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# Direct targets of the transcription factors ABA-Insensitive(ABI)4 and ABI5 reveal synergistic action by ABI4 and several bZIP ABA response factors

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**Abstract** The plant hormone abscisic acid (ABA) is a key regulator of seed development. In addition to promoting seed maturation, ABA inhibits seed germination and seedling growth. Many components involved in ABA response have been identified, including the transcription factors ABA insensitive (ABI)4 and ABI5. The genes encoding these factors are expressed predominantly in developing and mature seeds, and are positive regulators of ABA mediated inhibition of seed germination and growth. The direct effects of ABI4 and ABI5 in ABA response remain largely undefined. To address this question, plants over-expressing ABI4 or ABI5 were used to allow identification of direct transcriptional targets. Ectopically expressed ABI4 and ABI5 conferred ABA-dependent induction of slightly over 100 genes in 11 day old plants. In addition to effector genes involved in seed maturation and reserve storage, several signaling proteins and transcription factors were identified as targets of ABI4 and/or ABI5. Although only 12% of the ABA- and ABI-dependent transcriptional targets were induced by both ABI factors in 11 day old plants, 40% of those normally expressed in seeds had reduced transcript levels in both *abi4* and *abi5* mutants. Surprisingly, many of the ABI4 transcriptional targets do not contain the previously characterized ABI4 binding motifs, the CE1 or S box, in their promoters, but some of these interact with ABI4 in electrophoretic mobility shift assays, suggesting that sequence recognition by ABI4 may be more flexible than known canonical sequences. Yeast one-hybrid assays demonstrated synergistic action of ABI4 with ABI5 or related bZIP factors in regulating these promoters, and mutant analyses showed that ABI4 and these bZIPs share some functions in plants.

**Keywords** ABA · Arabidopsis · ABI4 · ABI5 · Transcriptional targets

## Introduction

Abscisic acid (ABA) regulates many agronomically important aspects of plant seed development, including synthesis of seed storage proteins and lipids, seed desiccation tolerance, dormancy, germination and the subsequent commitment to seedling growth (Finkelstein et al. 2002). Genetic studies, especially in Arabidopsis, have identified a large number of loci involved in ABA response. Digenic analyses indicate that these loci are likely to be acting in multiple overlapping response pathways. In addition to interactions among regulators of ABA response, there appears to be substantial cross-talk between signaling pathways regulating response to ABA and to other plant hormones, assorted stresses, and sugars (Acharya and Assmann 2009; Finkelstein and Gibson 2002; Shinozaki and Yamaguchi-Shinozaki 2007).

Three well-characterized positive regulators of ABA signaling are the transcription factors encoded by *ABI3*, *ABI4* and *ABI5*, which were initially identified in screens for mutants exhibiting ABA-resistant germination. These proteins are members of the B3-, APETALA2- (AP2), and basic leucine zipper- (bZIP) domain families, respectively, and regulate overlapping subsets of seed-specific and/or

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ABA-inducible genes (Finkelstein and Lynch 2000; Finkelstein et al. 1998; Giraudat et al. 1992; Lopez-Molina and Chua 2000). ABI5 has many close homologs, including ABA-Response Element Binding Factors (ABFs and AREBs) and *Arabidopsis thaliana* Dc3 Promoter Binding Factors (AtDPBFs), that also participate in ABA or stress signaling, primarily at later stages of growth (Choi et al. 2000; Kim et al. 2002; Uno et al. 2000).

Physiological studies have shown that the *ABI3*, *ABI4*, and *ABI5* loci have similar qualitative effects on seed development and ABA sensitivity, consistent with action in overlapping pathways, but that null mutations in *ABI3* are more severe than those in *ABI4* or *ABI5* (Finkelstein and Lynch 2000; Finkelstein et al. 1998; Parcy et al. 1994). Both ABI3 and ABI4 are expressed from globular stage embryogenesis onward, and can regulate expression of ABI5, which is activated by heart stage. All three are most highly expressed in mature seeds, but differ slightly in localization within the seeds (Brocard et al. 2002; Finkelstein and Lynch 2000; Lopez-Molina et al. 2001; Parcy et al. 1994; Penfield et al. 2006; Söderman et al. 2000).

Although *abi4* and *abi5* mutants were initially thought to have ABA- and seed-specific defects (Finkelstein 1994), they have also been shown to have defects in response to glucose, NaCl, and osmotic inhibition of germination and/or seedling growth, as well as displaying ABA-resistant seedling growth (Arenas-Huertero et al. 2000; Brocard et al. 2002; Laby et al. 2000; Quesada et al. 2000). Continued low levels of expression in vegetative growth are sufficient to modulate additional responses, as evidenced by ABI-dependent effects on lateral root growth, carbon metabolism, and retrograde signaling from plastids or mitochondria to the nucleus (Giraud et al. 2009; Koussevitzky et al. 2007; Rook et al. 2001; Signora et al. 2001).

In addition to mutant analyses, gain-of-function studies show extensive cross-regulation of expression among *ABI3*, *ABI4*, and *ABI5* (Söderman et al. 2000) and overexpression of any of these ABI transcription factors results in ABA or glucose hypersensitivity in vegetative tissues (Finkelstein et al. 2002). Overexpression of *ABI3* or *ABI4* is even sufficient to confer ABA-inducible vegetative expression of several "seed-specific" genes, which is partly dependent on increased *ABI5* expression (Söderman et al. 2000). *ABI5* overexpression can restore wild-type ABA sensitivity during germination and seedling growth to a weak *abi3* mutant (Lopez-Molina et al. 2002) and results in enhanced ABA induction of some genes normally regulated by ABA in vegetative tissues. These results suggest that seed-specific or ABA-inducible expression might be at least partially controlled by regulatory complexes containing combinations of these transcription factors.

ABI5 and its ABF/AREB/AtDPBF homologs bind to the class of G-box motifs known as ABA response elements (ABREs). Some members of this bZIP subfamily have been shown to regulate expression of themselves and each other, and function redundantly in response to some stresses (Finkelstein et al. 2005; Yoshida et al. 2010). ABI4 is also a member of a large transcription factor family which includes the Drought Response Element Binding factors (DREBs) and Ethylene Response Element Binding Proteins (EREBPs), but it differs from ABI5 in that it is an orphan member. Although classified as part of the DREB subfamily, based on up to 75% homology within the AP2-domain, there are no obvious candidates for factors with redundant function. In vitro selection of maize ABI4 binding sites identified a consensus of CACCK (Niu et al. 2002), which differs slightly from the DREB and EREBP consensus binding sites of RCCGAC (DRE) and TA-AGAGCCGCC (GCC box), respectively (Ohme-Takagi and Shinshi 1995; Sakuma et al. 2002).

Studies of sugar- and light-regulated gene expression have shown that ABI4 mediates ABA- and sugar-repression of photosynthetically associated nuclear genes, and that this is correlated with ABI4 binding to a motif designated the S-box [CACYKSCA] (Acevedo-Hernandez et al. 2005). The S-box is similar to the maize ABI4 binding consensus [CACCK] and is present in close association with G-boxes characteristically bound by bZIP factors. A different motif, consisting solely of the bases CCAC, has been correlated with ABI4-dependent retrograde signaling, particularly when adjacent to, or overlapping with, a G box motif (Koussevitzky et al. 2007). Although most of these studies focused on ABI4-repressed genes, ABI4-inducible gene expression has also been demonstrated to be dependent on sequences related to the S-box (Bossi et al. 2009).

Several studies have reported ABI-dependent gene expression based on altered transcript accumulation in mutants (Nakabayashi et al. 2005; Penfield et al. 2006), but these do not distinguish between direct and indirect regulation. To focus on direct targets of ABI4 and ABI5 regulation, we performed a preliminary microarray screen (assisted by the Laboratory for Functional Genomics at TAMU) comparing ABA-responsive gene expression in *35S:ABI* versus *abi* lines in 11 day old plants, a developmental stage when the *35S:ABI* expression is mainly ectopic and most of the normally redundantly functioning regulators are not active.

Our major hypothesis was that ABI4 and ABI5 might directly regulate some common targets, including signaling components generally assumed to act upstream of transcription factors, as well as novel components of ABA/ABI-dependent signaling. Preliminary analyses of replicate biological material for each genotype with the nearly full Arabidopsis genome Affymetrix ATH1 chips (approximately 24 K genes represented) identified slightly over 100

transcriptional target genes whose expression was enhanced in the *35S:ABI4* and/or *35S:ABI5* lines. Despite the similarity in growth phenotype of the *ABI4* and *ABI5* overexpression lines, only about 12% of these target genes were regulated by both *ABI4* and *ABI5*, and their common targets were mostly members of the *late embryogenesis abundant* (*lea*) class or encoded proteins of unknown function. The ABI-induced genes include both positive and negative regulators of ABA signaling, including a member of the clade of PP2Cs recently shown to interact directly with the PYR/PYL/RCAR class of ABA receptors. Surprisingly, although the ABI4-regulated genes were very tightly co-regulated in a manner distinct from the ABI5-regulated genes, most of their promoters lacked the previously identified ABI4 binding site, but were instead highly enriched for ABREs, i.e. bZIP binding sites. However, several of these promoters still bound to ABI4 in vitro, and could be activated synergistically by ABI4 and specific bZIPs.

## Materials and methods

### Plant materials

Arabidopsis plants were grown under continuous light. The *abi4-1* and *abi5-1* mutants were isolated as described in Finkelstein (1994). The overexpression lines used for the microarrays were those with the most active transgenes described in Brocard et al. (2002), and Söderman et al. (2000): line 114A for *35S:ABI4* and line 2D1 for *35S:ABI5*. The knockout lines used were SALK_043079 (*ABF1*) and SALK_075836 (*ABF3*) produced by Alonso et al. (2003). All mutants are in the Columbia background except *abi5-1*, which is in the Ws background; the original overexpression lines are both in the Ws background. Comparisons of bZIP mutant effects on seedling gene expression used the *abi5-7* mutant in the Col background, described in Nambara et al. (2002). Following loss of the *35S:ABI4* line due to inactivation of the transgene, we constructed a *35S:GFP:ABI4* fusion by insertion of an *ABI4* cDNA into the EcoRI site of pEGAD (accession no. AF218816) (Cutler et al. 2000). This fusion includes all but the first two and final amino acids of ABI4. This construct was introduced into Columbia ecotype and *abi4-1* plants by *Agrobacterium* mediated transformation using the floral dip method (Clough and Bent 1998), and transgenic lines were selected on the basis of BASTA resistance. Although GFP fluorescence was not detectable in lines carrying this construct, function of the transgene was tested in terms of its ability to complement ABA-resistance of the *abi4* mutant and confer ABA and glucose hypersensitivity in the wild type background.

For plants to be used for RNA extractions, seeds were surface-sterilized with 5% hypochlorite and 0.02% Triton X-100, then rinsed several times with sterile water before plating on GM (0.5× Murashige-Skoog salts, 1% sucrose) solidified with 0.55% agar. The seeds were stratified at 4°C for 3 day, then incubated at 22°C in continuous light (50–70 μE $m^{-2}$ $s^{-1}$) for 11 day prior to 4 h treatments with or without 50 μM ABA and/or 20 μM cycloheximide (CHX). To minimize disturbance of the roots, plants were treated by flooding the plates with 4 ml GM supplemented with ABA, CHX, or equal volumes of their respective solvents (50% DMSO for ABA, ethanol for CHX) for control treatments. Following incubation, plants were harvested, frozen in liquid nitrogen, and stored at −80°C until RNA was extracted.

Seed and seedling samples were sterilized as above, then plated on minimal nutrient salts solidified with 0.7% agar, with or without 5 μM ABA, 166.5 mM NaCl, 333 mM glucose or 333 mM sorbitol. Seedlings harvested at 2.5 day post-stratification were incubated on GM with or without 5 μM ABA. Germination and seedling growth assays testing ABA and stress sensitivities of various mutant lines used minimal nutrient salts solidified with 0.7% agar, with or without ABA concentrations ranging from 3 to 30 μM, 200 mM NaCl, 400 mM sorbitol or 333 mM glucose.

### RNA gel blots

Total RNA was isolated by hot phenol extraction, as previously described (Söderman et al. 2000), then size fractionated on MOPS [3-(*N*-morpholino)-propanesulfonic acid]-formaldehyde gels and transferred to nylon membranes (Osmonics, Westborough, MA) using 20× SSPE (1× SSPE is 0.115 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4) as blotting buffer. RNA was bound to the filters by UV cross-linking (120 mJ $cm^{-2}$ at 254 nm). Uniformity of loading and transfer was assayed qualitatively by methylene blue staining and quantitatively by hybridization to an rDNA probe. Transcripts from ABA-inducible genes were detected by hybridization to cDNA clones or PCR fragments as described by Söderman et al. (2000), labeled by random priming to a specific activity of $10^8$ cpm $\mu g^{-1}$. Hybridization conditions for abundant transcripts were 50% formamide, 5× SSPE, 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.1% SDS, and 200 mg $ml^{-1}$ of DNA at 43°C for 16–24 h in a Hyb-Aid rotisserie oven. Filters were washed twice at 60°C in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS and once at 60°C in 0.2× SSC and 0.1% SDS for 30–60 min. Low abundance transcripts were detected by hybridization to a random-priming labeled probe in 7% SDS, 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, and

1% BSA at 65°C for 16–24 h (Church and Gilbert 1984). The final wash for these filters was 40 mM Na phosphate buffer, pH 7.2, 1% SDS, and 1 mM EDTA at 60–65°C. Bound probe was detected by phosphoimager (BioRad) then visualized and quantified with Quantity One software.

## Promoter analysis

Promomer (http://bar.utoronto.ca/ntools/cgi-bin/BAR_Promoter.cgi) (Toufighi et al. 2005) was used to search the promoters of our microarray target sets for known transcription factor motifs. Promoters were defined as 1,000 bp upstream of the transcription start site as identified in the TAIR7 annotation, and searches were performed for motifs in the 5′ to 3′ orientation on both strands. *P*-value was calculated in MatLab using the formula *P*-value = 1 − sum(hygepdf(0:k,N,n,m)), where k = x − 1 (x = hits in target set), m = total genes in target set, n = hits in genome, and N = total genes in genome.

## Yeast assays

Promoter-lacZ reporter fusions were constructed by amplifying promoter fragments with primers including XhoI and Sph1 linkers and cloning into pGEM-T Vector System (Promega) After sequencing, promoters were subcloned into the XhoI/Sph1 sites of pLGΔ178, a derivative of the plasmid pLGΔ312 (Guarente and Mason 1983). Reporter clones were transformed into the yeast strain BY4705 (*MATα ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0*) (Brachmann et al. 1998) using the Alkali Cation transformation kit (Bio101) following the manufacturer's instructions; transformants were selected by complementation of uracil auxotrophy.

Fusions of the GAL4 activation domain and transcription factors to be tested (ABI4, ABI5, ABF1 and ABF3) were constructed using a CRE-lox system to recombine cDNAs in the pUNI51 vector to the pACT2lox vector (Liu et al. 1998). The ABF1 cDNA was stock # U19471, available through the ABRC; all others were constructed by subcloning previously isolated cDNAs into pUNI51. ABF3 was subcloned from a Gateway entry clone, ABRC stock # U15508. AD fusions were transformed into the yeast strain THY.AP4 (*MATa ura3 leu2 lexA::lacZ::trp1 lexA::HIS3 lexA::ADE2*) (Obrdlik et al. 2004) and transformants selected by complementation of the leu2 auxotrophy. To test potential activation by pairs of transcription factors, the pACT2lox vector was modified by in vivo recombination of an amplified TRP1 gene to replace the LEU2 gene, such that lines carrying two transcription factor fusions could be constructed by retransformation of the initial AD fusion line and selection for complementation of both leu and trp auxotrophy.

To test transactivation of the reporter fusions, yeast carrying the reporters and AD fusions or vector controls were mated by incubation overnight on YPD, then replica plated to YSM lacking uracil, leu and trp to select for diploids carrying all three plasmids. Activation of the lacZ reporter was pre-screened by colony lifts transferring yeast to Whatman #1 filters, which were then subjected to 3 rounds of freeze–thaw cycles in liquid nitrogen, then overnight incubation at 37°C on a 3MM filter saturated with Z buffer (113 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, pH 7), 200 μg/ml Xgal and 38 mM β-mercaptoethanol. Quantitative β-galactosidase assays were performed as described at http://labs.fhcrc.org/gottschling/Yeast%20Protocols/Bgal.html.

## EMSAs

Electrophoretic mobility shift assays (EMSAs) were performed with fusions of GST and the DNA binding domain of ABI4 (amino acids 1-114). The ABI4 DNA binding domain was amplified with primers containing the EcoRI site (forward: CGTACTGAATTCATGGACCCTTTAGCT TCCCAAC; reverse: CGTACTGAATTCTCAAGACGAA GGGGTTAGTTGAGCTG) and cloned into the EcoRI site of pBluescript. After sequencing, the ABI4 DNA binding domain was subcloned into the EcoRI site of pGEX, downstream of GST. The fusion protein was expressed in BL21 C+ cells using Overnight Express™ Instant TB medium (Novagen). Cells were harvested, resuspended in 1× PBS + 0.2 mM PMSF + 1 mg/ml lysozyme (Sigma), then frozen at −70°C for 30 min and thawed before sonicating repeatedly on ice. After sonication, Triton X-100 was added to 1% and the sonicate was incubated for 30 min at 4°C for 30 min. The soluble fraction was then incubated with Glutathione agarose (Sigma) for 30 min at 4°C and loaded onto an Econo-column (BioRad). Beads were washed 3× with PBS before GST-ABI4 was eluted three times with 10 mM glutathione in 25 mM Tris, pH 8.0. Eluted purified protein was stored at −80°C; small aliquots were diluted to make a working stock of 100 ng/μl in 20 mM Tris pH 8, 10% glycerol and 1 mM EDTA.

Fragments used as probes for EMSAs were upstream regions of genes that were highly induced by ABA in 11 day old 35S-ABI4 plants. Three of these had small intergenic regions such that probes were at most 316 bp long:

At1g32560 (nt −305 to +6 relative to trancription start site)
At4g16160 (nt −264 to +17 relative to trancription start site)
At1g27470 (nt −301 to +15 relative to translation start site)

For those with larger intergenic regions, 200–300 bp fragments containing most of the ABREs and/or CCAC motifs in each upstream region were amplified or digested from the yeast one-hybrid subclones. These included:

At2g25890 (nt −445 to −144 relative to transcription start site)
At3g17520 (nt −336 to −130 relative to transcription start site)
At4g25140 (nt −178 to −36 relative to transcription start site or +23 relative to translation start site)

In addition, the 5′upstream fragment of ABI4 described in Bossi et al. (2009) was used as a positive control.

These fragments were amplified and cloned as described for construction of the yeast one-hybrid reporter fusions. Fragments were re-amplified from subclones using vector-specific primers, then released from vector sequences by restriction digestion with XhoI and/or SalI. In addition, smaller fragments of these probes were produced by restriction digestion or PCR as indicated in the figure legends. Fragments were end-labeled by fill-in reactions in a volume of 20 µl including 30 ng fragment, 1× DNA polymerase buffer (Promega), 4–10 µCi 32P-dCTP, 3 units Klenow fragment of DNA polymerase and supplemented with 50 µM dTTP as needed, depending on the overhang sequence. After 10 min incubation at room temperature, reactions were diluted to 50 µl with TE and unincorporated dNTPs were removed by spin-column chromatography.

Binding reactions were performed in 15 µl volumes containing approximately 1 ng probe (2,000–4,000 cpm), 100 ng polydIdC, 1 µg BSA, with or without 100 ng GST or GST-ABI4 in 1× binding buffer (20 mM Tris pH 8, 7.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 10% glycerol). For competition assays, unlabeled probe fragments were included at 50–100× excess relative to the labeled probe. The non-specific competitors were either the 3′UTR of ABI4 described in Bossi et al. (2009) or bulk herring testes DNA. Reactions were incubated for 30 min at room temperature, then loaded immediately on a 4.5% acrylamide 1× TBE gel that had been pre-run at 120 V. Loading dye was added to the free probe sample and gels were run at 190 V until the BPB was approximately 1 cm from the bottom. Gels were lightly fixed with a mixture of 10% acetic acid and 10% methanol, then dried and exposed to a phosphorimager screen.

## Results

### Direct targets of ABI4 and ABI5 regulation

To emphasize the effects of ABI4 and ABI5 expression, we analyzed 11 day old seedlings, a developmental stage when seed specific transcription factors are not normally expressed but overexpression of ABI4 or ABI5 enhances ABA-inducible responses (Brocard et al. 2002; Söderman et al. 2000). Each ABI factor was constitutively overexpressed under control of the CaMV 35S promoter and ABA response was transiently induced by treating with 50 µM ABA for 4 h. Cycloheximide was added at the same time to limit expression of indirect targets. ABA-dependent gene expression was identified by comparing transcriptomes of *35S:ABI* seedlings treated with ABA and cycloheximide to seedlings treated with cycloheximide alone. ABI-dependent genes were then identified by comparison of *35S:ABI* versus *abi* mutant seedlings treated with both ABA and cycloheximide (Supp. methods). Direct ABI4 and ABI5 targets were defined as genes that were ABA induced at least twofold in the overexpression plants compared to the *abi* mutant plants. The absolute number of targets identified by this strategy depends on the strength of transgene expression, the developmental stage of the tissue, and the fold difference chosen as the criterion for ABA/ABI-regulation, and is therefore likely to be an underestimate, yet still informative regarding functional classes and structural characteristics of these transcriptional targets. In this study, the ABI4 and ABI5 overexpression lines each reproducibly induced less than 100 ABA- and ABI-dependent genes (95 and 59, respectively). There was relatively little overlap between the two sets of targets, with just 16 shared genes (Supp. Table 1).

The genes induced by ABI4 and by ABI5 represent many functional classes (Table 1). As expected, given the roles of ABI4 and ABI5 in seed development, a high percentage of the target genes were seed maturation related genes, including leas, oleosins and dehydrins. The majority of these seed maturation genes were induced only by ABI4 (18 genes) or by both ABI4 and ABI5 (10 genes). Only two seed maturation related genes were induced solely by ABI5, consistent with previous analyses of ABI5 overexpression (Brocard et al. 2002).

ABI4 and ABI5 also induced a variety of other gene targets. Both induced multiple transcription factors and signaling molecules, possibly involved in propagating the ABA signal. Interestingly, we also identified negative regulators of ABA signaling, including the protein phosphatase type 2C ABA hypersensitive at germination (AHG)1 (Nishimura et al. 2007). In addition, two ABI5 interacting factors previously characterized as negative regulators of ABA and stress signaling were identified as transcriptionally induced by ABI4: AFP2 and AFP3 (Garcia et al. 2008). These results show that negative feedback regulation is being induced alongside positive effector proteins.

### Validation of exogenous overexpression targets

To confirm that we had identified genes specifically induced by ABI4 or ABI5 overexpression and ABA

**Table 1** Functions of ABI/ABA induced genes. Function was determined based upon published reports, functions of homologs, and presence of known protein domains within gene. Genes were then sorted into related functional classes. The seed maturation category includes leas, oleosins, dehydrins and other genes expressed/required during seed maturation. Protein stability includes proteases and protease inhibitors not associated with seed maturation. Cell structure includes expansins and other genes involved in cell wall synthesis. The signaling category contained kinases and phospholipases. Stress/disease response genes included heat shock proteins, avirulence response genes and stress inducible genes. Metabolism included most other genes of known function not associated with another category

| Gene function | ABI4 | ABI4 specific | Shared | ABI5 specific | ABI5 |
|---|---|---|---|---|---|
| Seed maturation | 28 | 18 | 10 | 2 | 12 |
| Hormone response | 4 | 4 | 0 | 1 | 1 |
| Metabolism | 13 | 13 | 0 | 9 | 9 |
| Protein stability | 2 | 2 | 0 | 3 | 3 |
| Cell structure | 2 | 2 | 0 | 5 | 5 |
| Signaling | 2 | 2 | 0 | 3 | 3 |
| Stress/disease response | 9 | 8 | 1 | 4 | 5 |
| Transcription/DNA binding | 9 | 7 | 2 | 2 | 4 |
| Unknown | 26 | 23 | 3 | 14 | 17 |
| Total | 95 | 79 | 16 | 43 | 59 |

treatment, we examined the expression of some transcriptional target genes in 11 day old wild-type, mutant and overexpression seedlings treated with or without ABA and/or cycloheximide. These comparisons enabled us to distinguish those genes that were both ABA- and ABI-dependent from those that failed to meet one of these criteria, and to identify genes whose expression was not reduced by cycloheximide and were therefore most likely to be primary targets. We tested 39 genes, representing 29% of the genes induced only by ABI4, 19% of those induced specifically by ABI5, and 50% of those induced by both (Supp. Table 2).

Induction of ABI4 targets was found to be very sensitive to the relative strength of ABI4 overexpression. After the microarray and initial validation analyses, ABI4 overexpression in the line used for these studies was silenced in subsequent generations. Consequently, we created new overexpression lines, with a GFP-ABI4 fusion under the control of the 35S promoter, for use in continuing analysis of the identified targets. Although ABI4 function in this line was weaker than in the original *35S:ABI4* line (Supp. Fig. 1), possibly due to either reduced expression of the fusion gene or altered conformation of the fusion protein, some ABI4 transcriptional targets were still induced (Fig. 1). However, the induction of several targets was significantly weaker in the *35S:GFP:ABI4* line compared to the original *35S:ABI4* plants (Supp. Fig. 2A).

Of the 33 genes whose transcripts were detectable by RNA gel blots, 26 (79%) reproduced the pattern observed in the initial microarrays. However, only 16 (48%) met the more stringent criteria of depending solely on simultaneous ABA treatment and ABI over-expression for induction, and maintaining ABI-dependent induction levels in the presence of cycloheximide (Fig. 1, Supp. Fig. 2, Supp. Table 2).

## Endogenous expression of ABI4 and ABI5 targets

The goal of this study was to identify genes that were ABA/ABI transcriptional targets during normal plant development. ABI4 and ABI5 are most strongly expressed in seeds and young seedlings exposed to ABA or stresses such as NaCl or high glucose levels, so we compared expression in wild-type versus *abi* mutants at these stages. Analysis of publicly available microarrays using the Bio-Array Resource for Plant Functional Genomics (BAR) (Toufighi et al. 2005) confirmed that the majority (64%) of our target genes are expressed in either dry or 24 h imbibed seeds, with expression higher in dry seeds than in water-imbibed seeds for over 90% of seed-expressed genes (Supp. Table 3).

Furthermore, many of these genes have reduced expression in *abi4* and *abi5* mutants (Nakabayashi et al. 2005). Nearly 90% of the seed-expressed ABI4 targets identified in our microarrays were reduced at least twofold in *abi4* mutant seeds after 1 day imbibition, but less than a tenth were underexpressed in *abi4* dry seeds. Nearly half of the "ABI4-specific" targets were also underexpressed in *abi5* mutant seeds, predominantly at the dry seed stage. Although only 35% of our seed-expressed ABI5 targets were downregulated in *abi5* mutant seeds, nearly 40% of the "ABI5-specific" targets were downregulated in *abi4* mutant seeds. In contrast to the relatively few (12%) shared targets seen in our ectopic expression screen, 40% of the seed-expressed genes were downregulated in both *abi4* and *abi5* mutant seeds. Based upon these expression patterns,

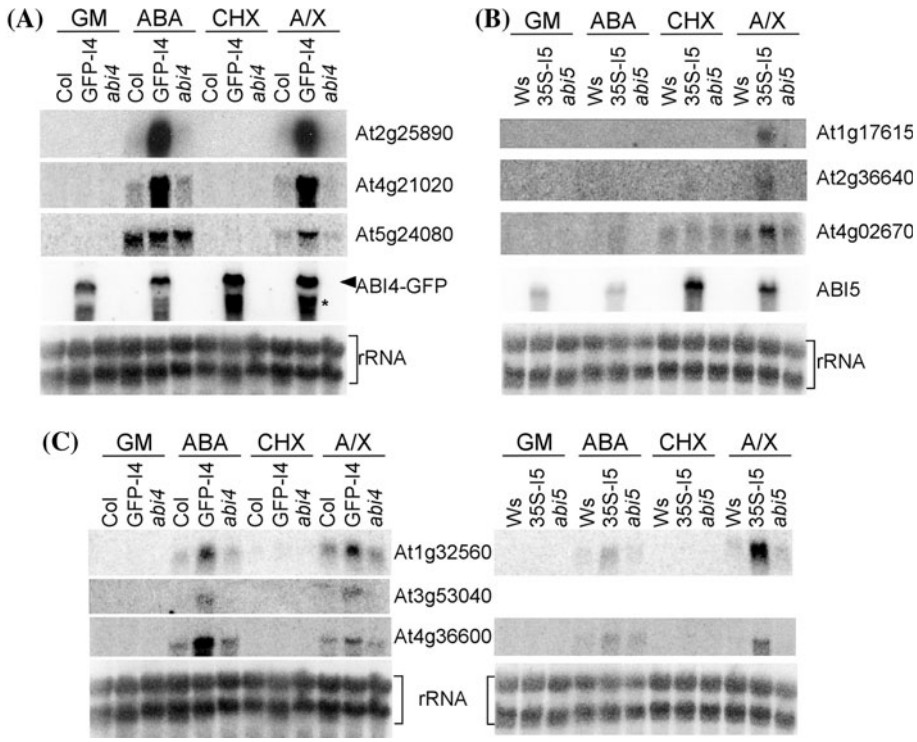


**Fig. 1** Confirmation of ABA and ABI dependent gene targets. Wild-type (Col or Ws), transgenic (35S-GFP-ABI4 or 35S-ABI5) and mutant (*abi4* and *abi5-1*) seedlings were grown for 11 days on GM, then treated for 3 h with either ABA, cycloheximide (CHX) or ABA + cycloheximide (A/X). Induction of ABI targets was assayed by Northern blot. **a** ABI4-regulated genes, **b** ABI5-regulated genes, and **c** genes regulated by both ABI4 and ABI5. For the ABI4-GFP transcript, the probe was specific to ABI4. An *arrowhead* indicates full length transcript and an *asterisk* indicates a truncated/partially degraded transcript

ABI4 preferentially affects continued expression during imbibition whereas ABI5 plays a slightly greater role in controlling expression of transcripts present in dry seeds.

The relative roles of these factors changes by 1 day after stratification, before any visible signs of germination have occurred. Comparisons of ABA- or stress-induced expression in wild-type versus *abi* mutant seeds at 1 day post stratification showed ABI5-dependent regulation by ABA and/or NaCl for 90% of the 20 seed-expressed genes tested (Fig. 2a, Supp. Table 4). In contrast, only 40% of these genes showed even slight defects in ABA or glucose induction between wild-type and *abi4* seeds (Fig. 2b, Supp. Fig. 3, Supp. Table 4). When incubated on GM, most seeds of all genotypes tested germinate by 2.5 days post-stratification, and similar fractions (10–15%) of wild-type and *abi* seeds begin germinating even in the presence of 5 uM ABA. Despite the similarity in growth of wild-type and mutants under these conditions, all transcripts tested decreased in at least one of the *abi* mutant lines (Fig. 3, Supp. Table 4).

The remaining seed expressed targets identified by our ectopic expression strategy, but not downregulated in *abi4* or *abi5* seeds, might be redundantly regulated by the ABI factor(s) and/or additional regulators such that single mutants show no significant change in transcript levels. Furthermore, at least some of the genes identified as ABI regulated in the imbibing *abi* versus wild-type seed studies are likely to be indirectly regulated. For example, one-sixth of the genes tested that had been identified as ABI-regulated in dry or imbibing seeds, and in 11 day old seedlings, showed greatly reduced ABA-induction in the presence of cycloheximide (Supp. Fig. 2b, Supp. Table 2). This pattern is consistent with a requirement for synthesis of an additional signaling factor for full expression.

## ABI4 and ABI5 target sets are differentially co-regulated

Using Genevestigator (Hruz et al. 2008) and BAR (Toufighi et al. 2005), we analyzed expression of target sets during development as well as in response to varied stresses and hormone treatments. As noted previously, most of the ABI4 and ABI5 target sets were expressed in mature seeds, although the ABI4 set was more enriched; 72% of ABI4 targets, but only 53% of ABI5 targets were expressed in seeds (Supp. Table 3). In addition, roughly two-thirds of ABI4 induced genes, including over 80% of targets shared with ABI5, were co-regulated under certain stresses in older plants (Table 2, Supp. Fig. 4). They were induced in 1–7 day old seedlings by ABA treatments ranging from 0.5 to 20 μM for a few hours to 2 days and by paclobutrazol (an inhibitor of GA biosynthesis), as well as by osmotic stress and salt stress in 16 day old plants. In contrast, less than a third of ABI5 specific targets were co-expressed with the ABI4 set under these conditions, suggesting that the ABI5 specific targets require higher ABA levels or different co-regulators for induction. Based upon these expression patterns, we

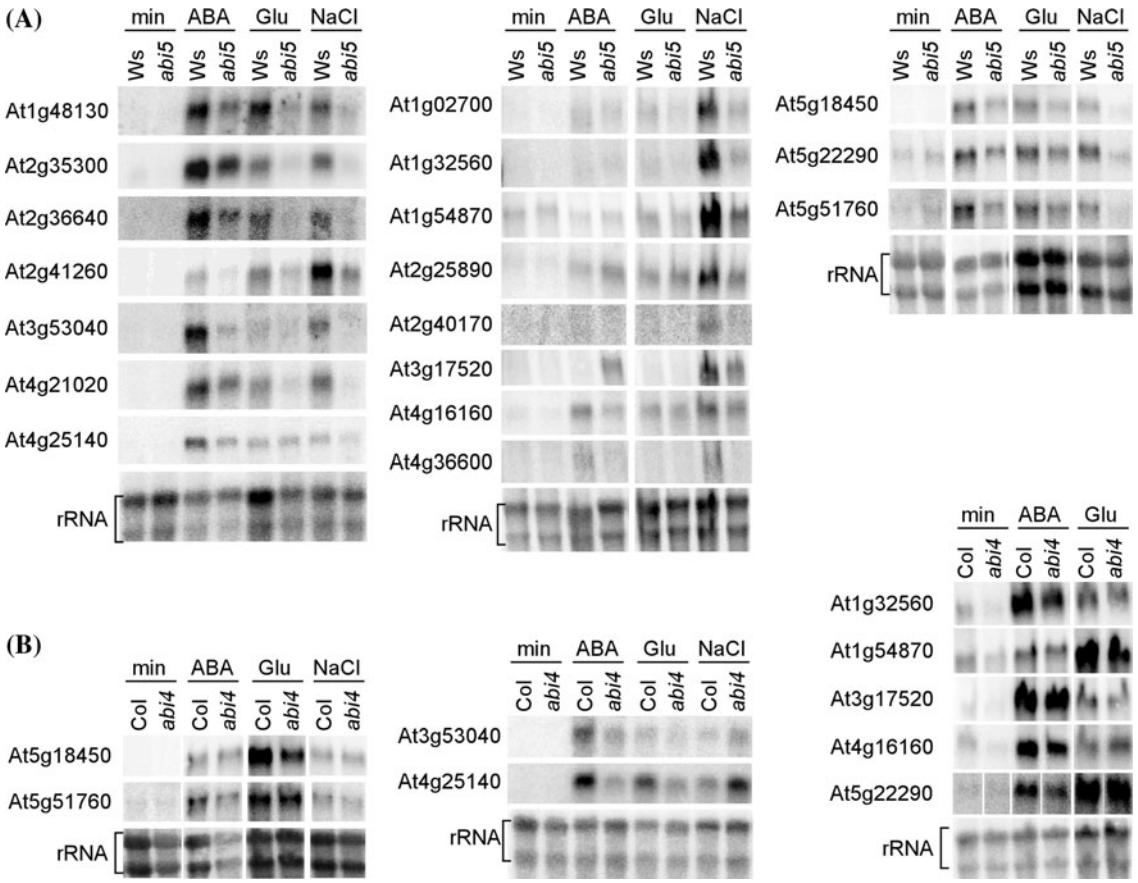


**Fig. 2** ABA and stress regulated gene expression post-stratification. Wild-type (Col or Ws) and mutant (*abi4* and *abi5-1*) seeds were stratified for 3 days on minimal media (min) with or without either 5 μM ABA, 330 mM Glucose or 165 mM NaCl, then germinated for 24 h. RNA was extracted and transcripts were analyzed by Northern blot. **a** Comparison of Ws and *abi5* seeds. **b** Comparison of Col and *abi4*

expected to find significant differences in the promoters of these gene sets.

## ABI4 and ABI5 target promoters share the same binding motifs

We used Promomer (http://bar.utoronto.ca/ntools/cgi-bin/BAR_Promoter.cgi) (Toufighi et al. 2005) to search the promoter regions of our ABI4 and ABI5 targets for enrichment of known activator binding site motifs (Table 3). The most highly enriched motifs for both sets were variations of the ABA response element (ABRE), which has previously been shown to be bound by ABI5 and related bZIP transcription factors (reviewed in Cutler et al. 2010). The stringent ABRE (YACGTGGC) was found in 33 and 37% of our ABI4 and ABI5 target genes, respectively. This is a significant enrichment over the 5% occurrence rate seen in a genome-wide promoter search. A slightly more degenerate version of the ABRE-like motif (BACGTGKM) is present in the majority of our target genes (75% of ABI4 targets and 71% of ABI5 targets), but in only 21% of all promoters in the genome. The enrichment was similar even when the shared target genes were not included (data not shown). The DRE core motif, associated with the DREB class of AP2 transcription factors (Sakuma et al. 2002), was also enriched in the ABI4 target promoters, but was even more enriched in those genes regulated by ABI5.

In contrast to the ABRE and DRE motifs, previously reported ABI4 binding sites were not significantly enriched in the set of direct ABI4 targets identified in our screen (Table 3). The S box motif was very slightly, but not significantly, enriched in this ABI4 target set compared to the genome or to ABI5 target genes. However, the S-box was still present in only 16% of the ABI4 target genes, suggesting that another motif may be responsible for ABI4 binding in the co-regulated ABI4 targets. The less stringent maize ABI4 consensus site was present in 80% of the ABI4-regulated promoters, but this was not a significant enrichment over its occurrence in the genome or in ABI5-regulated promoters. The CCAC motif is nearly ubiquitous, and it shows no significant enrichment in ABI4-regulated

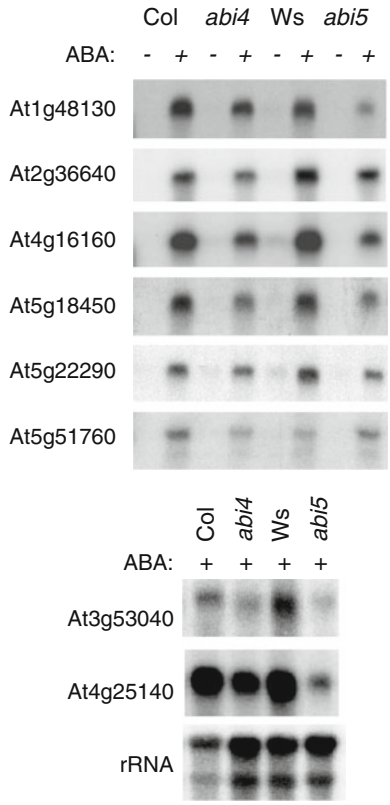


**Fig. 3** ABA-regulated expression during germination. Wild-type (Col or Ws) and mutant (*abi4* and *abi5-1*) seeds were stratified for 3 days on GM with or without 5 μM ABA, then incubated for 2.5 days prior to harvest. RNA was extracted and the indicated transcripts were analyzed by Northern blot. *Top panel* includes GM controls. *Lower panel* shows only ABA-treated samples, including rRNA loading control

genes. These results suggest that the currently described ABI4 consensus binding site(s) may be an underestimate of the variability possible in ABI4 recognition sites. To determine whether any novel motifs were highly enriched in the ABI4 target set, we analyzed these promoters with AlignACE, BioProspector, MotifSampler and Weeder (Hughes et al. 2000; Liu et al. 2001; Pavesi et al. 2004; Thijs et al. 2002), but all methods identified ABRE variants as the top-scoring motifs (data not shown).

## ABI4 and bZIPs synergistically activate promoters lacking previously characterized ABI4 binding sites

To determine whether ABI4-regulated promoters lacking known ABI4 binding sites but containing ABREs could interact with ABI4 or any bZIPs, we tested trans-activation of reporter genes in yeast. Promoters from those genes that were most strongly regulated by ABI4 and that lacked the S-box were cloned upstream of a $\beta$-galactosidase reporter and transformed into yeast. Plasmids encoding the GAL4 activation domain fused to full length ABI4, ABI5 or related bZIPs were transformed into yeast lines that were then mated to the reporter lines to create diploids with both reporter constructs and activator genes. The activator protein fusions rely on the DNA binding domain of the ABI or ABF protein for interaction with target promoters, but all are strong transcriptional activators due to the GAL4 activation domain,

As expected since these genes were largely unique to the ABI4 target set, ABI5 alone was unable to activate them in yeast. ABI4 alone also failed to activate expression from these promoters. However, some of the promoters were highly activated by either ABF1 or ABF3. Interestingly, we observed very strong synergy when both ABI4 and a bZIP (ABI5, ABF1 or ABF3) were co-expressed in yeast, with some promoters being activated up to 60-fold in the presence of both activators (Fig. 4). These results show that

**Table 2** Co-regulation of ABI4 targets by ABA and some stresses. Genevestigator (Hruz et al. 2008) was used to analyze target gene expression in response to ABA and stress treatment in previously published microarrays (Goda et al. 2008; Kilian et al. 2007; Nishimura et al. 2007; Penfield et al. 2006)

| Seedling | Treatment | Percent shared targets | Percent of ABI4 specific | Percent of ABI5 specific | Reference |
|---|---|---|---|---|---|
| 7 day | 10 uM ABA, 3 h | 69 | 59 | 23 | Goda et al. (2008) |
| 1 day | 20 uM ABA | 81 | 71 | 23 | Penfield et al. (2006) |
| 2 day—wildtype | 0.5 uM ABA | 88 | 70 | 23 | Nishimura et al. (2007) |
| 2 day—*ahg1* | 0.5 uM ABA | 94 | 73 | 33 | ibid |
| 2 day—*ahg3* | 0.5 uM ABA | 94 | 67 | 28 | ibid |
| 1 day | 20 uM PAC | 81 | 65 | 21 | Penfield et al. (2006) |
| 16 day shoots | 6–24 h 300 mM mannitol | 88 | 66 | 40 | Kilian et al. (2007) |
| 16 day roots | 6–24 h 300 mM mannitol | 69 | 65 | 33 | ibid |
| 16 day shoots | 6–24 h 150 mM NaCl | 56 | 47 | 30 | ibid |
| 16 day roots | 6–24 h 150 mM NaCl | 75 | 56 | 23 | ibid |

Genes were identified as regulated by a treatment if their expression was increased at least twofold as compared to the experimental control treatment. For each target gene set, the percent of genes that were induced by each stress treatment are shown

**Table 3** Distribution of potential binding sites for ABI4, ABI5 and related factors in the promoter regions of ABI target genes. Promomer (http://bar.utoronto.ca/ntools/cgi-bin/BAR_Promoter.cgi) (Toufighi et al. 2005) was used to search the promoters of our microarray target sets and of the whole genome for known transcription factor motifs

| Motif | Sequence | Whole genome (32,041 genes) | | ABI4 target set (95 genes) | | | ABI5 target set (59 genes) | | | Nakabayashi target set (504 genes) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Hits | % | Hits | % | *P*-value | Hits | % | *P*-value | Hits | % | *P*-value |
| ABRE-like | BACGTGKM | 6,618 | 21 | 71 | 75 | <E-10 | 42 | 71 | <E-10 | 203 | 40 | <E-10 |
| DRE core motif | RCCGAC | 7,340 | 23 | 36 | 38 | 7.1E-04 | 26 | 44 | 2.6E-04 | | | |
| ABRE | YACGTGGC | 1,501 | 5 | 31 | 33 | <E-10 | 22 | 37 | 4.1E-11 | 74 | 15 | <E-10 |
| S box | CACYKSCA | 3,801 | 12 | 15 | 16 | 0.152 | 7 | 12 | 0.560 | | | |
| Maize | CACCK | 24,337 | 76 | 76 | 80 | 0.213 | 50 | 85 | 0.072 | | | |
| CCAC | CCAC | 31,078 | 97 | 94 | 99 | 0.217 | 59 | 100 | 0.165 | | | |

Promoters were defined as the region 1,000 bp upstream of the transcription start, and searches were performed for motifs in the 5′ to 3′ orientation on both strands

ABI4 is able to activate gene promoters that lack the previously described ABI4 binding sites, but is not sufficient to do so alone in this heterologous system.

These results suggested that both ABF1 and ABF3 might also act redundantly with ABI4 and ABI5 in early seedling development. To test the roles of these bZIPs in regulating the ABI4 transcriptional target genes in planta, we compared these ABI4 target transcript levels in wild-type, *abi* and *abf* mutant seedlings. Because our previous studies had shown that *ABF1* and *ABF3* are not highly expressed in dry seeds, but take on a larger role in ABA response in young seedlings, we assayed response at 3 and 5 day post imbibition on media supplemented with 5 μM ABA. Under these conditions, the *abi* mutants were starting to germinate by 3 days, but germination of wild-type and the *abf* mutants were comparably inhibited (Supp. Fig. 5). Comparison of expression at 3 days showed that some of the ABI4-regulated transcripts were also decreased in *abf3* mutants (Fig. 5a). However, the magnitude of decrease was much less than the activation in yeast. For example, At3g53040 transcripts were reduced only about twofold in *abf3* seedlings, but induced nearly 60-fold by ABF3 alone in yeast.

To determine whether these relatively subtle effects on gene expression reflected redundant function of ABI4 and the ABFs, we analyzed double mutants. Comparison of digenic mutants with their respective parental lines showed alterations in different stress responses. The *abi4 abi5* mutants had significantly enhanced ABA resistance, and slightly increased glucose resistance (Fig. 5b, c). Although combination of *abf3* with *abi5* also enhanced ABA and glucose resistance, *abi4 abf3* mutants displayed ABA and glucose sensitivities similar to the *abi4* mutant (Fig. 5b, c; Finkelstein et al. 2005). Despite the negligible effect on ABA and glucose sensitivity, transcript levels were further reduced in the *abi4 abf3* double mutants for some of the genes whose promoters showed synergistic activation by ABI4 and bZIP factors in yeast, e.g. At3g53040 and At4g21020 (Fig. 5a). In the case of At3g17520, which appears regulated by ABI4 and all three bZIPs tested in yeast, transcript levels were significantly reduced only in the double mutant. In contrast to these reductions in expression, *ABF3* was over-expressed in *abi4* seedlings (Fig. 5d), similar to the previously described antagonistic regulation of *ABI5* and *ABF3* (Finkelstein et al. 2005).

## ABI4 directly binds DNA in the absence of additional transcription factors

Having identified genes that were regulated by ABI4 in plants and yeast, yet lacked the consensus ABI4 binding

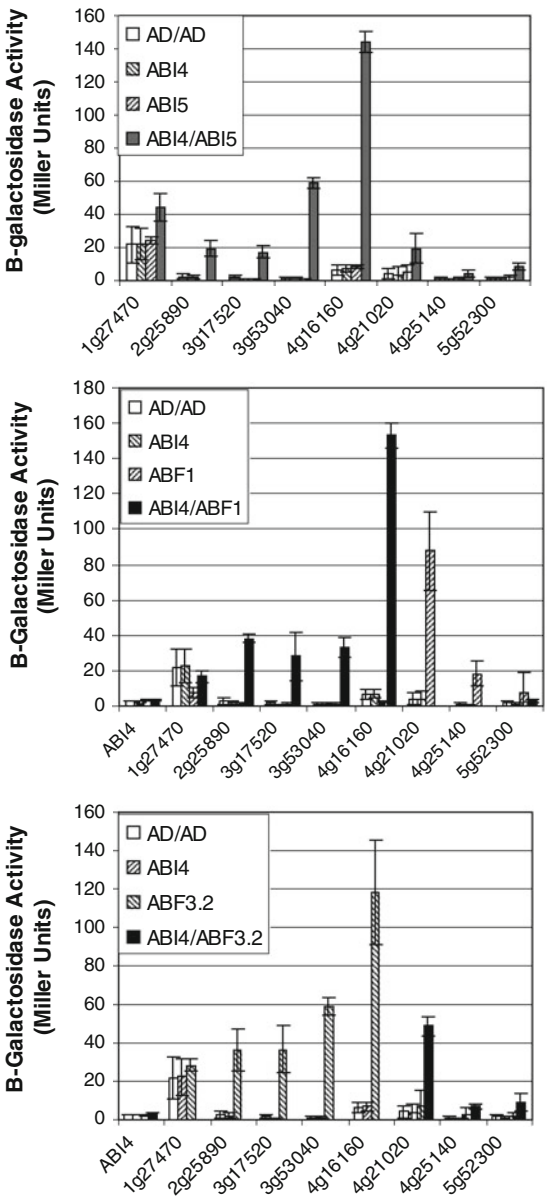


**Fig. 4** Synergistic activation of ABI4-regulated promoters by ABI4 and specific ABI5/ABF bZIPs in yeast. *Top*, *middle* and *bottom panels* show activation by combination of ABI4 with ABI5, ABF1 or ABF3, respectively. Promoters that were strongly activated by any single bZIP factor were not tested for synergistic activation with ABI4. All data presented are the averages ± standard deviation of assays on at least three samples, each representing many mating events

site, we next analyzed ABI4 binding in vitro. We tested 6 of these ABI4-regulated promoters by electrophoretic mobility shift assays (EMSAs) with GST alone or fused to the ABI4 DNA binding domain, using promoter fragments between 150 and 350 bp in length. A fragment of the ABI4 promoter previously characterized as containing ABI4 binding sites (Bossi et al. 2009) was included as a positive control. A GST fusion to the DNA-binding domain of ABI4, but not GST alone, bound the promoters of 4 ABI4 targets tested although only one of these (At1g32560) contained the maize ABI4 binding site (Fig. 6a). The fragments that failed to bind were truncations of those used in the yeast one-hybrid assays and may have been missing unidentified binding sites. Additional tests of smaller fragments of the At4g16160 promoter suggested that binding occurred within an approximately 50 bp region containing a CCAC sequence that overlaps with one of the promoter's four ABREs (Supp. Fig. 6). However, the ABI4 target promoter fragments appeared to bind less specifically than the ABI4 upstream fragment, in that binding was disrupted nearly as well by non-specific competitor as by cold probe DNA (Fig. 6b). Although the ABI4 upstream fragment contains a CE1-like ABI4 binding site, it lacks any ABREs and was not activated by ABI4 alone or in combination with any bZIPs in the yeast one-hybrid assay (Fig. 4).

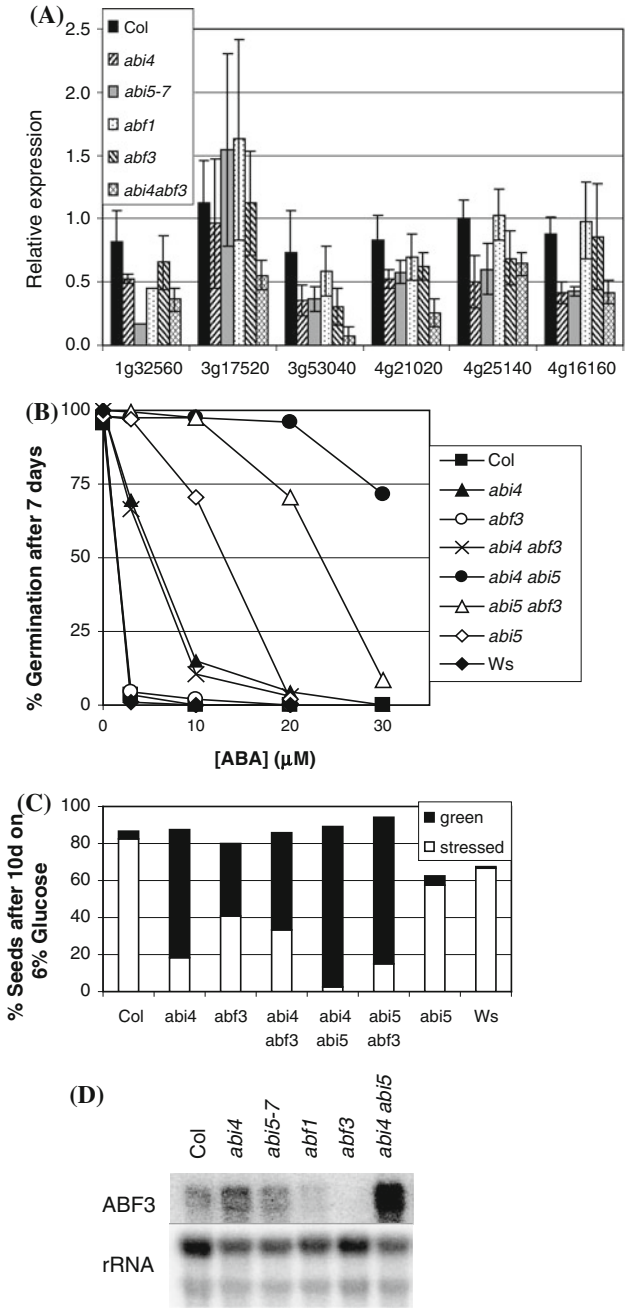


**Fig. 5** Redundant function of ABI4 and ABI5/ABF bZIPs in seedlings. **a** Comparison of ABA/ABI-dependent gene expression in wild-type (Col) and mutant seedlings at 3 day post-stratification on GM supplemented with 5 μM ABA. Expression levels shown are averages of duplicate assays of at least duplicate samples for each genotype, normalized relative to wild-type levels for each gene. **b** Comparison of ABA sensitivity in wild-type (Col and Ws), mono and digenic mutant lines, based on radicle emergence after 7 day on minimal media supplemented with the indicated concentrations of ABA. **c** Comparison of glucose sensitivity, based on production of arrested pink or white (stressed) versus green seedlings after 10 day on minimal medium supplemented with 6% glucose. **d** ABF3 transcript accumulation in the indicated genotypes, assayed by RNA gel blot

## Discussion

### Summary of ABI-regulated gene classes

Confirming our initial hypotheses, the apparent targets of ectopic ABI4 and ABI5 expression showed some overlap during vegetative growth in overexpression lines. However, although only 12% appeared co-regulated in 11 day old overexpression lines, nearly 40% of these genes were regulated by both ABI4 and ABI5/related bZIP factors in seeds and seedlings. This discrepancy in overlap could reflect a block to expression in the older plants or indirect regulation in seeds such that more targets appear shared. Nearly 60% of the genes induced in 11 day old plants by both ABI factors encode proteins involved in seed maturation or stress response. Seed maturation genes are also over four times as prevalent among the set regulated by ABI4 alone compared to the ABI5-regulated set. Of the remaining ABI-regulated genes, the largest class encodes proteins of unknown function. Each ectopically expressed ABI factor also regulated distinct sets of genes involved in metabolism, cell structure and protein stability.

The last major category of ABI-regulated genes included additional transcription factors, protein kinases, and a protein phosphatase (AHG1) that was recently found to interact directly with an ABA receptor (Park et al. 2009), thereby confirming the hypothesis that ABI transcription factors regulate some signaling components generally assumed to act upstream in ABA signaling. Within this category, ABI4 induced the expression of three genes involved in the repression of ABA signaling: the protein phosphatase AHG1 (Nishimura et al. 2007) as well as the ABI5 interacting proteins AFP2 and AFP3 (Garcia et al. 2008). Although ABI4 is a positive regulator of ABA response, these results indicate that it may also play a key role in the feedback regulation of ABA signaling.

### Advantages and limitations of ectopic expression for identifying ABI4 and ABI5 targets

A variety of microarray studies have analyzed the role of ABI transcription factors in gene regulation. These include the dry and imbibed seed comparisons for *abi4* and *abi5* mutants described above (Nakabayashi et al. 2005), comparison of endosperm versus embryo-expressed genes in germinating seeds (Penfield et al. 2006), ABI3-regulated genes in after-ripened or fresh (dormant) seeds (Carrera et al. 2008), targets of ectopically expressed maize VP1 (an ABI3 ortholog) in an *abi3* mutant background (Suzuki et al. 2003), and targets of ectopically expressed ABI3 (Nakashima et al. 2006). Comparison of these lists shows sub-

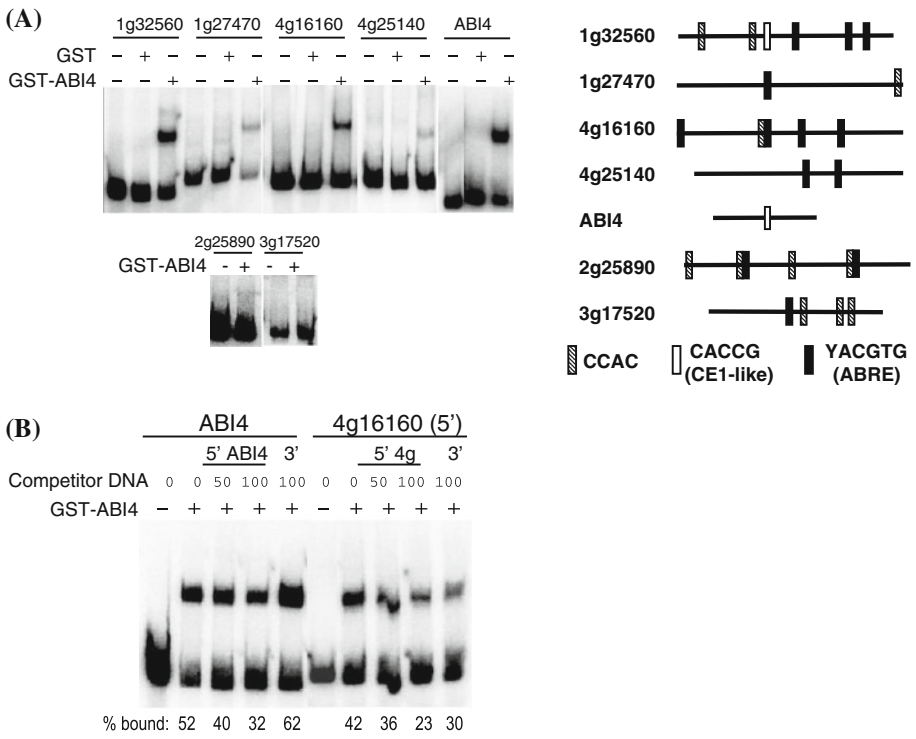


**Fig. 6** ABI4 binds to promoter fragments lacking any characterized ABI4 binding site. Electrophoretic mobility shift assays (EMSAs) with promoter fragments from At1g32560 (310 bp), At1g27470 (329 bp), At4g16160 (295 bp), At4g25140 (266 bp), At2g25890 (301 bp), At3g17520 (207 bp) or ABI4 (152 bp). The motifs contained within these fragments are indicated schematically. **a** Comparison of binding by GST versus GST-ABI4 DNA binding domain fusion demonstrates interaction with the ABI4 domain. **b** Competition with 50- or 100-fold molar excess of nonradioactive probe (5′ ABI4 or 5′ 4g16160) or the 3′ UTR of ABI4

stantial overlap among the genes regulated by these ABI transcription factors. Although the VP1/ABI3 ectopic expression studies used distinct microarrays, each representing approximately 7 K genes, both showed that nearly 5% of the genes assayed were regulated by VP1/ABI3. Further comparisons, using the Expression Browser on BAR to analyze publicly available microarray data, showed that roughly 80% of the genes identified as regulated by ABA and VP1 or ABI3 in the ectopic expression studies were underexpressed in *abi4* or *abi5* mutants at 24 h after imbibition, when compared to fresh wild-type seeds. Similarly, over 60% of the ABI4 and/or ABI5 targets identified in the present study are underexpressed in *abi3-4* mutants. A much smaller fraction appear underexpressed in all *abi* genotypes when compared to after-ripened wild-type seeds, presumably reflecting the higher commitment toward germination in all of these non-dormant seeds. The high degree of overlap among ABI-regulated genes probably reflects a combination of cross-regulation of these and other ABA response factors, and direct regulation of shared targets.

As discussed earlier, most of the genes induced by ectopic ABI expression are normally expressed in seeds, and many are regulated in seeds by the *ABI* loci, supporting the validity of our approach. The relatively smaller fraction of genes that were not downregulated in *abi* seeds may be redundantly regulated by other transcription factors present in seeds. For example, some of these genes are also regulated by related bZIP factors during ABA-inhibited germination (Fig. 5). Our method therefore allowed us to identify genes that are directly regulated by, though not solely dependent on, ABI4 and ABI5.

In contrast to seed-expressed genes regulated by multiple ABI factors, the apparent 35S-ABI transcriptional targets that are not expressed in seeds might depend on interacting factors absent in dry or imbibing seeds. Although initially described as seed regulatory factors, ABI4 and ABI5 have subsequently been shown to function well into vegetative growth where they affect lateral root development (Signora et al. 2001), photosynthetically active gene expression (Acevedo-Hernandez et al. 2005), and pathogen response (Kaliff et al. 2007). It is possible that some of the ABI targets identified by our screen are involved in these vegetative responses.

Despite identifying these additional targets, the number of genes induced in our ectopic expression study is much lower than the number of genes whose expression is reduced in *abi* seeds (approximately three and tenfold lower for *ABI4* and *ABI5*, respectively) (Nakabayashi et al. 2005); this could reflect indirect regulation in seeds, the strength of transgene expression, or a lack of interacting factors or chromatin accessibility required for expression in the older seedlings.

For example, promoter analyses suggest that our ABI5 regulated gene set is more enriched for direct ABI5 transcriptional targets than has been previously achieved using *abi5* mutant lines. Only 40.3% of genes identified as ABI5 dependent in dry seeds contain the degenerate ABRE-like motif (Table 3) (Nakabayashi et al. 2005). Although this is

a substantial enrichment over the genome-wide occurrence rate (21%), it is much lower than the 71% of 35S-ABI5 induced genes that contained this motif. This suggests that many genes identified as ABI5 dependent in loss of function mutant studies are not direct targets of ABI5. Our list of ABI target genes, though significantly smaller than those identified in mutant studies, may offer a more accurate representation of the direct transcriptional targets of ABI4 and ABI5.

Nakabayashi et al. found that 14.6% of the genes underexpressed in *abi5* mutant dry seeds were located near other downregulated genes, suggesting that their co-regulation was partly dependent on local chromosomal structure (Nakabayashi et al. 2005). Many seed specific genes are repressed during vegetative growth by chromatin remodeling that is dependent on PICKLE (Li et al. 2005), BRAHMA (Tang et al. 2008), Polycomb group proteins (Makarevich et al. 2006), trihelix repressors (Gao et al. 2009) and other factors. Previous studies have demonstrated that ectopic expression of B3 domain proteins such as the bean ABI3 homolog (PvALF) and, to a lesser extent, Arabidopsis FUS3 can modify chromatin structure to potentiate expression of seed genes in vegetative tissues upon subsequent induction by ABA (Li et al. 1999; Ng and Hall 2008). In addition, ABI3 (or its monocot ortholog VP1) and ABI5 (or its rice homolog TRAB1) display direct and synergistic interactions in two-hybrid analyses in yeast and in transient reporter activation assays in rice protoplasts, consistent with a role for ABI3 in induction as well as potentiation (Finkelstein et al. 2005; Gampala et al. 2002; Nakamura et al. 2001). Although ectopic ABI5 expression is not sufficient to activate several seed-specific promoters, partly due to the limited activation potential of ABI5 (Bensmihen et al. 2004), co-expression with PvALF renders phaseolin induction ABA-independent (Ng and Hall 2008). Our ectopic ABI5 expression lines lacked overexpressed ABI3 or similar seed transcription factors, and therefore may not have supported the vegetative induction of some genuine ABI5 targets. Consistent with this possibility, only two of the genes identified as induced by ABI5 overexpression in our microarrays (3.3%) were associated with the *abi5* downregulated gene clusters described in Nakabayashi et al. (2005).

### Mechanism of ABI4 activation of genes lacking consensus ABI4 binding site

The ABI4-regulated genes identified in ectopic expression lines correlated strongly with ABI4-dependent expression in seeds. However, most lacked either the closely related S box or maize ABI4 binding sites. These ABI4 binding sites were originally identified on the basis of in vitro binding studies and comparisons of ABI4-regulated genes, and were functionally tested by reporter gene activation in protoplasts or yeast one-hybrid assays (Acevedo-Hernandez et al. 2005; Niu et al. 2002).

The documented ABI4 binding sites appear to have relatively low affinity and specificity of binding, since EMSAs require ~100 ng of fusion protein to shift ~0.25 ng DNA as compared to less than 25 ng of purified protein for other transcription factor classes (Buratowski and Chodosh 2001). Furthermore, the documented sites include the motif in both the 5′ to 3′ and the 3′ to 5′ orientation, unusual for a non-palindromic recognition site (Acevedo-Hernandez et al. 2005). Our EMSAs demonstrated weakly selective binding to promoter fragments lacking the S-box [CACYKSCA] and related CE1-like binding site, indicating an even greater lack of specificity for DNA binding by ABI4.

Our functional assays were performed in yeast because this completely heterologous system lacks any additional plant factors that could contribute to promoter activation. For example, 35S-ABI4 expression in Arabidopsis results in ABA-inducible expression of ABI3, ABI5 and some related bZIPs (Brocard et al. 2002; Söderman et al. 2000). The presence of these additional factors in 11 day old plants or in transient assays in protoplasts could facilitate induction of ABI4 target genes that may require transcriptional activator synergy. In fact, all the target promoters we tested in yeast showed essentially no response to ABI4 alone, but several showed substantial synergy when ABI4 was combined with ABI5 or a related bZIP. The cooperative interaction of ABI4 and the bZIP transcription factors is also consistent with the significant enrichment of ABRE and ABRE-like motifs in the promoters of our *35S:ABI4* transcriptional target set. Furthermore, digenic mutant analyses showed that ABI4, ABI5 and ABF3 act redundantly in regulating some ABA responses in plants. Although we tested only ABF1 and ABF3 for synergy with ABI4, many of the ABI-regulated genes identified in our study were recently shown to be redundantly regulated by AREB1/ABF2, AREB2/ABF4, and ABF3 (Yoshida et al. 2010).

## Summary

Our results identified a relatively small number of direct transcriptional targets for ABI4 and ABI5, many of which are a subset of genes previously shown to be underexpressed in *abi4* and *abi5* mutants. These targets included both positive and negative regulators of ABA response. Although there was some overlap of these targets and both gene sets were highly enriched for ABREs in their predicted promoter regions, the patterns of co-regulation for ABI4- and ABI5-regulated genes were quite different.

Despite being tightly co-regulated in seeds, the promoters of the ABI4 target set were not significantly enriched for any previously described ABI4 binding sites, yet ABI4 was still able to bind some of these promoters in vitro. Finally, expression assays in a heterologous yeast system demonstrated that ABI4-regulated promoters could be synergistically induced by specific combinations of ABI4 and some bZIP factors, even when no canonical ABI4 binding sites were present, and that ABI4 binding alone was not sufficient for activation.

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