

# The *Arabidopsis thaliana* **ABSCISIC ACID-INSENSITIVE8** Locus Encodes a Novel Protein Mediating Abscisic Acid and Sugar Responses Essential for Growth <sup>W</sup>

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**Abscisic acid (ABA) regulates many aspects of plant growth and development, yet many ABA response mutants present only subtle phenotypic defects, especially in the absence of stress. By contrast, the *ABA-insensitive8 (abi8)* mutant, isolated on the basis of ABA-resistant germination, also displays severely stunted growth, defective stomatal regulation, altered ABA-responsive gene expression, delayed flowering, and male sterility. The stunted growth of the mutant is not rescued by gibberellin, brassinosteroid, or indoleacetic acid application and is not attributable to excessive ethylene response, but supplementing the medium with Glc improves viability and root growth. In addition to exhibiting Glc-dependent growth, reflecting decreased expression of sugar-mobilizing enzymes, *abi8* mutants are resistant to Glc levels that induce developmental arrest of wild-type seedlings. Studies of genetic interactions demonstrate that ABA hypersensitivity conferred by the *ABA-hypersensitive1* mutation or overexpression of *ABI3* or *ABI5* does not suppress the dwarfing and Glc dependence caused by *abi8* but partially suppresses ABA-resistant germination. By contrast, the ABA-resistant germination of *abi8* is epistatic to the hypersensitivity caused by *ethylene-insensitive2 (ein2)* and *ein3* mutations, yet *ABI8* appears to act in a distinct Glc response pathway from these *EIN* loci. *ABI8* encodes a protein with no domains of known function but belongs to a small plant-specific protein family. Database searches indicate that it is allelic to two dwarf mutants, *elongation defective1* and *kobito1*, previously shown to disrupt cell elongation, cellulose synthesis, vascular differentiation, and root meristem maintenance. The cell wall defects appear to be a secondary effect of the mutations because Glc treatment restores root growth and vascular differentiation but not cell elongation. Although the *ABI8* transcript accumulates in all tested plant organs in both wild-type and ABA response mutants, an *ABI8*- $\beta$ -glucuronidase fusion protein is localized primarily to the elongation zone of roots, suggesting substantial post-transcriptional regulation of *ABI8* accumulation. This localization pattern is sufficient to complement the mutation, indicating that *ABI8* acts either at very low concentrations or over long distances within the plant body.**

## INTRODUCTION

Abscisic acid (ABA) regulates many important events during both vegetative and reproductive growth of plants. These range from relatively slow effects, such as promotion of seed storage reserve synthesis, acquisition of desiccation tolerance and dormancy, and induction of stress tolerance, to rapid effects, such as stomatal closure (reviewed in Leung and Giraudat, 1998; Finkelstein and Rock, 2002). Many lines of evidence indicate that there are multiple mechanisms for both ABA perception and signaling. Genetic studies, especially in *Arabidopsis thaliana*, have identified a large number of loci involved in ABA response, and digenic analyses indicate that these loci are likely to be acting in multiple overlapping response pathways (reviewed in

Finkelstein et al., 2002). To date, >50 loci affecting ABA response have been cloned and found to encode proteins that affect processes including transcription, protein phosphorylation or farnesylation, RNA processing, and phosphoinositide metabolism (reviewed in Finkelstein et al., 2002; Himmelbach et al., 2003; Kuhn and Schroeder, 2003). In addition, many likely signaling intermediates correlated with ABA response (e.g., ABA-activated or -induced kinases or phospholipases and DNA binding proteins that specifically bind ABA-responsive elements) have been identified by molecular and biochemical studies (reviewed in Finkelstein et al., 2002; Himmelbach et al., 2003), but the relationships among most of these proteins are unclear. Mechanistic analyses of ABA response are further complicated by stage- and tissue-specific differences, such that a given locus may appear to function as both a positive and a negative regulator, depending on the response. For example, *enhanced response to ABA3 (era3)* mutants are hypersensitive to ABA inhibition of germination but resistant to inhibition of root growth by ABA (Ghassemian et al., 2000).

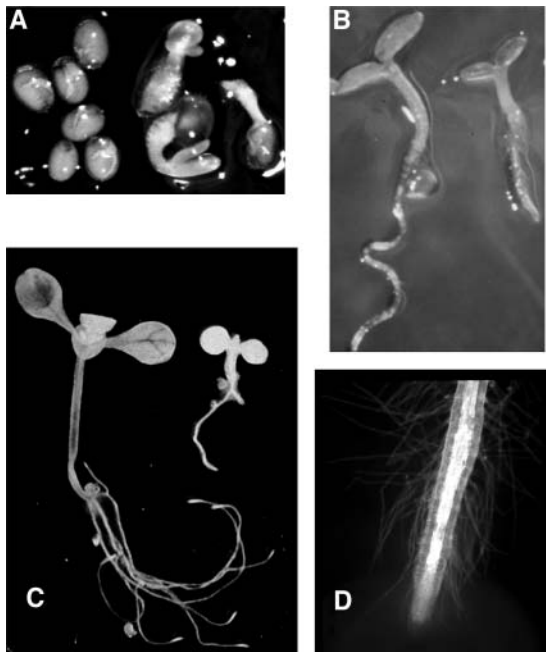
Although ABA response mutants were first identified in direct screens for loss of ABA response or ABA hypersensitivity, many additional mutations affecting ABA response have been identified in screens for defects in other signaling pathways. Consequently, there now is substantial evidence for cross talk between

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**Figure 1.** Morphology of *abi8* Mutant Seedlings.

Comparison of wild-type and mutant growth. The wild type is at left, and *abi8* is at right in (A), (B), and (C).

(A) Seeds incubated 4 d poststratification on 3  $\mu$ M ABA.

(B) 3-d-old seedlings grown on minimal medium.

(C) 11-d-old seedlings grown on GM.

(D) Tip of *abi8* root.

signaling pathways regulating response to ABA and assorted stresses (e.g., drought, salinity, and cold) (Ishitani et al., 1997), gibberellins (Lovegrove and Hooley, 2000), brassinosteroids (Steber and McCourt, 2001), jasmonic acid (Berger et al., 1996), auxin and ethylene (Wilson et al., 1990; Beaudoin et al., 2000; Ghassemian et al., 2000), sugars (Arenas-Huertero et al., 2000; Finkelstein and Lynch, 2000b; Huijser et al., 2000; Laby et al., 2000), and even meristem function (Ziegelhoffer et al., 2000). The fact that mutations in only some of the hormone response genes appear to affect multiple signaling pathways suggests that interactions among these pathways are relatively specific. Possible mechanisms of cross talk, particularly among ABA, sugar, and ethylene signaling, are discussed in many recent reviews (Mccourt, 1999; Sheen et al., 1999; Gibson, 2000; Coruzzi and Zhou, 2001; Gazzarrini and McCourt, 2001; Eckardt, 2002; Finkelstein and Gibson, 2002).

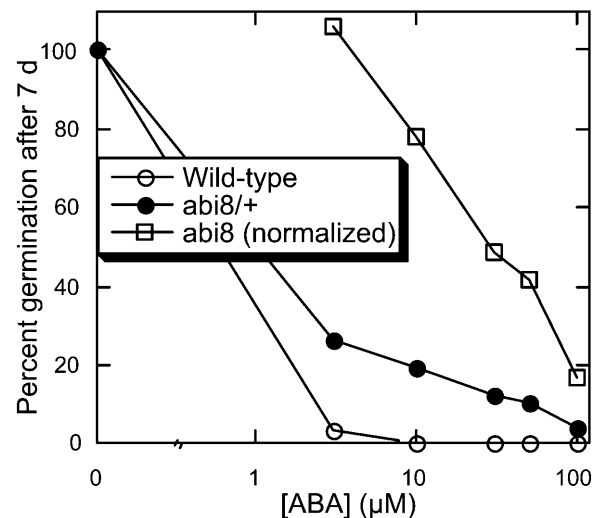
Despite recent major strides toward elucidating ABA signaling, our view of the relevant pathways still is fragmented. We have yet to identify sufficient signaling elements to constitute any complete pathway or network. Biochemical and genetic characterization of additional ABA response loci can help fill these gaps. Toward this end, we have been studying a severe ABA response mutant, *ABA-insensitive8* (*abi8*), isolated by screening for reduced sensitivity to ABA inhibition of germination. In addition to ABA resistance, *abi8* mutants have pleiotropic growth defects resulting in a severely dwarfed phenotype or death.

Consistent with this, molecular identification of the *ABI8* locus revealed that *abi8* is allelic to two dwarf mutants, *elongation defective1* (*eld1*) and *kobito1* (*kob1*) (Cheng et al., 2000; Pagant et al., 2002), whose hormone signaling defects were not recognized. This locus encodes a protein whose biochemical function is unknown and therefore is likely to represent a component of a novel signaling mechanism.

## RESULTS

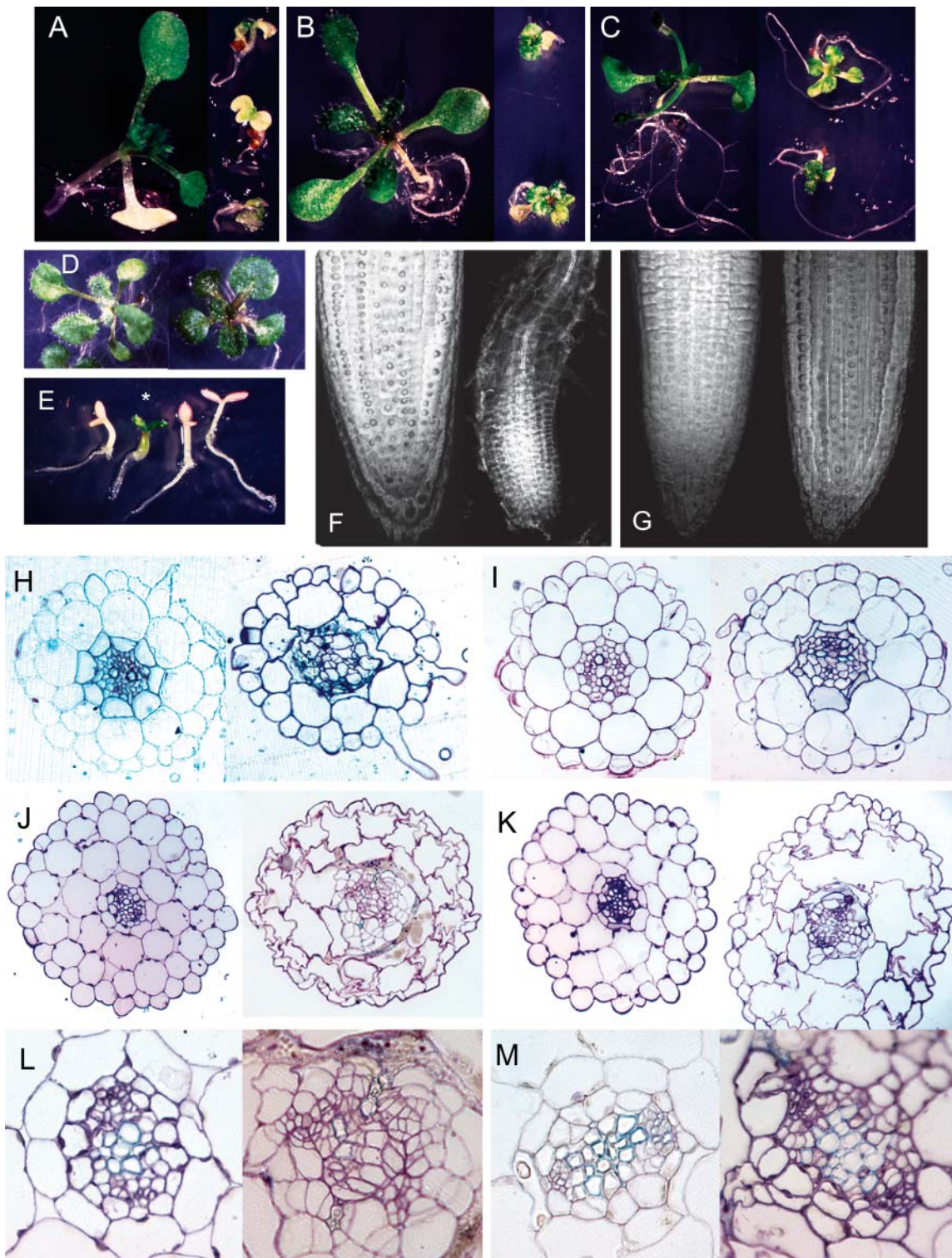
### Mutant Isolation

The *abi8* mutant was isolated on the basis of resistance to inhibition of germination by 3  $\mu$ M ABA (Figure 1). The mutation is fully recessive, such that heterozygotes have no apparent defects, but most homozygotes die as seedlings. The few survivors are severely stunted (1 to 2 cm tall at maturity), grow very slowly, flower many weeks later than wild-type plants grown under identical environmental conditions, and are male sterile (data not shown). Consequently, the mutant was backcrossed and maintained as a heterozygote (all physiological studies were performed with individuals identified in segregating populations). All of the characteristics described below cosegregated in lines that had been backcrossed three times. To determine the extent of resistance to ABA inhibition of germination, seeds of the wild type and *abi8/+* were incubated on media supplemented with up to 100  $\mu$ M ABA. The normalized *abi8* data points indicate the percentage of germination of the *abi8* segregants produced by an *abi8/+* parent. Although 3  $\mu$ M ABA inhibits germination of wild-type seeds, some *abi8* seeds germinate even in the presence of 100  $\mu$ M ABA (Figure 2).



**Figure 2.** ABA Sensitivity of Germination Inhibition for Wild-Type versus *abi8* Mutant Seeds.

Seeds were chilled for 3 d before incubation in continuous light at 22°C for 7 d. The normalized *abi8* data points indicate the percentage of germination of the *abi8* segregants produced by an *abi8/+* parent.



**Figure 3.** Growth of *abi8* Mutants Is Both Glc Dependent and Glc Resistant.

Comparison of wild-type and mutant growth. In all panels, the wild type is at left, *abi8* is at right, and each pair is shown at the same scale.

(A) to (D) Growth of 2- to 3-week-old plants on minimal medium (A), GM + 1% Suc (B), GM + 1% Glc (C), and minimal + 4% Glc (D).

(E) One-week-old progeny of *abi8*/+ cultured on minimal medium + 6% Glc. *abi8* segregant is designated by an asterisk.

(F) to (I) Root anatomy. Confocal longitudinal views of root tips grown on minimal medium (F) or minimal + 1% Glc (G). Toluidine blue-stained cross-sections of a differentiated zone of roots grown on minimal medium (H) or minimal + 1% Glc (I).

### Defects in Sugar Mobilization and Sensing

A striking feature of the mutant is that on a medium containing only essential mineral salts, radicle emergence is followed by swelling, rather than elongation, of the root and hypocotyl (Figure 1). Scanning electron microscopic comparisons of epidermal cell length showed that *abi8* hypocotyl cells were approximately sevenfold shorter than those of the wild type (data not shown). On this minimal medium, the root remains short, and lateral roots are rarely initiated. Even though abundant root hairs are produced, root surface area is greatly reduced. This stunting of root growth is exacerbated by even transient exposure to ABA, indicating that the mutant is not completely insensitive to ABA but responds inappropriately to it. Attempts to rescue the mutant by gibberellin, brassinosteroid, or indoleacetic acid application to stimulate cell elongation (and/or root growth, in the case of indoleacetic acid) were unsuccessful, indicating that the stunting did not reflect deficiencies in these hormones (data not shown). However, the growth defect is partially rescued by supplementing the medium with Glc but not sucrose (Figures 3A to 3C). Whereas root growth is much more vigorous, resulting in ~100% survival of seedlings, shoot growth is severely reduced on medium supplemented with only 1% Glc. Addition of 4% Glc mildly inhibits wild-type growth but further promotes *abi8* growth, such that both genotypes grow similarly under these conditions (Figure 3D). Anatomical comparisons showed that the mutation severely disrupts cell division patterns and organization of the stele and leads to loss of the root apical meristem in plants grown on minimal media (Figures 3F and 3H). By contrast, 1% Glc treatment led to restoration of the endodermal layer, properly oriented divisions in the vascular tissue, and maintenance of the root meristem (Figures 3G and 3I) but did not substantially increase elongation of individual cells (data not shown). Examination of hypocotyl anatomy in Glc-treated plants showed that despite the improved vascular development, mutant cells were ~2.5-fold shorter and much wider than those of the wild type and had some incomplete walls (Figures 3J to 3M), reminiscent of mutants with defects in cell walls.

Upon discovering that stunting was more severe after pre-selection on ABA, we began identifying *abi8* segregants based on their stunted growth when grown on hormone-free media supplemented with 1% Glc either continuously or after 3 to 5 d on minimal nutrient salts. Individuals cultured for several weeks on Glc-supplemented medium developed extensive roots, enabling them to routinely survive transfer to soil, but the shoots still grew very slowly and produced male sterile flowers. Although it was not possible to develop a source of homozygous seed, we were able to generate sufficient tissue for physiological and biochemical analyses of vegetative organs of mutant plants grown on either solid or liquid media supplemented with Glc.

The differential response to Glc versus sucrose suggested a defect in transport, uptake, or metabolism of sucrose pre-

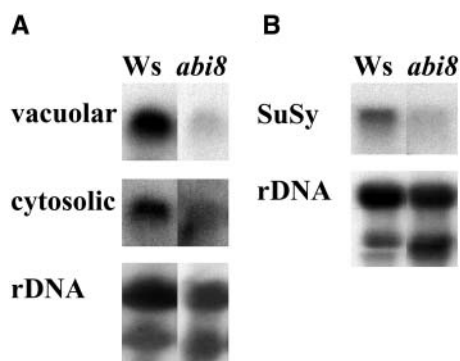
sumably supplied either exogenously or as a photosynthate. Sucrose is the most commonly translocated form of sugar in plants and may be cleaved by invertases present in the cell wall, cytosol, or vacuole or by sucrose synthase. Consistent with the poor growth of *abi8* in the presence of sucrose, total invertase activity, assayed by histochemical staining as described by Finkelstein and Lynch (2000b), was reduced slightly in *abi8* mutant roots (data not shown). Studies of antisense expression of assorted sugar-mobilizing enzymes have shown that cell wall invertases are the most critical isoforms for establishing sink strength and resulting growth of taproots, but vacuolar invertases and sucrose synthase also are important to root growth, contributing to the osmotic potential driving cell expansion (reviewed in Sturm and Tang, 1999). Using class-specific but not gene-specific probes, we found that both vacuolar and cytosolic invertase expression was reduced in mutant roots (Figure 4A). Expression of another sucrose-cleaving enzyme, sucrose synthase, also was reduced in mutant roots (Figure 4B). Cell wall invertase transcripts were undetectable in either genotype (data not shown), suggesting that they are less critical for promoting growth of seedling roots than they are for mature taproots.

To determine whether the sugar metabolism defects of the *abi8* mutant also are reflected in altered accumulation of soluble or insoluble sugars, we assayed levels of Glc, sucrose, fructose, and starch in young seedlings germinated on minimal medium. Under these conditions, the segregating progeny of *abi8* heterozygotes are readily distinguishable by 4 to 5 d postgermination and can be assayed without the complication of the contributions from the exogenous Glc that is required to maintain *abi8* seedling growth. Despite the decreased levels of invertase and sucrose synthase, there was only a small increase in starch accumulation and very little change in soluble sugars in the mutant (data not shown).

In addition to their roles in plant nutrition and in affecting the osmotic potential of growing cells, sugars are well known as signaling molecules in plants (reviewed in Rolland et al., 2002). Evidence for interactions between ABA and sugar signaling mechanisms has been provided by the isolation of new mutant alleles in ABA response or biosynthetic loci in screens for sugar-resistant seedling growth or gene expression (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001), induction of hypersensitivity to Glc by overexpression of ABI3, ABI4, or ABI5 (Finkelstein et al., 2002), Glc-induced expression of all three of these transcription factors and ABA biosynthesis (Arenas-Huertero et al., 2000; Brocard et al., 2002; Cheng et al., 2002), and suppression of ABA-inhibited germination by low levels of Glc (Finkelstein and Lynch, 2000b). To determine whether *abi8* also affects sugar signaling, seeds of *abi8/+* individuals were sown on medium containing 6% Glc. After 1 week, the wild-type segregants have arrested and turned pink, but the *abi8* mutants remain green and display their usual stunted growth (Figure 3E). Controls with isoosmotic sorbitol

Figure 3. (continued).

(J) and (K) Hypocotyl anatomy after 11 d of growth on minimal medium (J) or GM + 1% Glc (K). (L) and (M) High-magnification (100×) views of sections shown in (J) and (K), respectively.



**Figure 4.** Expression of Genes Encoding Sucrose-Cleaving Enzymes in Wild-Type versus *abi8* Roots.

Seedlings were grown on minimal media for 4 d to permit identification of *abi8* segregants based on their stunted growth and then transferred to minimal media with 1% Glc for 10 d before harvesting roots for RNA extraction. Blots were probed with clones corresponding to a vacuolar invertase (*At-β-fruct3*) and a cytosolic invertase (**A**) and a sucrose synthase (*SuSy*, *ATSUS1*) (**B**). Each lane contains 10 or 3 μg total RNA (**A**) and **B**, respectively); an rDNA probe was used to assay uniformity of loading.

indicated that the arrest of the wild type was not attributable to an osmotic effect, and *abi8* segregants were not significantly resistant to osmotic inhibition of growth (data not shown). This suggests that *ABI8* may be another member of the subset of ABA response loci and biosynthetic genes that affect sugar signaling.

### Defects in Stomatal Regulation

Stomatal closure is a well-known response to ABA, but only a subset of ABA-resistant mutants have defects in this response. To determine whether *abi8* mutants are defective in stomatal regulation, water loss in excised plants was measured after a 3-h incubation. Comparison of wild-type, *abi8*, and *abi8/+* heterozygotes shows that homozygous mutants lose at least twice as much water as the wild-type or heterozygous plants (Figure 5A). Because wilting also can result from defective vascular tissue (Postlethwait and Nelson, 1957; Rock and Ng, 1999), we tested stomatal response directly by measuring stomatal apertures of excised rosette leaves from 3- to 4-week-old plants that had been grown on agar medium supplemented with 1% Glc. Leaves were floated in opening solution for 3 h in light, followed by an additional 3 h in light with or without ABA or H<sub>2</sub>O<sub>2</sub>. Photoregulation was measured by comparing apertures of stomata examined either after 2 to 7 h of dark incubation of leaves or immediately after overnight incubation of intact plants in darkness (Figure 5B; data not shown). Although there was substantial variability in both wild-type and mutant responses, differences between wild-type and *abi8* apertures were significant for all except the light-incubated controls (Figure 5B). Whereas no closure of *abi8* stomata was observed in response to either ABA or darkness, partial closure was induced by H<sub>2</sub>O<sub>2</sub>, indicating that the mutant stomata still are capable of changing their apertures. However, the failure to promote full closure under any conditions

prevented us from testing whether the mutation also disrupts ABA inhibition of stomatal opening.

### *ABI8*-Dependent ABA-Regulated Gene Expression

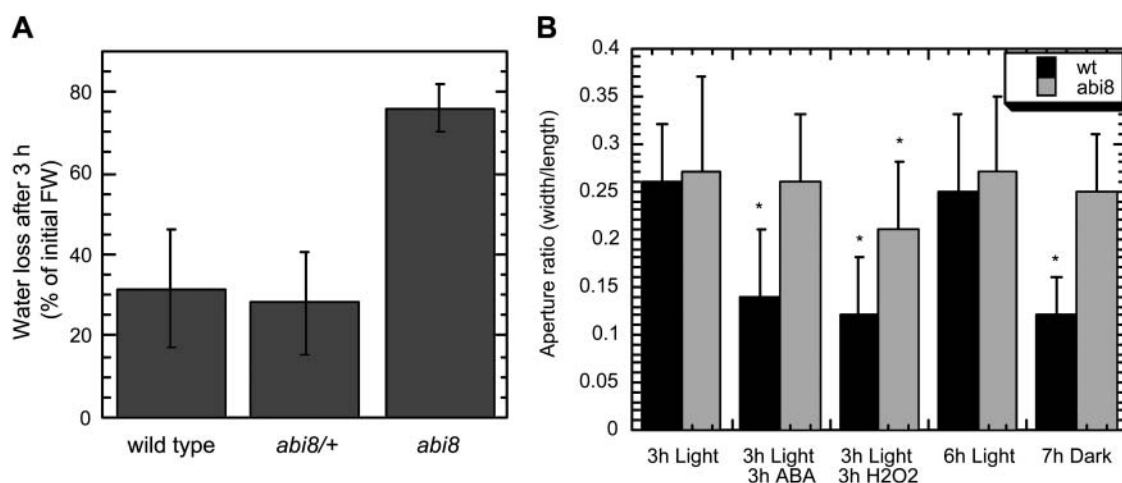
To determine whether *ABI8* is required for ABA-dependent gene expression, we compared ABA inducibility of a variety of ABA- and stress-induced transcripts in 2-week-old wild-type and *abi8* plants grown on agar medium supplemented with 1% Glc. Although some of these show reduced ABA response in the *abi8* mutant (*cor6.6*, *cor15a*, *dehydrin*, and *RAB18*), others show similar ABA response in wild-type and mutant segregants (*cor47* and *cor78*) or even constitutive expression (*osmotin*) in the mutant (Figure 6A). We also have compared reporter activity of *Dc3:GUS* and *RAB18:GUS* transgenes in seeds and found that GUS activity does not vary significantly among wild-type and mutant segregants (data not shown), indicating that *ABI8* function is not required for embryonic expression of these genes. Several light-regulated genes (e.g., ribulose biphosphate carboxylase small subunit [*RBCS*] and chlorophyll *a/b* binding protein [*CAB*]) have been shown to be ABA repressed in wild-type plants (Weatherwax et al., 1996). These genes were underexpressed in *abi8* mutants grown under control conditions and further repressed by ABA treatment (Figure 6B). These results support the conclusion that the *abi8* mutation alters, but does not eliminate, response to ABA.

### Genetic Interactions with Other Hormone-Signaling Components

To determine whether *ABI8* interacts genetically with some of the known ABA response loci, we constructed lines combining the *abi8* mutation and a variety of mutations or transgenes conferring ABA hypersensitivity or ABI-regulated GUS expression. These include *ABA-hypersensitive1* (*abh1*) (Hugouvieux et al., 2001) and hypersensitivity conferred by overexpression of ABI transcription factors (Parcy et al., 1994; Brocard et al., 2002). Comparison of germination sensitivities to ABA and seedling morphology in these lines have shown that overexpression of *ABI3* or *ABI5* does not restore normal seedling growth in *abi8* mutant segregants but results in intermediate ABA sensitivity at germination (Table 1). Similarly, seedlings of *abi8 abh1* double mutants display *abi8*-like dwarfing but are only weakly resistant to ABA inhibition of germination. The intermediate ABA sensitivities of the *abi8 35S:ABI* and *abi8 abh1* seeds suggest action in parallel pathways. Comparisons of ABI-regulated GUS expression show that *ABI3:GUS*, *ABI4:GUS*, and *ABI5:GUS* expression all appear normal in *abi8* mutant seedlings (data not shown), which also is consistent with action of *ABI8* in a separate pathway from the ABI transcription factors.

Many hormone-signaling mutants have defects in responding to multiple hormones, and several loci have been implicated in mediating cross talk between ABA and ethylene signaling. Because the abundant root hair growth and short swollen hypocotyls of *abi8* mutants are reminiscent of ethylene effects on seedlings, we tested whether inhibitors of ethylene synthesis or response (aminoethoxyvinylglycine and AgNO<sub>3</sub>, respectively) could suppress the stunted phenotype of the *abi8* plants (data





**Figure 5.** Stomatal Regulation in Wild-Type versus *abi8* Mutant Plants.

**(A)** Water loss in excised plants during 3-h incubation. Comparison of wild-type, *abi8*, and *abi8/+* heterozygotes (genotypes determined by analysis of *abi8* segregation in progeny).

**(B)** Stomatal apertures of rosette leaves from 3- to 4-week old plants floated in opening solution for 3 h in light, followed by an additional 3 h in light  $\pm 10 \mu\text{M}$  ABA or  $100 \mu\text{M}$  H<sub>2</sub>O<sub>2</sub> or measured after 7-h incubation in darkness.

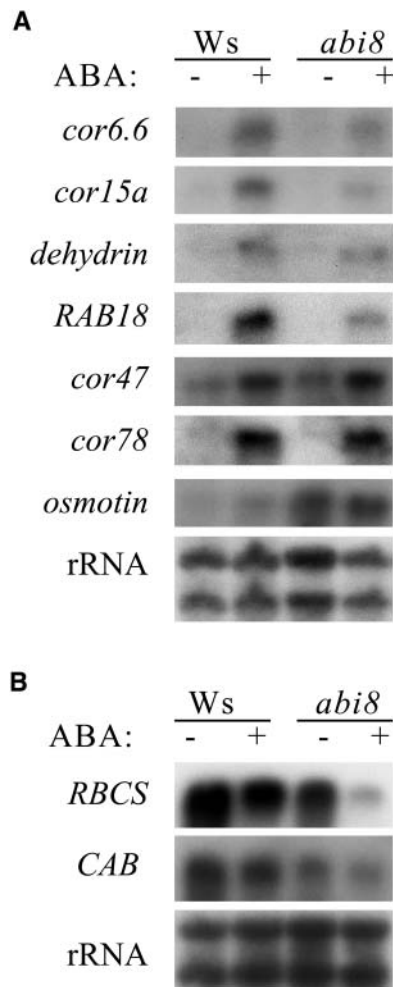
Asterisks designate samples with significantly different ( $P < 0.004$ ) apertures from light-incubated controls. Differences between wild-type and *abi8* apertures were significant for all except the light treatments.

not shown). Although these treatments resulted in slightly greater expansion of wild-type seedlings, the *abi8* mutants remained stunted, indicating that this phenotype is probably not attributable to defects in ethylene signaling (data not shown). However, because both of these inhibitors affect the initial steps in ethylene signaling (production or receptor binding of the hormone), we also constructed digenic mutants combining *abi8* with ethylene-insensitive (*ethylene-insensitive2* [*ein2*] and *ein3*) mutations affecting later steps in ethylene signaling. *EIN2* encodes a membrane protein related to mammalian NRAMP metal transport proteins (Alonso et al., 1999) that appears to mediate activation of *EIN3*. *EIN3* encodes a transcription factor that regulates expression of a class of transcription factors that bind ethylene-responsive elements of ethylene-induced genes (Chao et al., 1997; Solano et al., 1998). Mutations affecting *EIN2* have been isolated in screens for altered response to ABA (designated *era3*) (Beaudoin et al., 2000; Ghassemian et al., 2000), cytokinins (Cary et al., 1995), and auxin transport inhibitors (Fujita and Syono, 1996), suggesting that *EIN2* acts at a point of cross talk among hormone signaling pathways. Similarly, *ein3* mutants are slightly hypersensitive to ABA for inhibition of germination: only 4% of *ein3* seeds germinate after 5 d on media containing  $0.3 \mu\text{M}$  ABA, whereas 77% of wild-type seeds germinate under these conditions. In addition, both *ein2* and *ein3* mutations confer hypersensitivity to Glc for inhibition of seedling growth (Yanagisawa et al., 2003). Analyses of the progeny of *ein abi8/+* plants showed that both homozygous *ein2 abi8* and *ein3 abi8* segregants exhibited ABA-resistant germination and 1-amino-cyclopropane-1-carboxylic acid (ACC)-resistant cotyledon expansion but remained stunted (Table 2) and displayed Glc sensitivity that was intermediate between the parental lines (data

not shown). These results support the conclusion that the stunted phenotype of *abi8* mutants is not attributable to constitutive ethylene response but show that *abi8* acts epistatically to the *ein* mutations with respect to effects on ABA sensitivity of germination yet acts additively with respect to effects on Glc sensitivity of seedling growth.

#### ***ABI8* Encodes a Plant-Specific Protein of Unknown Biochemical Function**

Although isolated from a T-DNA insertion line collection, the *abi8* mutation did not cosegregate with any of the T-DNA markers. Consequently, we undertook a chromosome walk to identify the affected gene. We fine-mapped *ABI8* to within  $\sim 95$  kb on chromosome 3 and then tested nearly all of the predicted genes across the interval for complementation by wild-type genomic fragments introduced via floral-dip infiltration (Clough and Bent, 1998) of *abi8 LONG HYPOCOTYL2 (HY2)/ABI8 hy2* individuals. Complementation was observed with a 5-kb genomic fragment containing a single complete gene (At3g08550) that encodes a predicted 533-amino acid protein of unknown function. The *abi8* mutant allele contains a 5-bp deletion, resulting in a frame shift in the first exon at amino acid 85 and termination shortly thereafter, such that this allele is likely to be a biochemical null (Figure 7A). Basic Local Alignment Search Tool (BLAST) analyses have identified likely orthologs in *Nicotiana plumbaginifolia* and *Oryza sativa* (rice) (Figure 7B), and closely related genes are represented in EST collections from diverse plant species. Consistent with this proposed similarity of function, the *N. plumbaginifolia* gene was identified by virtue of an insertional mutant with a seedling lethal phenotype, *sdl-1* (N. Houba,



**Figure 6.** ABA-Regulated Gene Expression in Wild-Type versus *abi8* Mutant Plants.

RNA gel blot analyses of ABA effects on expression of ABA-inducible (A) and light-inducible/ABA-repressible (B) genes in 2-week-old plants. Blots were hybridized to indicated probes; loading uniformity was assayed by hybridization to rRNA.

unpublished data). Database searches indicate that *abi8* is allelic to two dwarf mutants, *eld1* and *kob1*, previously shown to disrupt cell elongation, cellulose synthesis, vascular differentiation, and root meristem maintenance (Cheng et al., 2000; Pagant et al., 2002). Although highly conserved with other plant proteins, database searches for domains of known function have shown only a suggestion of association with an unidentified membrane via a prokaryotic membrane lipoprotein lipid attachment site and possible targeting to the chloroplast. A variety of potential modifications are suggested by the presence of 4 *N*-glycosylation sites, 16 phosphorylation sites, 8 myristoylation sites, and an amidation site, but each of these motifs occur frequently in protein sequences, and their function must be verified experimentally.

*A. thaliana* contains two predicted genes that encode proteins that are 60 to 70% identical to ABI8 over 400 to 500 amino acids:

At3g57200 and At2g41450 (Figure 7B). One of these (At2g41450) encodes an additional 577 amino acids at the N terminus, which includes three conserved domains: a breast cancer C-terminal domain, an FBD domain (found in FBox and breast cancer C-terminal domain-containing plant proteins), and a domain present in *N*-acetyltransferases. Although there are no published reports of mutant phenotypes resulting from disruption of the *A. thaliana* homologs of *ABI8*, multiple insertion lines are available for both of these loci from the SALK SIGnAL collection (<http://signal.salk.edu>). We have identified lines carrying the desired insertions but see no evidence of a phenotype reminiscent of *abi8* (data not shown). In combination with the severity of the *abi8* phenotype, this suggests that these loci do not function redundantly.

### ABI8 Expression and Localization

At the level of mRNA accumulation, *ABI8* is expressed in all tissues tested throughout development in wild-type plants, but transcript levels are relatively low in developing siliques (Figure 8A). *ABI8* transcript levels show no consistent regulation by ABA exposure, although the gene appears slightly underexpressed in *abi1* mutants (Figure 8A). We have assayed tissue and sub-cellular localization by histochemical staining of transgenic plants containing fusions of an *ABI8* genomic fragment, including the entire 5' region and coding sequence, tagged at the C terminus with GUS. Despite the strong ABA resistance of mutant seeds, *ABI8*-GUS was undetectable histochemically in developing or mature seeds. However, fluorometric assays of extracts from mature seeds of independent transgenic lines showed

**Table 1.** Genetic Interactions between *abi8* and Other ABA Response Loci

Genotype	Germination (%) 0.3 $\mu$ M ABA		Germination (%) 1 $\mu$ M ABA		Dwarf (%) (n)	
		(n)		(n)		(n)
Ws	73.3	(359)	19.1	(393)	0	
<i>abi8/+</i>	96.4	(501)	49.2	(528)	22	(463)
<i>abi8</i>	100	(125)	100	(132)		
<i>35S:ABI3</i>	45.2	(126)	16.9	(136)	0	
<i>abi8/+ 35S:ABI3</i>	68	(409)	21.4	(252)	20.4	(216)
<i>abi8 35S:ABI3</i>	92.7	(82)	80.7	(88)		
<i>35S:ABI5</i>	2	(197)	0	(181)	0	
<i>abi8/+ 35S:ABI5</i>	26.7	(533)	15.6	(449)	24.1	(429)
<i>abi8 35S:ABI5</i>	54.1	(133)	62.5	(112)		
<i>abh1</i>	31.4	(118)	1.5	(134)	0	
<i>abi8/+ abh1</i>	15.4	(280)	4.3	(232)	23.2	(302)
<i>abi8 abh1</i>	57	(70)	17.3	(58)		

ABA sensitivity was assayed by measuring germination (scored as emergence of entire seedling from seed coat) after 4 d of incubation on 0.3  $\mu$ M ABA or 8 d of incubation on 1  $\mu$ M ABA. Among germinating progeny of *abi8/+ 35S:ABI* or *abi8/+* plants, *abi8* segregants were identified by their swollen radicles. The normalized percentage of germination was calculated relative to the anticipated fraction of double homozygotes. Dwarfing as a result of the *abi8* mutation was assayed in progeny of *abi8/+* plants grown on hormone-free medium; dwarfing was not observed in lines lacking the *abi8* mutation. The results are expressed as a percentage of the total number of seeds plated (n).

**Table 2.** Genetic Interactions between *abi8* and Ethylene Response Loci

Genotype	Seedling Morphology on ACC Triple Response				ABA Sensitivity	
	–	+	<i>abi8</i> -Like	(n)	Germination	(n)
Ws	0	100	0	(100)	0	(315)
<i>abi8/+</i>	0	71.4	28.6	(56)	23	(513)
<i>ein2</i>	100	0	0	(100)	0	(100)
<i>abi8/+ ein2</i>	75.4	0	24.7	(292)	28	(500)
<i>abi8/+</i>	0	73.9	26.1	(46)	22.3	(417)
<i>ein3</i>	100	0	0	(100)	0	(205)
<i>abi8/+ ein3</i>	73.4	0	26.6	(726)	19.7	(680)

Ethylene sensitivity was assayed by plating seeds on 10  $\mu$ M ACC and scoring seedling morphology after 4 d in the dark at 22°C; the triple response was scored as maintenance of an apical hook, failure to expand cotyledons, and radial swelling. *abi8*-like seedlings were distinguished from those displaying the triple response by their reduced apical hooks and enhanced radial swelling of the hypocotyl. The *ein abi8* double mutants show complete loss of the apical hook. ABA sensitivity was assayed by measuring germination and hypocotyl swelling after 8 or 9 d of incubation on 3  $\mu$ M ABA for the *ein2* and *ein3* monogenic and digenic lines, respectively. The results are expressed as a percentage of the total number of seeds plated (n).

that GUS activity ranged from 10 to 70 units/h/seed above the background detected in nontransgenic control seeds. Furthermore, stratification and subsequent incubation on media containing 1  $\mu$ M ABA was sufficient to both delay germination and induce ABI8-GUS accumulation near the radicle tip (Figure 1 in supplemental data online), consistent with a role for ABI8 in inhibiting the radicle growth required for germination.

Although green fluorescent protein (GFP)-KOB1 expressed under control of the 35S promoter of *Cauliflower mosaic virus* recently was shown to localize to the plasma membrane of elongated root cells (Pagant et al., 2002), we found that the ABI8-GUS fusion protein expressed under control of its own promoter is localized primarily to the elongation zone of roots, where it accumulates in a punctate pattern within the cytoplasm (Figure 8B). A similar cytoplasmic localization of the GFP-KOB1 fusion was observed in the cell division zone of the root, indicating that membrane association is not obligate. Furthermore, ABI8-GUS transgenes exhibiting this localization pattern are sufficient to complement the mutation (Table 3), indicating that there may be substantial post-transcriptional control of ABI8 accumulation, such that minor variations in transcript levels may not be functionally significant. Consistent with the possibility of post-transcriptional control, RNA gel blot analyses indicate that the transgene is strongly expressed in both roots and shoots (Figure 8C) even though the fusion protein is undetectable in most tissues (Figure 8B), even by fluorometric assays (data not shown). The high abundance of transgene transcripts seen in Figure 8C might reflect the presence of multiple fusion genes in the transgenic lines but does not result in high-level protein accumulation.

## Interactions with Stress Signaling

The discovery that *abi8* was allelic to a cellulose-deficient mutant raised the possibility that some of the signaling defects also might be derived from the alterations in cellulose synthesis capacity. Recent studies have shown that defects in cellulose synthesis, induced either genetically or chemically, can lead to overproduction of jasmonate (JA) and ethylene and activate stress responses dependent on these hormones (Ellis et al., 2002). To determine whether the cell wall defects of the *abi8* mutant also affect this aspect of stress signaling, we tested *abi8* effects on expression of two genes regulated by JA, ethylene, and cellulose deficiency: *VSP2* and *PDF1.2*. *VSP2* encodes a vegetative storage protein, whereas *PDF1.2* encodes a plant defensin involved in fungal resistance. Expression of both genes is induced, to different degrees, by either JA or disruption of cellulose synthesis. *PDF1.2* expression also is induced by ethylene, but *VSP2* is repressed by ethylene (Ellis et al., 2002). RNA gel blot analyses indicated that ABA acts antagonistically to ethylene, in that it slightly induced *VSP2* but repressed *PDF1.2* expression in Wassilewskija (Ws) seedlings (Figure 9). By contrast, *abi8* seedlings strongly expressed *PDF1.2* in the absence of ABA but still could repress *PDF1.2* and hyperinduced *VSP2* in response to ABA. These results suggest that ABA regulation of *VSP* and *PDF1.2* expression was functional or even enhanced in the *abi8* mutant. Although the high basal level of *PDF1.2* expression in the mutant might be explained by cell wall-based signaling, *VSP* expression was not similarly altered, again demonstrating that not all aspects of the *abi8* phenotype can be ascribed to cellulose deficiency alone.

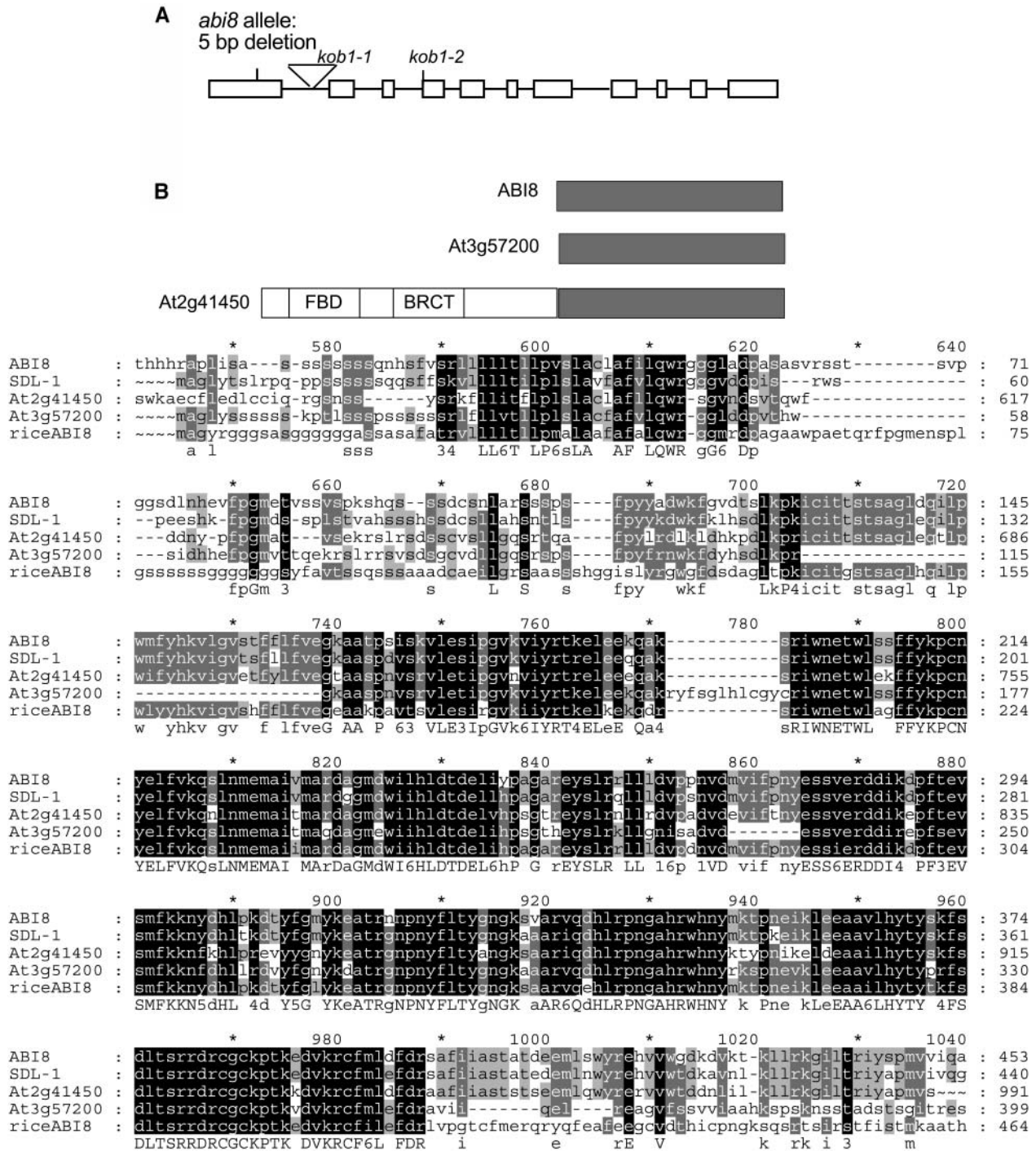
## DISCUSSION

At least five independent mutations in *ABI8/ELD1/KOB1* genes have been identified in screens for ABA response defects or dwarf plants (Cheng et al., 2000; Pagant et al., 2002; K. Lertpiriyapong and Z.R. Sung, personal communication). The *kob1-1* and *kob1-2* alleles had a T-DNA insertion in an intron and a defect affecting splicing, respectively, but still were capable of making a small amount of properly spliced transcript. By contrast, the *abi8* allele results in loss of >80% of the C-terminal portion of the protein and is likely to be a biochemical null mutant. The *eld1* alleles also are nulls (K. Lertpiriyapong and Z.R. Sung, personal communication). All of the mutants are morphologically similar, in that they are sterile and have severely stunted growth because of reduced cell elongation that is not rescued by treatment with any known hormones or inhibitors of hormone synthesis/response. However, the *kob1-1* allele is less dwarfed than the other reported mutants. Because of the distinct nature of the screens, characterization of the various alleles has emphasized different aspects of their physiological and molecular defects.

### Growth Defects in *abi8/eld1/kob1* Mutants

The severe stunting of *eld1* root growth was shown to reflect terminal differentiation of the root apical meristem and cessation of cell division at the root tip, with only occasional activation of cell

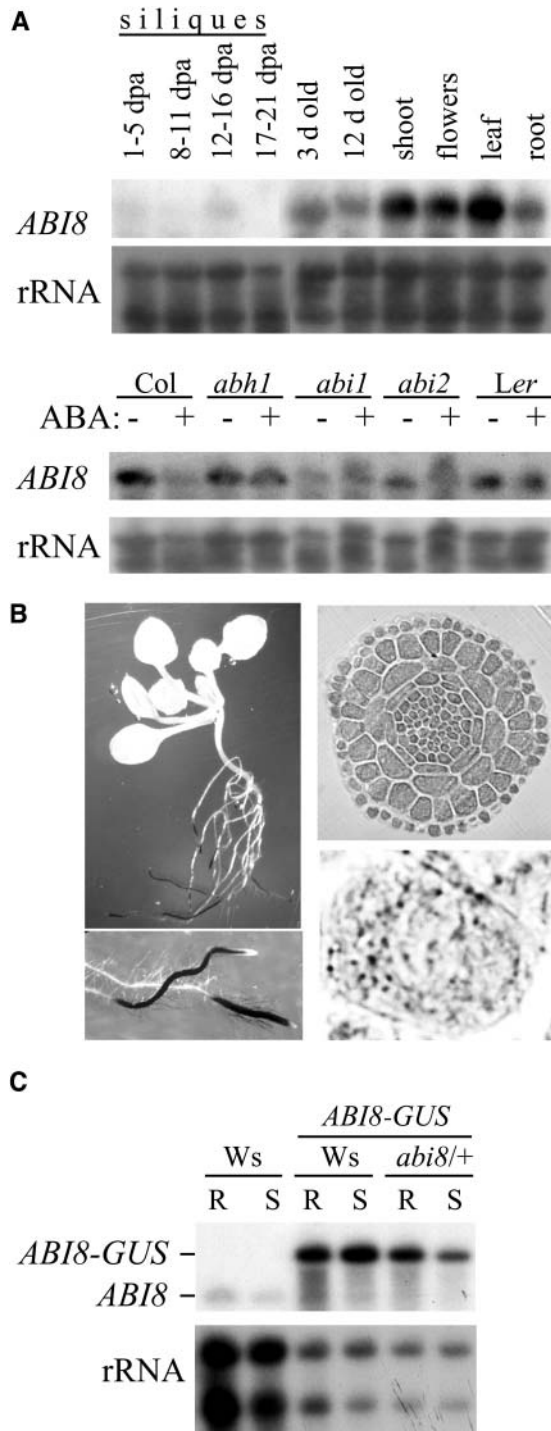




**Figure 7.** *ABI8* and Homologous Genes.

**(A)** Schematic of *ABI8* exon/intron structure and site of *abi8* mutation.

**(B)** Alignments of predicted amino acid sequences with those from homologous genes. Schematic of *A. thaliana* genes (top); sequence alignment of *A. thaliana*, *O. sativa*, and *N. plumbaginifolia* genes (bottom). Residues conserved across all five sequences are shaded black; residues conserved across three or four sequences are shaded gray. Consensus sequences are shown underneath; conserved groups are as follows from top to bottom: 1 = DN, 3 = ST (hydroxylated), 4 = KR (basic), 5 = FYW (aromatic), and 6 = LIVM (aliphatic or M). Alignment was performed by the Pileup program of the University of Wisconsin Genetics Computer Group software package; shading of conserved residues was accomplished with the GeneDoc program.



**Figure 8.** *ABI8* Expression.

**(A)** RNA gel blot analyses of *ABI8* transcript accumulation in assorted tissues and genotypes. ABA response in different genotypes was compared in 2-week-old plants exposed to 50  $\mu$ M or no ABA for the last 2 d of culture. *Col*, Columbia ecotype; *Ler*, Landsberg erecta ecotype.

**(B)** Localization of GUS activity derived from an *ABI8-GUS* fusion, including the entire *ABI8* coding sequence, under control of the *ABI8*

division in newly emerging lateral roots (Cheng et al., 2000). In this regard, it is noteworthy that the *eld1* mutant roots have reduced expression of *cdc2a* (Cheng et al., 2000), a cell cycle marker that reflects competence for cell division and whose activity is inhibited by the ABA-induced INHIBITOR OF CYCLIN DEPENDENT KINASE1 (Wang et al., 1998). In this respect, the mutants appear to display a constitutive ABA response. Both *eld1* and *kob1* mutants were described as having a greatly reduced zone of elongation, such that differentiation of root hairs was observed almost all of the way to the root tips; *abi8* mutants also display this characteristic. All of the mutants have small cuboidal root and hypocotyl cells; the reduced cell elongation is correlated with an  $\sim$ 45 to 60% decrease in cellulose synthesis in the *kob1* mutants, leading Pagant et al. (2002) to suggest that reduced cellulose synthesis is a primary effect of the *kob1* mutations.

Although the *eld1* stele was characterized as disrupted by somewhat randomly arrayed vascular differentiation, which also is observed in *abi8* mutants, this was not described for the *kob1* mutants possibly because they are weaker alleles. Growth of *abi8* mutant roots also arrests on standard growth media (minimal nutrient salts or Murashige and Skoog salts with 1% sucrose), but this arrest is prevented by inclusion of Glc (0.5 to 4%) in the medium. This result indicates that the mutation does not force the roots to arrest but may alter their ability to produce or respond to signals promoting growth. Microscopic examination of *abi8* roots and hypocotyls showed that the improved root growth on Glc reflected maintenance of the root apical meristem and improved vascular development but that the mutant cells still were much shorter than those of the wild type. These results suggest that the cellulose synthesis defect still inhibits cell elongation, but tissue differentiation and overall morphology are subject to additional regulation.

Both *eld1* and *kob1* mutants were shown to have ectopic accumulation of wall components, such as suberin and lignin, throughout the hypocotyl and in the cotyledons, reflected in waxy white patches on the cotyledons (Cheng et al., 2000; Pagant et al., 2002). The *abi8* mutant shows similar waxy patches when grown under conditions that lead to growth arrest but not when growth is partially rescued by inclusion of at least 1% Glc (Figure 3), indicating that accumulation of these secondary products also may be a secondary effect of the mutation.

#### ***ABI8/ELD1/KOB1* Affect ABA and Glc Signaling**

In addition to observing the growth defects described above, characterization from the perspective that *abi8* represents an ABA response mutant has uncovered defects in ABA signaling in germination, stomatal regulation, and regulation of gene expression. However, this is not a complete lack of response because some ABA-regulated genes show normal ABA response,

promoter. Left, entire plant and root tip; right, cross-section through root tip of 2-week-old plant and high-magnification view of a single cell showing the punctate staining pattern.

**(C)** RNA gel blot analyses of *ABI8* and *ABI8-GUS* transcript accumulation in transgenic lines. R, roots; S, shoots.

**Table 3.** Complementation of *abi8* in *ABI8-GUS* Transgenic Lines

Genotype	Germination		Kanamycin R	
	(%)	(n)	(%)	(n)
<i>abi8/+</i>	25	(271)	NA <sup>a</sup>	
<i>abi8/+ ABI8-GUS/0</i>	4.9	(144)	77	(81)
<i>abi8/+ ABI8-GUS</i>	0	(208)	100	(200)

The *abi8* phenotype was assayed by measuring germination and hypocotyl swelling after 7 d of incubation on 3  $\mu$ M ABA. Presence, number of insertion sites, and homozygosity of transgenes was assayed by scoring kanamycin resistance (kanamycin R). The results are expressed as a percentage of the total number of seeds plated (n).

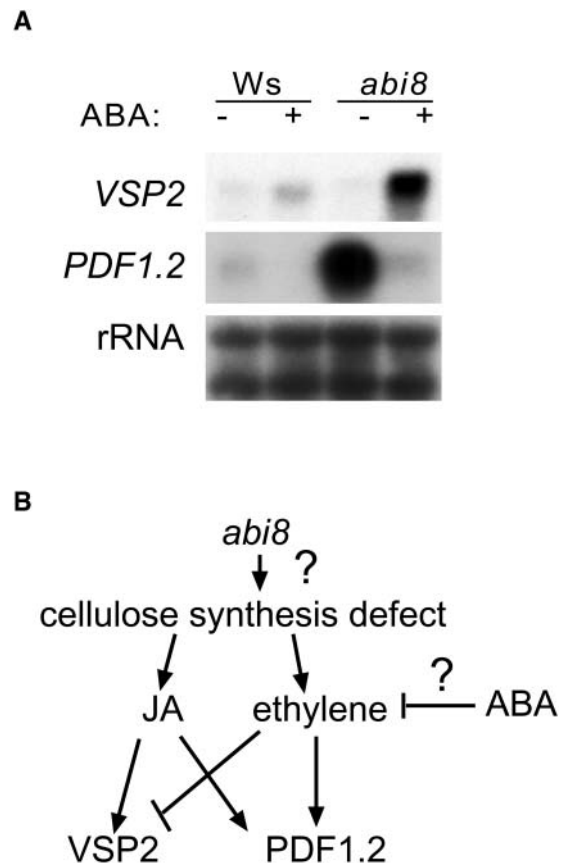
<sup>a</sup>NA, not applicable.

whereas others become constitutively expressed and/or hyper-responsive. Similarly specific but distinct effects on ABA-regulated gene expression have been described for many other ABA response mutants, including all of the other *abi* mutants and *abh1* (Hugouvieux et al., 2001; Finkelstein et al., 2002; Hoth et al., 2002; Suzuki et al., 2003). Furthermore, some of these previously documented specificities also appear contradictory, including opposing effects of *abi3* and *abi5* mutations on expression of specific genes expressed late in embryogenesis (Parcy et al., 1994; Finkelstein and Lynch, 2000a) or decreased expression of several ABA-inducible genes in the ABA-hypersensitive *abh1* mutant (Hugouvieux et al., 2001). Thus, *abi8* mutants resemble many other ABA response mutants in displaying differential defects in ABA response. Our studies also have shown that *abi8* growth is not only dependent on low concentrations of Glc but also is resistant to the inhibitory effects of high Glc, suggesting a defect in sugar signaling and/or transport. Whereas other ABA response or biosynthetic mutants have been found to be resistant to high Glc, this mutant differs from the others in that it also is Glc dependent. It also appears to act in a distinct pathway from the other *ABI* loci implicated in Glc response.

The fact that the impaired growth is far more severe than that observed in other wilty mutants with defects in ABA biosynthesis (*aba*) or signaling (*abi1*) is consistent with the observation that the *abi8* mutation disrupts additional processes required for growth, such as cellulose synthesis, vascular differentiation, and expression of invertases or sucrose synthases, that would affect the osmotic potential of elongating root cells. These results suggest that ABA and/or Glc signaling regulates expression of these sugar-mobilizing enzymes. Although Glc regulation of these enzymes has been described in many other plant species (Koch, 1996), ABA regulation is not well characterized. These enzymes are generally encoded by multigene families, with specific family members exhibiting opposite responses to sugars, such that some are induced by feast and others by famine conditions (Koch, 1996), and it is not clear which of the *A. thaliana* genes are regulated by Glc and/or ABA. BLAST analyses indicate that all of these sugar-mobilizing enzymes belong to multigene families with six to nine members per family in *A. thaliana*, only a few of which have been analyzed extensively (Schwebel-Dugue et al., 1994; Mercier and Gogarten, 1995; Haouazine-Takvorian et al., 1997; Tymowska-Lalanne and Kreis, 1998). Our studies did not attempt to discriminate among these multitudes of family

members but clearly showed that the most abundantly expressed member(s) were dependent on *ABI8* for normal expression.

Investigation of stomatal regulation revealed that the mutant fails to close its stomata in response to ABA or darkness and only weakly responds to H<sub>2</sub>O<sub>2</sub>. The partial response to H<sub>2</sub>O<sub>2</sub> suggests that *abi8* mutants may have defects in ABA-induced production of reactive oxygen species, but this does not explain their complete lack of response to ABA or the failure to respond to darkness. Another possibility is that the defect in dark response may be another aspect of the constitutively photomorphogenic phenotype described for the *eld1* allele (Cheng et al., 2000). In this regard, it is noteworthy that genetic interactions affecting photomorphogenesis have been demonstrated between *abi3* and *de-etiolated1* mutants (Rohde et al., 2000) and that *abifusca3* and *abi leafy cotyledon1* digenic mutants reveal cryptic effects resulting in a mildly deetiolated phenotype (Nambara et al., 2000; Brocard-Gifford et al., 2003). Additional interactions between darkness and ABA accumulation and signaling have



**Figure 9.** Interactions Affecting Expression of Genes Induced by Cellulose Deficiency.

**(A)** RNA gel blot analyses of ABA effects on gene expression in 2-week-old plants. Blots were hybridized to indicated probes; loading uniformity was assayed by hybridization to rRNA.

**(B)** Proposed model of signaling interactions regulating VSP2 and PDF1.2 expression.

been demonstrated in wild-type plants (Weatherwax et al., 1996). Although the defects in stomatal regulation also might reflect unidentified defects in guard cell structure (e.g., possibly affecting the arrangement of cellulose microfibrils that determines guard cell turgor and shape), *abi8* guard cells look surprisingly normal, in that they are approximately the same size as those of wild-type plants (data not shown). Furthermore, the partial response to H<sub>2</sub>O<sub>2</sub> is not consistent with a solely structural defect, as observed when disruption of pectic cross-linking results in locking of stomatal aperture (Jones et al., 2003).

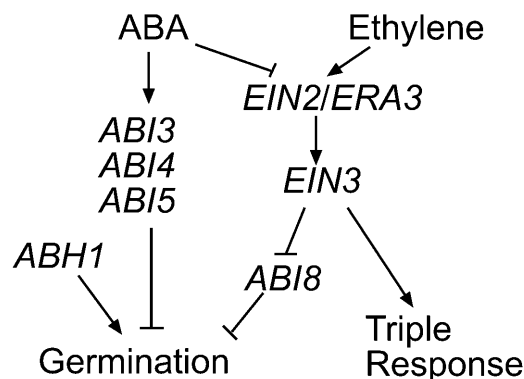
### Interactions with Other Signaling Loci

In addition to describing multiple defects in response to ABA and Glc, our genetic studies have demonstrated epistatic or additive relationships between *abi8* and several loci known to affect ABA, and in some cases Glc or ethylene, signaling. At least a dozen loci have been identified that disrupt response to at least two of these signals. In general, ABA and Glc responsiveness are correlated, whereas ethylene acts antagonistically to these signals at germination and in early seedling growth. The loci shown to be associated with both ABA and sugar response have been limited so far to those encoding ABA biosynthetic enzymes (*ABA1*, *ABA2/GIN1/ISI1/SIS4*, and *ABA3/GIN5/LOS5*), the ABI transcription factors (*ABI3*, *ABI4/GIN6/ISI3/SIS5/SUN6*, and *ABI5*) and related proteins (*ABF3* and *ABF4*), and *EIN2/ERA3* and *CTR1* (reviewed in Leon and Sheen, 2003). Among these, the ABI transcription factors appear to function in the same signaling pathway mediating ABA response (Finkelstein et al., 2002), and expression of all three is induced by Glc in an apparently hexokinase-dependent manner (Leon and Sheen, 2003). The *EIN2/ERA3* and *CTR1* loci also have been shown to function in a single pathway, in this case, mediating ethylene response via effects on *EIN3* and resulting ethylene-dependent gene expression (Wang et al., 2002). This pathway recently was shown to be modulated by hexokinase-dependent degradation of *EIN3* (Yanagisawa et al., 2003). Although *ctr1* alleles have been isolated as enhancers of ABA resistance in *abi1-1* mutants (Ghassemian et al., 2000), similar to the interactions reported for *abi1-1* and *abi* transcription factor mutations (reviewed in Finkelstein and Rock, 2002), genetic studies testing potential

interactions among the ABI transcription factors and the products of these ethylene response loci have not been reported.

Our studies place *ABI8/ELD1/KOB1* function downstream of *EIN2* and *EIN3* and possibly in a separate pathway from those requiring action of *ABH1* and the ABI transcription factors in regulating ABA effects on germination (Figure 10). Epistasis analyses among *abh1*, *era1*, and several *abi* mutants indicated that *ABH1* also acts in a distinct pathway from *ERA1* and the ABI transcription factors (Hugouvieux et al., 2001; Brady et al., 2003). However, the additive effects of *abi8* and *ein* mutations on Glc and ethylene response indicate that these loci mediated response to these signals via distinct pathways, suggesting that *ABI8* defines a novel signaling pathway affecting Glc response. The consistently dwarfed phenotype of all of the lines carrying the *abi8* mutation might reflect defects in cellulose synthesis that are not affected by any of the other loci tested in the double mutant or transgenic lines.

The discrepancy between transcript and protein accumulation indicates that comparisons of *ABI8* transcript levels in various mutants may not be a good indicator of *ABI8* activity and that *ABI8* accumulation is likely to be subject to control at the level of translation and/or stability. Furthermore, differences in *ABI8/ELD1/KOB1* protein accumulation or localization may be mediated by additional unidentified factors. For example, tissue-specific interactions with other protein(s) might explain why the 35S::GFP-KOB1 product was cytoplasmic in the root tip but localized to the plasma membrane elsewhere (Pagant et al., 2002). Even though these putative additional factors have not been identified yet, the observed genetic interactions and physiological responses clearly place *ABI8/ELD1/KOB1* in an ABA- and Glc-regulated signaling network, suggesting that many of the growth defects may reflect signaling defects and not direct effects on cellulose biosynthesis. Furthermore, this suggests that ABA and/or Glc signaling may interact with several processes with which these signals had not been associated previously, including promotion of cellulose biosynthesis and organizing vascular differentiation. Although it may seem contradictory to ascribe the severity of the phenotype to signaling defects that are not observed in the *aba* or other *abi* mutants, this might be explained by the substantial redundancy in both ABA biosynthetic and signaling pathways. Alternatively, *ABI8/ELD1/KOB1* may integrate stress signaling in response to ABA, ethylene, JA, Glc, and cell wall-derived compounds.



**Figure 10.** Proposed Model of Genetic Interactions between *ABI8* and Some Other ABA Response Loci Affecting Germination.

### Potential Function of *ABI8/ELD1/KOB1*

The cloning and molecular analyses of *ABI8/ELD1/KOB1* have raised many new questions. The predicted amino acid sequence reveals no domains of known biochemical function, and the genetic analyses place its action in a network of signaling factors. Subcellular localization of an overexpressed GFP-KOB1 fusion showed that it was in the plasma membrane of cells in the root elongation zone but showed a punctate intracellular distribution in the cell division zone at the root tip (Pagant et al., 2002). By contrast, accumulation of a complementing *ABI8*-GUS fusion expressed under control of the *ABI8* promoter was limited to the root elongation zone and the more terminal portions of the zone of differentiation, where it was concentrated in punctate patches

in the cytoplasm similar to those described for KOB1 in the root tip. These results raise the question of whether the observed plasma membrane localization of 35S:GFP-KOB1 is functionally significant or a default localization for an ectopically expressed protein with a lipid attachment site. The punctate distribution displayed by both the physiologically functional ABI8-GUS fusion and the root tip-localized GFP-KOB1 may depend on an interaction with an anchoring cytoplasmic partner that is absent throughout most of the root.

The discrepancy between the extremely limited vegetative expression pattern of the ABI8-GUS fusion protein and its ability to complement the mutation's effects in tissues throughout the plant body suggests that ABI8 effects on shoot tissue may depend on either very low levels of protein or a long-distance signaling mechanism that preconditions shoots to respond to ABA and/or other signals. Fluorometric assays of ABI8-GUS activity in mature seeds demonstrated that it accumulates to levels one to three orders of magnitude lower than those observed for promoter fusions with the ABI transcription factors (Söderman et al., 2000; Brocard et al., 2002; R.R. Finkelstein, unpublished data). However, ABI8-GUS activity increased significantly in seeds stratified in the presence of ABA, consistent with a role for ABI8 in maintaining developmental arrest in imbibing seeds. This expression was strongest in the radicle tip, where it might be involved in preventing production of hydrolases needed for emergence and/or reactivation of cell cycling in postgerminative growth.

As suggested with respect to *cev1*, a cellulose synthase mutant, disruption of cell wall synthesis may cause release of cell wall-derived compounds, such as oligosaccharides, that may act as long-distance signals themselves. Alternatively, ABI8 might aid transport of water and available signals simply by promoting vascular differentiation. However, several lines of evidence indicate that it is unlikely that the defects of *abi8/eld1/kob1* mutants can be explained fully in terms of effects on root and hypocotyl vascular development and the resulting capacity for long-distance transport. First, even isolated leaves of *abi8* plants with rescued vascular development have altered stomatal response to applied ABA. In addition, these rescued plants display aberrant ABA regulation of gene expression. Furthermore, vascular development is disrupted in mutant tissues in which ABI8 appears to be not usually expressed, based on ABI8-GUS activity (e.g., the hypocotyls). ABI8 accumulation also may be transiently induced in specific tissues in response to as yet unidentified signals. In summary, the *abi8/eld1/kob1* mutants provide evidence linking ABA and/or Glc signaling to promotion of cellulose biosynthesis and organizing vascular differentiation and provide an opportunity to decipher the function of a novel essential protein and possibly a novel signaling mechanism.

## METHODS

### Plant Material

The *abi8* mutant was isolated from a T-DNA insertion line collection in the Ws background produced by Feldmann (1991) and made available by agreement with DuPont. The *abh1*, *ein2-1*, and *ein3-1* mutants are described by Roman et al. (1995) and Hugouvieux et al. (2001). The

35S:ABI lines are described by Parcy et al. (1994) (2x35S:ABI3, isolate C7A19) and Brocard et al. (2002) (35S:ABI5, isolate 2A4). The *ABI4:GUS* fusion includes 3 kb of upstream genomic sequence and four codons of the *ABI4* sequence in a translational fusion, as described by Söderman et al. (2000). The *RAB18:GUS* and *Dc3:GUS* fusions are described by Ghassemian et al. (2000) and Chak et al. (2000), respectively. The *ABI8-GUS* fusion was constructed by engineering a *SacII* site into the final codon of *ABI8* and then fusing a 4.3-kb fragment containing 1.3 kb of upstream sequence and the entire *ABI8* coding sequence, including introns into pBI101.3. The transgene was introduced into *abi8/+* plants by *Agrobacterium tumefaciens*-mediated floral-dip transformation (Clough and Bent, 1998). Primary transformants were selected for growth in the presence of 40  $\mu\text{g}/\text{mL}$  of kanamycin; their progeny were tested for segregation of kanamycin resistance and the *abi8* phenotype.

For RNA isolation from vegetative tissues, plants were grown 12 d on 0.5  $\times$  MS nutrients (Sigma, St. Louis, MO) supplemented with 1% sucrose and 0.55% agar and then transferred to fresh media with or without 50  $\mu\text{M}$  ABA for 2 additional d before harvest. For RNA isolation from leaves, flowers, and siliques, plants were grown in soil in continuous light at 22°C. Leaves were harvested during rosette stage, and flowers include open flowers and buds. Siliques were harvested in pools corresponding to four developmental stages: early embryogenesis (1 to 5 d post anthesis [dpa]), maturation (8 to 11 dpa), postabscission (12 to 16 dpa), and late embryogenesis (17 to 21 dpa). For RNA isolation from roots and shoots, 2-d-old plants were transferred into Gamborg B5 salts (Gamborg, 1968) supplemented with 1% Glc and grown hydroponically for 3 weeks under agitation in continuous light at 22°C before harvest. All growing tissues were weighed, flash frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$  until extraction. Dry seed were stored at room temperature.

### Positional Cloning

After preliminary linkage analysis indicated that the *abi8* mutation was near the top of chromosome 3, plants heterozygous for the *abi8* mutation were outcrossed to Landsberg marker lines carrying the *hy2* and *glabrous1* mutations. Mapping populations were produced by identifying F2 families segregating *abi8* and then scoring the resulting F3 families at *abi8* and *hy2* to identify recombinants. Additional recombinants were identified by screening F2 individuals for recombinations between two flanking markers scorable by PCR: the cleaved amplified polymorphic sequence (CAPS) marker 17D8L and the simple sequence length polymorphism marker nga126 (Bell and Ecker, 1994). Fine-mapping within these recombinant populations made use of additional CAPS markers, restriction fragment length polymorphisms, and single nucleotide polymorphisms. Reaction and cycling conditions for CAPS markers were as described by Konieczny and Ausubel (1993).

### Double Mutant Construction

To construct double mutants, *abi8/+* plants were crossed with *abh1*, *ein2*, or *ein3* plants. Progeny of *abi8/+ abh1* double mutants were identified on the basis of 100% Basta resistance and *abi8* segregation. Seed of *abi8/+ ein* individuals were identified on the basis of their phenotype on ACC in the dark (75% of plants with long hypocotyl, no plant showing ethylene triple response) and *abi8* segregation. Homozygous double mutants were not fertile. To introduce transgenes into the *abi8* background, transgenic lines were crossed onto *abi8/+* individuals, which were identified at seed maturity by segregation of the *abi8* mutation in the selfed progeny. Transgenic F1s were selected on the basis of kanamycin resistance, and their progeny were tested for segregation of the *abi8* allele. Kanamycin-resistant F2s were selected and their progeny scored to identify homozygous transgenic lines segregating the *abi8* mutation.

### Germination 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, or sorbitol. The dishes were incubated for 3 d at 4°C to break any residual dormancy and then transferred to 22°C in continuous light (50 to 70  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ); germination was scored after 4 to 9 d.

### Stomatal Aperture Measurements

Stomatal apertures were measured essentially as described previously by Kwak et al. (2002), except that the pH of the opening solution (10 mM Mes-Tris, 5 mM KCl, and 50  $\mu\text{M}$   $\text{CaCl}_2$ ) was decreased from 6.15 to 5.6 to promote ABA uptake. Leaves were incubated 3 h in light to promote stomatal opening and then for an additional 3 h with or without 10  $\mu\text{M}$  ABA or 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . For testing photoregulation, leaves were incubated continuously in the dark. After these treatments, leaves were fragmented in a Waring blender, and epidermal fragments were quickly mounted for photomicrography. Each experiment was replicated three times. Average stomatal aperture ratios (pore width:length) were determined from measurements of 59 to 225 stomata per treatment; statistical analyses were conducted using Student's *t* test, assuming an unequal variance.

### RNA Gel Blot Analysis

RNA was isolated from immature siliques as previously described (Söderman et al., 2000). RNA was extracted from vegetative tissues by either a hot-phenol extraction procedure or Plant RNeasy (Qiagen, Valencia, CA) minipreps (Söderman et al., 2000). RNA concentrations were estimated based on absorbance at 260 and 280 nm.

Total RNA (5 to 10  $\mu\text{g}$  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  $\times$  SSPE (1  $\times$  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  $\text{mJ cm}^{-2}$  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 by Söderman et al. (2000), labeled by random priming to a specific activity of  $10^8$  cpm  $\mu\text{g}^{-1}$ . The *cor6.6*, *cor15a*, and *cor78* cDNA clones are described by Hajela et al. (1990); *osmotin* (242K20T7), *dehydrin* (ATTS0613), cytoplasmic invertase (158A15T7), and sucrose synthase (182C20T7) cDNA clones are ESTs provided by the ABRC; and the *RAB18* cDNA was an EST (PAP023) provided by M. Delseny. The *RBCS* (At5g38430), *CAB* (At5g01530), *PDF1.2* (At5g44420), and *VSP2* (At5g24770) transcripts were detected by hybridization to PCR fragments amplified from *Ws* genomic DNA. Hybridization conditions were 50% formamide, 5  $\times$  SSPE, 5  $\times$  Denhardt's solution (1  $\times$  Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.1% SDS, and 200  $\text{mg mL}^{-1}$  of DNA at 43°C for 16 to 24 h in a Hyb-Aid rotisserie oven. Filters were washed twice at 60°C in 2  $\times$  SSC (1  $\times$  SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS and once at 60°C in 0.2  $\times$  SSC and 0.1% SDS for 30 to 60 min. *ABI8* transcripts were detected by hybridization to a random-priming labeled cDNA clone in 7% SDS, 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, and 1% BSA at 65°C for 16 to 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 to 65°C.

### Microscopy

For comparisons of root differentiation, 7- or 11-d-old wild-type and *abi8* seedlings, grown on minimal medium or minimal medium supplemented with 1% Glc, were fixed for 24 h in a 4% formalin/5% acetic acid/50% alcohol solution, followed by several rinses in 1  $\times$  PBS. Seedlings were stained with 10  $\mu\text{g mL}^{-1}$  propidium iodide for 1 h with gentle agitation, followed by several rinses in 1  $\times$  PBS. Whole seedlings were mounted in glycerol or wintergreen oil and viewed on a Bio-Rad 1024 laser scanning confocal microscope (Hercules, CA). Images were processed with Adobe Photoshop (Mountain View, CA).

For GUS localization, 15-d-old seedlings were infiltrated with 5-bromo-4-chloro-3-indolyl- $\beta$ -glucuronic acid for 10 min, followed by an overnight incubation in the dark at 37°C, as described by Brocard et al. (2002). Seedlings then were fixed for 4 h at 4°C in 4% glutaraldehyde and 0.05 M sodium cacodylate buffer, pH 7.0, with a change of fresh fixative after the first 2 h. Seedlings then were dehydrated in a graded series of ethanol and propylene oxide, gradually infiltrated with Spurr's resin, and finally embedded in 100% resin and incubated at 65°C for several days. Three-micrometer-thick sections were cut using the LKB Bromma 2088 Ultratome V (Leica Instruments, Bannockburn, IL). Sections were attached to glass slides with heat and viewed on an Olympus BX51 microscope (Tokyo, Japan). Images were taken with an Optronics Magnafire camera and processed with Adobe Photoshop.

Seedling anatomy was compared in 11-d-old wild-type and *abi8* seedlings grown on minimal medium or minimal medium supplemented with 1% Glc. Seedlings were fixed as described above with the addition of a postfixation treatment in 2% osmium tetroxide and 0.05 M sodium cacodylate buffer for 1 h at room temperature before the seedlings were dehydrated. Two- to three-micrometer-thick sections were cut using the LKB Bromma 2088 Ultratome V. Cross-sections were attached to glass slides with heat, stained with 0.5% toluidine blue O, and then viewed with an Olympus BX51 microscope. Images were taken with an Optronics Magnafire or Microfire camera and processed with Adobe Photoshop.

### Quantification of Soluble Sugars and Starch

Starch and soluble sugar (sucrose, fructose, and Glc) levels in 7-d-old plants grown on minimal medium were determined as previously described by Chia et al. (2000). After extraction of soluble sugars, the extract was divided into three fractions for parallel determinations of sucrose, fructose, and Glc levels. Sucrose was digested with 400 units invertase and 1 unit phosphoglucosomerase, followed by measurement of released Glc by the Infinity Glc reagent (Sigma). Fructose and Glc levels were determined by digestion of extracted soluble sugars with 1 unit phosphoglucosomerase or no enzyme, respectively, followed by measurement of released Glc by the Infinity Glc reagent. Control experiments indicated that the Glc reagent was not contaminated with phosphoglucosomerase, such that the measured Glc levels did not include any contribution from endogenous fructose levels (data not shown).

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