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Redundant and distinct functions of the ABA response loci *ABA-INSENSITIVE(ABI)5* and *ABRE-BINDING FACTOR (ABF)3*

Ruth Finkelstein[1,*], Srinivas S.L. Gampala[2,4], Tim J. Lynch[1], Terry L. Thomas[3] and Christopher D. Rock[2]

[1]*Molecular, Cellular, and Developmental Biology Department, University of California at Santa Barbara, Santa Barbara, CA, 93106, USA (*author for correspondence; e-mail finkelst@lifesci.ucsb.edu);* [2]*Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409;* [3]*Department of Biology, Texas A&M University, BSBE 201, College Station, TX 77843;* [4]*Current address: Department of Plant Biology, Carnegie Institution of Washington, 260 Panama St, Stanford, CA, 94305, USA*

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Abstract

Abscisic acid-responsive gene expression is regulated by numerous transcription factors, including a subgroup of basic leucine zipper factors that bind to the conserved *cis*-acting sequences known as ABA-responsive elements. Although one of these factors, ABA-insensitive 5 (ABI5), was identified genetically, the paucity of genetic data for the other family members has left it unclear whether they perform unique functions or act redundantly to *ABI5* or each other. To test for potential redundancy with *ABI5*, we identified the family members with most similar effects and interactions in transient expression systems (*ABF3* and *ABF1*), then characterized loss-of-function lines for those loci. The *abf1* and *abf3* monogenic mutant lines had at most minimal effects on germination or seed-specific gene expression, but the enhanced ABA- and stress-resistance of *abf3 abi5* double mutants revealed redundant action of these genes in multiple stress responses of seeds and seedlings. Although *ABI5*, *ABF3*, and *ABF1* have some overlapping effects, they appear to antagonistically regulate each other's expression at specific stages. Consequently, loss of any one factor may be partially compensated by increased expression of other family members.

Abbreviations: ABA, abscisic acid; ABI, ABA-insensitive; ABF, ABRE binding factor; ABRE, ABA-responsive element; AD, GAL4 activation domain; AREB, ABA response element binding factor; AtDPBF, *Arabidopsis thaliana* Dc3 promoter binding factor; BD, GAL4 binding domain; bZIP, basic leucine zipper; COR, cold responsive; EEL, enhanced Em levels; Em, early methionine-labeled; GBF, G-box binding factor; GM, germination medium; GUS, beta-glucuronidase (*uidA*); LEA, late embryogenesis abundant; RAB, responsive to ABA; RD, responsive to dehydration; TILLING, Targeting Induced Local Lesions in Genomes; VP1, Viviparous 1

Introduction

The plant hormone abscisic acid (ABA) is an important regulator of plant growth and development, affecting such diverse processes as seed maturation and germination, cell division and elongation, and responses promoting tolerance of abiotic stresses (Finkelstein and Rock, 2002; Himmelbach *et al.*, 2003). Many of these processes involve changes in gene expression and many

ABA-regulated genes have been identified (Busk and Pages, 1998; Rock, 2000; Finkelstein *et al.*, 2002; Hoth *et al.*, 2002;). Promoter analyses in diverse species have identified a conserved cis-acting element, designated the ABA-responsive element (ABRE), that is a subset of the 'G-box' sequence bound by basic leucine zipper (bZIP) class transcription factors (Busk and Pages, 1998). In addition, convergent genetic and biochemical studies have identified ABA-INSENSITIVE 5 (ABI5) and closely related bZIP factors as trans-acting factors that regulate ABRE-containing genes (Kim *et al.*, 1997; Choi *et al.*, 2000; Finkelstein and Lynch, 2000; Uno *et al.*, 2000; Lopez-Molina *et al.*, 2001; Kim *et al.*, 2002).

Several members of the Arabidopsis ABI5-homologous clade of bZIPs were initially identified on the basis of their ability to bind ABREs in yeast one-hybrid assays or *in vitro*, and were consequently designated ABFs or AREBs for *A*BRE *b*inding *f*actors or *A*BA *r*esponse *e*lement *b*inding factors, respectively (Choi *et al.*, 2000; Uno *et al.*, 2000). Five additional factors, designated At-DPBFs, were identified by homology to clones encoding the sunflower *D*c3 *p*romoter *b*inding *f*actors (DPBFs) (Kim *et al.*, 1997, 2002). Comparison of their predicted amino acid sequences showed that several of these independently identified loci were identical: *ABF2/AREB1, ABF4/AREB2, ABI5/AtDPBF1, AREB3/AtDPBF3* and *ABF3/AtDPBF5* (Kim *et al.*, 1997, 2002), although *ABF3.2* and *AtDPBF5/ABF3.1* appear to be splice variants derived from a single locus (Brocard *et al.*, 2002; Kim *et al.*, 2002). *In vitro* DNA binding assays showed that these factors had similar binding specificity and several could form heterodimers, creating a strong potential for redundant function (Kim *et al.*, 2002). Among these, ABI5 was shown to form heterodimers with AtDPBF3 and AtDPBF4. Five additional family members were identified by homology during annotation of the genome (Jakoby *et al.*, 2002). However, in the absence of any functional information, it was not possible to predict the specific physiological role(s) of any of these factors.

As a first step toward defining the roles of the *ABF/AREB/DPBFs*, their expression patterns were characterized to provide a developmental context that specified which responses they might mediate. Consistent with the fact that the clones encoding the ABFs and AREB factors came from stressed vegetative tissue, expression analyses indicated that all were inducible to varying degrees by ABA, salt, cold or drought stress (Choi *et al.*, 2000; Uno *et al.*, 2000). However, many of the initial studies were done with disparate stages of growth or different concentrations or durations of stress treatments such that it was impossible to directly compare expression of all family members under any conditions. Since then, more global analyses have been performed by microarray studies of stress- and ABA-treated tissue or developing seeds (Ruuska *et al.*, 2002; Seki *et al.*, 2002; Zimmermann *et al.*, 2004) (http://www.weigelworld.org/resources/microarray/AtGenExpress/), or RNA gel blot analyses of expression in developing wild-type seeds (Bensmihen *et al.*, 2002) or *ABI*-regulated expression in vegetative tissue or maturing seeds (Brocard *et al.*, 2002; Brocard-Gifford *et al.*, 2003). In addition, developmental regulation of specific loci has been analyzed by promoter-GUS fusions (Kang *et al.*, 2002; Kim *et al.*, 2004). The gist of these studies is that, although each locus shows some developmental or stress-specificity and may show opposing *ABI*-dependence, many of these loci are expressed concurrently and thus might function redundantly.

Multiple *abi5* mutants have been isolated independently in screens for ABA-resistant germination (Finkelstein, 1994), ABA-resistant seedling growth (Lopez-Molina and Chua, 2000), and reduced expression of a GUS reporter driven by the promoter from the ABA-dependent *l*ate *e*mbryogenesis *a*bundant (lea) *AtEm1* gene (Carles *et al.*, 2002). However, no mutant alleles of the other 12 members of the Arabidopsis *ABI5/ABF/AREB/DPBF* clade of bZIPs (Jakoby *et al.*, 2002) have been identified in any forward screens for loss of ABA response. Possible explanations for this include redundancy, antagonistic function of distinct family members, or a failure to use an appropriate response for screening. Reverse genetic studies, making use of sequence-indexed loss-of-function lines or overexpression lines, can distinguish among these possibilities. Until recently, the only loss-of-function line described was the e*nhanced* E*m* le*v*els (*eel)* mutant (Bensmihen *et al.*, 2002). *EEL* is expressed in maturing seeds (Bensmihen *et al.*, 2002), shows enhanced ABA-induction in *35S:ABI3* and *35S:ABI4* lines (Brocard *et al.*, 2002), is identical to *AtDPBF4*, and its product is capable of forming heterodimers with ABI5 that bind the *AtEm1* promoter (Bensmihen

et al., 2002; Kim *et al.*, 2002). These characteristics would be consistent with redundant function of *EEL* and *ABI5*. However, the enhanced *Em* levels in the mutant indicate that EEL actually antagonizes ABI5 function to delay *Em* expression until very late in embryogenesis.

In contrast to *EEL*, overexpression of either *ABF3* or *ABF4* was reported to increase sensitivity to ABA, NaCl and osmotic stress at germination, to enhance drought tolerance and increase the basal level of expression for several stress-induced genes (Kang *et al.*, 2002), suggesting that they might be additional positive regulators of ABA response. Similarly, *ABF2* overexpression alters sensitivity to ABA and several stresses, conferring increased tolerance of some stresses but inhibiting growth in the presence of even mild exposure to others (Kim *et al.*, 2004). Despite the dramatic effects of overexpression of these *ABFs*, recent analyses of knockout lines showed that *ABF2* functions primarily in seedling Glc response, whereas *ABF3* and *ABF4* contribute differentially to ABA, drought, and salt stress response in vegetative growth (Kim *et al.*, 2004). Thus, while such overexpression studies demonstrate the potential effects of these regulators, they may overestimate their role, e.g. in tissues where they are not normally expressed.

An alternate and potentially more rapid approach to address function of these factors is to test their ability to transactivate specific reporter genes in transiently transgenic protoplasts, then analyze corresponding loss-of-function lines. In order to determine whether any of the *ABI5*-related gene(s) act redundantly with *ABI5* in their normal developmental contexts, we have identified knockout lines for the loci showing the strongest transactivation of an *Em* promoter and constructed double mutants combining *abi5* with *abf3*, the only mutation conferring even slight ABA-resistance. Our results extend those recently reported for the *abf3* mutant (Kim *et al.*, 2004).

Materials and methods

Plant materials

Arabidopsis plants were grown in growth chambers under continuous light. The *abi5-1* mutant was isolated from a mutagenized Ws population as described in (Finkelstein, 1994). Seeds of insertion and TILLING (Targeting Induced Local Lesions in Genomes) lines were obtained from the Arabidopsis Biological Resources stock center. Homozygous insertion lines were identified by screening for 100% kanamycin resistance and/or confirmed by PCR amplification of T-DNA junction fragments, and the absence of an uninterrupted endogenous gene product. The junction fragments were subsequently sequenced. The knockout lines used were SALK_043079 (*ABF1*) and SALK_075836 (*ABF3*), both in the Columbia background; the NPTII gene is no longer active in the *abf1* line. The *abi5 abf3* double mutants were selected from F2 populations on the basis of ABA-resistant germination and kanamycin resistance; genotypes were confirmed by PCR and segregation analysis in the F3 generation. The TILLING mutant used was *abf3*-CS91025; the progenitor line for this mutant is 'Big Mama,' an *erecta* mutant in the Col background (Till *et al.*, 2003).

Maize mesophyll protoplasts were isolated from 20-h illuminated leaves of 10-day-old maize seedlings that had been kept in the dark at 25 °C. The middle part of the second leaves (about 6 cm in length) was cut into 0.5 mm strips with a razor blade and digested with gentle shaking in an enzyme solution containing 1% (w/v) cellulose RS, 0.1% (w/v) macerozyme R10 (Yakult Honsha, Nishinomiya, Japan), 0.6 M mannitol, 10 mM MES (pH 5.7), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.1% BSA (w/v) for 3 h at room temperature. Protoplasts were released by shaking on a rotary shaker at 80 rpm for 10 min and were filtered through a 70 μm nylon filter. Protoplasts were collected by centrifugation at 100 g for 2 min, washed in cold 0.6 M mannitol solution, centrifuged, and resuspended at 2×10^6/ml in cold 0.6 M mannitol. Electroporation conditions were 400 V/cm, 200 μF, 10 msec, and two pulses with a Biorad GenePulser apparatus. Each sample contained 5×10^4 protoplasts and about 50 μg DNA in 0.3 ml of 0.6 M mannitol and 20 mM KCl.

Embryonic rice (*Oryza sativa*) callus cultures (Radon 6 from the International Rice Research Institute, Los Baños, Philippines) were obtained. Embryonic rice callus cultures were grown as suspensions in liquid culture as well as on phytagel plates containing MS medium supplemented with 2.0 mg/l 2,4-D. Cultures were propagated and

digested for making protoplasts as previously described except that 10 mM HEPES (Sigma, St. Louis, MO, USA), pH 5.6, was substituted for phosphate in the Krens' F medium, and 2% (weight/volume; w/v) cellulase YC, 0.35% (w/v) macerozyme, and 0.1% (w/v) pectolyase Y23 were used for overnight digestion (Karlan Research Products, Santa Rosa, CA, USA). Protoplasts were transformed with various mixtures of DNA reporter and effector constructs using polyethylene glycol precipitation. Transformed protoplasts were incubated with or without 100 μM ABA for 16 h in the dark in Krens solution before quantifying β-glucuronidase (GUS) and luciferase (LUC) reporter enzyme activities as previously described (Gampala *et al*., 2002). ABA was dissolved and stored in absolute ethanol at −20 °C as a 0.1 M stock solution. Prior to use, required dilutions of ABA were made in Krens solution, and control samples received the same volume of solvent as in ABA treatments.

Plasmid constructs

Plasmid pBM207 contains the wheat (*Triticum aestivum*) Early Methionine-labeled (*Em*) promoter driving the expression of GUS, encoded by *uidA* from *Escherichia coli*. Plasmid pDH359 contains *ABI5* cDNA driven by *Ubiquitin* (*Ubi*) promoter. Plasmid pCR349.13S contains the *CaMV35S* promoter driving the *VP1* sense cDNA. Plasmid pDirect2.6 contains the *Ubi* promoter in a reverse orientation and was used as control construct to balance the total amount of input plasmid DNA between various treatments. Plasmid pAHC18 contains the *Ubi* promoter driving firefly (*Photinus pyralis*) *LUC* cDNA and was included in transformations to provide an internal reference for non-ABA-inducible transient transcription in reporter enzyme assays. *ABF1*, *ABF3*, *ABF4*, *AREB3*, and *EEL* were amplified by PCR using gene-specific primers from an Arabidopsis cDNA library (Minet *et al*., 1992) and were cloned into plasmid pDH349 (Gampala *et al*., 2002) containing the maize *Ubi* promoter and nopaline synthase 3′ termination signals. Primers used for PCR amplification are listed in Table 1.

Yeast two-hybrid assays

Translational fusions between ABI3, ABI5 and the related bZIP factors and the GAL4 activation and DNA-Binding Domains were constructed in the pGAD-C(x) and pGBD-C(x) vectors, respectively (James *et al*., 1996). The BD-ABI5 construct encoded all but the first eight amino acids of ABI5, thus including all conserved domains, as previously described (Nakamura *et al*., 2001). The cDNAs used for construction of the BD-AtDPBF fusions were described in (Kim *et al*., 2002). The cDNAs used for construction of the remaining BD-ABF fusions are described above. The newly constructed BD fusions included full length clones encoding AtDPBF3 (AREB3) and AtDPBF4 (EEL), and all but the first 6 amino acids of ABF1, the first 3 amino acids of ABF3.1 or ABF3.2, and the first amino acid of ABF4, thereby including all conserved domains. All gene fusions were transformed into yeast (*Saccharomyces cerevisiae*) strain PJ69 (James *et al*., 1996) and β-galactosidase activity was quantified as previously described (Nakamura *et al*., 2001).

Germination and seedling growth assays

Germination assays were performed with seeds that were surface sterilized in 5% hypochlorite and 0.02% Triton X-100 and then rinsed several times with sterile water before plating on minimal medium (Haughn and Somerville, 1986) containing 0.7% (w/v) agar supplemented with different concentrations of ABA, Glc, NaCl or sorbitol. The

Table 1. Gene-specific PCR primers used to clone Arabidopsis ABI5-like cDNAs used herein.

Gene	Primer sequence (5′- > 3′; F = forward, R = reverse)	
ABF1	F: cccaagcttggatccaaagggtctgattcgtttgt	R: cggggtaccgttaacgtcacatcttctctatagct
ABF3	F: ccgctcgagggatccgaagcttgatcctcctagtt	R: cggggtaccgatatcagatacaagataaattcact
ABF4	F: cccaagcttggatccgaacaagggttttagggctt	R: cggggtaccgatatcgttgccactcttaagtaata
AREB	F: cccactagtggatccatggattctcagaggggtat	R:cggggtaccgatatctcagaaaggagccgagcttg
3 EEL	F: cccggtaccggatccacagtttctaaggcaaaata	R: cggaggcctgaattcacttgaactagtgtttgtac

dishes were incubated for 3 days at 4 °C to break any residual dormancy and then transferred to 22 °C in continuous light (50–70 μE /m^2/s^1); germination was scored daily up to 7 days. For assays of seedling sensitivity to Glc, seedling development and color was scored after 10–12 days.

For root growth assays for all except TILLING lines, seeds were germinated on GM (0.5×Murashige-Skoog salts, 1% sucrose), then 2.5 d old seedlings were transferred to fresh media supplemented with ABA, NaCl, or sorbitol. Plates were incubated vertically for an additional 5 days before measuring root growth. For TILLING lines, 4-day-old seedlings were transferred to media (0.5×MS salts, 2% sucrose, 1% phytagel) with or without ABA for 3 additional days before measuring growth.

RNA isolation and RNA gel blot analysis

RNA was isolated from dry seeds and vegetative tissues as previously described (Soderman *et al.*, 2000). RNA concentrations were determined based on absorbance at 260 and 280 nm. Total RNA (2–10 μg per lane) was size fractionated on MOPS 3-(*N*-morpholino)-propanesulfonic acid]-formaldehyde gels and then 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 hybridization to an rDNA probe. Transcripts from ABA-inducible genes were detected by hybridization to cDNA clones as described (Soderman *et al.*, 2000), labeled by random priming to a specific activity of 10^8 cpm/μg. The *ABI5* cDNA clone is described in (Finkelstein and Lynch, 2000), the *cor78* cDNA clone was described by (Hajela *et al.*, 1990); the *AtEm1* and *RAB18* cDNAs were provided by M. Delseny; the *RD29B* cDNA (U15808) is an expression vector subclone derived from RIKEN cDNA clone RAFL05-11-I09 (Yamada *et al.*, 2003). The *ABF3* (At4g34000) transcripts were detected by hybridization to an *AtDPBF5* cDNA (Kim *et al.*, 2002). The *ABF1* (At1g49720) transcripts were detected by hybridization to a PCR fragment amplified from Ws genomic DNA, corresponding to nt 587-941 of the coding sequence, a relatively gene-specific region of exon 1. Hybridization conditions for *LEA* and *dehydrin* 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 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. *ABI5, ABF1*, and *ABF3* 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. Hybridization was quantified by Phosphoimager analysis; abundance of individual transcripts was normalized relative to rRNA present in each lane.

Results

Effects of bZIP overexpression in protoplasts

Many of the ABI5-related bZIPs are expressed during seed development and/or early seedling growth (Bensmihen *et al.*, 2002; Kim *et al.*, 2002) and are therefore candidate regulators of physiological events at the transition between these stages. ABA promotes acquisition of desiccation tolerance during seed maturation and maintenance of this state in seeds that have begun to germinate (Finkelstein *et al.*, 2002). Although the biochemical basis of desiccation tolerance is not fully understood, expression of genes encoding the hydrophilic class of proteins known as LEAs is correlated with this tolerance. To identify the bZIPs that were most likely to function in ABA response at this stage, six of the most closely related members of the Arabidopsis ABI5 clade were tested for their ability to transactivate the *LEA* class wheat *Em* promoter in transient overexpression assays. These studies used protoplasts derived from either rice embryonic suspension cultures or maize mesophyll cells from 10-day-old seedlings, heterologous expression systems that were well-established in the lab (Gampala *et al.*,

2002). This comparison showed that all except ABF1 enhanced *Em*-GUS expression in maize protoplasts by 2.5–4-fold in the presence of 100 μM ABA and 5–17-fold on hormone-free medium. The strongest ABA-dependent induction in maize protoplasts was achieved with ABF3.2, which gave nearly 7-fold induction vs. 2–4-fold for the remaining bZIPs (Figure 1A, bars marked 'minus 35S:VP1'). Similar results were seen with rice protoplasts (Supplementary data Figure 1). The inductive effect of ABF3.2 was antagonized by the dominant negative abi1-1 protein (Figure 1B), as had previously been shown for ABI5 (Gampala *et al.*, 2002).

Previous studies have demonstrated ABA-dependent synergistic effects between ABI5 and ABI3 or their orthologs (Hobo *et al.*, 1999; Gampala *et al.*, 2002), so the various bZIPs were also tested for possible synergism with VIVIPAROUS1 (VP1), the maize ortholog of ABI3. Again the greatest enhancement of ABA-dependent VP1 transactivation was seen with ABF3.2 in maize and rice (Figure 1A; Supplementary data Figure 1). Surprisingly, although ABF1 did not enhance ABA inducibility of the *Em::GUS* reporter in maize (Figure 1A) or rice (Finkelstein *et al.*, 2002); Supplementary data Figure 1] when presented alone, it showed a very strong synergism

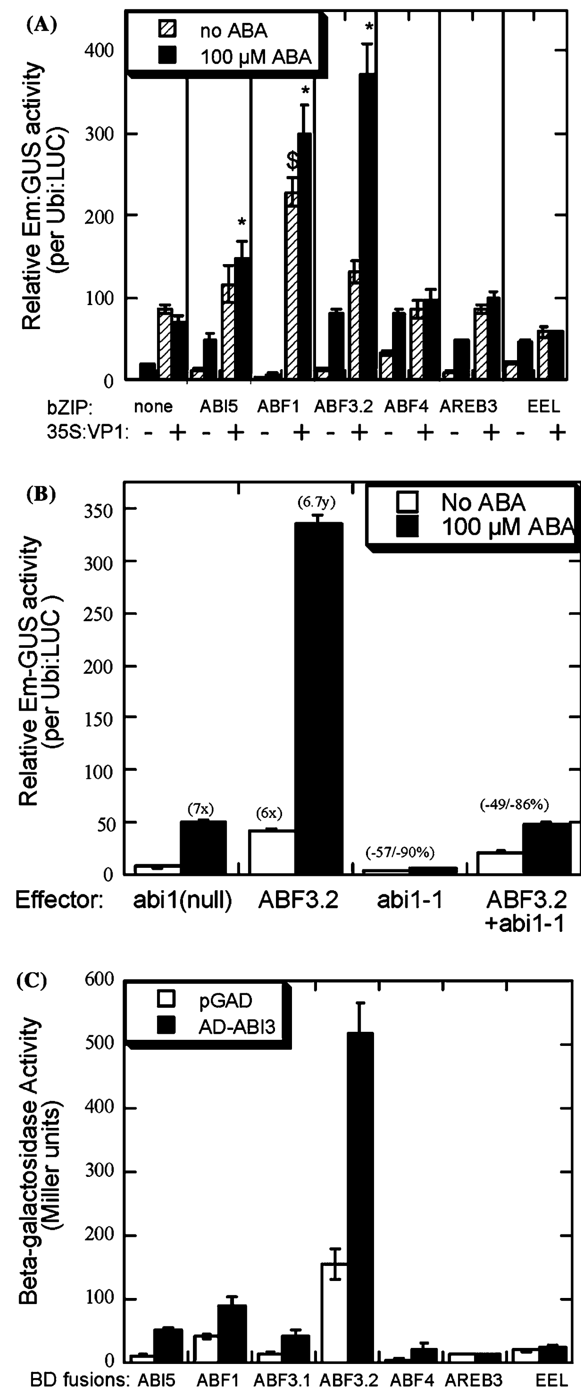


Figure 1. Interactions of bZIPs and other ABA response factors in heterologous systems. Overexpressed ABI5-like family members interact synergistically with ABA and VP1 to transactivate the wheat *Em* promoter. Maize mesophyll protoplasts were transformed with either *Em*-GUS construct alone or in combination with *Ubiquitin* (*Ubi)*-bZIP and/or *35S*-VP1 or *Ubi*-vector alone. Dollar sign ($) or asterisk (*) represent significantly different from ABF3.2 + VP1 or VP1 + ABA transactivation alone, respectively; $P < 0.0004$ (two-sided Student's *t*-test, equal variance assumed). Error bars are ± SEM, four replicates per sample. (B) Over-expressed abi1-1 significantly antagonizes *Em*-GUS expression. Inhibition was observed both in presence and absence of ABA or ABF3.2, $P < 0.0003$ (two-sided Student's *t*-test, equal variance assumed). Numbers in parentheses (x) represent fold induction over 'No ABA' control and numbers in parentheses (y) represent fold transactivation by over-expressed ABF3 compared to treatment with 100 μM ABA alone. Negative percentages in parentheses indicate the percent inhibition of *Em*-GUS expression relative to controls. Control samples were co-transformed with a *35S-Ppdk*-ABI1^{null} expression construct encoding the phosphatase inactive G174D mutant (Sheen, 1998). Error bars are ± SEM, four replicates per sample. (C) Yeast two-hybrid assays of interactions between ABI3 and seven members of the ABI5-like bZIP family. Activity of a beta-galactosidase reporter was measured in yeast containing pairwise combinations of bZIP fusions to the GAL4 DNA binding domain (BD fusions) and either full length ABI3 fused to the GAL4 activation domain (AD-ABI3) or the AD vector control (pGAD). Results are the average of assays with three independent transformants for each combination; error bars represent S.D.

with VP1 (Figure 1A). Consistent with the interactions observed in protoplasts, yeast two-hybrid assays indicated that ABI3 interacted directly with ABF1, ABF3.1, ABF3.2, and ABF4, as well as with ABI5 (Figure 1C). However, the intrinsic activating activity of these bZIP factors varied greatly and the relative strength of the interactions differed between yeast and plant cells. For example, activity of a GAL4 binding domain (BD)-ABF1 fusion was enhanced only 2-fold by a GAL4 activation domain (AD)-ABI3 fusion in yeast, but ABF1 activity in maize protoplasts was enhanced greater than 40-fold by co-transformed VP1. Conversely, BD-ABF4 activity was enhanced nearly 10-fold by AD-ABI3 in yeast, but ABF4 and VP1 showed only a slight interaction in maize protoplasts, and only in the absence of exogenous ABA. Furthermore, although only the ABF3.2 variant was tested in protoplasts, this variant was greater than 10-fold more active in yeast than ABF3.1. The discrepancy between relative activities in yeast and plants might reflect requirements for plant-specific protein modification or interaction with other plant proteins.

These results suggested that the best candidates for possibly redundant mediators of seed or seedling ABA response were ABF3 and ABF1. Although seed transcript levels for these genes were below the limit of detection on RNA gel blots (Bensmihen *et al.*, 2002), they have been detected through multiple stages of seed development by microarray studies (Zimmermann *et al.*, 2004), albeit at ~30–50-fold lower levels than peak expression reported for *ABI5* in these stages. Furthermore, the *ABF3.1* splice variant was initially identified from a cDNA library derived from embryonic tissue (Kim *et al.*, 2002). Consequently, they are reasonable candidates for regulators of gene expression in seeds, in addition to their previously implicated role in vegetative stress response.

Identification of mutants

Whereas over-expression analyses are valuable indicators of the potential functions of a given gene, mutant analyses provide information regarding their essential functions. To test the importance of *ABF3* and *ABF1*, T-DNA insertion lines at each locus were obtained from the SIGnAL collection (Alonso *et al.*, 2003). In addition, TILLING lines were obtained for *ABF3* (Till *et al.*, 2003), one of which was predicted to result in a severe loss of function. The positions of the insertions or point mutation are indicated in Figure 2. The T-DNA insertion in *ABF1* is in the first exon and the *abf3* insertion is in an intron in the 5′UTR; both insertions might be expected to disrupt transcript accumulation. Consistent with this, comparison of *ABF* transcript levels in ABA-treated wild-type vs. mutant plants showed that the insertions greatly reduced expression of the disrupted *ABF*. The TILLING line CS91025 encodes a truncated product that terminates at amino acid 66, in the C1 domain, and is therefore expected to be a functional null, regardless of whether any transcript is produced.

Germination sensitivity to ABA and stresses

Mutations in *ABI5* have been shown to reduce sensitivity to ABA, NaCl and osmotic inhibition of germination (Finkelstein, 1994; Carles *et al.*, 2002). To determine whether *ABF3* or *ABF1* contribute to these responses, despite not having been uncovered by numerous forward genetic

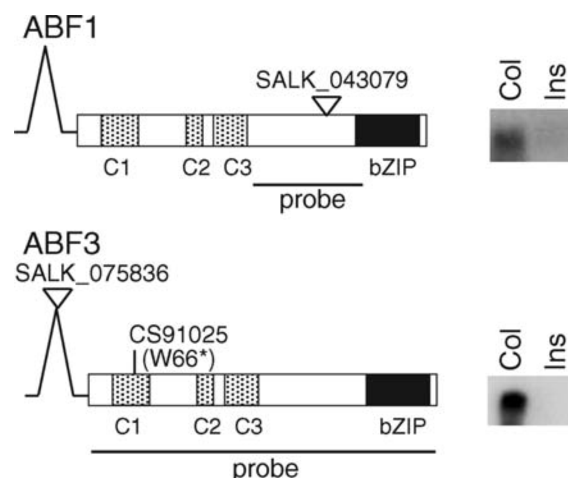


Figure 2. ABF1 and ABF3 transcript structure, and location and effects of mutations. Transcript structure schematics show conserved coding regions (C1–C3; bZIP) as shaded boxes, 5′UTR with intron as bent line. Inverted triangles are locations of T-DNA insertions and CS91025 is a TILLING nonsense point mutant. Although *ABF3* can be alternatively spliced to produce different C-termini, both mutations result in loss of both products. RNA gel blots compare 11-day-old wild-type (Col) vs. insertion line (Ins) mutants harvested following 6 h exposure to 50 μM ABA; each is hybridized to a probe corresponding to the disrupted gene.

screens based on ABA- or NaCl-resistant germination, we tested the mutants' sensitivity to ABA and NaCl at this stage. In addition, we tested sensitivity to sorbitol to differentiate between potential differences in NaCl sensitivity reflecting altered osmotic sensitivity vs. ionic effects. Compared to the *abi5* mutant, which itself has the weakest germination phenotype of the known *ABI* loci (Finkelstein, 1994), neither the *abf1* nor *abf3* knockout line showed significant resistance to ABA, NaCl, or sorbitol. However, as described recently in (Kim *et al.*, 2004), the *abf3* knockout and null TILLING lines display extremely weak ABA resistance to inhibition of seed germination (Supplemental data Table 1). To test the possibility that *abf3* and *abi5* function redundantly in this or other responses, we constructed and analyzed *abf3 abi5* digenic mutants. The double mutant germinates to a much greater degree on moderate ABA concentrations, e.g. 5–10 μM (Figure 3A). However, unlike combinations between *abi5* and *abi1*, *abi3-1* or the *leafy cotyledon* class mutants (Finkelstein 1994; Brocard-Gifford *et al.*, 2003), the *abf3 abi5* digenic mutants are not resistant to very high ABA levels (e.g. >30 μM). The double mutant also displays enhanced NaCl- and sorbitol-resistant germination, but interpretation of this result is complicated by the greater NaCl and sorbitol-resistance of the Col background introduced with the *abf3* mutation (Figure 3B, C). These results support the idea of redundant function of *ABI5* and *ABF3*, but do not rule out the possibility that additional members of the family may also contribute to these responses.

Seedling growth

Stress effects on seedling growth can be scored at a very early stage, as in the 'post-germination developmental arrest' by ABA described in (Lopez-Molina *et al.*, 2001), or by their effects on subsequent shoot development (formation of true leaves, stress-induced anthocyanin accumulation) and root growth. The mono- and digenic

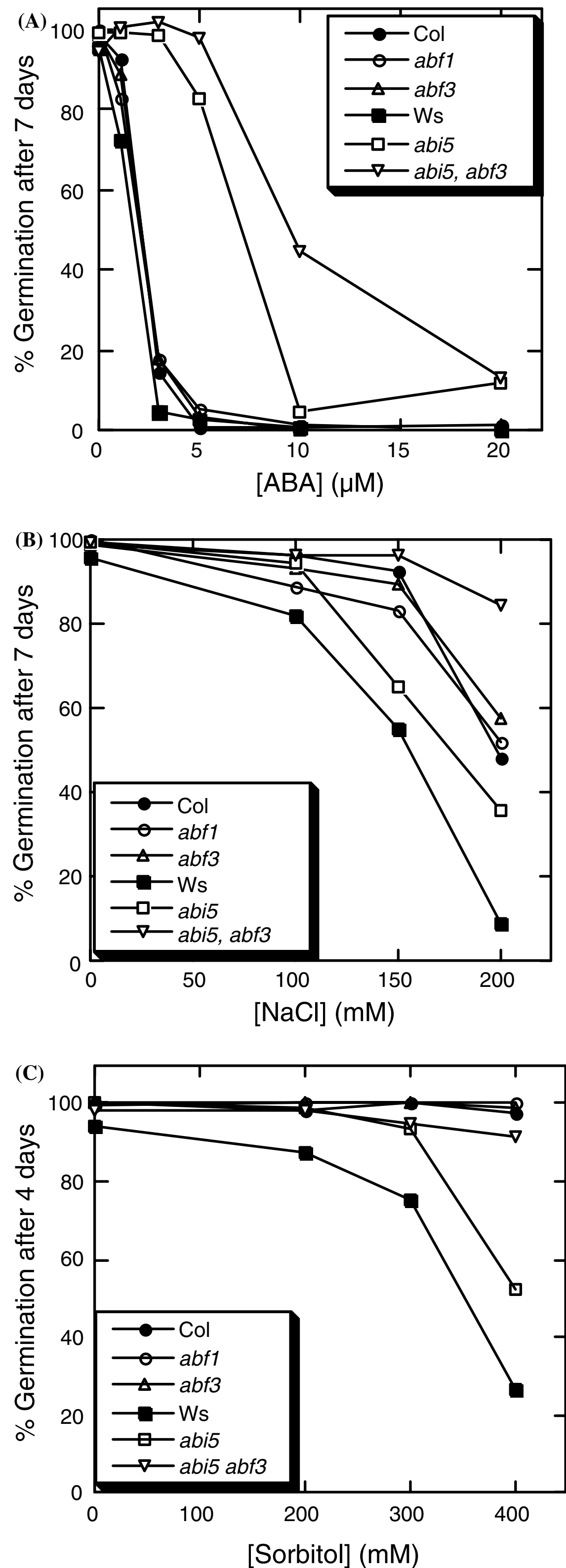


Figure 3. ABA, salt and osmotic sensitivities of germination in *abf* mutants. Mono- and digenic mutants of the indicated genotypes were sown on minimal media supplemented with (A) ABA, (B) NaCl, or (C) sorbitol. Radicle emergence was scored as germination. Results are average of 2–4 independent assays, each with 30–170 seeds/genotype for each treatment.

mutants described above were compared for their sensitivities to abiotic stress or hormone treatments.

To test sensitivity to ABA for post-germination arrest, seeds were stratified for 3 day on germination medium (GM) and tested for their response to ABA when transferred to hormone-containing medium after 0–5 day on GM. In contrast to *abi5*, which was resistant to ABA from day 0 onward, the *abf3* mutants were indistinguishable from wild type in that growth was arrested when exposed to ABA within the first 1.5 day (Supplemental data Figure 2).

Stress sensitivity of root growth was assayed by comparing root length of seedlings following transfer to fresh GM supplemented with ABA, NaCl or sorbitol. The *abf1* mutant was indistinguishable from wild type in these assays (Figure 4A and B). Although the *abf3* knockout mutants displayed no substantial difference from wild type in their response to NaCl or sorbitol (Figure 4B), two independent *abf3* lines were significantly resistant to ABA inhibition of root growth (Figure 4A and C). Although *abi5* mutants have near normal ABA sensitivity for this response, the *abi5 abf3* double mutant again showed enhanced resistance compared with the *abf3* mutant, consistent with redundant function (Figure 4A).

Stress sensitivity of shoot growth was compared by measuring the proportion of arrested seedlings with significant anthocyanin accumulation ('pink') following continuous incubation on a high concentration of Glc (333 mM; 6% [w/v]). Iso-osmotic sorbitol did not induce this response (data not shown), indicating that the Glc effect is not simply osmotic. Differential Glc sensitivities of the wild-type backgrounds complicate analysis of the mutant data. However, despite the apparent lack of effect of the *abf3* mutation on Glc sensitivity (Figure 5), the digenic mutant displayed greatly enhanced Glc-resistance compared to the mild effects of the *abi5* mutation alone.

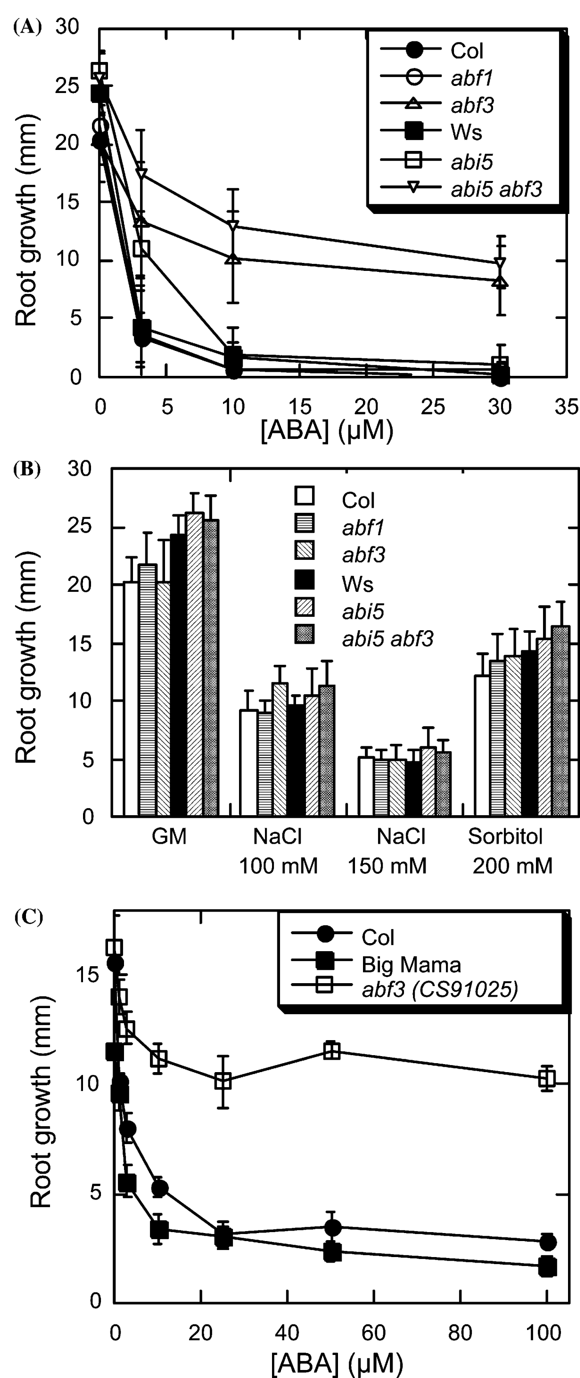


Figure 4. ABA, salt and osmotic sensitivities of root growth in *abf* mutants. Mono- and digenic mutants of the indicated genotypes were stratified on hormone-free medium and incubated 2.5 d at 22 °C to permit germination before transferring seedlings to media supplemented with (A) ABA or (B) NaCl or sorbitol for 5 additional days before measuring root length. The *abf* mutants represented in (A) and (B) are the SALK insertion lines. ABA sensitivity of the TILLING mutant allele of *abf3* was tested by measuring root elongation of 4 d old seedlings during 3 day exposure to ABA (C). All values shown are the average of at least 10 individuals, ± S.D. except (C) ± SEM, seven individuals.

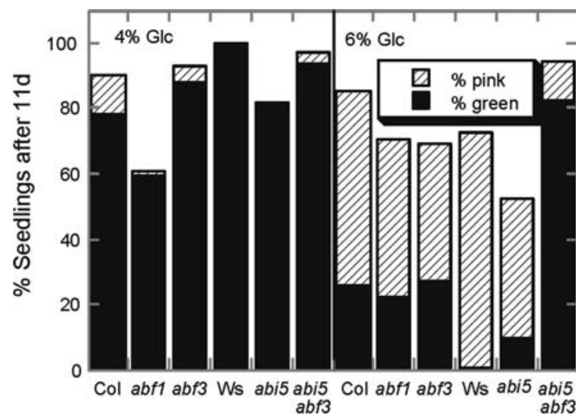


Figure 5. Glucose sensitivity of seedlings. Mono- and digenic mutants of the indicated genotypes were incubated for 11 d on media supplemented with 4% or 6% (333 mM) Glc. Seedlings were scored as stressed/arrested (pink), unstressed/growing (green), or ungerminated. Results are average of two independent assays, each with 30–160 seeds/genotype for each treatment.

Effects on ABA-inducible and seed-specific gene expression

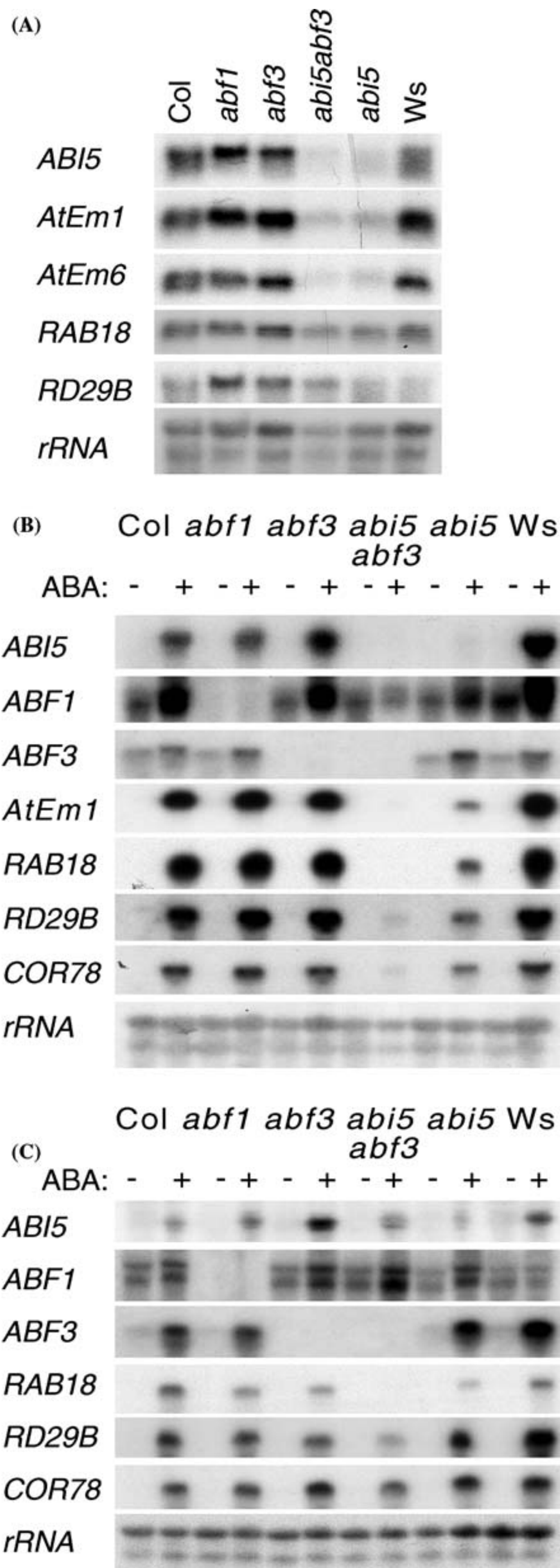
Numerous ABA-inducible genes have been identified that serve as good markers of ABA response at distinct stages of development (Rock, 2000). Among these, the Group 1 and 2 LEAs *AtEm1*, *RAB18*, and *RD29B* (Wise, 2003) are expressed in maturing seeds and their expression is maintained in young seedlings whose growth is arrested by ABA. *RAB18, RD29B* and additional dehydrins, such as *COR78/RD29A*, are also strongly ABA-induced in vegetative tissue. We compared expression of these markers in the various wild-type and mutant lines at representative stages: dry seeds, 5 day post-stratification with or without 3 μM ABA, and 10 day plants with or without brief exposure to 50 μM ABA.

As previously demonstrated (Finkelstein and Lynch, 2000), *abi5* mutants had greatly reduced seed expression of *AtEm1* and the related *AtEm6*, but had relatively minor reductions in *RAB18* transcripts and no change in *RD29B* expression. Mutations in *ABF3* did not substantially alter LEA expression and did not enhance the *abi5* effect in digenics (Figure 6A), despite the overexpression effects on *Em*-GUS activity observed in the transient assays (Figure 1). In contrast, levels of all tested transcripts in *abf1* mutant seeds averaged 15–30% higher than in wild-type, depending on the transcript. The lack of effect of the *abf3* mutation is consistent with the low expression of *ABF3* transcripts in maturing seeds. Whereas the enhanced accumulation of *LEA* transcripts in the *abf1* mutant is reminiscent of the *eel* mutant (Bensmihen *et al.*, 2002), it differs from the *eel* phenotype in that *ABI5* expression also increases slightly (~30%) in the *abf1* mutant.

Although expression of both *ABF1* and *ABF3* was induced in 5d seedlings incubated from stratification onward on GM supplemented with 3 μM ABA (Figure 6B), the *abf* mutant seedlings continued to express *AtEm1* and the *dehydrins* under these conditions. These results suggest that *ABF1* and *ABF3* are still not essential for *AtEm1* or *dehydrin* expression in young seedlings. In contrast, lines that contain the *abi5* mutation, and are therefore not arrested, had reduced levels of these ABA-inducible transcripts (Figure 6B). It is noteworthy that *ABF3* appeared hyper-induced by ABA in *abi5* mutants, to roughly 60% higher levels than wild-type, consistent with the cross-regulation suggested by the decrease in *ABF3* induction previously described in *35S:ABI5* lines (Brocard-Gifford *et al.*, 2003). Similarly, *ABI5* expression appeared hyper-induced in *abf3* seedlings, suggesting that this cross-regulation is reciprocal, not hierarchical. In contrast, expression of *ABF1* was slightly under-induced in *abi5* mutants.

Finally, we compared 10 day plants exposed to ABA for only 6 h, such that they are developmentally similar but physiologically different. At this stage, ABA-induced accumulation of *RAB18* and *RD29B* was reduced slightly in all mutants tested, relative to their wild-type backgrounds. Although *ABI5* expression was greatly reduced by this stage compared to its expression in seed or seedlings (Brocard-Gifford *et al.*, 2003), the digenic mutant accumulated substantially less *RAB18* or *RD29B* transcript than the monogenic lines, indicating redundant effects of *ABI5* and *ABF3* (Figure 6C). In contrast, *COR78/RD29A* expression was almost

Figure 6. ABF-dependent gene expression. RNA gel blot analyses with the indicated probes, comparing expression in mono- and digenic mutants at three stages: (A) Dry seed, (B) 5d plants incubated on GM with (+) or without (−) 3 μM ABA from stratification onward, (C) 10 d plants treated with (+) or without (−) 50 μM ABA for the last 6 h before harvest.



unchanged in all mutant lines. *ABF3* no longer appeared repressed by *ABI5* at this stage (compared with Ws control), but *ABI5* expression was still hyper-induced in *abf3* mutants (~75% higher than wild-type) (Figure 6C). Interestingly, *ABF1* was hyper-induced at the 10 day seedling stage of development in *abf3, abi5*, and especially *abf3abi5* double mutants.

Discussion

Attempts to predict ABF function

Genomic analyses of gene function often rely on similarities of sequence and presumed structure or expression patterns to predict functional relationships, but these predictions must be tested genetically. BestFit analyses (Devereux *et al.*, 1984) indicate that the bZIP showing the greatest overall amino acid similarity to ABI5 is ABF2, whereas that showing the greatest similarity within the bZIP domain is AtDPBF2, and EEL shows relatively low similarity by either comparison (Supplemental data, Tables 2 and 3). The bZIP domain is likely to be most critical for determining DNA binding specificity and potential for heterodimerization with other bZIPs, but strong homology is not required for heterodimer formation. Overall homology is likely to reflect potential for interactions with other protein classes. For example, interactions with ABI3 have been mapped to the C2 domain of ABI5 (Nakamura *et al.*, 2001). Superficial analysis of the homologies described above might lead to predictions that ABF2 would function most similarly to ABI5, whereas EEL would not necessarily have the same physiological effects because it might not interact with the same additional factors. Consistent with the latter prediction, mutant analyses indicate that *EEL* acts antagonistically to *ABI5*, apparently because EEL competes with ABI5 for binding to the ABA-inducible *AtEm1* promoter and can form heterodimers with ABI5 (Bensmihen *et al.*, 2002), yet does not physically interact with ABI3 (Figure 1C). In contrast to the first prediction, and despite the fact that it is highly expressed in radicles of dry seeds, *ABF2* has no effect on germination but functions primarily in glucose response of seedlings (Kim *et al.*, 2004).

Functional tests of physiological roles of ABFs

We used transactivation of an *ABI5*-regulated gene as a functional assay to identify *ABF3* and *ABF1* as candidates for redundant function, then tested their roles by analysis of loss of function lines. Although the transient assay systems combined target promoters and potential regulators from diverse species (wheat, Arabidopsis, and maize), and tested their function in protoplasts from developmentally distinct sources (rice embryonic suspension cultures and maize mesophyll), the results demonstrated that the regulatory relationships are conserved and that the activity of some specific regulators is sufficient to induce target gene expression. Furthermore, apparent synergistic action of VP1 and a given bZIP in the protoplast system correlated qualitatively with the apparent interactions in the yeast two-hybrid system. In both cases, the strongest interactions were seen with ABF3.2, ABF1, and ABI5. Although genetic data is not yet available for all of these loci, to date some of the protoplast results have correlated with the results of mutant analyses. For example, overexpression of EEL antagonized VP1 transactivation of wheat *Em:GUS* in maize protoplasts (Figure 1A), possibly by disrupting interactions with an endogenous bZIP, consistent with its role in delaying/repressing Arabidopsis *Em* expression (Bensmihen *et al.*, 2002). However, whereas ABF3.2 conferred the strongest inducibility of *Em-GUS* expression in the protoplasts, *abf3* mutants showed essentially normal *AtEm1* expression, apparently due to redundancy with *ABI5*. The dramatic effects of ABF3.2 over-expression in the transient assay (Figure 1A) are similar to those described for stably transformed *ABF3* over-expression lines (Kang *et al.*, 2002). These results illustrate both the limitation and the value of the over-expression approach: over-expression may over-emphasize the role of a particular regulator, especially at a stage when it is not normally expressed or is acting redundantly. However, it can also identify regulators whose importance might be obscured by redundancy in analyses of monogenic mutants.

Expression of endogenous ABI5-*related bZIPs*

To better understand the functions of these regulators in their normal developmental contexts, we need to know when they are expressed, whether they regulate each other's expression, and whether they interact to regulate genes such as the *LEAs* and *dehydrins*. The first of these questions is addressed by comprehensive microarray studies of all family members, except *ABF2*, that have been made available via GENEVESTIGATOR (Zimmermann *et al.*, 2004). Although transcripts for all *ABF/AREB/AtDPBFs* are detectable throughout development, peak expression of some family members is as much as 30–500-fold above their basal levels. As previously documented for seed development (Bensmihen *et al.*, 2002), *AtDPBF2* reaches a peak in mid-seed development, *EEL* increases concurrently with *AtDPBF2* but remains high until almost seed maturity, and *ABI5* increases sharply in maturing seeds. Comparison of transcript accumulation in developing seeds identified *AtbZIP13*, which is most similar structurally to *GBF4*, as the family member with most similar expression pattern to *ABI5* (Bensmihen *et al.*, 2002), but this comparison did not include post-germination expression. Furthermore, AtbZIP13 is one of the least similar to ABI5 structurally, in that it lacks the conserved C1 domain and has only limited homology to the C2 and C3 domains present in the N-half of most other family members (Bensmihen *et al.*, 2002). Consequently, this similarity of expression is unlikely to be a good predictor of physiological function.

ABF1, *ABF3* and *ABF4* rise at most 4-fold above their basal levels during seed development, reaching only 2–20% the peak levels seen for *AtDPBF2, EEL* or *ABI5*, and are therefore less likely to be major regulators of gene expression during seed maturation. In contrast, these *ABFs* are strongly induced by a variety of stresses during vegetative growth. Based on microarray studies of stressed hydroponically grown plants (AtGenExpress), *ABF1* appears most strongly induced by cold treatment, whereas *ABF3* is the member of this clade that is most strongly induced by osmotic, salt or drought stress (Genevestigator). Our studies showed that *ABF1* and *ABF3* are expressed in 5-day-old seedlings and are induced by exposure to ABA, but that *ABI5* is still the most strongly expressed family member at this stage. By 10 day, *ABF3* and, to a lesser extent, *ABF1* expression have surpassed that of *ABI5*. Transcriptome profiling of ~10 day seedlings

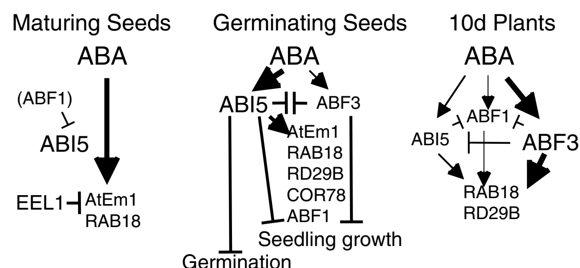


Figure 7. Model of changing regulatory relationships among the bZIPs and their roles in ABA signaling at different stages of development.

treated for 1–3 h with ABA (Goda, unpublished, from Genevestigator) (Zimmermann *et al.*, 2004) support our results, demonstrating that transcript levels for *ABF1* and *ABF3* are approximately 3–20-fold higher, respectively, than those for *ABI5* in ABA-treated seedlings. These profiling studies also identify *ABF4* as showing similar ABA-inducibility to *ABF1* at this stage, consistent with a role for *ABF4* in ABA signaling in seedlings, as suggested by our functional assay results showing interactions between ABF4 and ABA (in the absence of VP1) (Figure 1A) and the effects of both loss- and gain- of ABF4 function reported by (Kang *et al.*, 2002; Kim *et al.*, 2004).

Consistent with these expression patterns, *abi5* mutants have the most significant defects in dry seed phenotypes such as *LEA* gene expression, or ABA or stress sensitivity of germination (Figure 7). Similarly, *ABI5* was still the major regulator of LEA and dehydrin expression in 5 day seedlings, although some redundancy with *ABF3* was observed at this stage. By 10 day, even the reduced *ABI5* expression is still necessary for full induction of the *dehydrins*, but *ABF3* and *ABF1* are becoming important for *dehydrin* induction. However, at this stage, both *ABF3* and *ABI5* appear to slightly inhibit *ABF1* induction by ABA. Given that *ABI5* and these *ABFs* can regulate some of the same genes in over-expression assays, and *ABI5* and *ABF3* function redundantly, it was initially surprising that the *abf3* knockout phenotype was so mild and that ABF1 function was not activated by ABA in transient assays. However, these bZIPs antagonistically cross-regulate each other such that a loss of *ABI5* function results in increased *ABF3* expression, and vice versa. Consequently, single mutants have compensatory increases in redundant regulators, which are lost in the double mutants. We speculate that ABF1 over-expression in transient assays in maize and rice (Figure 1, Supplementary data) may affect ABA sensitivity through cross regulation of ABI5 and/or ABF3 orthologs in these species.

Our genetic studies did not attempt to distinguish between the ABF3 splice variants because the knockout and non-sense lines would be deficient in both forms. However, the two forms differ in the 'zipper' portion of the bZIP domain such that they might have different dimerization capacities, and they have vastly different intrinsic transcriptional activation functions in the yeast assay. Consequently, any developmental differences in splice variant abundance are likely to be significant for the physiological function of the *ABF3* locus.

Summary

These studies indicate that *ABI5* and *ABF3* act redundantly in some seedling ABA and stress responses, but their relative importance varies among responses. For example, *ABI5* is a much more critical determinant of germination sensitivity to ABA or other stresses, consistent with its much stronger expression in mature seeds, whereas *ABF3* is more important for ABA sensitivity of root growth in seedlings. In addition, *ABF3* functions in drought response during vegetative growth, although its contribution to stomatal regulation is minor compared to that of loci such as *ABI1* (Koornneef *et al.*, 1984; Kim *et al.*, 2004). ABI5, ABF1, and both ABF3 variants are capable of interacting synergistically with ABI3/VP1, but the opportunity for *in planta* interactions are limited to tissues where both factors are expressed. Consequently only ABI3 and ABI5 are likely to interact in maturing seeds, whereas ABI3 might interact with several of these bZIPs in seedlings arrested by stress or ABA treatments. Recent results have demonstrated that ABI3 is expressed in lateral root primordia and shoot meristems (Rohde *et al.*, 2000; Brady *et al.*, 2003), suggesting that observed knockout and overexpression phenotypes in vegetative tissues (Kang *et al.*, 2002; Kim *et al.*, 2004; this report) may be due in part to interactions with ABI3. The antagonistic cross-regulation of expression for these factors may

provide a mechanism for enhancing the importance of each factor, and corresponding factor-specific responses, at distinct stages or in response to different stresses. This proposed combinatorial mechanism of "sharpening" expression domains is similar to the function of pair-rule transcription factors in Drosophila embryo development (Lawrence and Johnston, 1989). Finally, the limited ABA resistance of even the double mutants suggests that additional factors, e.g. the other ABI transcription factors or additional bZIPs, may help mediate these responses.

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