

Regulation and Role of the Arabidopsis *Abcisic Acid-Insensitive 5* Gene in Abscisic Acid, Sugar, and Stress Response¹

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Abscisic acid (ABA) and stress response from late embryonic growth through early seedling development is regulated by a signaling network that includes the Arabidopsis *ABA-insensitive (ABI5)* gene, which encodes a basic leucine zipper transcription factor. We have characterized genetic, developmental, and environmental regulation of *ABI5* expression. Although expressed most strongly in seeds, the *ABI5* promoter is also active in vegetative and floral tissue. Vegetative expression is strongly induced by ABA, and weakly by stress treatments during a limited developmental window up to approximately 2 d post-stratification, but ABA and some stresses can induce expression in specific tissues at later stages. *ABI5* expression is autoregulated in transgenic plants and yeast (*Saccharomyces cerevisiae*), and stress response appears to involve *ABI5*-dependent and -independent mechanisms. To determine whether *ABI5* is necessary and/or sufficient for ABA or stress response, we assayed the effects of increased *ABI5* expression on growth and gene expression. Although overexpression of *ABI5* confers hypersensitivity to ABA and sugar, as previously described for *ABI4* and *ABI3* overexpression lines, it has relatively limited effects on enhancing ABA-responsive gene expression. Comparison of expression of eight *ABI5*-homologous genes shows overlapping regulation by *ABI3*, *ABI4*, and *ABI5*, suggestive of a combinatorial network involving positive and negative regulatory interactions.

The phytohormone abscisic acid (ABA) regulates many agronomically important aspects of plant growth and development, including seed maturation, dormancy, stress tolerances, and water relations (for review, see Leung and Giraudat, 1998; Rock, 2000; Finkelstein et al., 2002). All of these processes are regulated by additional signals, including other phytohormones, stage-specific regulators, and abiotic stresses. Studies of ABA-deficient mutants have shown that ABA is an essential mediator in triggering some plant responses to abiotic stresses, including drought, salinity, and cold (Rock, 2000; Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong and Zhu, 2001). Dehydration and low temperatures result in elevated levels of ABA, which, in turn, trigger the synthesis of some proteins responsible for drought or freezing tolerance. However, although most of the drought- and salt-induced genes studied to date can be induced by ABA, many aspects of stress response are also mediated by ABA-independent mechanisms (Shinozaki and Yamaguchi-Shinozaki, 2000). These results indicate that ABA participates in only part of a network of stress-signaling mechanisms. Furthermore, even the ABA-dependent portions of this network rely on both independent and partially redun-

dant signaling components, some of which are produced only in specific developmental stages or tissues of the plant.

Components of this signaling network have been identified by a combination of biochemical, cell biological, and forward and reverse genetic approaches. To date, nearly 50 loci in Arabidopsis alone have been demonstrated to function in various aspects of ABA response (for review, see Finkelstein et al., 2002). Their products include transcription factors, protein phosphatases and kinases, RNA binding/processing proteins, GTP-binding proteins, enzymes of phospholipid or phosphoinositide metabolism, and proteins regulating vesicle trafficking or membrane localization of specific proteins. Some of these loci have also been identified independently via diverse genetic screens including defects in response to other phytohormones (ethylene, auxin, or brassinosteroids), abiotic stresses (osmotic, salt, cold, or UV light), sugars (Glc or Suc), or even meristem defects. The repeated identification of a few loci affecting response to multiple signals has led to the suggestions that these genes are points of "cross talk" among signaling pathways or nodes in a regulatory web (for review, see McCourt, 1999; Coruzzi and Zhou, 2001; Gazzarrini and McCourt, 2001; Finkelstein and Gibson, 2002; Finkelstein et al., 2002).

A critical stage in the plant life cycle is the production of viable seeds "programmed" to initiate vegetative growth when conditions are favorable for seedling establishment. ABA contributes to this pro-

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cess by promoting accumulation of storage reserves and acquisition of desiccation tolerance in maturing seeds, and by inhibiting germination and the ensuing loss of desiccation tolerance (for review, see Finkelstein et al., 2002). Among the Arabidopsis genes controlling this aspect of ABA response are those encoding three transcription factors that appear to act combinatorially: *ABA-insensitive* (*ABI3*), *ABI4*, and *ABI5*. Although initially described as seed-specific regulators, all three have subsequently been shown to function in various aspects of vegetative growth, including some ABA-regulated gene expression, root branching, plastid differentiation, vegetative meristem quiescence, and floral induction (Rohde et al., 1999, 2000; Söderman et al., 2000; Signora et al., 2001). *ABI4*, and to a lesser extent *ABI5*, are also required for the developmental arrest and anthocyanin accumulation induced by exposure to high concentrations of sugars within the first 2 d post-stratification (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000).

ABI5 differs from *ABI3* and *ABI4* in that all tested aspects of the null mutant phenotypes are relatively mild. Furthermore, unlike these relatively unique members of the B3- and APETALA2-domain families, it encodes a member of a small subfamily with extensive homology outside the defining domain of its transcription factor class, the basic Leu zippers (bZIPs). BLAST analyses of the Arabidopsis genome have shown that the *ABI5*-homologous subfamily of Arabidopsis comprises nine genes, each with three conserved charged domains in the amino-half and a bZIP domain near the C terminus (Finkelstein et al., 2002). These have been designated ABA response element binding (AREB), ABA-responsive promoter elements (ABRE) binding factors (ABFs), and *Arabidopsis thaliana* Dc3 promoter binding factors (AtDPBFs). To date, only *ABI5* has been identified by mutation (Finkelstein, 1994; Lopez-Molina and Chua, 2000). However, seven of the other subfamily members have been identified by binding and trans-activation of ABREs and/or homology to sunflower (*Helianthus annuus*) genes encoding ABRE-binding proteins (Kim et al., 1997; Kim and Thomas, 1998; Choi et al., 2000; Uno et al., 2000; Kim et al., 2002). Furthermore, most have been shown to be ABA- and/or stress-inducible, leading to the suggestion that this subfamily might function redundantly to mediate response to ABA or abiotic stresses. However, it is equally possible that at least some of the different family members might function independently in stage- or stress-specific responses.

In the present work, we have extended our studies of *ABI5* regulation to define the stage and tissue specificity of expression under stressed and unstressed conditions. We found that *ABI5* is autoregulated and that its promoter is active throughout the plant's life in a variety of specific tissues, but that expression and inducibility by ABA and stress are

highest at the transition from mature seeds to seedling growth. In addition, we tested whether overexpressing *ABI5* would alter growth responses to ABA or sugar, and we compared ABA-responsive gene expression in transgenic lines overexpressing *ABI3*, *ABI4*, or *ABI5*. These studies show that increased *ABI5* expression is sufficient for hypersensitivity to inhibition of growth by ABA or sugar, but affects only a subset of *ABA/ABI3/ABI4*-regulated genes, including a complex array of positive and negative effects on expression of the *ABI5*-homologous bZIPs. Our results are consistent with the hypothesis that *ABI5* participates in combinatorial regulation of ABA and stress responses involving positive and negative interactions with an array of other regulators that differ among stage-, tissue-, or stress-specific responses.

RESULTS

Autoregulation of *ABI5* Expression

Our initial studies demonstrated that *ABI5* transcript accumulation was regulated by ABA and most of the *ABI* loci (Finkelstein and Lynch, 2000). In the case of *abi5* mutants, it was not clear whether the reduced transcript accumulation reflected autoregulation of *ABI5* transcription or decreased accumulation of a poorly translated transcript. To distinguish between these possibilities, activity of the *ABI5* promoter was assayed in fusions with reporter genes that function in plants or yeast (*Saccharomyces cerevisiae*). An *ABI5:: β -glucuronidase* (*GUS*) transgene containing approximately 3 kb of genomic DNA was used to construct a translational fusion including the first 28 codons of *ABI5*. Transgenic wild-type plants carrying a single copy of the transgene were backcrossed to *abi5-1* mutants (which are biochemical nulls) so that *ABI5* promoter activity could be compared in isogenic lines, with or without *ABI5* function. Comparison of *GUS* activity in extracts from mature dry seeds showed an approximately 4-fold reduction in the *abi5* mutant, demonstrating that *ABI5* activity is required for full expression, but that *ABI5* is still expressed in the mutant (Fig. 1).

To determine whether *ABI5* expression is sufficient for activation of its promoter, a yeast one-hybrid assay was used to test β -galactosidase activity produced from a series of *ABI5* promoter fusions to the reporter gene *lacZ*. Although the *ABI5::GUS* translational fusion had led to high-level expression of *GUS* in plant tissues, a comparable fusion with the *lacZ* gene was completely inactive in yeast (data not shown), possibly due to the presence of a large intron in the 5'-untranslated region (UTR). Therefore, we constructed transcriptional fusions whose *ABI5* promoter fragments terminated upstream of this intron, but included three (*ABI5pDra::lacZ*) or four (*ABI5pHinc::lacZ*) predicted *ABI5* binding sites (Fig. 2A), based on the DPBF core binding sequence defined by Kim et al. (1997). Expression of *ABI5* did

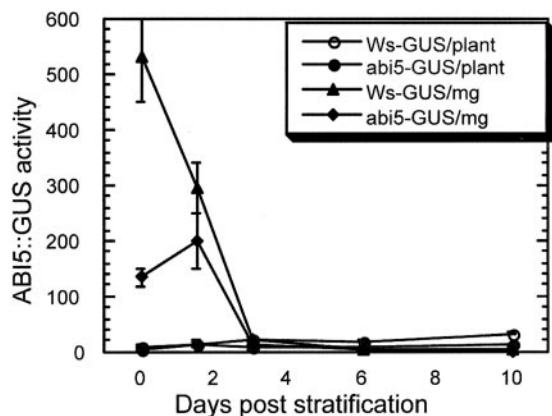


Figure 1. *ABI5*- and developmental regulation of *ABI5::GUS* activity during early seedling growth. GUS activity was measured in dry seeds or germinating seedlings at 1.5, 3, 6, and 10 d post-stratification. Extracts were derived from isogenic *ABI5::GUS* transgenic lines, differing only at the *ABI5* locus. Activity (nanomoles MU produced per hour) is expressed per plant and per milligram of fresh weight. Values shown are the mean \pm SD of eight replicate assays.

not trans-activate the *ABI5::lacZ* fusions in yeast (data not shown), possibly because the phosphorylation required for activation (Uno et al., 2000) might not occur in yeast. However, cell lines combining the reporter genes with a GAL4 activation domain (GAL4AD)-*ABI5* fusion had 3- to 12-fold higher β -galactosidase activity than lines expressing only the GAL4AD protein (Fig. 2B), indicating that the binding domain of *ABI5* can target trans-activation of the *ABI5* promoter. In contrast, although ectopic expression of *ABI3* or *ABI4* leads to increased *ABI5* expression in transgenic plants (Finkelstein and Lynch, 2000; Söderman et al., 2000), GAL4AD fusions to these transcription factors did not activate the *ABI5* promoter.

Developmental and Environmental Regulation of *ABI5* Expression

Studies of *ABI5* expression to date have shown that transcript levels are highest in mature seeds and flowers, with transcript levels in unstressed vegetative tissues ranging from low to undetectable (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). To analyze the tissue specificity of *ABI5* promoter activity, *ABI5::GUS* activity was assayed at various stages through development and in response to assorted stresses. Although previously described as almost undetectable within 2 d after pollination (Lopez-Molina and Chua, 2000), histochemical staining of GUS activity is observed throughout the seed at heart stage (Fig. 3A). Expression continues at low levels throughout the growing embryo (Fig. 3, B and C), reaching a peak at seed maturity (Fig. 3D). *ABI5::GUS* expression also occurs throughout the silique wall, especially along the suture joining the carpels (Fig. 3F). *ABI5::GUS* activity per milligram of

fresh weight declines rapidly following germination, dropping by approximately 100-fold by 6 d post-stratification due primarily to dilution as seedling fresh weight increases (Fig. 1), possibly accounting for the previous characterization of *ABI5* transcript levels as undetectable in 5-d-old seedlings (Lopez-Molina and Chua, 2000). However, total GUS activity per plant approximately doubles over this period, reflecting de novo expression in unstressed vegetative tissue. Vegetative *ABI5::GUS* expression shows the highest concentrations in root tips, nodes, and major leaf veins of seedlings (Fig. 3E). In older plants, expression is observed in minor veins and at discrete points along the margins of leaves and in flowers, especially in stigmas, maturing anthers, and pedicels (Fig. 3, G and H).

Induction of *ABI5* accumulation by ABA was shown to be restricted to a narrow developmental window of approximately 1 to 2 d post-stratification (Lopez-Molina et al., 2001). A similar window has been described for sugar sensitivity of seedling growth (Gibson et al., 2001). Because sugar-induced developmental arrest is slightly dependent on *ABI5* function, the window of sugar sensitivity might reflect a limited period of sugar-responsive *ABI5* expression. If *ABI5* expression is primarily subject to developmental control, other abiotic stress signals (salt, osmotic, drought, or cold) might also become ineffective at approximately the same stage. To test these hypotheses, *ABI5::GUS* activity was assayed in transgenic lines exposed to stresses at different stages

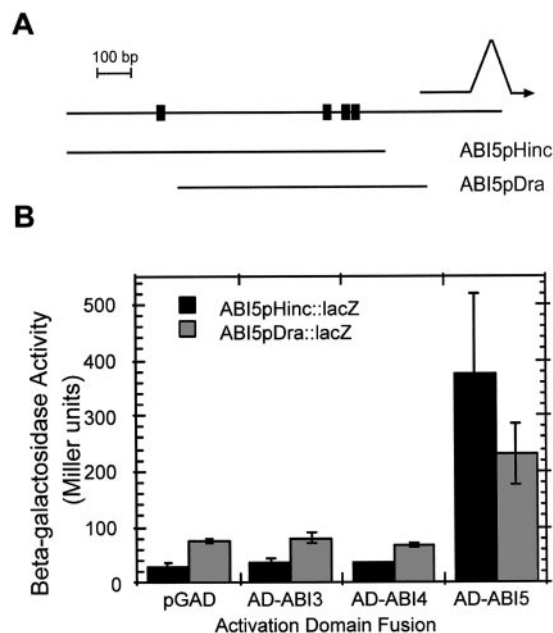
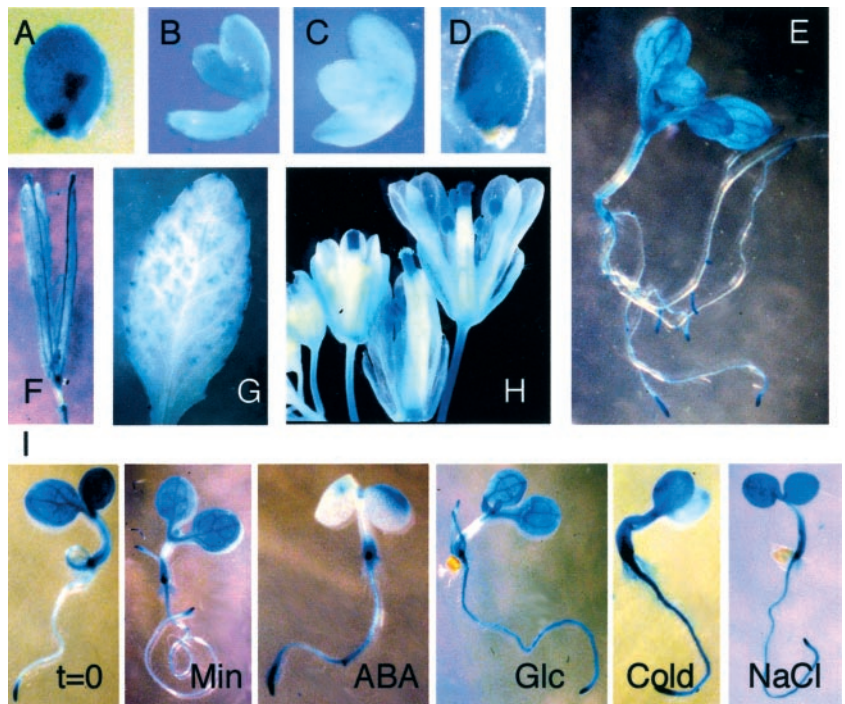


Figure 2. *ABI5* promoter regulation in yeast. One-hybrid analysis with *ABI5* promoter fragments fused to *lacZ* reporter. A, Promoter fragments used in fusions. Black boxes represent DPBF core recognition sequences. Arrow represents the transcription unit. B, *ABI5* promoter-driven β -galactosidase activity produced by GAL4AD fusions to *ABI* transcription factors (AD-*ABIx*) or vector control (AD).

Figure 3. Localization of *ABI5::GUS* activity during development or stress response of wild-type plants. A, Seed with heart-stage embryo; B, cotyledon stage embryo; C, mid-maturation stage embryo; D, mature seed; E, 10-d seedling; F, silique; G, leaf from 3-week-old plant; H, flowers; and I, 3-d-old seedlings treated as indicated: t = 0, or 2 d of exposure to minimal medium (Min); ABA, 50 μM ABA; Glc, 250 mM Glc; Cold, 4°C; NaCl, 125 mM NaCl.



in seedling development. The salt, sugar, and sorbitol stress treatments were designed to be osmotically equivalent and growth inhibiting, but not lethal. All stresses were imposed for 2 d except for drought, which was imposed for 2 h. Exposure to ABA, cold, Glc, or NaCl at 1.5 d post-stratification delayed the decline (cold, Glc, and NaCl treatments) and/or induced de novo *ABI5::GUS* expression, reflected in increased GUS activity per milligram (ABA treatment; Fig. 4). At later time points, GUS activity per fresh weight is still higher in most of the stressed plants than in the unstressed controls. Although this partly reflects the decreased growth of the stressed plants, comparison of histochemical staining shows that Glc, cold, NaCl, and drought treatment of 3-d seedlings all result in de novo *ABI5::GUS* expression throughout the root, whereas ABA treatment leads to very strong expression in primary and lateral root tips (Fig. 3I and data not shown). In contrast, seedlings stressed at 6 or 10 d post-stratification showed minimal differences in tissue distribution of *ABI5::GUS* staining relative to controls (data not shown). However, plants appear to regain some sensitivity to ABA and Glc at 10 d post-stratification (Fig. 4), resulting in increased *ABI5*-regulated GUS expression overall. Although the applied concentrations of NaCl and sorbitol were sufficient to inhibit growth, they had a relatively minimal impact on total *ABI5::GUS* activity. Drought treatment also had almost no impact on total *ABI5::GUS* expression at any time point (Fig. 4). Comparison of *ABI5::GUS* expression in wild-type and *abi5* backgrounds shows that GUS activity is consistently lower in the mutants, suggesting that most of these stress responses are

mediated by a combination of *ABI5*-dependent and -independent mechanisms.

Effects of Ectopic *ABI5* Expression

ABI5 function is required for normal regulation of a variety of genes expressed during mid-late embryogenesis and, to a lesser extent, during seedling growth (Finkelstein and Lynch, 2000). Constitutive overexpression of two other transcription factors regulating many of the same genes, *ABI3* and *ABI4*, confers ABA-inducible expression of seed-specific genes in vegetative tissues (Parcy et al., 1994; Söderman et al., 2000). This is accompanied by hyperinduction of *ABI5* expression (Söderman et al., 2000), suggesting that *ABI5* expression might be an important mediator of these effects on gene expression. To determine whether overexpression of *ABI5* is sufficient to confer similar changes in developmental specificity of gene expression, we assayed expression of several marker genes in 2-week-old *35S::ABI5* plants exposed to 50 μM ABA for the last 2 d of growth. Despite having moderate to high levels of *ABI5* expression (Fig. 5A), none of the seed-specific marker genes were expressed at detectable levels (Fig. 5B), suggesting that *ABI5* must act in combination with the products of regulatory genes normally expressed during seed development. Consistent with this, *ABI3* and *ABI4* were not overexpressed in these transgenic lines (Fig. 5C). Although *ABI5* overexpression did not result in ABA-inducible expression of seed protein genes in vegetative tissues, the *35S::ABI5* lines displayed slightly increased induction of some ABA-inducible transcripts that are nor-

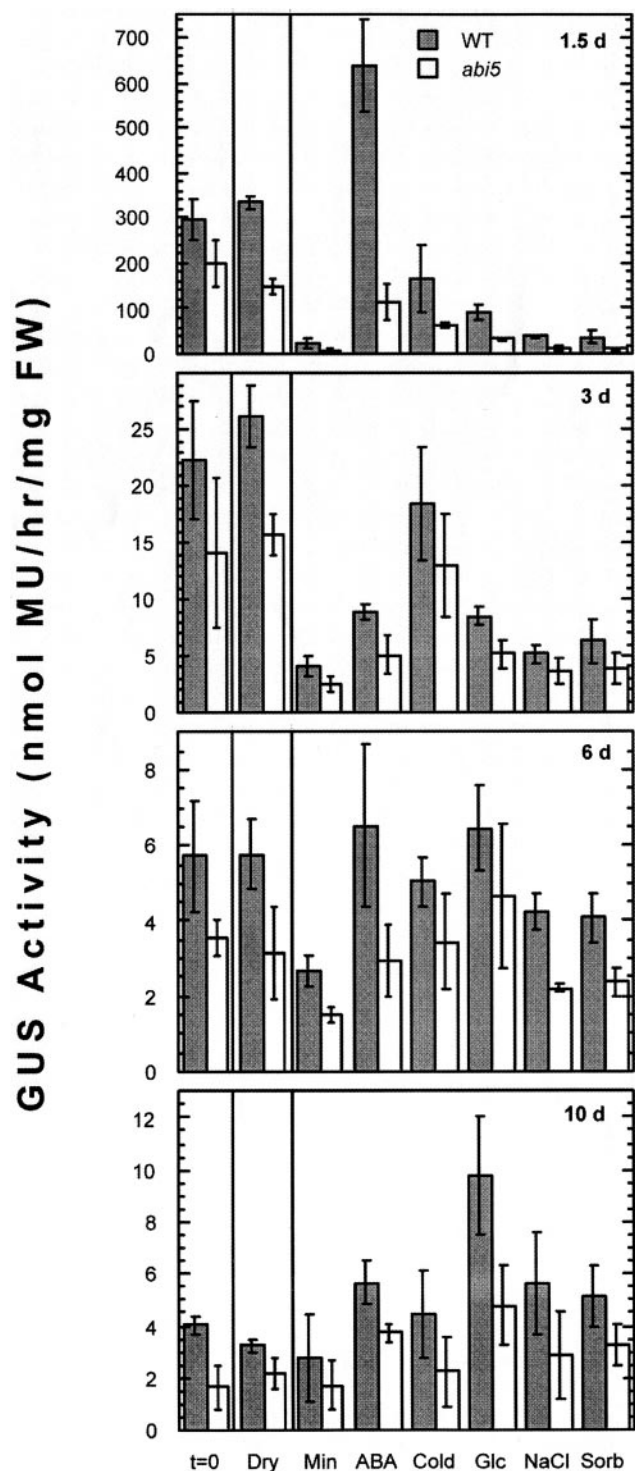


Figure 4. Stress induction of *ABI5* promoter activity in seedlings. GUS activity was measured in seedlings at 1.5, 3, 6, and 10 d post-stratification that were harvested immediately ($t = 0$), dried on absorbent paper in a closed petri dish for 2 h prior to harvest (Dry), or incubated on control medium (min) or exposed to $50 \mu\text{M}$ ABA, 125 mM NaCl, 250 mM sorbitol, 250 mM Glc, or 4°C for 2 d prior to harvest. Extracts were derived from isogenic *ABI5::GUS* transgenic lines, differing only at the *ABI5* locus. Activity (nanomoles MU per hour) is expressed per milligram of fresh weight. Values shown are the mean \pm SD of duplicate assays on two to four samples per treatment.

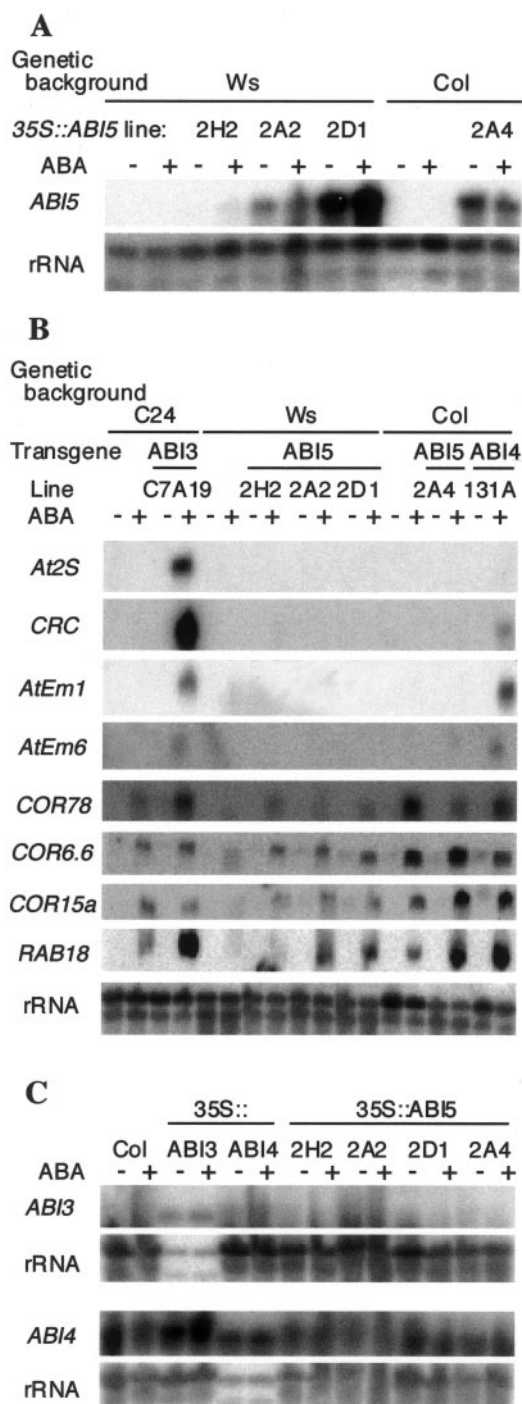


Figure 5. ABA-responsive gene expression in *ABI* overexpression lines. Plants were grown for 12 d on GM, then transferred to fresh media $\pm 50 \mu\text{M}$ ABA for 2 d prior to harvest. RNA gel blots were hybridized to probes corresponding to the indicated genes. A, *ABI5* expression in four independent *35S::ABI5* lines and their corresponding wild-type progenitors. Each lane contains $1 \mu\text{g}$ of total RNA. B, ABA-inducible marker gene expression in *35S::ABI5* lines, representative strong *ABI3* and *ABI4* overexpression lines, and all corresponding wild types. Each lane contains $5 \mu\text{g}$ of total RNA. C, *ABI3* and *ABI4* expression in *35S::ABI5* and wild-type lines. For *ABI3* hybridization, *35S::ABI3* lanes contain $0.5 \mu\text{g}$ and others contain $10 \mu\text{g}$ of total RNA. For *ABI4* hybridization, *35S::ABI4* lanes contain $1 \mu\text{g}$ and others contain $15 \mu\text{g}$ of total RNA.

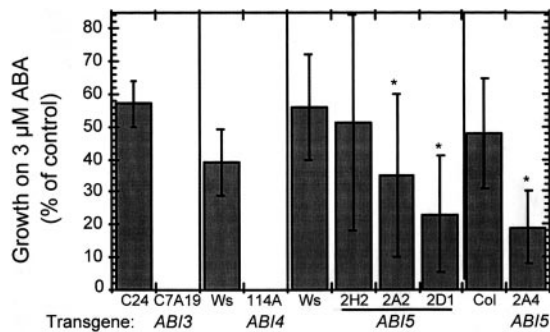


Figure 6. Effects of *ABI* transcription factor function on ABA hypersensitivity of root growth. After 2 d of growth on hormone-free medium, seedlings were transferred to fresh media containing 0 or 3 μM ABA. New growth was measured after 4 d and is expressed as a percentage of growth on the control medium. Each set represents the average of at least 10 replicates within an individual experiment; sds are expressed as percentages of the control growth. An asterisk indicates 35S::ABI5 lines with significantly increased ABA sensitivity ($P = 0.034, 0.0005, \text{ or } 4 \times 10^{-5}$ for lines 2A2, 2D1, and 2A4, respectively, based on Student's *t* test).

mally expressed in seedling tissues (e.g. *Cor6.6*, *Cor15a*, and *Rab18*). *Cor78* expression did not show a consistent response to *ABI5* overexpression.

Whereas overexpression of *ABI5* had only limited effects on ABA-inducible expression of a small set of ABA-responsive marker genes (Fig. 5B) and growth under osmotic stress (data not shown), it was sufficient to confer hypersensitivity to ABA for inhibition of root growth (Fig. 6). This finding is similar to results described for overexpression of hemagglutinin peptide-tagged *ABI5* (Lopez-Molina et al., 2001). The transgenic lines with the strongest *ABI5* expression had roughly twice the ABA-induced reduction in growth relative to wild type, whereas overexpression of *ABI3* or *ABI4* essentially blocks growth under the same conditions, equivalent to at least a 3-fold increase in sensitivity. In addition, overexpression of *ABI5* conferred hypersensitivity to sugars for inhibition of seedling growth and induction of anthocyanin accumulation (Fig. 7). The degree of hypersensitivity to ABA and sugar was correlated with the level of *ABI5* expression in the transgenic lines. These results suggest that growth control by ABA and sugars may be more tightly correlated with *ABI5*-regulated expression of genes other than those shown in Figure 5.

The differential response of seed-specific and seedling-inducible genes to *ABI5* overexpression (Fig. 5) suggests that developmental specificity may be conferred by other factors regulating these genes. *ABI5* is a member of a small subfamily of bZIP proteins, some of which are good candidates for factors contributing to ABA/*ABI5*-regulated gene expression. For example, some of the ABF/*AREB*s are correlated with ABA-inducible gene expression in vegetative tissues (Choi et al., 2000; Uno et al., 2000), whereas some *AtDPBF*s are expressed in seeds and have been shown to form heterodimers with *ABI5* (Kim et al., 2002). The overlap in

target genes regulated by these transcription factors and the potential for heterodimerization among them has led to the suggestion that these family members might interact and/or have partially redundant functions. Consistent with this, overexpression of *ABF3* and *ABF4* was recently reported to result in hypersensitivity to ABA, salt, and Glc, as well as producing similar and distinct effects on ABA-regulated gene expression (Kang et al., 2002). Although loss-of-function lines are required to test the specific roles of individual family members, evidence of coordinate expression or regulation could provide further support for the hypothesis of functional redundancy within this family.

To determine whether any of the *ABI5* family members are crossregulated by the *ABI* transcription factors, transcript accumulation was compared between wild-type and lines strongly overexpressing *ABI3*, *ABI4*, or *ABI5*, treated with or without ABA (Fig. 8). Whereas portions of the encoded proteins are highly conserved at the amino acid level, identity at the nucleotide level is lower and all hybridizations gave distinct patterns, indicating that these results do not reflect cross-hybridization within this gene family. However, some of the probes appeared to hybridize to two transcripts, possibly reflecting alternative splicing. In contrast to *ABI5*, most of the other *ABI5* subfamily members were not overexpressed in any of the transgenic lines. *AtDPBF2*, the gene with highest homology to *ABI5*, was not detectably expressed in any of these tissues (data not shown). *ABF2/AREB1* even appears underinduced in all of the transgenic

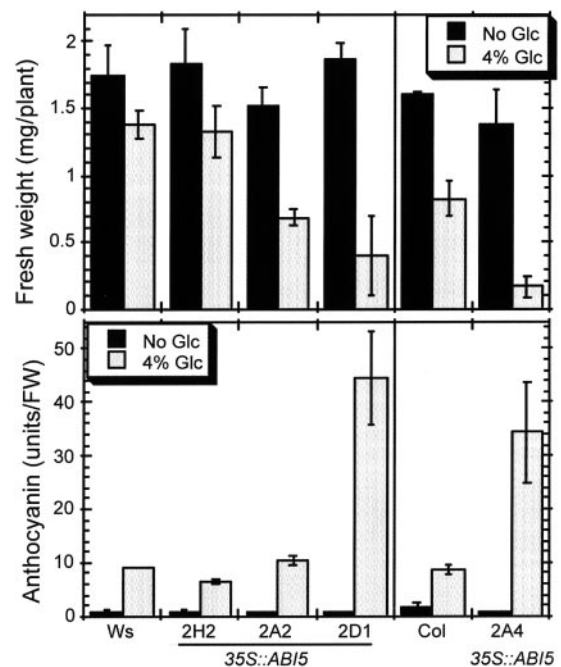


Figure 7. *ABI5* overexpression increases seedling sensitivity to sugar. Seedlings of the indicated genotypes were harvested after 9 d of growth on minimal medium, with or without 4% (w/v) Glc. A, Fresh weight per plant. B, Anthocyanin content per fresh weight. Values shown are the mean \pm SD of two to four assays per treatment.

lines, whereas *ABF3* and/or *AtDPBF5* (alternative splicing products of the same gene, differing only by approximately 90 nucleotides of coding sequence and their 3'-UTRs) are strongly repressed only in the *35S::ABI5* line. In contrast, *AtDPBF4* is hyperinduced in the *ABI3* and *ABI4* overexpression lines, but not the *ABI5* overexpression line. The observed cross-regulation by *ABI3* and *ABI4*, two known regulators of seed gene expression, suggests that *AtDPBF4* may also regulate seed gene expression. *ABF1* may also be slightly hyperinduced in the *35S::ABI3* line, but this could also reflect a subtle difference between the C24 and Wassilewskija (*Ws*) ecotypes. The only family member identified solely as a predicted gene (*At5g42910*) is repressed by ABA in wild type, and even its basal expression appears to be reduced in the *35S::ABI* lines. These results indicate that the crossregulation is relatively specific and may reflect functional relationships among genes involved in coordinate regulation.

DISCUSSION

Comparison of *ABI5* transcript accumulation in mature mutant and wild-type seeds has demonstrated that normal accumulation depends on wild-type function of *ABI1*, *ABI2*, *ABI3*, and *ABI5*, as well as normal ABA biosynthesis (Finkelstein and Lynch, 2000). In addition, *ABI5* expression can be induced by ABA in vegetative tissue, and this response is greatly amplified in lines expressing *ABI3* or *ABI4* under control of the cauliflower mosaic virus 35S promoter (Finkelstein and Lynch, 2000; Söderman et al., 2000). These results collectively indicated that ABA and all of the *ABI* loci could regulate *ABI5* expression, but did not address whether the regulation was direct or indirect. We have examined regulation of *ABI5* promoter activity using fusions to reporter genes and found that this promoter is active in more tissues than those of *ABI3* or *ABI4* (Parcy et al., 1994; Rohde et al., 1999; Söderman et al., 2000), and that *ABI5* is necessary for full *ABI5* expression in plants. Furthermore, a *GAL4AD-ABI5* fusion was sufficient to activate *ABI5::lacZ* expression in yeast in the absence of any other plant transcription factors. Although a nonhybrid *ABI5* did not regulate its promoter in yeast, analyses of *GAL4BD* fusions to various domains of *ABI5* have shown that full-length *ABI5* does not function as a transcriptional activator in yeast (Nakamura et al., 2001), possibly because it requires modification such as phosphorylation and/or a conformational change to become active. Taken together, these results imply direct autoregulation of *ABI5* expression, but also demonstrate *ABI5*-independent regulation in plants. In contrast, *ABI3* and *ABI4* were not sufficient to trans-activate the *ABI5* promoter in yeast, suggesting that their effects in plants are indirect, multifactorial, or require some form of post-translational modification that does not occur in

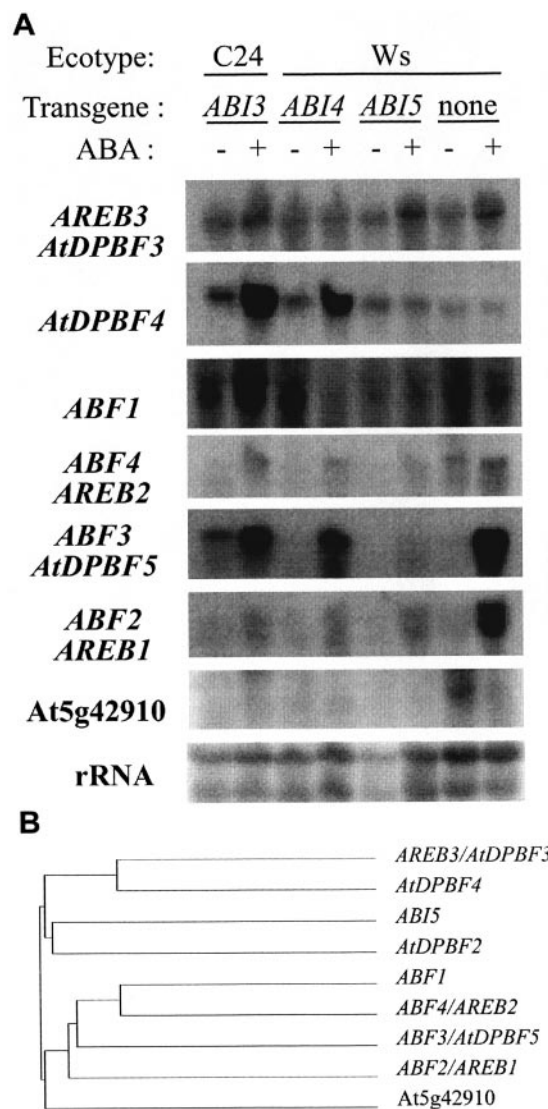


Figure 8. *ABI5*-homologous bZIP expression in *ABI* overexpression lines. A, RNA gel-blot analyses of three strong *35S::ABI* lines (isolates C7A19, 114A, and 2D1 for *ABI3*, *ABI4*, and *ABI5* overexpression, respectively) hybridized to cDNAs or PCR fragments corresponding to the indicated genes. All reported names are shown for genes isolated independently in multiple labs. Each lane contains 7 μ g of total RNA. B, Tree depicting homology relationships among Arabidopsis *ABI5*-homologous family members. The corresponding AGI or GenBank accession numbers for the named genes, followed by the bZIP designations proposed by Jakoby et al. (2002), are given in parentheses: *AtDPBF2* (At3g44460; AtbZIP67); *AtDPBF3/AREB3* (At3g56850; AtbZIP66); *AtDPBF4* (At2g41070; AtbZIP12); *AtDPBF5/ABF3* (At4g34000; AtbZIP37); *ABF1* (At1g49720; AtbZIP35); *ABF2/AREB1* (AF093545; AtbZIP36); and *ABF4/AREB2* (At3g19290; AtbZIP38). Although *ABF2* and *AREB1* have been independently cloned as cDNAs, the corresponding genomic sequence has not yet been identified.

yeast. These results suggest that *ABI5* expression is activated by *ABI3* and/or *ABI4* or other stage/tissue-specific regulators, and can then contribute to maintaining its expression by autoregulation.

ABI5 is expressed in a variety of distinct cell types through development, showing some overlap with *ABF3* and *ABF4* expression in vegetative and floral tissues (Kang et al., 2002), and it is likely that its physiological role varies among tissues and stages. The highest *ABI5* expression is observed in mature dry seeds, where it has been shown to regulate some late embryogenesis abundant genes (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Delseny et al., 2001), at least some of which are correlated with desiccation tolerance (Swire-Clark and Marcotte, 1999). A similar role might be played in anthers, where maturing pollen must also acquire desiccation tolerance. In seedlings, expression is highest in root tips (primary and lateral), nodes, and major veins; when effective, ABA or stress treatments accentuate these expression patterns and may slightly expand the distribution of *ABI5* expression to include tissues that might otherwise be growing rapidly, e.g. those present throughout new leaves. *ABI5* has been proposed to act as a mediator of ABA repression of growth at the onset of seedling development (Lopez-Molina et al., 2001), possibly via effects on cell-cycle machinery (Wang et al., 1998), and might continue this role in selected tissues well after commitment to seedling growth. Specific effects of ABA on promoting initiation of lateral roots, but inhibiting their elongation, have been implicated in response to drought (Vartanian et al., 1994). More recently, ABA, *ABI5*, and *ABI4* have been shown to mediate the inhibitory effect of NO_3^- on lateral root development, possibly via effects on sensing the C/N balance regulating lateral root growth (Signora et al., 2001). Localized expression of *ABI5* might allow it to function as a sensor, becoming stabilized and active by ABA-dependent changes in phosphorylation state under stress conditions. In addition, *ABI5* expression might be high under unstressed conditions in tissues that have low rates of cell division, such as maturing vascular tissue.

As previously described for *ABI5* protein accumulation (Lopez-Molina et al., 2001), *ABI5* promoter activity was highly inducible by ABA when applied within 2 d post-stratification, but was only weakly ABA responsive at later time points. Cold and Glc slightly induced *ABI5::GUS* expression at most time points, especially in roots, indicating that these stress responses were not limited to the same developmental window as ABA response. In contrast, the NaCl, sorbitol, and drought treatments had little or no effect on *ABI5::GUS* activity per milligram of fresh weight, suggesting that the reported increase in *ABI5* protein accumulation in response to salt or drought might be a largely posttranscriptional effect, possibly mediated by phosphorylation-induced changes in protein stability (Lopez-Molina et al., 2001).

Previous studies of the ABI transcription factors have shown that overexpression of *ABI3* or *ABI4* resulted in hypersensitivity to ABA inhibition of root

growth and ABA-induced vegetative expression of genes normally expressed primarily in seeds, including *ABI5* (Parcy et al., 1994; Finkelstein and Lynch, 2000; Söderman et al., 2000). Observation of additional crossregulation among these *ABI* loci led to the suggestion that these transcription factors act combinatorially to regulate subsets of ABA-inducible and/or seed-specific genes. Subsequent studies demonstrated direct physical interactions between *ABI5* and *ABI3* or their rice (*Oryza sativa*) homologs in yeast (Hobo et al., 1999; Nakamura et al., 2001), as well as synergistic interactions between *ABI5* and VP1 or their rice homologs in mediating ABA-activated gene expression in transient expression assays in plant protoplasts (Hobo et al., 1999; Gampala et al., 2002). *ABI5* overexpression has been shown to resemble *ABI3* overexpression in that it confers hypersensitivity to ABA inhibition of root growth and promotes slightly more efficient water retention (Parcy et al., 1994; Parcy and Giraudat, 1997; Lopez-Molina et al., 2001). Furthermore, overexpression of *ABI3* or *ABI5* confers increased sensitivity to Glc inhibition of seedling growth (Finkelstein et al., 2002) even though *abi3* and *abi5* mutants have not been recovered in sugar-sensing screens. Similar results have recently been described for overexpression of *ABF3* and *ABF4* (Kang et al., 2002). In combination with the observation that *abi5* mutants are mildly sugar resistant (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000), these results suggest that *ABI5* can mediate sugar response, but that its activity is not normally a limiting factor, possibly because other functionally redundant proteins can partially compensate for loss of *ABI5* function. Further evidence for a role in sugar response is provided by the observation that *ABI5* expression is induced by Glc. However, even the transgenic lines with the strongest *ABI5* expression were not as hypersensitive to ABA or sugar as the lines that strongly overexpress *ABI3* or *ABI4* (Fig. 6; Finkelstein et al., 2002). Furthermore, we have found that *ABI5* overexpression does not increase vegetative expression of *ABI3*, *ABI4*, or the seed-specific genes regulated by the combinatorial activity of these transcription factors. The specific genes regulated by *ABI5* in any given tissue might be determined by combinatorial interactions between *ABI5* and *ABI3* or other appropriate and available transcription factors.

Some of the likely candidates for transcription factors that interact with *ABI5* are other members of the *ABI5*-homologous subfamily of bZIP proteins. However, despite the fact that most of these proteins were initially identified on the basis of binding to ABREs, and some can confer increased ABA-inducible reporter gene expression in protoplasts, they show widely varying degrees of induction by ABA and the ABI transcription factors (Choi et al., 2000; Uno et al., 2000; Finkelstein et al., 2002). In fact, different *ABI5*-homologous family members show either positive or

negative response to overexpression of any given ABI transcription factor, which may reflect similar or antagonistic interactions, respectively. The structural similarities of this subfamily are not paralleled in their expression patterns. Given that the ABI transcription factors are not expressed constitutively throughout development, the *ABI5*-homologous genes that they regulate are also likely to vary in their developmental specificity. Consistent with this, the reported ABA inducibility of individual family members varies substantially, possibly reflecting differential responses of 3- to 5-week-old plants exposed to ABA hydroponically (Choi et al., 2000; Uno et al., 2000) or 2-week-old plants grown on solid medium (Fig. 7). It is noteworthy that the two family members displaying the strongest ABA inducibility in 2-week-old wild-type plants, *ABF2/AREB1* and *ABF3/AtDPBF5*, differ greatly in their regulation by the *ABI* genes. These results collectively argue against simple functional redundancy within the *ABI5*-homologous family. However, they do not exclude the possibility that some family members will interact, synergistically or antagonistically, to regulate specific target genes. Hetero- and homodimerization among sunflower homologs of this family have already been documented (Kim and Thomas, 1998), and there is ample precedent for positive and negative interactions among dimerizing bZIP proteins (for review, see Hurst, 1995).

In summary, we have demonstrated that *ABI5* expression extends throughout the life of the plant in a variety of specific tissues and in response to a variety of stresses, rather than being limited to the stages associated with its previously characterized role in mediating seed maturation and ABA response in very young seedlings. Despite the breadth of its expression pattern, loss of *ABI5* function has relatively subtle phenotypic effects, suggesting that it plays a minor or a functionally redundant role in many tissues. Comparison of *ABI3*, *ABI4*, and *ABI5* overexpression lines has revealed similar and distinct effects of these transcription factors, consistent with complex regulatory interactions among them and other factors such as the other members of the *ABF/AREB/AtDPBF* family. The fact that *ABI5* overexpression is sufficient for many aspects of ABA- and sugar-hypersensitive growth, but only a subset of ABA-inducible genes, should help provide us with a way to identify the ABA-regulated genes that are most tightly correlated with growth effects.

MATERIALS AND METHODS

Plant Material

The *abi5-1* mutant line was isolated from the *Ws* background, as described by Finkelstein (1994). The *35S::ABI3* line (C7A19 isolate) was constructed in the C24 background, as described by Parcy et al. (1994). The *35S::ABI4* lines (114A and 131A isolates) were constructed in the *Ws* and Columbia backgrounds, respectively, as described by Söderman et al. (2000). *ABI5::GUS* and *35S::ABI5* transgenic lines were constructed in the Columbia

and *Ws* backgrounds by vacuum infiltration (Bechtold et al., 1993) with *Agrobacterium tumefaciens* carrying the plasmids described below. An *ABI5::GUS* transgenic line in the *Ws* background was crossed to the *abi5* mutant, and ABA- and kanamycin-resistant F_2 individuals were selected and screened for homozygosity of the transgene and the *abi5* mutation in the F_3 generation.

For RNA isolation from 2-week-old plants, seeds were surface sterilized in 5% (v/v) hypochlorite and 0.02% (v/v) Triton X-100, and then rinsed two to four times with sterile water before being plated on GM (Valvekens et al., 1988) composed of 0.5× Murashige-Skoog nutrients (Murashige and Skoog, 1962) and 1% (w/v) Suc solidified with 0.55% (w/v) agar. The dishes were incubated 3 d at 4°C to break any residual dormancy, and were then transferred to 22°C in continuous light (50–70 $\mu\text{E m}^{-2} \text{sec}^{-1}$). After 12 d, plants were transferred to fresh GM with 1% (w/v) Suc, 0.7% (w/v) agar, and 0 or 50 μM ABA (mixed isomers; Sigma, St. Louis) for an additional 2 d before harvest. All tissues for RNA isolation were weighed, flash frozen in liquid nitrogen, and stored at -70°C until extraction.

For histochemical staining of GUS activity in developing seeds, plants were grown in soil in continuous light at 22°C. To test for induction of *ABI5* promoter activity by specific stresses, seedlings were grown aseptically on Murashige-Skoog medium with 1% (w/v) Suc and 0.55% (w/v) agar. After 1.5, 3, 6, or 10 d at 22°C in continuous light, seedlings were harvested immediately or transferred to minimal medium (Haughn and Somerville, 1986) with 0.7% (w/v) agar and 50 μM ABA, 0.125 M NaCl, 0.250 M sorbitol, 0.250 M Glc, or no supplement for an additional 2 d before harvest. Cold stress was imposed by incubation for 2 d in a dimly illuminated cold room at 4°C. Drought stress was imposed by letting the seedlings dry for 2 h on 3MM paper (Whatman, Beverly, MA) in a closed petri dish. GUS activity was assayed histochemically and fluorometrically in replicate samples.

For Glc sensitivity assays, seeds were grown aseptically on minimal medium (Haughn and Somerville, 1986) containing 0.7% (w/v) agar with or without 4% (w/v) Glc. The dishes were incubated 3 d at 4°C, and were then transferred to 22°C in continuous light. Plants were harvested and weighed after 9 d, and then stored at -70°C until used for extraction of anthocyanins.

Transgene Construction

An *ABI5* overexpression transgene was constructed by fusing an *ABI5* cDNA into pGA643 (An et al., 1988) with 23 bp of 5'-UTR between the cauliflower mosaic virus 35S promoter and the initiating codon of *ABI5*. An *ABI5::GUS* transgene was constructed in pBI101 as a translational fusion containing approximately 3 kb of upstream genomic sequence and the first 28 codons of *ABI5*. Although there was substantial quantitative variability among the transgenic lines, the qualitative expression patterns were very similar. The data presented displays results with one representative transgene in wild-type and *abi5* backgrounds. Binary plasmids carrying the transgenes were introduced into *A. tumefaciens* line GV3101 by direct transformation, followed by selection for growth on appropriate antibiotics (kanamycin for pBI101 derivatives, and tetracycline and kanamycin for pGA643 derivatives).

One-Hybrid Assays of Transcription Activation Function

Two overlapping restriction fragments from the promoter region of *ABI5* were fused upstream of the *lacZ* gene in the plasmid pLGΔ178, a derivative of the plasmid pLGΔ312 (Guarente and Mason, 1983). These fragments were a 1,028-bp *HincII* fragment that ended 109 bp upstream of the transcription start site of *ABI5* and an 819-bp *DraI* fragment that ended 33 bp downstream of the transcription start site of *ABI5*. Each construct was used to cotransform the yeast cell line PJ69-4A where the second plasmid was pGAD (a plasmid containing the activation domain of GAL4), or its derivatives encoding *ABI3*, *ABI4*, or *ABI5* fused in frame with the activation domain of GAL4. All transformations were performed using the EZ transformation kit (Bio 101, Vista, CA) following the manufacturer's instructions. The activation domain and pLGΔ178 plasmids were maintained by growing the yeast on media lacking Leu and uracil.

Quantitative assays of GAL4AD-driven β -galactosidase gene expression were performed as described at <http://www.fhrc.org/labs/gottschling/yeast/Bgal.html>. All data presented are the averages \pm SD of assays on at least three independent transformants.

Measurement of GUS Activity

Soluble extracts of seeds were assayed fluorometrically for GUS activity using 4-methylumbelliferyl glucuronide (Rose Scientific Ltd., Edmonton, AB, Canada) as substrate as described in (Jefferson et al., 1987).

GUS activity in intact plants was detected histochemically by infiltration with 5-bromo-4-chloro-3-indolyl- β -glucuronic acid as described in (Jefferson et al., 1987). Plant material was cut and incubated in GUS staining solution containing 50 mM sodium phosphate, pH 7.0, 0.1% (v/v) Triton X-100, K₃/K₄ FeCN 0.5 mM, and 1 mM 5-bromo-4-chloro-3-indolyl- β -glucuronic acid at 37°C overnight. Tissues were cleared of chlorophyll in ethanol. Photographs of whole-mounted tissues were taken using a stereomicroscope.

RNA Gel-Blot Analysis

RNA was isolated from vegetative tissue by hot phenol extraction as described previously (Finkelstein, 1993). RNA concentrations were estimated based on absorbance at 260 and 280 nm.

Total RNA (5–20 μ g lane⁻¹) was size fractionated on MOPS-formaldehyde gels (Sambrook et al., 1989), and was then transferred to Magna Nylon membranes (Osmonics, Westborough, MA) using 20 \times SSPE as blotting buffer. RNA was bound to the filters by UV-crosslinking (120 mJ cm⁻² at 254 nm). Uniformity of loading and transfer was assayed qualitatively by methylene blue staining of the filters (Herrin and Schmidt, 1988). Transcripts from *At2S3*, *CRC*, *AtEm1*, *ABI3*, and the *RAB18* homolog were detected by hybridization to cDNA clones as described by Söderman et al. (2000), labeled by random priming to a specific activity of 10⁸ cpm μ g⁻¹. The *COR6.6*, *COR15a*, and *COR78* transcripts were detected by hybridization to cDNA clones as described by Hajela et al. (1990). The *AtEm6* mRNA was detected by hybridization to a genomic clone encompassing the entire transcribed region and 0.8-kb 5'-flanking sequences. The *ABI5* probe was a PCR-amplified genomic fragment excluding most of the conserved bZIP domain. The *ABI4* probe was an *EcoRI* fragment from a cDNA clone encompassing all but the first two and final codons of the coding sequence. Hybridization probes for the *AtDPPF* transcripts were full-length cDNA clones as described by S. Kim, J. Ma, P. Perret, Z. Li, and T. Thomas (unpublished data); the remaining *ABF/AREB* family members were detected by hybridization to exon-specific PCR fragments corresponding to the following segments of the coding sequences (ABF1, nucleotides 286–941; ABF2, nucleotides 324–1,050; ABF4, nucleotides 2–1,016; and At5g42910, nucleotides 60–881). Hybridization conditions were 50% (v/v) formamide, 5 \times SSPE, 5 \times Denhardt's, 0.1% (w/v) SDS, and 200 μ g mL⁻¹ DNA at 43°C or 7% (w/v) SDS, 0.5 M sodium-phosphate, pH 7.2, 1 mM EDTA, and 1% (w/v) bovine serum albumin at 65°C for 16 to 24 h (Church and Gilbert, 1984) in a Hyb-Aid rotisserie oven. Filters were washed twice at 60°C in 2 \times SSC and 0.1% (w/v) SDS, and once at 60°C in 0.2 \times SSC and 0.1% (w/v) SDS for 30 to 60 min.

Root Growth Assays

For root growth assays, seeds were surface sterilized before being plated on GM containing 0.7% (w/v) agar. Petri plates were incubated for 3 d at 4°C and were then transferred to 22°C in continuous light. After 2 d, germinated seedlings were transferred to GM supplemented with 0 or 3 μ M ABA. Plates were incubated vertically, with seedlings placed with their root tips pointing up such that new root growth would occur along the surface of the plates in the opposite direction from the original growth. New root growth was measured after 4 d, and then average growth was calculated for each genotype and treatment and was expressed as a percentage of the growth on hormone-free media.

Measure of Anthocyanin Content

Anthocyanins were extracted as described by Chia et al. (2000) by homogenization in methanol/HCl (99:1), using between 20 and 150 μ L solvent mg⁻¹ fresh weight. Extracts were cleared by centrifugation, and then absorbances were measured at 530 and 657 nm. Relative anthocyanin concentrations were calculated and expressed relative to tissue fresh weight according to the formula: [OD530 - (0.25 \times OD657)]/[extract volume]/fresh weight.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requester.

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