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ABI5-binding proteins (AFPs) alter transcription of ABA-induced genes via a variety of interactions with chromatin modifiers

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Abstract

Key message **Overexpression of ABI5/ABF binding proteins (AFPs) results in extreme ABA resistance of seeds via multiple mechanisms repressing ABA response, including interactions with histone deacetylases and the co-repressor TOPLESS.**

Abstract Several ABI5/ABF binding proteins (AFPs) inhibit ABA response, resulting in extreme ABA resistance in transgenic Arabidopsis overexpression lines, but their mechanism of action has remained obscure. By analogy to the related Novel Interactor of JAZ (NINJA) protein, it was suggested that the AFPs interact with the co-repressor TOPLESS to inhibit ABA-regulated gene expression. This study shows that the AFPs that inhibit ABA response have intrinsic repressor activity in a heterologous system, which does not depend on the domain involved in the interaction with TOPLESS. This domain is also not essential for repressing ABA response in transgenic plants, but does contribute to stronger ABA resistance. Additional interactions between some AFPs and histone deacetylase subunits were observed in yeast two-hybrid and bimolecular fluorescence assays, consistent with a more direct mechanism of AFP-mediated repression of gene expression. Chemical inhibition of histone deacetylase activity by trichostatin A suppressed AFP effects on a small fraction of the ABI5-regulated genes tested. Collectively, these results suggest that the AFPs participate in multiple mechanisms modulating ABA response, including both TOPLESS-dependent and -independent chromatin modification.

Keywords ABA-INSENSITIVE(ABI5) · AFP · Arabidopsis · Germination · Histone deacetylase · Chromatin

Abbreviations

ABA	Abscisic acid
ABI	ABA-insensitive
AFP	ABI five binding protein
ABF/AREB	ABRE binding factor
ABRE	ABA-responsive element
AD	GAL4 activation domain
Em6	*Arabidopsis* early methionine-labelled 6
BiFC	Bimolecular fluorescence complementation
BD	GAL4 binding domain
bZIP	Basic leucine zipper
EAR domain	Ethylene-responsive element binding factor-associated amphiphilic repression domain
GM	Germination media
HDA or HDAC	Histone deacetylase
Lea	Late embryogenesis abundant
Min	Minimal media
MODD	Mediator of OsbZIP46 deactivation and degradation

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NINJA	Novel Interactor of JAZ
RAB18	Responsive to ABA 18
SAP18	Sin3-associated protein 18
TPL/TPR	Topless and topless-related
TSA	Trichostatin A

Introduction

The phytohormone abscisic acid (ABA) promotes production of mature, desiccation tolerant seeds, and inhibits their subsequent germination under conditions unfavorable for seedling growth, two processes critical for reproductive success (reviewed in Finkelstein 2013). The transition from seed development to vegetative growth is accompanied by massive shifts in gene expression as the germinating seedlings mobilize the reserves accumulated during maturation and prepare to become photosynthetic. These shifts are mediated by a combination of chromatin remodeling and changes in available transcription factors. Abscisic acid signaling in this process involves interactions among positive regulators of gene expression and negative regulators of their stability or action. For example, positive regulators include the ABA-INSENSITIVE(ABI)5/ABA Response Element (ABRE)-binding factor (ABF/AREB) clade of basic leucine zipper (bZIP) transcription factors that are induced by ABA produced during seed development or in response to dehydrating stresses. These bZIPs must be phosphorylated to be active and are inhibited by protein phosphatases and ubiquitin ligases that affect their activity and stability, respectively (Supp. Fig. 1).

ABA response is also inhibited by a small family of ABI5 interacting proteins, designated ABI5 Binding Proteins (AFPs). These AFPs were identified through yeast two-hybrid screens using ABI5 as "bait" (Garcia et al. 2008; Lopez-Molina et al. 2003). The AFPs are differentially regulated: all family members are induced by ABA or stresses such as salinity or dehydration, but at different stages in seed or seedling development (Garcia et al. 2008). AFPs also interact directly with additional ABFs/AREBs, and transcripts for several AFPs are regulated by ABI and ABF transcription factors. However, in contrast to the ABI5/ABF/AREB clade of bZIPs, most AFPs act as repressors of ABA and stress responses, possibly acting as indirect sensors of declining ABA levels. The ratio of AFP1 and AFP2 to ABI5 protein is highest at low concentrations of ABA, suggesting that these proteins function in a feedback loop to allow seedlings to escape from ABA inhibition of growth. Although the AFP proteins contain three domains that are highly conserved across multiple plant species, initial bioinformatics studies did not identify any known functional domains that could provide clues to their mechanism of action.

To determine whether AFPs altered ABI5 expression or accumulation, ABI5 transcript and protein levels were measured in seeds and seedlings with loss or gain of AFP function. Although both of these genotypes had similar levels of ABI5 transcripts as wild-type, the AFP1 over-expressors were ABA resistant and had much lower levels of ABI5 protein, whereas *afp1* mutants were hypersensitive to ABA and had increased ABI5 accumulation (Lopez-Molina et al. 2003). Most of these measurements compared seedlings that had already germinated with seeds whose germination was inhibited, such that they did not distinguish between reduced ABI5 levels as a cause or effect of germination. However, the apparent half-life of ABI5 nearly doubled in the (hypersensitive) *afp1* mutant and decreased in the over-expression line, suggesting that AFP destabilized ABI5, presumably by promoting its degradation via the proteasome. In contrast, similar studies with a different *afp1* allele and with *afp2* mutants did not detect any differences in ABI5 accumulation before germination was completed (Garcia et al. 2008). Furthermore, the latter studies showed that AFP2 and ABI5 proteins are both nuclear-localized and stable when ABA is present, indicating that more than co-localization is required to promote ABI5 degradation (Garcia et al. 2008).

More recently, characterization of a homolog of the AFPs, Novel Interactor of JAZ (NINJA), raised the possibility of a different mode of action by demonstrating that NINJA acts as a transcriptional co-repressor via interactions with TOPLESS (TPL) through NINJA's Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) domain (Pauwels et al. 2010). Two of the AFPs also interacted with TPL in a yeast two-hybrid assay, suggesting that they might also act in transcriptional repression. TPL interacts physically with diverse transcriptional repressors, histone deacetylases (HDACs), and at least genetically with a histone acetyl transferase, suggesting that it acts as a co-repressor by modifying chromatin structure (Causier et al. 2012; Kagale and Rozwadowski 2011; Ke et al. 2015; Liu et al. 2014; Tai et al. 2005). This hypothesis was supported by a recent study in rice showing that a rice homolog of the AFPs, MODD (Mediator of OsbZIP46 deactivation and degradation), negatively regulates ABA response by interactions with both a TPR/HDA complex and OsbZIP46, the closest homolog to Arabidopsis ABF1, resulting in decreased histone acetylation of OsbZIP46 target genes (Tang et al. 2016). MODD also interacted with an E3 ligase leading to decreased stability of OsbZIP46. The combined effect of these interactions was to attenuate ABA response, similar to the role of the AFPs in Arabidopsis.

In this paper, we have directly tested whether the AFPs act as transcriptional repressors in a heterologous system, using reporter genes in yeast regulated by either constitutive

yeast promoters or ABF-inducible ABRE-regulated promoters from Arabidopsis. Our studies show that, although all four AFPs share the EAR domain implicated in interaction with TPL, only three of these AFPs have significant intrinsic repressor activity. Furthermore, the EAR domain is not necessary for repressing either the constitutive promoter or ABF-activation of ABRE-containing promoters in yeast. Consistent with this, overexpression in Arabidopsis of either AFP1 or AFP2 lacking their EAR domains is sufficient to confer resistance to ABA inhibition of germination. In addition, we found that AFP2 can interact directly with histone deacetylase subunits, as well as with the other AFPs and itself, providing another possible mechanism for modifying gene expression.

Materials and methods

Yeast transcriptional repression assays

The yeast cell line Y122 was a gift from Dr. Xinnian Dong (Duke University). The GAL4 binding domain vectors pGBD and pAS2 were converted from trp selection to his selection by targeted recombination in yeast co-transformed with the vector linearized within the TRP1 gene and a PCR product comprising the HIS3 gene flanked by sequences homologous to the TRP1 gene (primer sequences listed in Supp. Table 1). Choice of vectors was determined by available full-length cDNAs and absence of restriction sites that would interfere with conversion of trp to his selection. The pAS2 vector can be recombined with cDNAs in the pUNI51 vector (Yamada et al. 2003), by Cre-lox recombination as described at http://signal.salk.edu/pUNI51.html. Partial AFP1 cDNAs were constructed by PCR amplification or restriction digestion of specific domains, which were then subcloned into pUNI51. Gene fusions were transformed into the yeast cell line PJ69-4A (James et al. 1996), using the EZ yeast transformation kit, according to manufacturer's instructions (Bio101, Vista, CA, USA). Y122 yeast carrying vector or fusion clones were grown in YSM lacking histidine (his) and uracil (ura) to mid-log phase, then lysed for beta-galactosidase assays as described at https://labs.fhcrc.org/gottschling/Yeast%20Protocols/Bgal.html. Activities were normalized relative to vector controls in any given assay set. BD-fusion expression was compared by immunoblots with anti-GAL4 DBD (Millipore 06-262), probed with fluorescent anti-rabbit Ab (Licor) and detected with an Odyssey imager.

GAL4 AD fusions to ABI5, ABF1 and ABF3 on a plasmid with a TRP1 gene were constructed previously and transformed into the AP4 background, which could be mated to BY4705 cells carrying ABA-responsive promoter fusions to the lacZ gene on a plasmid complementing ura3 auxotrophy to construct yeast one-hybrid cell lines (Reeves et al. 2011). FLAG-tagged AFPs or AFP domains were constructed in a vector derived from pACT2lox, in which the GAL4 activation domain had been replaced with the FLAG tag from pHB3-FLAG, and transformed into the AP4 cells with the AD-bZIP fusions. Diploid yeast containing all 3 plasmids were selected on YSM lacking trp, leu and ura. The FLAG- fusions were quantified immunologically with anti-FLAG antibodies (Sigma F1804), probed with fluorescent anti-mouse Ab (Licor) and detected with an Odyssey imager, to identify lines with comparable expression of the FLAG-AFP fusions. Domain-specific subclones in the pUNI vector were constructed by ligation of specific fragments produced by either restriction enzyme digestion or PCR.

Yeast two-hybrid constructs and assays

Fusions between the *GAL4* activation domain (AD) and full-length *AFP* cDNAs were constructed using the pACT-2lox vector and pUNI cDNA clones. Translational fusions between GAL4 BD and TPL/TPR coding sequences (Causier et al. 2012) were a gift from Dr. Barry Causier (U. of Leeds). HDA19 and SAP18 cDNAs (U17853 and U12539, respectively) were obtained from the ABRC and recombined with the pACT2lox vector.

BD fusions were transformed into yeast line PJ69-4A selecting for growth on yeast synthetic medium (YSM) without trp. AD fusions were transformed into Y187, selecting for growth on YSM without leu. Interactions were tested by matings between pairs of lines carrying BD- and AD-fusions; following overnight incubation on YPD plates, matings were replica plated to YSM lacking leu and trp to select for diploids or YSM also missing histidine and adenine to screen for diploids that had activated the HIS3 and ADE2 reporter genes.

Bimolecular fluorescence complementation

Split YFP fusions were constructed using the Gateway compatible pSITE-nEYFP-C1 (GenBank Acc# GU734651) and pSITE-cEYFP-C1 (Acc# GU734652) vectors and either Gateway entry clones or PCR products with attL ends added as described in (Fu et al. 2008), following manufacturer's instructions for LR clonase reactions (Invitrogen). Recombinant clones were selected on the basis of replication in *E.coli* TOP10 cells (Invitrogen) and spectinomycin resistance, then transformed into *Agrobacterium tumefaciens* strain GV3101 for infection of plant tissue. For BiFC assays, pairs of *Agrobacterium* lines expressing nEYFP and cEYFP fusions were combined with GV3101 expressing the P19 protein of tomato bushy stunt virus to enhance transient expression, as

described in (Voinnet et al. 2003). Cultures of all bacteria to be used in infiltration were grown overnight in selective media, diluted to an OD_{600} of 1.0 in 10 mM $MgCl_2$, 10 mM MES pH 5.6, 0.2 mM acetosyringone and rocked at room temperature for 2–3 h prior to mixing and infiltration of leaves on 2–3 week old *Nicotiana benthamiana* plants. Fluorescence was scored 2–4 days later, using an Olympus AX70 microscope.

Plant materials and transgenes

All Arabidopsis plants were grown in pots in growth chambers under continuous light at 22 °C. The *afp1-4* (SALK_098122) and *afp2-1* (SALK_131676) mutant lines, both in the Col-0 background, were described in (Garcia et al. 2008). The *abi5-1* and *abi5 abf3* digenic mutants are described in (Finkelstein et al. 2005). The 35S:YFP:AFP fusions were constructed in pEG104 (Earley et al. 2006), using either Gateway entry clones or PCR products with attL ends added as described above. MYC9 fusions were constructed by recombination of pUNI cDNA clones with pKYLX-myc9-loxP (ABRC stock #CD3-677).

Binary plasmids carrying transgenes were introduced into *A. tumefaciens* line GV3101 by direct transformation, followed by selection for growth on kanamycin. Plant transformation of both Col and *afp* mutants was performed using *Agrobacterium tumefaciens* in the floral dip method (Clough and Bent 1998). Transgenic YFP fusion plants were selected on the basis of BASTA resistance and MYC fusion lines were selected by Kanamycin resistance. All lines with confirmed transgene expression were tested for altered ABA sensitivity.

Germination assays

Germination assays were performed with seeds surfaced sterilized in 5% hypochlorite and 0.02% Triton-X solution and then rinsed several times with sterile water before plating on minimal mineral salts (Haughn and Somerville 1986) or germination medium (0.5x Murashige and Skoog salts (Murashige and Skoog 1962) 1% sucrose, 0.05% MES, pH 5.8) containing 0.7% (w/v) agar supplemented with different concentrations of ABA or TSA. Plates were incubated overnight at 4 °C to break residual dormancy and then transferred to 22 °C in continuous light (50–70 $\mu E\ m^{-2}\ s^{-1}$). Germination, identified as radicle emergence, was scored at the indicated time points. Photos of seedlings were taken with an Olympus stereomicroscope, using a Lumenera digital camera with Infiniti software. Images were processed with FiJi (Schindelin et al. 2012).

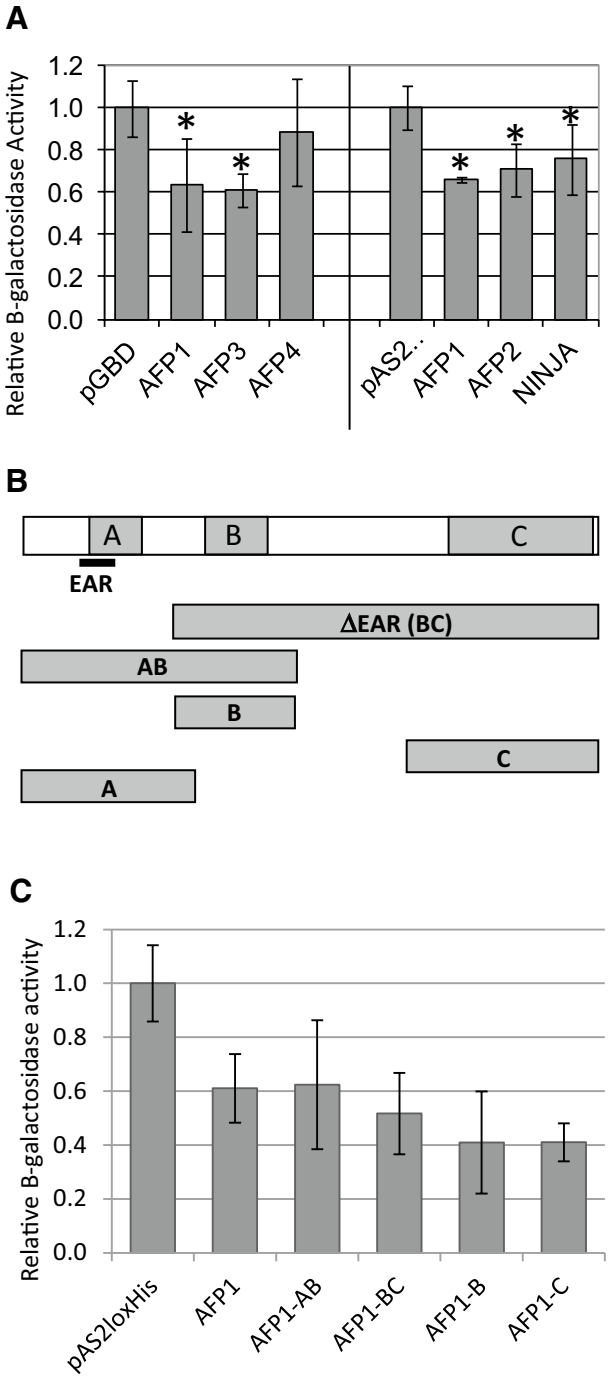
RNA accumulation analysis

RNA was isolated from seeds and seedlings using LiCl and hot phenol extractions, respectively, as described in (Finkelstein et al. 2005). For 1–3 days post-stratification time course in Col seedlings displayed in Supp. Fig. 3B, transcript levels in total RNA (1–5 $\mu g\ lane^{-1}$) were analyzed by Northern blots, as described in (Reeves et al. 2011). Hybridization was quantified by Phosphoimager analysis; abundance of individual transcripts was normalized relative to rRNA present in each lane. For 1–2 days time course and dry seeds of all transgenic lines and their progenitor controls, transcript levels were measured by qRT-PCR. DNA-free total RNA was prepared by incubation with RQ1 DNase (Promega) and RNAsin for 30 min. at 37 °C, followed by inactivation of the DNase by addition of EGTA and incubation for 5 min. at 65 °C, then clean-up over Qiagen RNeasy or Zymo-Clean columns according to manufacturers' instructions. Approximately 0.5 ug of RNA was used as template for cDNA synthesis by MMLV reverse transcriptase (Promega), using a 10:1 mix of random hexamers and oligo dT as primers. cDNA concentrations were checked by qRT-PCR, using EvaGreen master mix (Midwest Scientific) in an iQ5 cycler (Bio-Rad), according to manufacturer's instructions. Primers used for normalizing were selected for uniform expression in seeds or young seedlings subjected to a variety of treatments: AT5G46630 and AT1g13320 (Czechowski et al. 2005). Equal amounts of cDNA were used as templates for reactions with all other primer sets (listed in Supp. Table 1), quantified relative to a standard curve spanning the range of concentrations present in all genotypes, as described in (Carr and Moore 2012).

YFP fusion expression

YFP fusion proteins were viewed in whole seedlings, using an Olympus AX70 microscope. For semi-quantitative analyses, protein extracts were analyzed by immunoblotting. Protein samples were isolated by homogenizing seeds or seedlings in 1X Laemmli buffer (Laemmli 1970) using plastic pestles in microfuge tubes. Samples were centrifuged for 5–10 min at 4 °C to remove debris. Protein samples were separated by SDS–PAGE on 10% polyacrylamide gels, then prepared for immunoblot analysis by electroblotting to a nitrocellulose membrane for 1 h at 4 °C in 25 mM Tris, 192 mM glycine, 20% v/v methanol. Membranes were blocked using Odyssey or Casein blocking buffer (LI-COR Biosciences, Lincoln, NE) and then incubated with primary monoclonal anti-GFP (1:10,000, UBPBio, Aurora, CO) overnight at 4 °C followed by secondary IRDye 800 conjugated affinity purified IgG (mouse) (LI-COR Biosciences) as specified by the LI-COR protocol. Antibody binding was

Fig. 1 Repression of constitutively active gene by BD-AFPs and –NINJA. **a** Full length AFPs introduced as BD fusions in either of two vectors modified to complement HIS auxotrophy: pGBD and pAS2-His. Activities in multiple independent assays were normalized relative to the vector control levels in any given assay set. *Error bars* represent standard deviation of at least two assays performed with triplicate samples. *Statistically different from vector control ($P < 0.01$, based on two-tailed Student's *t* test). **b** Schematic of AFP domain structure and extent of coding sequence in each fusion. Domains A, B and C are as described in Garcia et al. (2008). EAR domain identified by Pauwels et al. (2010) is indicated by *bold underline*. AFP1-ΔEAR = aa 93-345 (end), AFP1-AB = aa1-172, AFP1-A = aa1-95, AFP1-B = aa93-172, AFP1-C = 233-345 (end). **c** Repression by BD fusions to indicated domains of AFP1. Relative expression of fusion proteins is shown in Supp. Fig. 2A, indicating that the –BC domain fusion is slightly underexpressed. *Error bars* represent standard deviation of four assays performed with triplicate samples. Student's *t* test shows all constructs are significantly repressive



visualized using the 800 channel of the Odyssey Infrared Imaging System (LI-COR Biosciences).

Results

Some AFPs have intrinsic repressor activity

The discovery that the AFPs' closest homolog acted in transcriptional repression led us to test whether the AFPs could perform a similar function. We chose to use a heterologous assay system to determine whether the AFPs could act as transcriptional repressors in the absence of any other plant proteins. The yeast cell line Y122 contains a repressor reporter construct comprised of a beta-galactosidase gene under constitutive control of a double GCN4 promoter element that is 72 bp downstream of 5 copies of the GAL4 upstream activating sequence (UAS) (Saha et al. 1993). Potential repressors are targeted to the GAL4 UAS as fusions to the GAL4 DNA binding domain (DBD). Comparison of beta-galactosidase activity with lines containing only a GAL4 DBD showed that all except AFP4 have some repressor activity, resulting in a 30–40% decrease in reporter expression (Fig. 1a). The AFPs share three highly conserved domains, originally designated A, B and C (Garcia et al. 2008); the EAR domain overlaps the A domain (Fig. 1b), e.g. amino acids 44–56 and 46–82, respectively, of AFP1. Mapping of domains within AFP1 required for repression showed that all domains tested could confer repressor function to a GAL4 DBD fusion, but that the EAR domain was not required for this activity (Fig. 1c; Supp. Fig. 2A). Surprisingly, several of the subdomains were more effective repressors than full-length AFP1, suggesting that AFP1 may interact with multiple transcriptional repressors through various domains, which may not all be fully accessible in the full-length fusion.

AFP1 and AFP2 repress bZIP activation of ABRE-regulated genes

To further test whether the AFPs could repress ABA-regulated promoters in a heterologous context, we introduced them into a yeast one hybrid assay with previously characterized reporters and activators. The bZIPs in the ABI5/ABF/AREB clade bind to "G-box" motifs known as ABREs to regulate ABA- and stress-regulated

genes. Previous studies have identified numerous genes expressed in seeds and seedlings that are directly regulated by some members of this bZIP clade (Finkelstein et al. 2005; Nakabayashi et al. 2005; Yoshida et al. 2010), and we have established several yeast one-hybrid reporters comprised of proximal promoters of such genes regulating the lacZ gene (Reeves et al. 2011). Co-expression of a GAL4 AD fusion to any of the ABI5/ABF/AREB clade bZIPs provides a constitutively active transcriptional inducer. To determine whether the AFPs could repress this activation, we expressed FLAG-tagged fusions of AFP1 or AFP2 along with the AD-bZIP fusions and tested their effects on trans-activation of several ABRE-containing promoters (Fig. 2a). These studies showed that both AFPs resulted in a roughly four- to ten-fold decrease in activation of multiple promoters by the ABFs and ABI5. Mapping studies testing the effects of various domains of AFP1 emphasized the importance of the C domain for effective repression by AFP1 (Fig. 2b; Supp. Fig. 2B), consistent with its role in interacting with

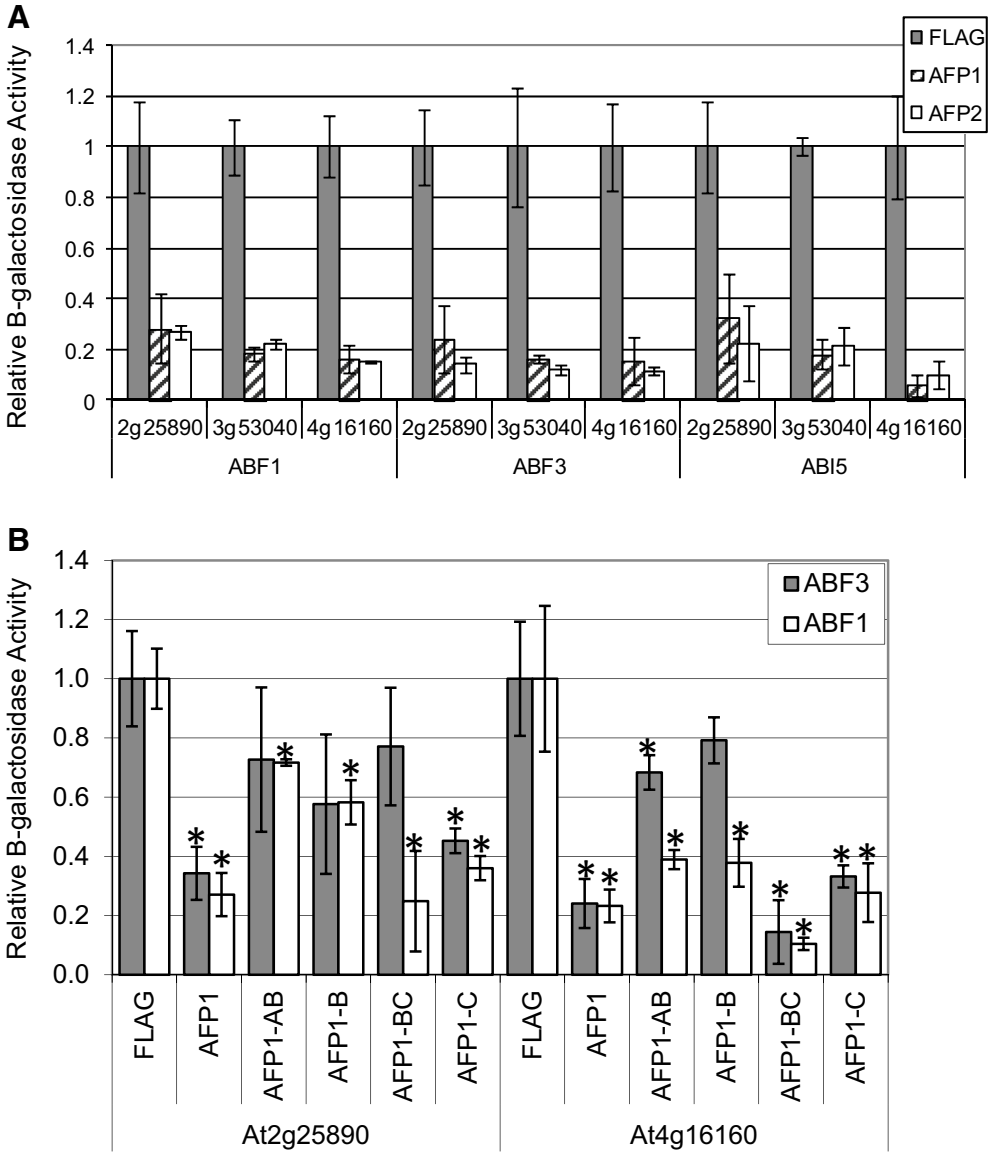


Fig. 2 AFPs repress bZIP-induced activation of ABRE containing pro-lacZ fusions. **a** Relative activity of LacZ reporters regulated by the indicated bZIP-regulated promoters in lines containing FLAG-AFP fusions or the vector control (dACT) and GAL4 AD fusions to the indicated bZIPs (ABF1, ABF3 and ABI5). Activities were normalized relative to the vector control levels in any given assay set. *Error bars* represent standard deviations in at least 3 assays with triplicate samples. **b** Comparison of repression by distinct domains of AFP1 (as defined in Fig. 1 legend) of two reporter fusions activated by either ABF1 or ABF3. *Error bars* represent standard deviations in at least 1 assay of triplicate samples. *Statistically different from vector control ($P < 0.01$, based on two-tailed Student's *t* test)

the bZIPs, whereas constructs containing the AB or B domains had milder repressive effects.

Several possible mechanisms could account for this repression: competition for binding to the promoters of the reporter constructs, sequestering of the bZIPs by binding to the AFPs, or recruitment of additional repressor proteins such as TPL or chromatin modifying proteins. To determine whether the AFPs might bind to these promoters, we tested AD-AFP fusions for possible activation of the reporter constructs, but found that both the AD alone and the AD-AFP1 fusion failed to activate the reporter. Although TPL is only distantly related to the yeast co-repressor Tup1, EAR domain proteins have been found to interact directly with histone deacetylase complex subunits (reviewed in Kagale and Rozwadowski 2011), which are conserved across eukaryotes and therefore likely candidates for mediating repression.

Interactions between AFPs and repressor proteins

AFP2 and AFP3 were previously reported to interact with TPL (Pauwels et al. 2010), and the AFP3/TPL interaction was confirmed in a large-scale yeast two-hybrid screen (Causier et al. 2012). We performed additional yeast two-hybrid tests to determine whether any of the other AFPs could interact with TPL, its homologs (TOPLESS-related, TPRs), or histone deacetylase subunits (Fig. 3). These studies showed strong interactions between TPL and either AFP2 or AFP3, but a weaker interaction between TPL and AFP1. In addition, AFP2 interacted with all four TPR proteins and AFP3 interacted with all except TPR4. AFP1 also interacted very weakly with TPR1, 2 and 3. Although none of the AFPs interacted with HDA19 in yeast two-hybrid assays, AFP2 interacted strongly with the Sin3-Associated Protein (SAP)18 subunit of the histone deacetylase complex. Domain mapping studies indicated that, as expected for interactions with the EAR domain, the A domain of the AFPs was required for interactions with TPL and the TPRs. However, the interaction with SAP18 appeared to require multiple domains of AFP2.

To determine whether the interactions observed in yeast could occur in plants, bimolecular fluorescence complementation (BiFC) experiments were conducted by *Agrobacterium*-infiltration of *Nicotiana benthamiana* using either AFP1 or AFP2 fused to the C-terminus of nEYFP, paired with cEYFP fusions to bZIPs, TPL, or histone deacetylase complex subunits (Fig. 4). These confirmed previously reported interactions between AFP1 and ABF3, AFP2 and ABF1, and both AFPs with ABI5. In addition, AFP1 and AFP2 interacted with TPL in this assay. Both AFPs also interacted weakly with HDA19, and AFP2 interacted with SAP18. All observed interactions were in the nuclei. Collectively, these studies show some specificity of interactions between AFPs, TPL/TPRs and histone deacetylase subunits, and are consistent with formation of repressive complexes containing these proteins.

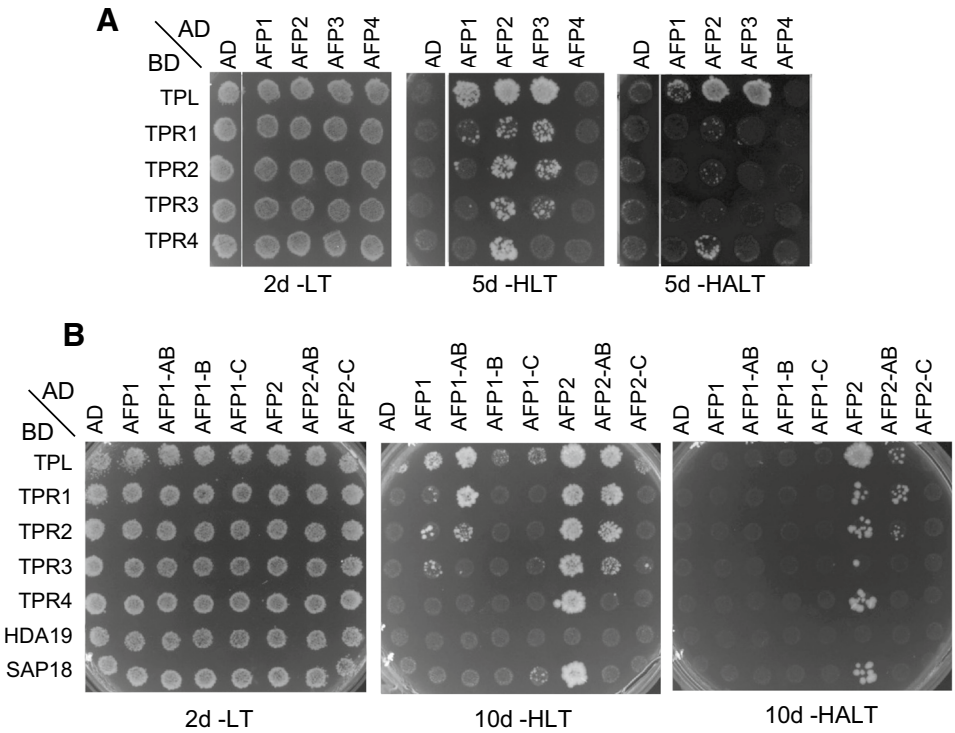


Fig. 3 Yeast two-hybrid interactions between AFPs and TPL/TPRs, SAP18 and HDA19. **a** Interactions between GAL4 AD alone (AD) or fusions to the AFPs and GAL4 BD fusions to TOPLESS (TPL) or its homologs (TPRs). **b** Mapping AFP domains required for interaction with TPL, TPRs, or two subunits of the histone deacetylase complex (HDA19 and SAP18). Growth on –LT selects for diploids carrying both AD and BD fusion vectors. Growth on –HLT or –HALT requires GAL4-dependent activation of the HIS and ADE reporters. AFP1 domains as defined in Fig. 1 legend, AFP2 –AB = aa1-149, AFP2-C = aa149-348 (end)

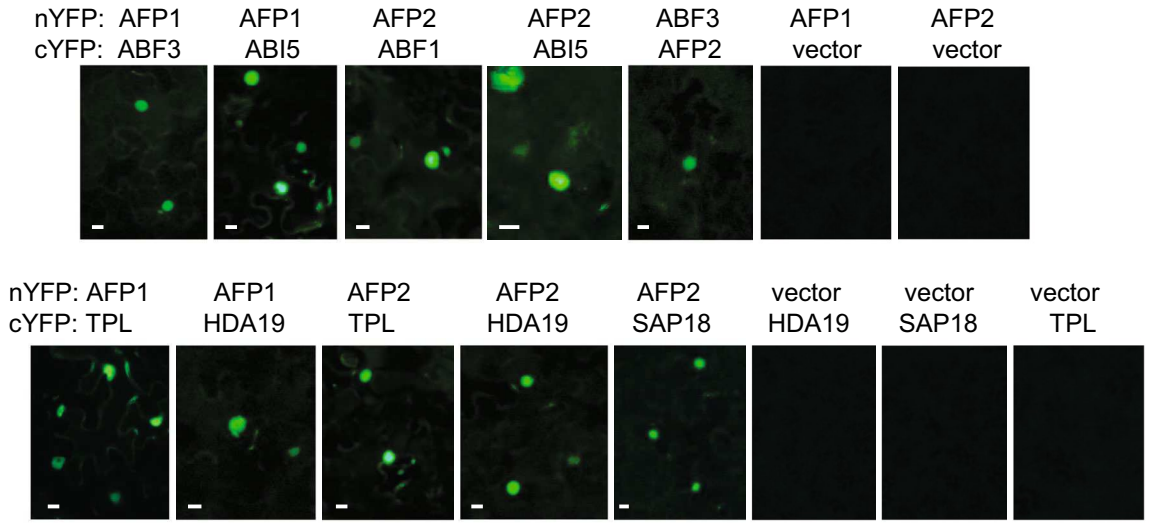


Fig. 4 BiFC interactions between AFPs, bZIPs and chromatin modifying proteins. Agrobacteria carrying plasmids encoding the indicated pairs of fusions to the N-terminal (nYFP) or C-terminal (cYFP) portions of yellow fluorescent protein were co-infiltrated into *N. benthamiana* leaves. Micrographs of the lower epidermis were taken 2–3 days after infiltration. *Bar* 10 µm

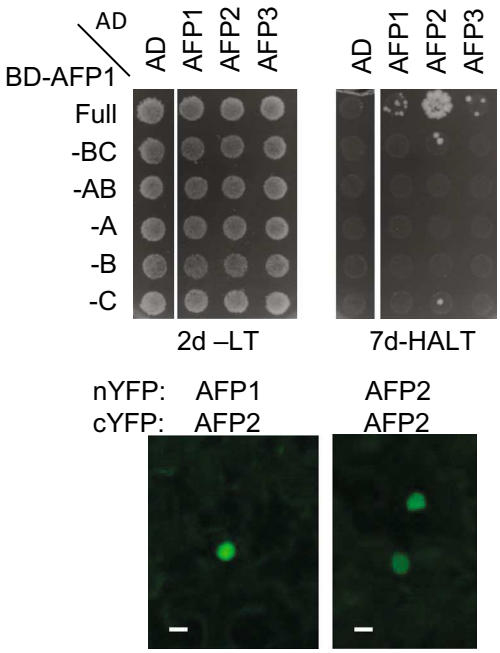


Fig. 5 Yeast two-hybrid and BiFC interactions among AFPs. Abbreviations for fusions and media are the same as in Figs. 3 and 4 legends. *Bar* 10 µm

Homo- and heterodimerization of AFPs

We had previously found that AFP2 interacted strongly with itself, AFP1 and AFP3 in yeast two-hybrid assays (Garcia et al. 2008). AFP1 also forms homodimers and interacts with AFP3 in yeast, but not as strongly as the interactions with AFP2 (Fig. 5). The C domain, previously shown to be required for interactions with ABI5 and other ABF-clade bZIPs, was also required for these interactions, which were slightly enhanced by the B domain. The strongest interactions, AFP2 homodimers and AFP1/2 heterodimers, are also seen in BiFC assays (Fig. 5).

Over-expression of full-length or truncated AFPs in plants confers ABA resistance at germination

Previous studies have shown that AFP1 over-expression lines are resistant to ABA inhibition of germination (Lopez-Molina et al. 2003). To better monitor expression levels and localization, we constructed YFP- and MYC_9- fusions for both AFP1 and AFP2. Most of the YFP fusion lines had much stronger phenotypes than the MYC_9 fusions, producing hemizygous seeds with as much as 200-fold decreased sensitivity to ABA relative to their progenitor lines (Fig. 6a; Supp. Fig. 3). The lines with the strongest phenotypes produced homozygous seeds that failed to complete maturation, resulting in green desiccation intolerant seeds (Supp. Fig. 4). However, these seeds could be rescued by excision and transfer to culture media just prior to complete drying. Surprisingly, ABA resistance was not tightly correlated with AFP protein levels (Fig. 6b). Two possible explanations are: (1) additional factors might be limiting and (2) reduced viability due to excessive accumulation might detract from further enhancing resistance.

The yeast reporter expression assays described above indicated that individual domains of the AFPs were sufficient to reduce expression of these reporters so long as they were targeted to the promoters, by either GAL4-BD fusions or association with bZIPs. To determine whether these

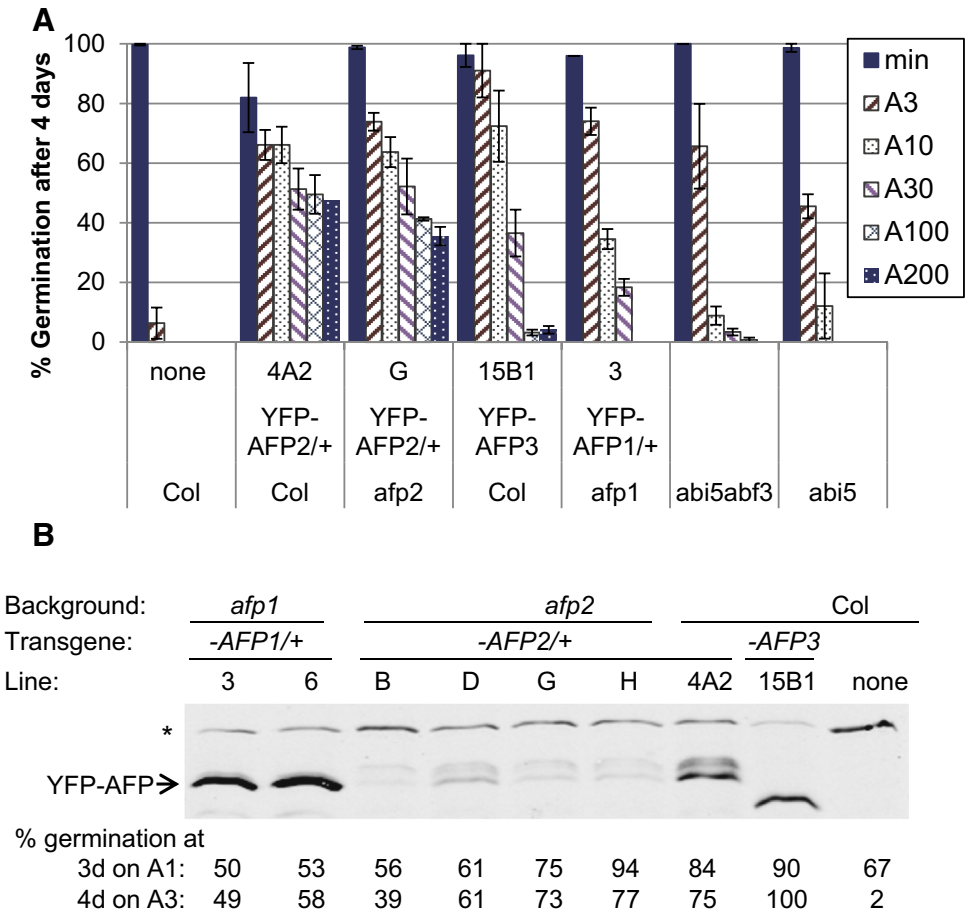


Fig. 6 Effects of overexpressing AFPs in plants. **a** ABA resistant germination conferred by over-expression of AFP1or AFP2 compared to previously characterized *abi* single and double mutant lines. Germination was scored on minimal media with no ABA (min) or 3–200 µM ABA (A3–A200). *Error bars* represent s.e. of 2–13 replicate assays with each line. **b** Immunoblot using an anti-GFP antibody, of extracts from indicated lines harvested after 6 days on 1 µM ABA. Germination data was scored earlier on these or parallel samples (A1 = 1 µM ABA, A3 = 3 µM ABA). YFP-AFP fusions run at ~80 kD, with AFP2 appearing as a doublet. Light cross-reacting band (*), also seen in extract from non-transgenic line, reflects total protein loaded

truncated proteins also repressed bZIP functions in plants, we over-expressed various domains of the AFPs, fused to YFP tags, in both wild-type and *afp* mutant backgrounds. Although expression levels, and consequently phenotypes, varied substantially among independent transgenic lines, the ΔEAR (BC)-, and C-domain fusions for both AFP1 and AFP2 conferred at least mild ABA-resistance at germination (Fig. 7a, b; Supp. Fig. 5). In contrast, only the AFP1-AB domain conferred even mild resistance to ABA and the AFP2-A domain alone conferred none. Comparison of YFP fluorescence indicates that the A and AB domain fusions are relatively highly expressed (Fig. 7c), indicating that the limited effect of these fusions was not due to a lack of expression. The B domain contains a nuclear localization signal, which presumably enhances the potential to interact with the transcription factors and histone deactylase subunits in the nuclei. Although the C domain fusion lacks this signal, some of the fusion protein was observed in nuclei, possibly because this domain is involved in interactions with bZIP proteins such as ABI5, which are nuclear-localized themselves. The ABA-resistance conferred by the BC and C domain fusions suggests that the interactions with TPL and its homologs via the AFPs' EAR domains are not absolutely essential for the AFPs to function as repressors of ABA response.

AFP over-expression alters ABI5-induced gene expression

ABI5 is highly expressed during seed maturation, and can be again when seeds are exposed to ABA or dehydrating stresses that lead to ABA re-accumulation during the first 2 days post-imbibition (Finkelstein and Lynch 2000; Lopez-Molina et al. 2001). During these phases of growth, ABI5 directly regulates expression of numerous Late embryogenesis abundant (*Lea*) genes and some signaling proteins (Reeves et al. 2011), such that transcript levels of many *Lea* genes are greatly reduced in *abi5* mutant seeds (Supp. Fig. 6A). ABI5 is also at least indirectly required for activation of hundreds more genes, many of which are grouped in clusters potentially regulated coordinately by changes in chromatin structure (Nakabayashi et al. 2005). Consequently, transcript levels for the *AFPs* and several ABA/ABI5-regulated seed-expressed genes also decrease and re-accumulate following imbibition in the presence of ABA, as shown for wild-type seeds (Supp. Fig. 6b).

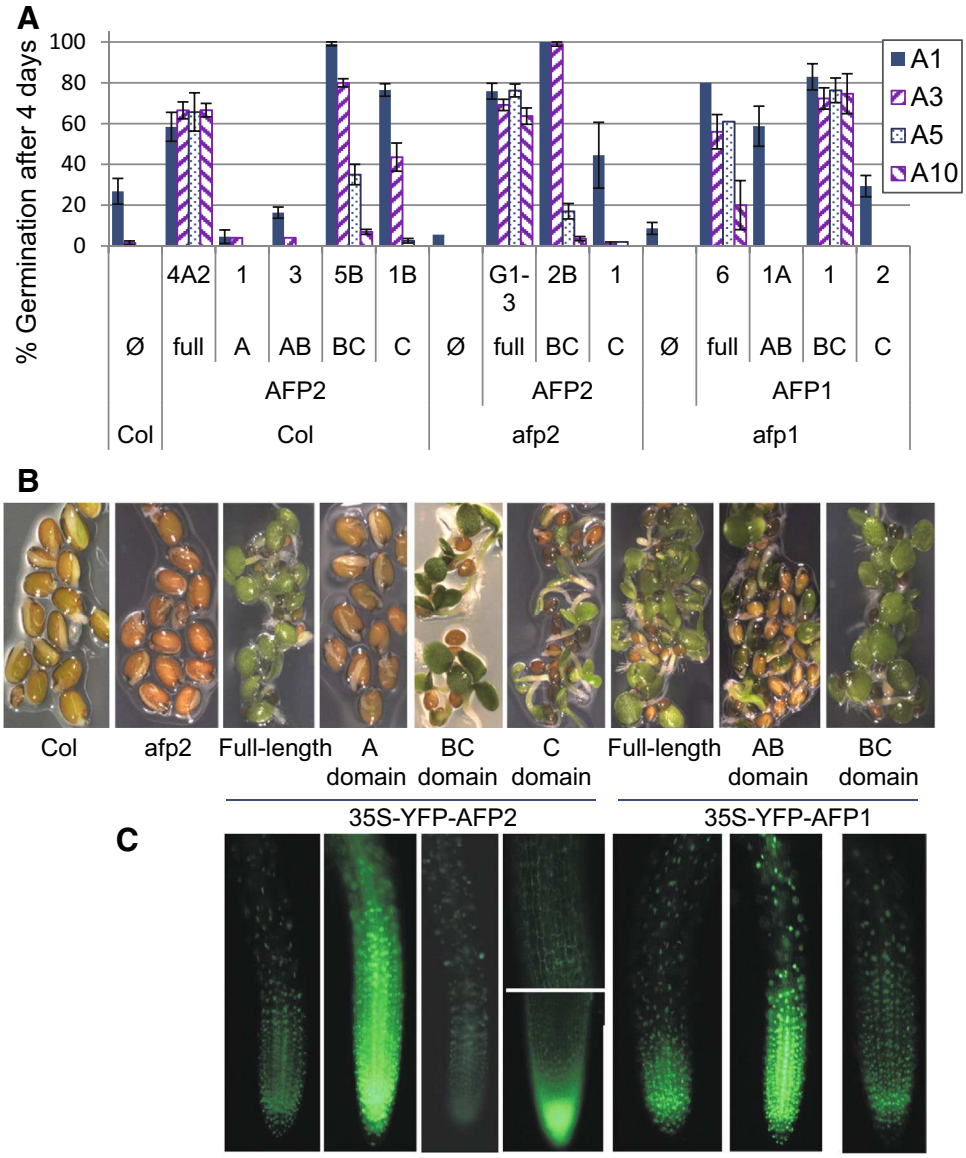


Fig. 7 Effects of overexpressing indicated AFP truncations in plants (domains as shown in Fig. 1b). **a** Reduced sensitivity to ABA for germination inhibition. Germination was scored on minimal media supplemented with 1–10 µM ABA (A1–A10). AFP2-A = aa 1–101, AFP2-B = aa 94–149, AFP2-AB = aa 1–149, AFP2-C = aa 149–348 (end), AFP2-BC = aa 94-end, AFP1-AB = aa 1–172, AFP1-BC = aa 93–345 (end). *Error bars* represent s.e. of 2–24 replicate assays with each line. **b** Seedling growth after 4 days on 1 µM ABA. **c** YFP fusion fluorescence in root tips after 4 days on hormone-free medium; upper image for 35S-YFP-AFP2-C domain is from hypocotyl

Conversely, over-expression of AFP1 or AFP2 reduces seed expression of genes encoding transcription factors such as ABI5 and some ABI5-regulated DREB2 family members, whereas AFP3 overexpression represses these *DREB2* genes, but does not alter *ABI5* transcript accumulation (Fig. 8). Effects of AFP overexpression on seed expression of some Lea, dehydrin or storage reserve genes vary among AFPs, with AFP2 most consistently repressive. AFP2 and AFP1 also negatively regulate each other, such that AFP2 overexpression reduces *AFP1* transcript levels 4–5-fold, and *AFP2* transcripts are reduced roughly two-fold in AFP1 overexpressers (Fig. 8c). Expression of the lipid storage body gene *oleosin1* is surprisingly high in the homozygous AFP2 over-expression lines, possibly because these seeds fail to complete maturation and may represent an earlier stage when reserve accumulation is closer to peak levels.

As shown for seedlings in Fig. 6b, seed expression of the YFP fusions varies substantially (Supp. Fig. 7), and is poorly correlated with the effects on ABI5-regulated genes. The degree of ABI5-regulated transcript reduction varies among independent transgenic lines, but correlates with the reduced ABA sensitivity observed in germination assays. As for the germination assays, comparisons of homo- and hemi-zygous siblings show a greater reduction of some ABI5-regulated transcripts in the homozygotes because the segregating wild-type progeny in the hemizygotes "inflate" the apparent expression of these transcripts. Although the *afp* mutants might be expected to increase expression of ABI5-regulated genes, only a subset of these transcripts

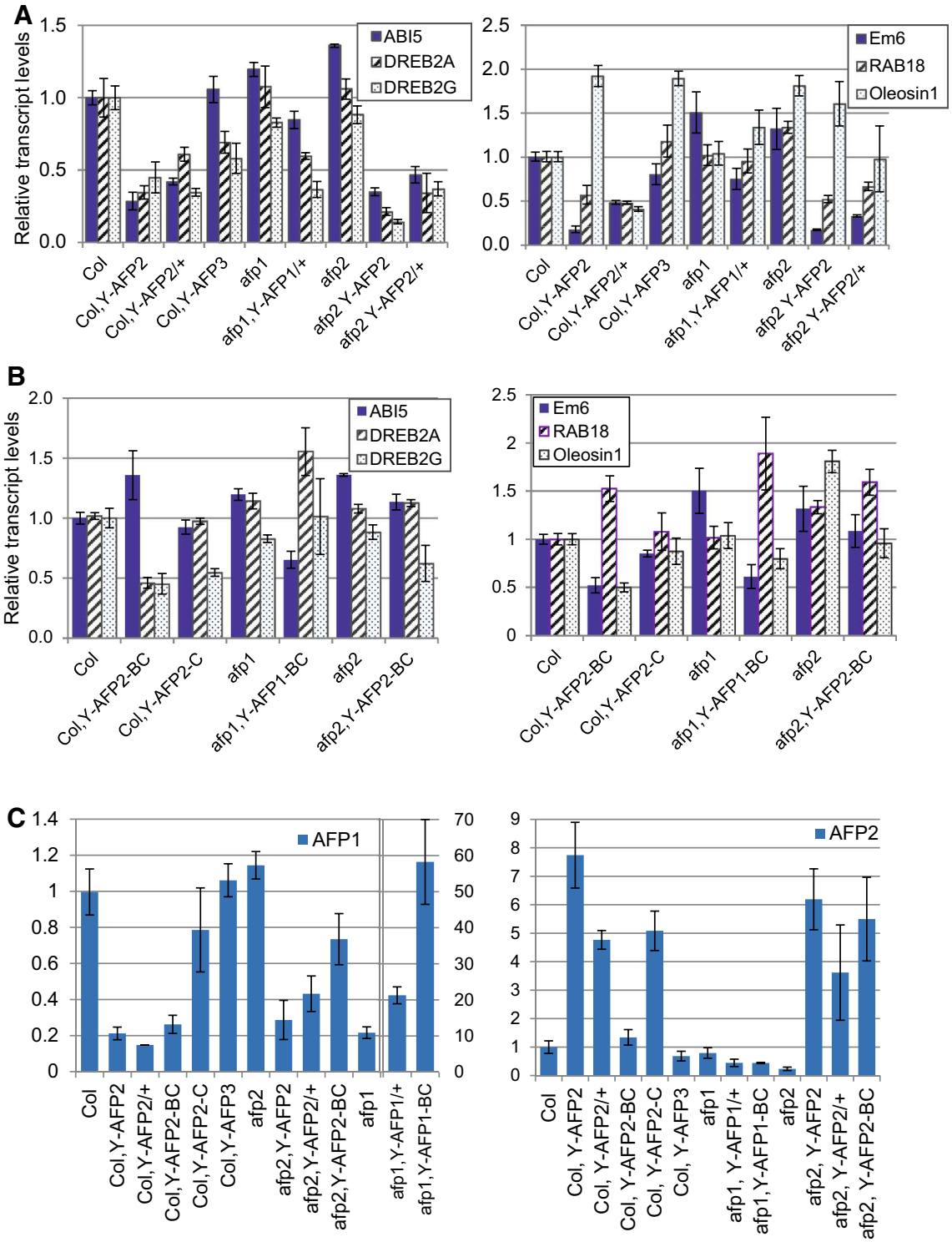


Fig. 8 Transcript levels of regulators (*ABI5* and *DREB2s*) and ABA-regulated genes (*Em6*, *RAB18* and *oleosin*) in seeds over-expressing YFP-AFP fusions (**a**) or AFP truncations lacking the EAR domain (**b**). Comparison of AFP transcript levels in the lines analyzed in **a** and **b** (**c**). Col, Y-AFP2 = line#4A1; Col, Y-AFP2/+ = line#4A2; Col, Y-AFP3 = lines#14E1&15B1; afp1, Y-AFP1/+ = line#3; afp2, Y-AFP2 = line#H1; afp2, Y-AFP2 = line#H5/6; Col, Y-AFP2-BC = line#5BD; Col, Y-AFP2-C = line #1B; afp1, Y-AFP1-BC = line#1C; afp2, Y-AFP2-BC = line#5A. Transcripts were measured by qRT-PCR, normalized relative to expression of *AT5G46630*, a gene expressed uniformly in diverse seed samples. *Error bars* represent S.E. of at least duplicate assays of at least 2 independent samples

(e.g. *ABI5, RAB18* and *oleosin1* in *afp2* mutants) show significant increases; this could reflect the facts that the mutants are only knockdown lines, these loci act redundantly (Garcia et al. 2008), and their products repress each other's expression.

To address the role of the TPL/TPR interactions in mediating AFP-dependent repression, we tested whether deletions lacking the EAR domain required for interaction with the TPL/TPRs also reduced seed expression of ABI5-induced genes. Similar to effects on germination and reporter gene expression in yeast, overexpression of BC or even C domain fusions was sufficient to repress some of these genes (Fig. 8b), but the effects varied depending on the gene and genetic background. For example, the AFP2-BC domain reduced expression of *DREB2G* in both wild-type and mutant backgrounds, but reduced *DREB2A* only in the wild-type background, and had no significant effect on *ABI5* in either background. In contrast, the AFP1-BC domain repressed *ABI5* expression, but not the *DREB2s*. Similarly differential effects were seen for the Lea gene *Em6*, whose expression was reduced by AFP2-BC in a wild-type background and by AFP1-BC in an *afp1* background. However, the dehydrin gene responsive to ABA *(RAB)18* was slightly overexpressed in some of these lines. These results are consistent with distinct mechanisms of AFP-regulation of different genes, possibly reflecting the variety of complexes that may be comprised of different combinations of bZIP, AFP, and TPL/TPR family members.

Role of chromatin modification in AFP-dependent repression of ABA-regulated genes

Late embryonic gene expression is terminated during germination, in part by chromatin modifications including histone deacetylation (Tai et al. 2005). To test whether AFP repression of embryonic genes was dependent on HDAC activity, seeds were imbibed in the presence or absence of trichostatin A (TSA), an HDAC inhibitor. Dose response and time course experiments were conducted to identify a TSA concentration that produced a similar delay in germination as treatment with 3 µM ABA (Fig. 9a; Supp. Fig. 8A, B). Comparison of total vs. acetylated histone levels showed that 10 µM TSA treatment effectively inhibited deacetylation in both genotypes (Supp. Fig. 8C). After 1 day, almost no germination was seen for either genotype on any media, but *Em6* and *ABI5* transcript levels had already declined to <1 or 6%, respectively, of their levels in dry wild-type seeds (Fig. 9b). Although these transcripts were present at similar or even lower levels in the hemizygous YFP-AFP2/+ line at 1 day post-imbibition (Fig. 9c), on most media this appeared to be a higher fraction of the dry

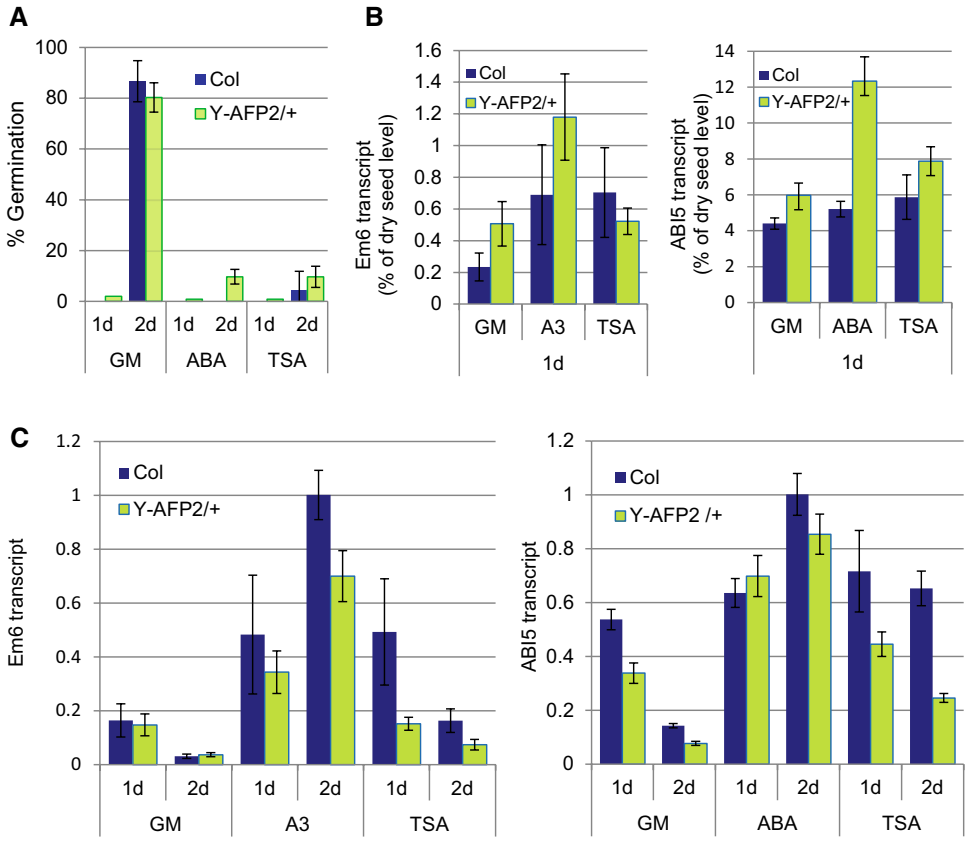


Fig. 9 Effects of AFP2 overexpression on seed gene expression and sensitivity to ABA or trichostatin A (TSA). **a** Germination of indicated genotypes after 1 or 2 days incubation on germination media (GM), with or without 3 µM ABA or 10 µM TSA. **b** Transcript levels of indicated genotypes expressed as % of level in dry seeds after 1 day on media as in (**b**). **c** Transcript levels after incubation as in (**a**), normalized relative to expression in Col seeds after 2 days on 3 µM ABA. Transcripts were measured by qRT-PCR, normalized relative to expression of a constitutively expressed gene. *Error bars* represent S.E. of triplicate measurements of at least 2 independent samples

seed level (Fig. 9b), reflecting the reduced initial level in these seeds. Extended exposure to ABA led to increases in these transcripts, but was less effective in the YFP-AFP2/+ line. Even the reduced levels shown for the YFP-AFP2/+ lines are overestimates because half the seeds in these populations are either wild-type segregants or homozygous transgenic seeds whose low viability limits degradation of these transcripts.

As previously described (Tai et al. 2005), TSA delayed both germination and the decline in seed-expressed transcripts, but its effects differed from those of ABA in that these transcripts were not re-induced during extended incubations on TSA despite showing a similar degree of germination inhibition (Fig. 9). *Em6* transcript levels initially declined more rapidly in TSA-treated seeds overexpressing AFP2 than in wild-type seeds, such that they were roughly three-fold higher in wild-type than AFP2 overexpression seeds after 1 day on TSA. However, by 2 days the levels were similar in both genotypes. In contrast, *ABI5* transcripts did not decline in wild-type seeds between 1 and 2 days on TSA, but were substantially lower and declining in AFP2 overexpression seeds cultured on TSA. Although *Em6* is regulated by ABI5 and their transcript levels are correlated in response to ABA (Fig. 9; Supp. Fig. 6), they respond differently to TSA and neither appears repressed by AFP2 via an HDAC-dependent mechanism.

To further test the importance of HDAC activity in AFP-repression of ABA-induced genes, we compared wild-type and AFP2 overexpressing seeds incubated for 39 h on media with or without ABA or TSA. At this stage, HDAC activity has already peaked in the absence of inhibitor (Tai et al. 2005) and seeds of both genotypes on media without either inhibitor are starting to germinate, whereas <2% of those on either ABA or TSA are germinating (Fig. 10a). We focused on the *DREB2s* because comparison of dry seed transcript levels indicated that they were repressed by AFP2, possibly in a manner independent of the EAR domain/TPL interaction (Fig. 8). Although AFP2 overexpression inhibits post-imbibition activation of both *DREB2s* by ABA (Fig. 10b), *DREB2A* showed greater induction by TSA than by ABA in the AFP2 overexpressors. Relief of this AFP2-dependent repression by an HDAC inhibitor is consistent with HDAC-mediated repression of *DREB2A* in the AFP2 overexpression line.

Discussion

The AFPs were initially identified based on either protein–protein interactions with ABI5 or as ABA-induced genes (Garcia et al. 2008; Huang and Wu 2007; Lopez-Molina et al. 2003). Genetic studies showed that most of the family members (AFP1, AFP2 and AFP3) were negative regulators of ABA response, leading to the suggestion that they functioned in a feedback loop to attenuate ABA response (Garcia et al. 2008), but their mechanism of action was unclear. The discovery that NINJA, the closest homolog of this protein clade, interacted with TPL to act as a co-repressor of JA-induced gene expression led to the suggestion that the AFPs have an analogous function in repressing ABA-induced gene expression (Pauwels et al. 2010). Recent studies of the rice AFP homolog MODD show that these interactions and functions are conserved across angiosperms (Tang et al. 2016).

Intrinsic repressor activity of AFPs

We have found that, among the AFPs, only the three that behave genetically as repressors of ABA response also have intrinsic transcriptional repressor activity as BD-fusions in yeast one-hybrid assays and can interact with TPL and some of the TPRs. Furthermore, although sufficient, the domain that interacts with TPL is not necessary for repressor activity in yeast, implying that these may act through multiple mechanisms. Consistent with this possibility, some of the AFPs with repressor activity also interact with two

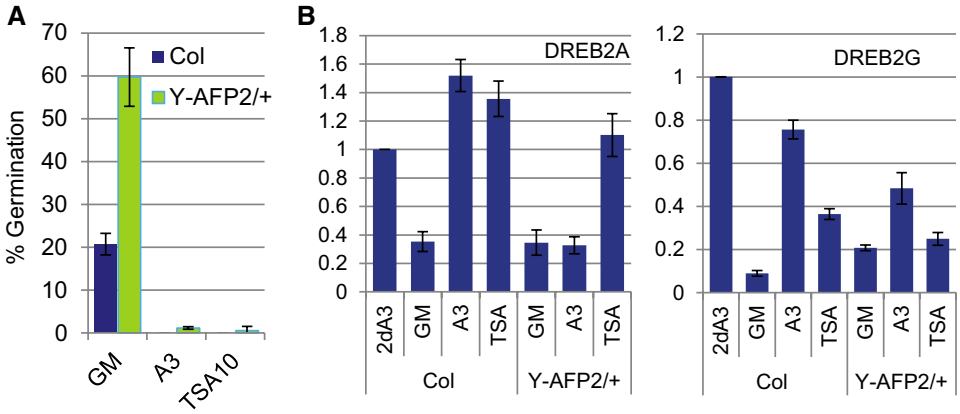


Fig. 10 Effects of AFP2 overexpression on seed gene expression and sensitivity to ABA or trichostatin A (TSA). **a** Germination of indicated genotypes after 39 h incubation on GM, with or without 3 µM ABA or 10 µM TSA. **b** *DREB2* transcript levels in indicated genotypes incubated on media as in (**a**), expressed relative to level in Col after 2 days on 3 µM ABA. Transcripts were measured and normalized as described in Fig. 9

subunits of the histone deacetylase complex in yeast two-hybrid and/or BiFC assays. Both of these assays are subject to both false positives and negatives, and some discrepancies between their results are not unusual (Lumba et al. 2014). However, the additional interactions seen in BiFC assays could reflect indirect interactions in a repressive protein complex and were not seen in negative controls. Further indirect support for these interactions is provided by the functional evidence that AFPs are repressive in yeast, which lack a TPL ortholog but do have the conserved HDAC complex, the precedent that other EAR domain containing repressors interact with HDAC (reviewed in Kagale and Rozwadowski 2011), and the corresponding interactions documented for rice homologs of AFPs, TPRs, and HDAs (Tang et al. 2016).

Recent studies of another class of EAR-domain proteins, the AUX/IAA repressors of auxin signaling, have shown that oligomerization is required to effectively inhibit auxin response factor (ARF) activity (Korasick et al. 2014). Our studies show that the AFPs with the strongest repressive effects are those that strongly interact as homo- and heterodimers, AFP2 and AFP1, consistent with the possibility that they also act as multimers. Although structurally very different from the AUX/IAA proteins, the AFPs are predicted to encode intrinsically disordered proteins (Supp. Fig. 9). Other proteins with this characteristic have been found to oligomerize and interact with many different partners, thereby forming scaffolds for possibly multiple protein complexes (Turoverov et al. 2010). Intrinsically disordered proteins are now recognized as critical regulators of signaling and, especially in plants, adaptation to stress and other environmental signals (Pietrosemoli et al. 2013; Sun et al. 2013).

Our yeast one-hybrid assays indicated that the AFPs' repression of bZIP-dependent activation of ABA-regulated promoters was not mediated by binding directly to those promoters. However, these assays did not rule out the possibility that the AFPs were sequestering the bZIPs, thereby preventing them from binding to the ABREs.

Physiological role(s) of AFPs

Previous studies showed that all of the *AFPs* are induced by ABA and dehydrating stresses, but with different developmental timing (Garcia et al. 2008). All are expressed in seeds, but *AFP2* transcripts are most abundant at this stage. Following imbibition, *AFP2* is initially most strongly induced by ABA or stresses, but *AFP1, AFP3* and *AFP4* become more strongly induced by 5 days post-stratification. The rice *MODD* gene is most homologous to *AFP3* and is also induced by ABA or drought in 3 week old plants; consistent with this, gain and loss of function lines have strong effects on vegetative traits such as drought tolerance (Tang et al. 2016).

The overlap in expression of *AFP1* and *AFP2* is reflected in redundant action of these factors, such that double mutants are more hypersensitive to ABA and stress for inhibition of germination and seedling growth than single mutants (Garcia et al. 2008). The redundancy and antagonistic regulation of these AFPs is reminiscent of the redundancy and antagonistic regulation of the bZIPs whose activity they regulate (Finkelstein et al. 2005). In both cases, this provides a mechanism for transitioning between overlapping regulatory circuits appropriate to different developmental stages.

Overexpression of AFP1, AFP2 or AFP3 confers resistance to extremely high ABA concentrations, up to a 200-fold reduction in seed response that exceeds the effects of mutations in any single locus affecting ABA response. Although *AFP3* is one of the few genes induced by ABA in nearly all contexts (reviewed in Finkelstein 2013), we focused on *AFP1* and *AFP2* because of their stronger effects at germination.

To address the mechanism of this resistance, fusions of specific AFP domains were tested for their effects on ABA response in transgenic Arabidopsis. These studies showed that the EAR domains of either AFP1 or AFP2 were neither necessary nor sufficient for repressing ABA inhibition of germination in transgenic plants, whereas the C domain and especially the BC domain fusions did confer resistance to ABA. Similar to results in yeast, this could reflect sequestration through C domain interactions with bZIPs or modifiers of chromatin structure such as HDACs.

The transition from seed development and maturation through dormancy to germination involves major changes in gene expression regulated by chromatin remodeling (Bouyer et al. 2009; Footitt et al. 2015; Han et al. 2012; Ryu et al. 2014; Schneider et al. 2016; Tai et al. 2005; van Zanten et al. 2013; Veerappan et al. 2012; Wang et al. 2016; Zhang and Ogas 2009). Changes in histone modifications such as acetylation contribute to this regulation, and these are mediated by multiple histone deacetylases (HDACs) and acetylases. Genetic studies have shown that distinct HDACs have opposing effects on the overall process of germination and appear to affect different subsets of genes (Tanaka et al. 2008; van Zanten et al. 2014). For example, during germination HDA6 and HDA19 repress embryonic properties including expression of storage protein genes and the major transcription factors conferring embryonic identity (LEC1, LEC2, FUS3 and ABI3), whereas HDA9 represses seedling traits such as expression of photosynthetic genes in maturing seeds. Consistent with these differences, these reports conflicted regarding the effects on germination and seedling growth due to chemical inhibition of HDACs by TSA. The present study

replicates previous results showing inhibition of germination by TSA, and further shows that the AFP overexpression lines are slightly resistant to this inhibition.

Following seed imbibition, seed ABA levels decrease dramatically, permitting germination of nondormant seeds, but will subsequently increase due to de novo synthesis in dormant seeds (reviewed in Finkelstein 2013; Finkelstein et al. 2008). Exposure to dehydrating stresses or ABA also leads to increased ABA levels that block germination. These fluctuations in ABA content are also reflected in changes in transcript levels for many seed-expressed ABA-regulated genes including *Em6, oleosins, ABI5* and *AFP2*. All of these decline rapidly in the first 24 h post imbibition even in the presence of ABA, but can be re-induced by continued ABA exposure.

The hypothesis that AFPs repress ABA/ABI5-regulated genes at least in part through changes in histone acetylation predicts that these target genes will be under-expressed in the AFP over-expression lines, but that TSA inhibition of HDAC activity will restore their expression (Supp. Fig. 1). Consistent with this, transient assays with rice protoplasts co-expressing a MODD-GAL4DB fusion and a 35S:GAL4-LUC reporter showed that 20 μM TSA was sufficient to mostly overcome repression due to MODD (Tang et al. 2016). Furthermore, all four bZIP target genes tested in rice (2 *Leas* and 2 *RABs*) appeared to have MODD-mediated repression via altered histone acetylation. In contrast, although many seed-expressed genes show reduced expression in seeds and ABA-treated seedlings of the AFP over-expression Arabidopsis lines, only one locus tested (*DREB2A)* maintained higher expression when treated with TSA. This diversity of regulatory patterns and responses to TSA is consistent with control by multiple chromatin remodeling mechanisms, some of which are susceptible to the same inhibitor yet affect distinct processes. Whereas this study focused on the role of AFP interactions with proteins affecting histone acetylation, their effects on additional well-characterized mechanisms of chromatin remodeling regulating the seed to seedling transition such as nucleosome positioning, histone ubiquitination and methylation have yet to be explored.

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Author contributions RRF conceived and performed experiments, wrote the manuscript, and repeatedly applied for funding. TJL conceived and performed experiments and provided feedback. BJE and DRM performed experiments and provided feedback.

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