM Tris, pH 7.4) with a very low solute concentration, His_6::TaABF1 sometimes became insoluble during buffer exchange from denaturing to native conditions (Figure 3B).

To minimize the amount of His_6::TaABF1 that became insoluble in native conditions, a screen of 7 buffers was completed to determine optimum native buffer solute composition for His_6::TaABF1 solubility. The buffers tested were 20 mM Tris (pH 7.4) with 100, 200, or 300 mM NaCl, and 50 mM Tris with 0, 100, 200, or 300 mM NaCl. The charges on the salt ions, in theory, should increase protein stability in solution, thus increasing solubility. For all seven buffers, about half of the His_6::TaABF1 remained in solution after the spin column buffer exchange from denaturing to native conditions (Figure 3C). Adding arginine and glutamine to the purification buffer did not improve solubility (data not shown).

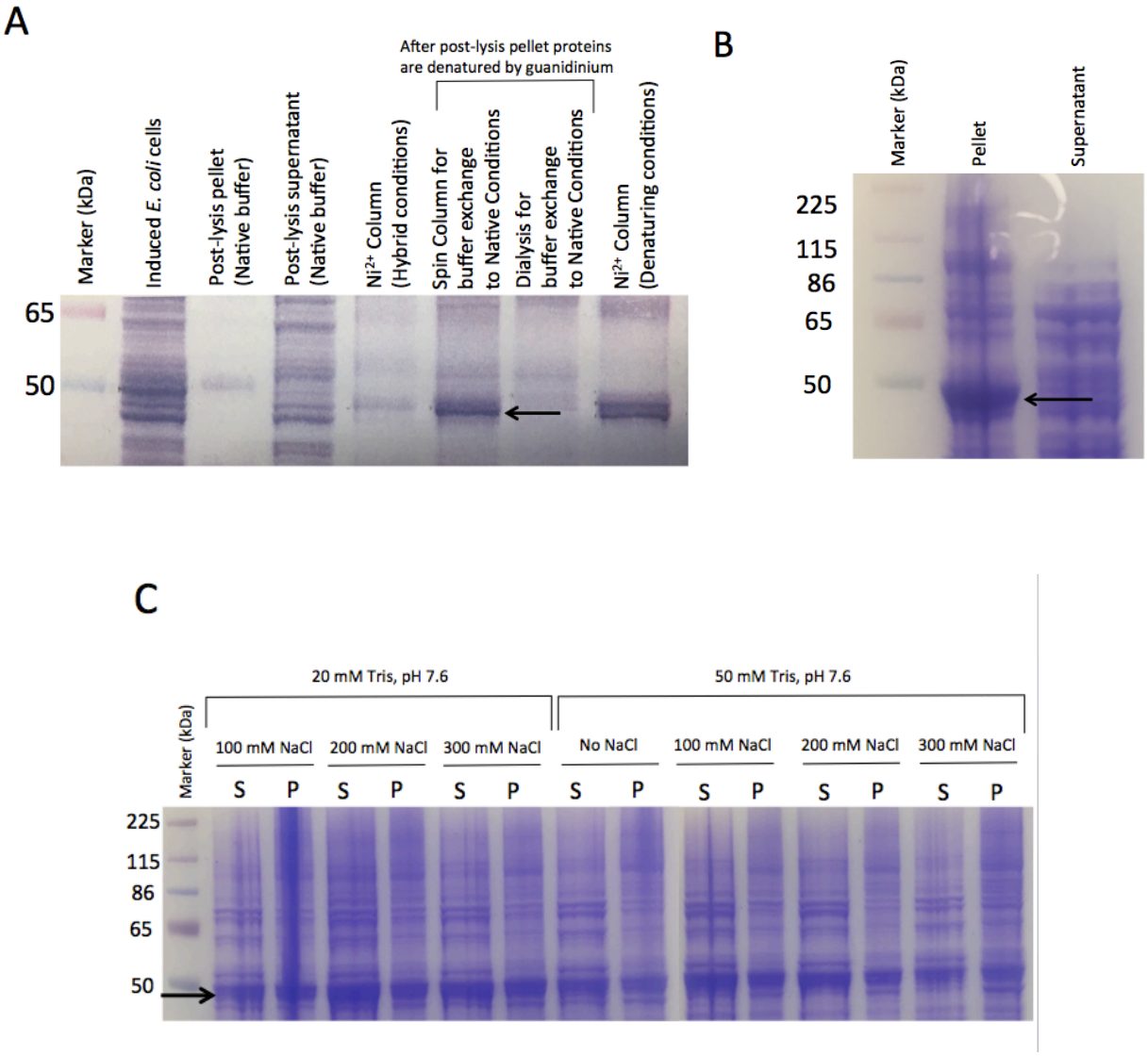

Figure 3. SDS-PAGE analysis for development of TaABF1 purification scheme. **(A)** SDS-PAGE separation of proteins that result from different attempted His_6::TaABF1 purification schemes. Total protein is stained with Coommassie reagent. Supernatant and pellet from the first centrifugation following *E. coli* cell lysis. (post-lysis pellet and post-lysis supernatant) reveal that His_6::TaABF1 is initially insoluble in native buffer. Native conditions: 20 mM Tris, pH 7.4 **(B)** An attempt to refold His_6::TaABF1 by spin column buffer exchange from guanidinium buffer to 20 mM Tris (pH 7.4) in which TaABF1 (black arrow) remained insoluble (pellet). Little, to no TaABF1 remained soluble (supernatant) **(C)** His_6::TaABF1 (black arrow) re-folding by spin column buffer exchange into 7 native buffer types: 20 mM or 50 mM Tris (pH 7.4) with 0 mM, 100 mM, 200 mM, or 300 mM NaCl. About 50% of TaABF1 protein (black arrow) became insoluble during the buffer exchange from denaturing to native conditions. After centrifugation, the soluble purified protein in the supernatant (S) and insoluble purified protein in the pellet (P) were separated on an SDS-PAGE gel.

Next, because the His_6::TaABF1 did not consistently travel the same distance during SDS-PAGE separation and because protein yield when using the nickel columns was sometimes low, we wanted to confirm that His_6::TaABF1 was successfully recovered during the purification process and confirm that the histidine tag was still attached to the protein. Therefore, western blots for TaABF1 and for the histidine tag were completed. Some TaABF1 degradation occurred in all samples, even in the non-lysed *E. coli* cells. However, in all samples, there was a visible TaABF1 band at approximately 50 kDa, the size of full-length His_6::TaABF1, and the histidine tag was present at all locations where the TaABF1 was also present (Figure 4).

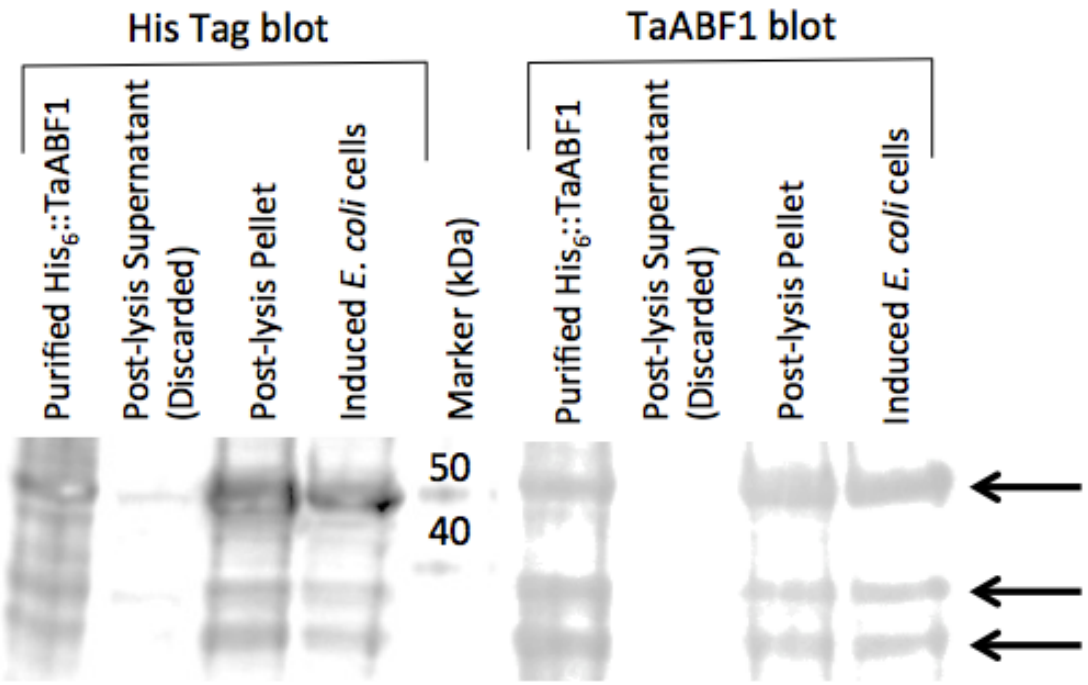

Figure 4. Western blot for TaABF1 and His tag. Western blot analysis was completed to determine the presence of TaABF1 and its histidine tag (both, black arrows) in the induced *E. coli* cells before cell lysis, in the supernatant and pellet after centrifugation following *E.coli* cell lysis, and in the final, purified His_6::TaABF1 sample. Harris et al. demonstrated high specificity of anti-TaABF1 antibodies previously (2013).

Protein can be recovered from the phosphorylation assay

To obtain protein kinases for the phosphorylation assay, the aleurone layers of wheat grains were isolated and ground with a mortar and pestle to produce the aleurone extract that contained wheat protein kinases. The aleurone extract was combined with His_6::TaABF1 and the mixture was incubated to allow potential phosphorylation of His_6::TaABF1 by the kinases. After incubation, protein mixtures were either nickel column re-purified to isolate the histidine-tagged TaABF1 then separated by SDS-PAGE, or were separated by SDS-PAGE without nickel column re-purification (Figure 5). Following separation, the TaABF1 band (Figure 5A, arrow) was excised from the gel, subject to tryptic digestion, and then analyzed by liquid chromatography-mass spectrometry (LC-MS). The recovery shown (Figure 5A, arrow) was the greatest yield of TaABF1 obtained after the nickel column re-purification.

Recovery from the phosphorylation assay, however, was not consistent. The same procedures were followed for each re-purification, but there frequently was not enough TaABF1 recovered during re-purification to visualize a TaABF1 band after SDS-PAGE separation (Figure 5B, box). There was sometimes enough His_6::TaABF1 protein present after the phosphorylation assay in the non-re-purified samples to be visualized on the gel (Figure 5B, arrow), so that the band could be excised for tryptic digestion, but this band was not always present (Figure 5A). Lastly, some aleurone proteins that are about 50 kDa, the size of His_6::TaABF1, bound to the nickel column and eluted from the column with His_6::TaABF1 during the final elution (figure 5).

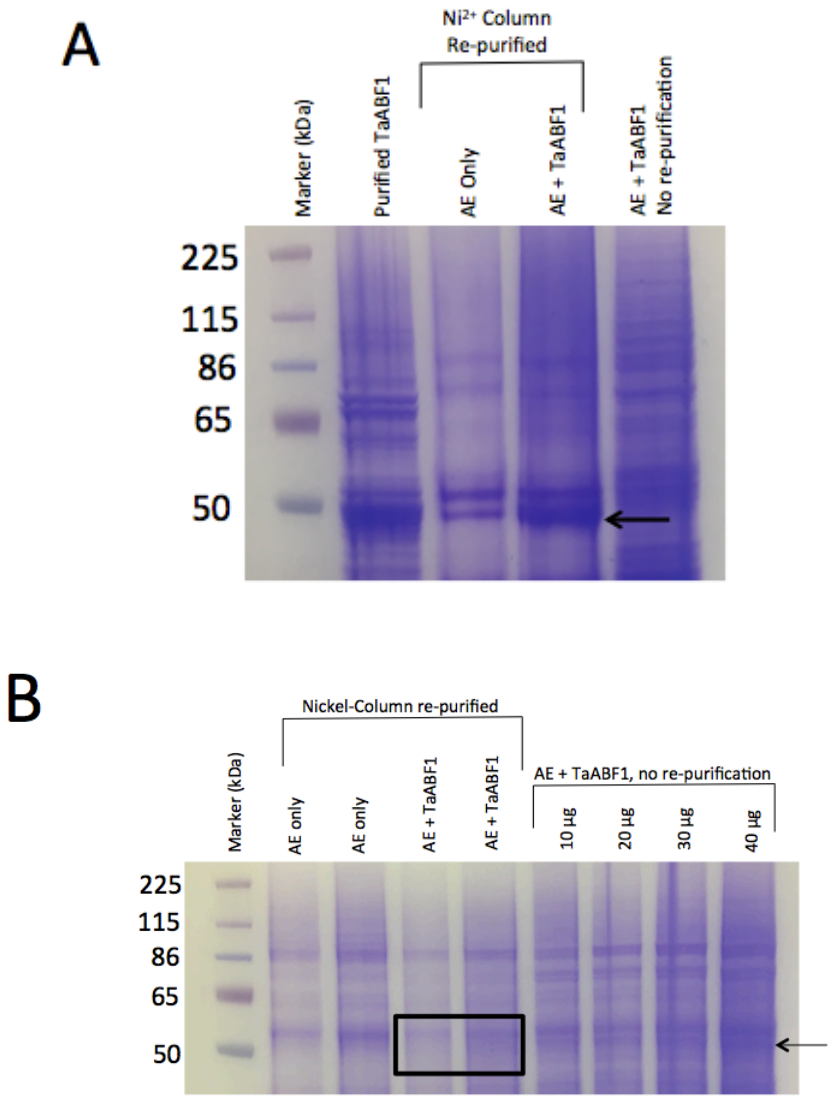

Figure 5. SDS-PAGE separation after phosphorylation assay. Control samples with only aleurone extract (AE) and samples of AE mixed with His_6::TaABF1 were re-purified with a nickel column (Ni^{2+} re-purified) before SDS-PAGE separation. An additional sample of AE mixed with His_6::TaABF1 was not re-purified and was separated on the SDS-PAGE gel from the original mixture. **(A)** Representative gel from nickel column re-purification during which His_6::TaABF1 was recovered. His_6::TaABF1 (black arrow) from the re-purification was excised from the gel for tryptic digestion and LC-MS analysis. For comparison, the original purified, un-phosphorylated His_6::TaABF1 was run in lane 2. **(B)** Representative SDS-PAGE gel from when His_6::TaABF1 was not recovered during the re-purification process (black box). Here, a faint His_6::TaABF1 band can be seen in the non-re-purified sample (black arrow), which was excised from the gel for tryptic digestion.

Test peptides and peptides from tryptic digestion of BSA can be identified by LC-MS

To design an LC-MS protocol that could be used to successfully identify protein fragments, we first analyzed synthetic test peptides. The synthetic test peptides, with known masses and known concentrations, were successfully identified. Numerous synthetic test peptides were analyzed, and results from a representative mixture of three synthetic test peptides T145, T368, and P22 (Table 4) are presented (Figure 6). The peaks for each of these three peptides are distinct in the total ion chromatogram (Figure 6A), and expected masses (Table 4) were observed for all three synthetic peptides. As an example, an enlargement of the mass peaks for the T145 test peptide is provided (Figure 6B). For the synthetic test peptides, the strongest LC-MS signals resulted from loading 2.0 µg of protein, and a significant signal was still visible when 0.5 µg was loaded, amounts that should also be attainable with successful nickel-column re-purification of His_6::TaABF1.

Table 4. Representative synthetic test peptides for LC-MS analysis

Test Peptide	Amino Acid Sequence	$(m + H)^+$	$(m + 2H)^{2+}$
T145	CAARPSQQPPVQPSVPA	1732.0	866.5
P22	RDVRTKEHFAVKFIERGHK	2353.3	1177.15
T368	EQSKENVNAKKGAPLSR	1855.99	928.49

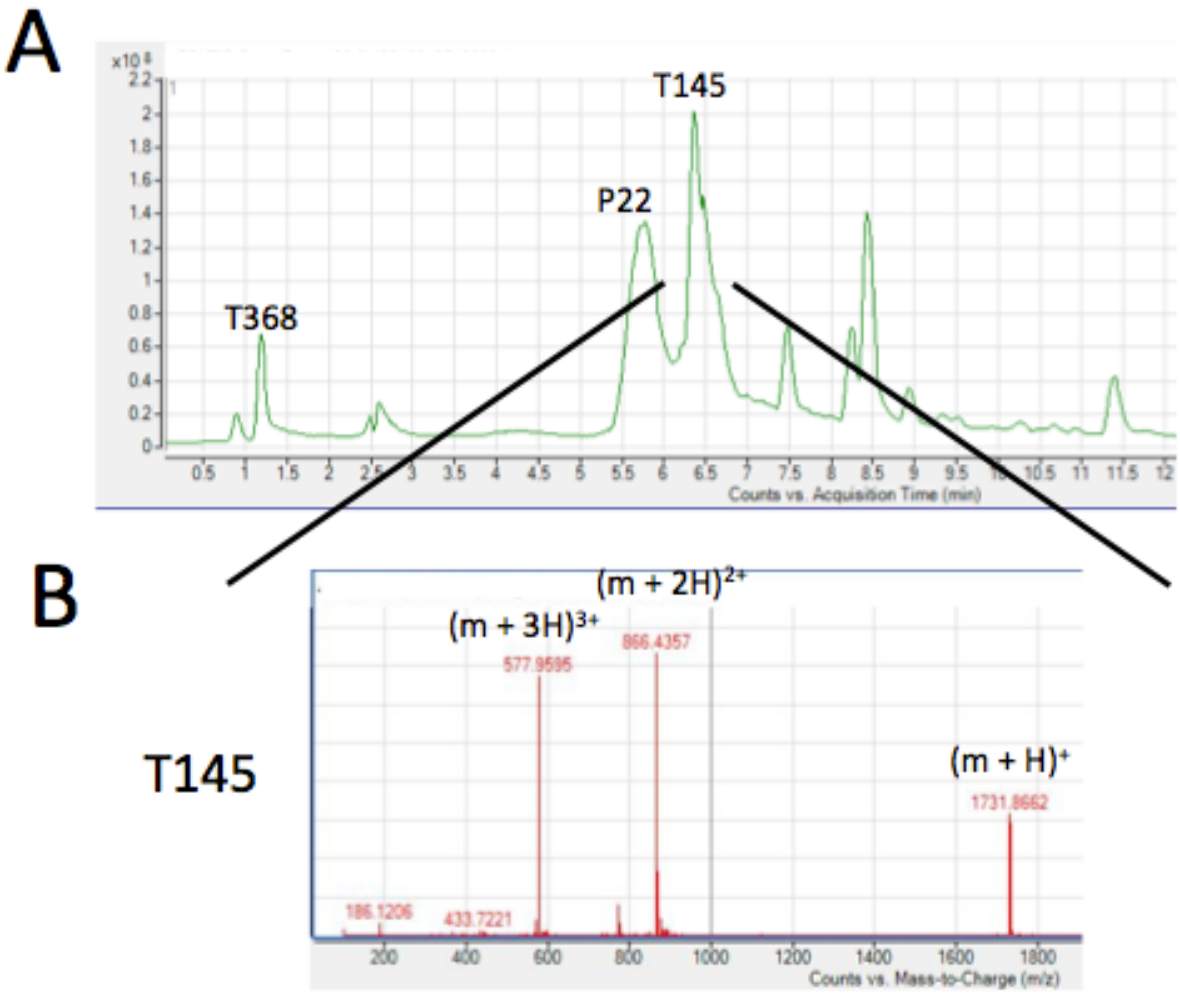

Figure 6. Representative analysis of synthetic test peptides by LC-MS. (A) Total ion chromatogram with peaks for T368, P22, and T145 synthetic peptide, eluting t 1.25, 5.5, and 6.5 minutes, respectively. **(B)** Mass peaks for the T145 synthetic test peptide.

To further test the tryptic digestion and LC-MS protocol, we digested a control protein, bovine serum albumin (BSA), with trypsin and analyzed the peptide fragments by LC-MS. Expected BSA fragments were identified with an average mass accuracy to the thousandths place of the mass to charge ratio (Table 5).

Table 5. Observed and expected masses for BSA tryptic digestion fragments

Peptide	Observed m/z	Expected m/z	Difference
B29	356.6841	356.6905	-0.0064
B66	582.3109	582.3189	-0.008
	1163.6126	1163.6306	-0.018
B101	545.33	545.3405	-0.0105
B161	464.2489	464.2503	-0.0014
	927.4908	927.4934	-0.0026
B198	379.7083	379.7151	-0.0068
	758.4102	758.4229	-0.0127
B205	649.3179	649.3338	-0.0159
B223	353.678	353.6812	-0.0032
	706.346	706.3552	-0.0092
B236	689.366	689.3729	-0.0069
B249	461.7432	461.7476	-0.0044
B257	789.4571	789.4716	-0.0145
B341	752.349	752.3573	-0.0083
B402	653.3555	653.3617	-0.0062

Purified TaABF1 is not phosphorylated in E. coli and can be identified by LC-MS

To ensure that we could use LC-MS to identify TaABF1, not just BSA or synthetic test peptides, we analyzed the peptides that result from the tryptic digestion of purified, un-phosphorylated His_6::TaABF1. For the purified un-phosphorylated His_6::TaABF1, most, but not all, expected protein fragments could be identified by LC-MS (Table 6). Further, LC-MS analysis of the un-phosphorylated protein confirmed that the *E. coli* cells do not phosphorylate His_6::TaABF1. Specifically, all fragments containing the key serine residues (Figure 7A), S36, S37, S113, and S115, could be detected and these residues were neither singly nor doubly phosphorylated (Figure 7C-E).

Table 6. Observed and expected masses for un-phosphorylated TaABF1 tryptic digestion fragments

Peptide	Observed m/z	Expected m/z	Difference
T11	439.8799	439.869	0.0109
	659.3132	659.3035	0.0097
T22	456.9050	456.9088	0.0038
	684.8661	684.8595	0.0066
	1368.7189	1368.7117	0.0072
T35	796.3840	796.3849	0.0009
	1194.0712	1194.0774	0.0062
T111	715.8663	715.8548	0.0115
T164	838.4639	838.4633	0.0006
T179	501.3145	501.3143	0.0002
T353	344.7385	344.7338	0.0047
	688.4696	688.4603	0.0093
T360	747.4153	747.4069	0.0084

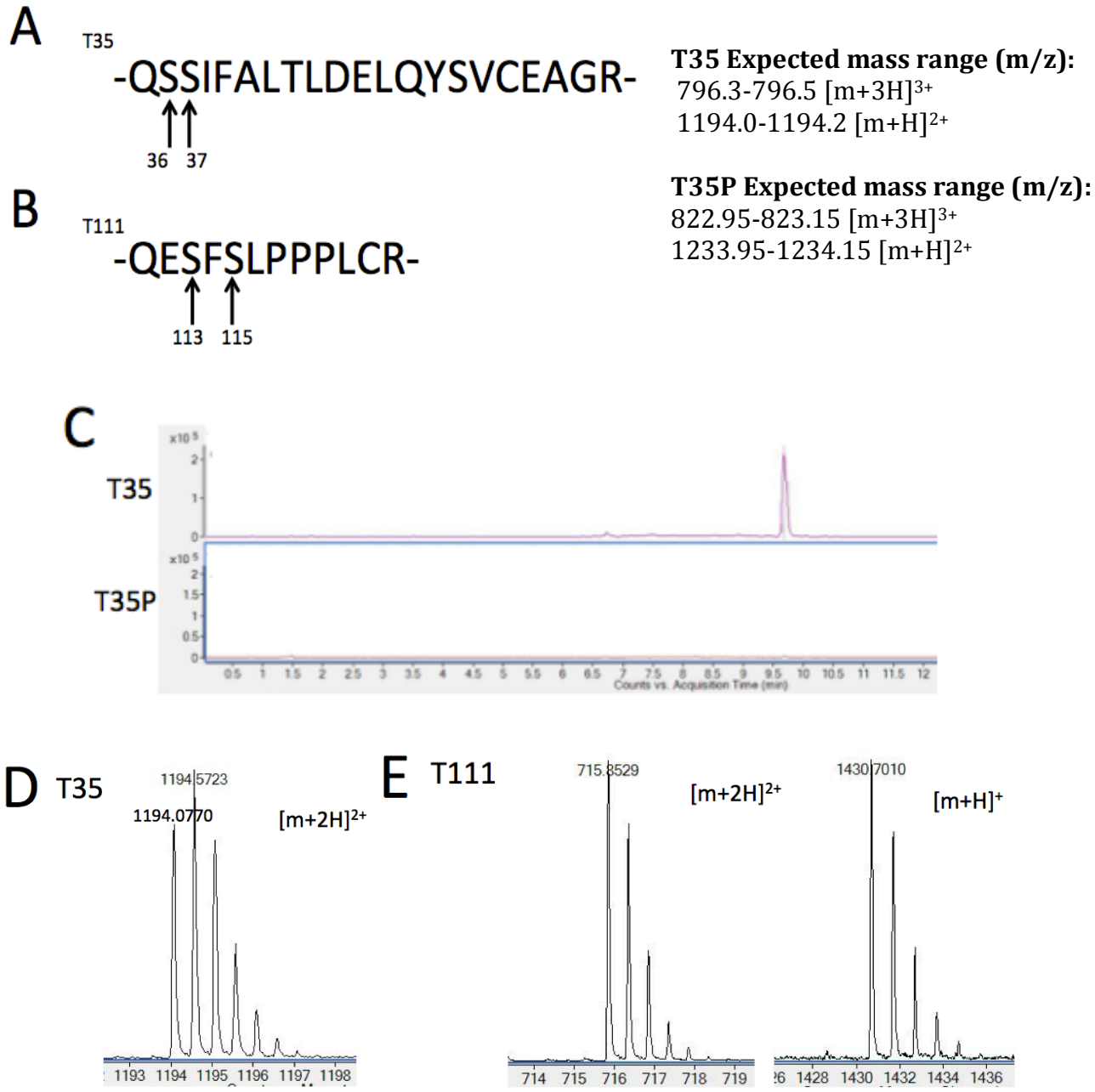

Figure 7. LC-MS analysis of peptide fragments after TaABF1 tryptic digestion. (A) Digestion fragment T35. **(B)** Digestion fragment T111. **(C)** Representative extracted ion chromatograph for T35 expected mass range (796.3-796.5 m/z 1194.0-1194.2 m/z), and singly phosphorylated (T35P) expected mass range (822.95-823.15 m/z+; 1233.95-1234.15 m/z). There is no T35P peak present. Extracted ion chromatograph for doubly phosphorylated T35PP also shows no peaks in the expected mass range (849.6-849.8 m/z; 1260.6-1260.8 m/z) (data not shown) **(D)** Counts vs. mass to charge ratio for T35 fragment shows mass peaks for un-phosphorylated S35, S36. There were no mass peaks for phosphorylated S35, S36. **(E)** Counts vs. mass to charge ratio for T111 fragment with mass peaks for un-phosphorylated S113, S115. There were no mass peaks for phosphorylated S113, S115.

We have yet to identify TaABF1 by LC-MS after the phosphorylation assay

LC-MS analysis after the phosphorylation assay has proven to be more difficult. Several attempts have been made to analyze the His_6::TaABF1 recovered after the phosphorylation assay. We have used TaABF1 bands excised from the post-phosphorylation assay SDS-PAGE separation for both nickel column re-purified TaABF1 as well those from the non-re-purified samples. However, no TaABF1 peptides were detected by LC-MS in any of these trials. In one trial, there was not an internal control peak at 842.51 (m/z, m+H) for the trypsin fragment that should result from trypsin proteins digesting other trypsin proteins, suggesting that the trypsin did not cleave. However, this internal control peak was present for all other trials.

Discussion

The activity of many plant ABFs is controlled by phosphorylation (Casaretto and Ho 2005; Chae et al. 2007; Kobayashi et al. 2005; Shutz et al. 2008), and ABFs share high levels of sequence consensus and predicted structural/functional homology (Li et al. 2016). Thus, it is highly probable that the activity of TaABF1 is also regulated via post-translational phosphorylation. It has been shown that, in response to varying hormone conditions in cereal grains, the phosphorylation status of TaABF1 may change (Johnson 2008) and that mimicked phosphorylation of S113 inhibits TaABF1 activity while mimicked phosphorylation of S36, S37, S113, and S115 increases TaABF1 activity (Johnson, unpublished data). However, it is still unclear whether phosphorylation occurs at these (or other) specific residues *in vivo*.

As we work to determine *in vivo* TaABF1 phosphorylation status, we were able to express a recombinant histidine tagged version of the TaABF1 protein in *E. coli*. However, it has

proven to be difficult to purify His_6::TaABF1, as it is initially insoluble in native buffer conditions (Figure 3A). We are able to solubilize His_6::TaABF1 somewhat by denaturing it in guanidinium buffer then using spin columns for a buffer exchange back to native conditions. Other attempted re-folding methods included purification using the ProBond nickel column system under denaturing or hybrid conditions, and removal of guanidinium by dialysis, but the spin columns were both the easiest method and produced the highest soluble protein yield (Figure 3A). With all methods, including the spin column method, some His_6::TaABF1 protein still becomes insoluble during the buffer exchange.

The solubility of His_6::TaABF1, however, was inconsistent in native conditions (Figure 3B). When determining native buffer conditions, our aim was to refold the His_6::TaABF1 in a buffer that contained the lowest possible Tris and NaCl concentrations necessary for His_6::TaABF1 to be soluble. We sought low concentrations of Tris and NaCl for two reasons: first, to mimic the environment within the cereal grain and second to avoid interfering with kinase activity during the phosphorylation assay. Increased solute concentrations, including high NaCl concentrations, have previously been shown to decrease the activity of some kinases, even in moderate concentrations of less than 200 mM NaCl (Ugwu and Apte 2004). However, negatively charged ions are necessary for the stability of some proteins in solution, including His_6::TaABF1, which does not remain very soluble in 20 mM Tris (pH 7.4) without NaCl (Figure 3B). Plant cells, however, do not utilize NaCl and their growth can be inhibited by high sodium concentrations; thus, they have very low NaCl concentrations (Blumwald et al. 2000). Therefore, the goal is to mimic the low-NaCl environment of a plant cell with the native buffer and simultaneously keep His_6::TaABF1 in solution.

Next, we compared a few different native buffers in an attempt to increase protein solubility and maximize protein yield. Adding arginine and glutamine, a method that has been shown to improve protein solubility and long-term stability (Golonav et al. 2004), did not improve the solubility of His_6::TaABF1. We then examined buffers with different solute concentrations of Tris and NaCl (Figure 3C). Though all of these buffers allowed only about half of the His_6::TaABF1 to remain soluble, we selected 20 mM Tris (pH 7.4) with 100 mM NaCl for native conditions. It was the buffer with the lowest solute concentrations of those tested, and the concentration and protein yield for this buffer was only slightly less than that of the buffer with the highest solute concentrations; therefore it was selected over other buffers. It is important that we maximize protein yield and concentration of purified His_6::TaABF1 so that we can increase the likelihood of recovering protein from the phosphorylation assay. If we start the phosphorylation assay with more His_6::TaABF1, then it is more likely that we will be able to recover enough protein during the re-purification process for successful LC-MS analysis.

We then sought to confirm that His_6::TaABF1 was present in the final soluble, purified protein solution and that the histidine tag was not being cleaved. We were initially concerned that the histidine tag might have been cleaved, because His_6::TaABF1 appeared to travel slightly different distances when separated on SDS-PAGE depending on the sample that was run (Figure 3A). Western blotting confirmed that TaABF1 and the histidine tag were present, and that the histidine tag was at all of the same locations as the TaABF1 protein, suggesting that the tag is not being cleaved (Figure 4). The un-cleaved tag is necessary for successful nickel-column re-purification to recover the His_6::TaABF1 after the phosphorylation assay. The western blot revealed slight degradation of His_6::TaABF1 present at all stages from the induced *E. coli* cells to the final purified product, but the dominant species still appeared to be the ~50 kDa, full-

length His_6::TaABF1. Because guanidinium affects protein separation by SDS-PAGE, it is probable that the slightly different migrations of His_6::TaABF1 on the gel were caused by the buffer exchanges yielding slightly different guanidinium concentrations in the different samples.

The purified His_6::TaABF1 was then incubated with kinases that were extracted from the aleurone layer of imbibed wheat grains that had not been exposed to GA or ABA, to determine whether the kinases would phosphorylate the His_6::TaABF1. After the phosphorylation, we compared the recovery of His_6::TaABF1 either by re-purifying using nickel affinity chromatography and separating proteins by SDS-PAGE or by separating proteins by SDS-PAGE without re-purification (Figure 5). We found that protein could be visualized on a gel more consistently without nickel column re-purification, but that it was not enough protein to be able to detect significant LC-MS peaks after tryptic digestion. The nickel column re-purification was less consistent, and the His_6::TaABF1 bands were sometimes very faint or not present. Even when a visible His_6::TaABF1 band appeared on the gel after the nickel-column re-purification, it was not enough protein to detect by LC-MS after tryptic digestion. The nickel column re-purification may be inconsistent due to protein loss during wash steps and varied starting concentrations of purified His_6::TaABF1 in the phosphorylation assay. Some loss of protein during re-purification is expected and unavoidable, but too-low recovery may be overcome by using a larger starting concentration of His_6::TaABF1or a larger starting volume for the entire phosphorylation assay, so that there is a greater chance that, despite protein loss, more total protein will be obtained from the re-purification. Alternatively, we may want to experiment with different buffers in an attempt to reduce the presence of contaminating aleurone proteins in the final elution from the nickel columns. If we have fewer contaminating proteins, then when we

load a specific amount of protein on a gel, we will be confident that a larger proportion of these proteins are His_6::TaABF1 and not contaminating proteins.

Although we have not successfully identified phosphorylated His_6::TaABF1 using LC-MS as a result of challenges during re-purification and one trial in which the trypsin did not appear to cleave any protein, we have successfully identified synthetic test peptides (Table 4, Figure 6) as well as the peptides that result from the tryptic digestion of BSA (Table 5) and purified, un-phosphorylated His_6::TaABF1 (Table 6, Figure 7). Because we can identify these peptides, we know that our LC-MS procedure should allow us to detect the peptides of trypsin-digested His_6::TaABF1 after the phosphorylation assay, assuming that we are able to recover enough protein from the phosphorylation assay and assuming that the trypsin properly cleaves.

Importantly, we are able to identify the T35 and T111 peptides from the tryptic digestion of purified, un-phosphorylated His_6::TaABF1. These peptides contain the serine residues of interest—S36, S37, S113, and S115—and are un-phosphorylated. Because these peptides are un-phosphorylated after initial His_6::TaABF1 purification, we know that the *E. coli* are not phosphorylating the protein and that any phosphorylation that we may observe after the phosphorylation assay would be due to the aleurone kinases (Figure 7C-E). Thus, the procedures for LC-MS should prove to be successful once we are able to recover a greater amount of protein from the phosphorylation assay.

Thus far, we have shown that His_6::TaABF1 is able to be purified and solubilized, and that peptides from the tryptic digestion of purified His_6::TaABF1 can be identified by LC-MS and are not phosphorylated. We are still working to recover enough His_6::TaABF1 after the phosphorylation assay to determine the phosphorylation status of His_6::TaABF1 after exposure to kinases from the aleurone layer of the wheat grain. The next step, after optimization of the re-

purification yield, is to experiment with the addition of GA and/or ABA to wheat grain imbibing buffer to see if the presence of these hormones has an effect on the phosphorylation status of the His_6::TaABF1. In longer-term future directions, we will work to identify which kinases, such as PKABA1 or other SnRK2s, are responsible for the phosphorylation of TaABF1.

Broadly, in order to understand the mechanisms of TaABF1 regulation and how TaABF1 responds to hormonal changes within cereal grains, we want to identify whether TaABF1 residues are phosphorylated *in vivo*, which TaABF1 residues are phosphorylated, which kinases are responsible for the phosphorylation, and how that phosphorylation may affect TaABF1 function. Such an understanding could prove useful in elucidating how an environmental stress stimulus can affect hormonal changes within cereal grains and how those changes, in turn, could cause a response in ABFs. As a result of this knowledge, we could devise new mechanisms of agricultural crop control, and hopefully, be able to produce larger cereal grain crop yields.

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