A Screen for Genes That Function in Abscisic Acid Signaling in *Arabidopsis thaliana*

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ABSTRACT

The plant hormone abscisic acid (ABA) controls many aspects of plant growth and development under a diverse range of environmental conditions. To identify genes functioning in ABA signaling, we have carried out a screen for mutants that takes advantage of the ability of wild-type Arabidopsis seeds to respond to (−)-(R)-ABA, an enantiomer of the natural (+)-(S)-ABA. The premise of the screen was to identify mutations that preferentially alter their germination response in the presence of one stereoisomer *vs.* the other. Twenty-six mutants were identified and genetic analysis on 23 lines defines two new loci, designated *CHOTTO1* and *CHOTTO2*, and a collection of new mutant alleles of the ABA-insensitive genes, *ABI3*, *ABI4*, and *ABI5*. The *abi5* alleles are less sensitive to (+)-ABA than to (−)-ABA. In contrast, the *abi3* alleles exhibit a variety of differences in response to the ABA isomers. Genetic and molecular analysis of these alleles suggests that the *ABI3* transcription factor may perceive multiple ABA signals.

THE plant hormone abscisic acid (ABA) controls numerous physiological processes, ranging from inhibition of germination and the establishment of seed dormancy to adaptive responses to a variety of abiotic stresses (Zeevaart and Creelman 1988, see review; Leung and Giraudat 1998). Recent genetic studies in Arabidopsis have demonstrated that plant responsiveness to ABA is controlled by a number of molecular processes including transcription (Giraudat *et al.* 1992; Finkelstein *et al.* 1998; Finkelstein and Lynch 2000b; Lopez-Molina and Chua 2000), RNA processing (Hugouvieux *et al.* 2001), post-translational modifications (Leung *et al.* 1994, 1997; Meyer *et al.* 1994; Cutler *et al.* 1996), and metabolism of a second messenger (Xiong *et al.* 2001). Elucidation of the relationships by which these and other factors act to transmit the ABA signal is essential for understanding the disparate roles of ABA on plant growth and development.

We have chosen the Arabidopsis seed as a model system for studying the role of ABA-mediated signal transduction in the control of seed dormancy and germination for a number of reasons. An allelic series of mutations that decrease ABA biosynthesis demonstrate that the level of seed dormancy in Arabidopsis is dependent on embryonic ABA concentrations (Karssen *et al.* 1983). Thus, seed germination is an excellent biological assay for ABA responsiveness. Moreover, because exogenously applied ABA can inhibit wild-type germination, mutations that decrease seed responsiveness to ABA can be easily identified and, to date, five loci (*abi*; abscisic acid-insensitive) have been characterized. Dominant mutations in two genes that encode homologous type 2C protein phosphatases, designated *ABI1* and *ABI2,* reduce ABA sensitivity in both embryonic and adult plants (Leung *et al.* 1994, 1997; Meyer *et al.* 1994). By contrast, recessive mutations in the *ABI3*, *ABI4*, and *ABI5* genes mostly affect seed responses and identify three different classes of transcription factors (Giraudat *et al.* 1992; Finkelstein *et al.* 1998; Finkelstein and Lynch 2000b; Lopez-Molina and Chua 2000). Although the relationship between these proteins is unclear, recently it has been shown that ABI3 and ABI5 interact in a yeast two-hybrid protein assay (Nakamura *et al.* 2001). Furthermore, Soderman *et al.* (2000) reported that ectopic expression of the *ABI3* or *ABI4* gene increases in the accumulation of *ABI5* mRNA, and these genes could act cooperatively *in vivo.*

Critical to our understanding of how ABA activates seed dormancy and inhibits germination is the identification of all the genes that are involved in the transduction of the hormone signal. To identify new factors involved in ABA signaling and expand the collection of mutant alleles that alter seed ABA sensitivity we have taken advantage of the ability to separate ABA enantiomers from a chemically synthesized mixture of the naturally occurring (+)-(S)-ABA [(+)-ABA] and its mirror image (−)-(R)-ABA [(−)-ABA]. The two molecules are very similar in shape, differing only in the disposition

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of the methyl groups on the ring (Figure 1). The vinyl methyl and the gem dimethyl groups are reversed in the mirror image forms. Comparing the structures of the enantiomers in the conformation adopted by ABA in the crystal structure, the 7′- and 9′-methyl groups are almost identical, with the major difference being the location of the axial 8′ methyl group.

The premise of the screen is that it might be possible to identify mutants that differentiate between (+)-ABA and (−)-ABA. The choice of these compounds was based on the observation that natural genetic variation may have caused subtle differentiation between these two stereoisomers in a number of plant species. In wheat embryos, for example, (−)-ABA effectively induces gene expression of *dhn* and *lea* genes, but the effects of this enantiomer on another ABA inducible gene *Em* is relatively minor (Walker-Simmons *et al.* 1992). In Arabidopsis protoplasts, (−)-ABA fails to induce the ABA responsive gene *Rab18*, but can increase the conversion of natural ABA to its breakdown product phaseic acid (Windsor and Zeevaart 1997; Jeannette *et al.* 1999). The screen was modeled after the successful screens for mutations that confer a seed ABA-insensitive phenotype to ABA stereoisomer mixtures (Koornneef *et al.* 1984; Finkelstein 1994). Genetic manipulation of the germination response to one ABA isomer *vs.* the other should lead to a finer scale dissection of the ABA response, which will be useful in identifying molecules that, although essential to ABA signaling, may have only a minor effect on it. For example, if activation of a redundant ABA response has a preference for one stereoisomer over another, then mutations that disrupt its function may change the ratio of response to the two isomers. Here we report isolation and characterization of (−)-ABA-insensitive mutants based on inhibition of germination. Although all these mutants also exhibit insensitivity to (+)-ABA, in contrast to wild type the degree of insensitivity to these stereoisomers is different among mutants. The mode of ABA action in hypothetical ABA signaling pathways is discussed.

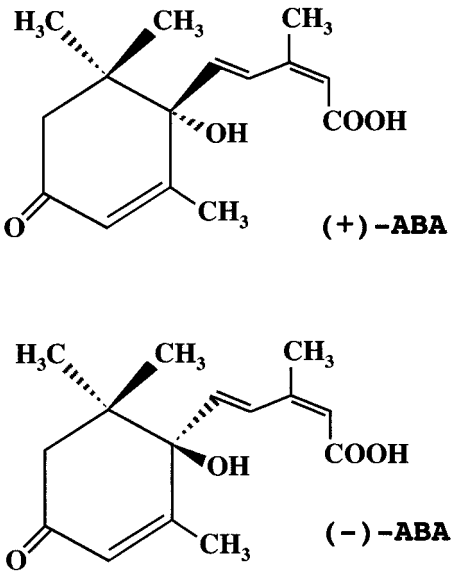

Figure 1.—Structure of (+)-(S)-ABA and (−)-(R)-ABA. (−)-ABA is the unnatural stereoisomer of natural (+)-ABA.

MATERIALS AND METHODS

Plant materials and growth conditions: *Arabidopsis thaliana* M2 ecotype Columbia seeds mutagenized by ethyl methanesulfonate (EMS), fast neutron irradiation, and gamma ray irradiation were purchased from Lehle Seeds (Round Rock, TX). Strain names containing E, F, and G designated mutant lines isolated from EMS-, fast neutron-, and gamma ray-mutagenized M2 populations, respectively. The M2 pools designated as E31 to E48 or F4 contain *gl1* mutation as a genetic marker. Strain T45-3 is a mutant strain isolated from T-DNA insertion lines; however, this strain does not contain T-DNA (data not shown). The *abi4-1* and *abi5-1* mutants used for the allelism tests were obtained from Dr. Ruth Finkelstein (Finkelstein 1994). Sterilizing seed and growing plants under sterile conditions was done as previously described (McCourt and Keith 1998).

Mutant screen and germination test: (+)-ABA and (−)-ABA were purified by chiral HPLC (Dunstan *et al.* 1992). Seeds were chilled for 4 days and transferred to continuous light conditions at room temperature (24°). For germination tests, the percentage of germination was scored each day with expansion and greening of the cotyledons used as a criterion for germination. At least three independent experiments were performed and the representative results were shown. For sugar addition experiments 2% glucose was added to a standard 0.5× MS plate with or without 1 μM (+)-ABA. Seeds were chilled for 4 days and transferred to room temperature with continuous light conditions for a week and scored as described above.

Mapping of the *cho* mutations: Mutant lines were crossed to Landsberg *erecta*. SSLP and CAPS markers were used to map the mutations (Konieczny and Ausubel 1993; Bell and Ecker 1994). Information on the markers was obtained from the TAIR web site (http://www.arabidopsis.org/aboutcaps.html).

DNA sequencing: The *abi3* gene was amplified by PCR and cloned into pT7Blue T-Vector (Takara, Kyoto, Japan). Double-stranded DNA was sequenced on both strands by DNA sequencer ABI377 (Applied Biosystems, Foster City, CA). Two independent clones were sequenced on both strands to identify the *abi3* mutations.

RESULTS

Isolation of mutants that are insensitive to (−)-ABA: Genes encoding factors that respond to the unnatural stereoisomer (−)-ABA are also expected to function in response to the naturally occurring (+)-ABA (Figure 1). Therefore, mutations in these genes should be expected to also have some altered responsiveness to (+)-ABA. On the basis of this premise we first compared the germination response of Columbia wild-type seed to the different stereoisomers. Wild-type seed shows 100% inhibition at concentrations ≥2.4 μM (+)-ABA while concentrations >5 μM are required to give similar results when (−)-ABA is used (Figure 2). Similar germina-

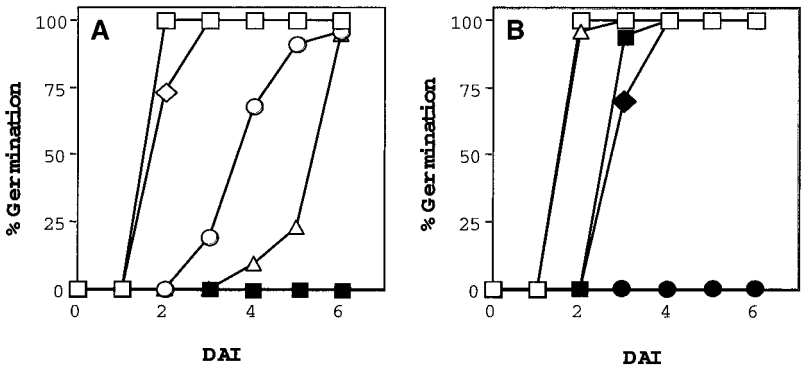

FIGURE 2.—Inhibition of wild-type germination by (+)-ABA or (−)-ABA. Open squares, 0 μM; open diamonds, 0.3 μM; open circles, 0.6 μM; open triangles, 1.2 μM; solid squares, 2.4 μM; solid diamonds, 3 μM; solid circles, 5 μM. (A) Inhibition of wild-type germination by (+)-ABA. Wild-type seeds were sown on various concentrations of (+)-ABA, chilled for 4 days, and cultured under continuous light conditions at room temperature. Percentage of germination is plotted against days after imbibition (DAI). (B) Inhibition of wild-type germination by (−)-ABA. Wild-type seeds were chilled for 4 days and cultured under continuous light conditions at room temperature. Percentage of germination is plotted onto DAI.

tion curves in response to (+)- or (−)-ABA were observed with Landsberg *erecta* wild-type seeds (data not shown). These results indicate that (−)-ABA is sensed, although not as efficiently, by Arabidopsis seeds at the level of germination. To test if the (−)-ABA response is through a known ABA response pathway, *ABI1-1*, *ABI2-1*, and *era1-2* seeds were tested for their sensitivity to the ABA isomers. The *ABI1-1* and *ABI2-1* seeds were able to germinate on 10 μM (−)-ABA while the *era1-2* mutant seed was able to germinate on 0.3 μM (−)-ABA (data not shown), demonstrating that (−)-ABA can signal through similar ABA response pathways as does (+)-ABA.

On the basis of these observations we screened ~360,000 M2 seeds derived from 75,000 M1 EMS, 29,000 M1 fast neutron-, and 13,500 M1 gamma ray-irradiation mutagenized seeds for mutants that were able to germinate in the presence of 10 μM (−)-ABA. Seeds that germinated on this concentration of (−)-ABA were propagated to the M3 generation. After retesting, 26 M3 lines that represented at least 23 independent mutations were advanced for further analysis. Mutations were deemed independent if the ABA-insensitive seed was isolated from separate M2 seed pools. Using the criterion of 50% inhibition of germination, 20 of 26 mutant lines were identified as insensitive to either 3 μM (+)-ABA or 10 μM (−)-ABA (Table 1). (−)-ABA-insensitive mutants that are able to germinate in the presence of 10 μM (−)-ABA were categorized into two classes on the basis of the ability of germination on (+)-ABA. One class, designated as class I ABA-insensitive mutants, shows (+)-ABA insensitivity on 3 μM (+)-ABA, and the second class, designated as class II insensitive mutants, fails to germinate on 3 μM (+)-ABA. Aside from (−)-ABA-insensitive mutants, the third class (class III) does not germinate on 10 μM (−)-ABA but germinates faster than wild type on 1 μM (+)-ABA, a concentration that under our assay conditions delays wild-type germination.

The class I ABA-insensitive mutants were expected to be allelic to the known *abi* loci because this class permits germination on both (+)-ABA and (−)-ABA. Subsequent genetic analysis demonstrated that these 20 lines were recessive and fall into three complementation groups (Table 1): 4 *abi3* (4 independent), 12 *abi4* (10 independent), and 4 *abi5* (3 independent). By contrast, 6 lines of class II ABA-insensitive mutants that fail to germinate on 3 μM (+)-ABA were able to germinate faster than wild type on 1 μM (+)-ABA (data not shown). Therefore, these lines also have reduced sensitivity to both (+)-ABA and (−)-ABA, but the degree of insensitivity to the two stereoisomers is different from that in wild type. The 6 class II lines are recessive and define two *abi3* alleles and two new loci, designated as *chotto1* (*cho1*) and *chotto2* (*cho2*; Table 1). Three independent *cho1* alleles were identified and this locus was mapped onto the bottom of chromosome 5 with tight linkage to the CAPS marker ASB2 (seven recombinants in 640 chromatids). Although the *abi2* locus is also located on this region, the *cho1* locus is genetically separable from the *abi2* locus and subsequent sequencing of the ABI2 gene in the *cho1* mutant showed no mutation in the ABI2 gene (data not shown). The single *cho2* mutation was mapped onto the bottom of chromosome 4 and is linked to the CAPS marker AG (six recombinants in 24 chromatids). No known *abi* loci were mapped close to this region, suggesting *cho2* also defines a new ABA response gene.

ABI transcription factors participate in differential responses to ABA stereoisomers: As mentioned, all 20 strong ABA-insensitive mutants turned out to have mutations in the previously characterized three ABA response genes (Table 1). These genes encode different transcription factors with *ABI3*, *ABI4*, and *ABI5* belonging to the B3, AP2, and bZIP families, respectively, based on the conserved DNA-binding domain (GIRAUDAT *et al.* 1992; FINKELSTEIN *et al.* 1998; FINKELSTEIN and LYNCH 2000b). Although mutations in these genes define a role for these transcription factors on ABA responsiveness in seeds, a detailed study using multiple alleles has not been reported to measure how various mutations within each gene influence ABA response. For example, although null alleles of *abi3* have a multitude of late embryogenic defects (NAMBARA *et al.* 1994, 1995), the *abi3-1* allele,

TABLE 1

List of the (−)-ABA-insensitive mutants

Strain	% germination		Allele no.
	10 μM (−)	3 μM (+)	
E4-6	100	100	*abi3-8*
E2-21	100	100	*abi3-9*
E41-6	100	100	*abi3-10*
E18-11	100	50	*abi3-12*
E8-4	100	100	*abi4-3*
E14-7	100	100	*abi4-4*
E21-8	100	100	*abi4-5*
E21-9	100	100	*abi4*(sib)
E21-12	100	100	*abi4*(sib)
E32-17	100	100	*abi4-6*
E33-3	100	100	*abi4-7*
E35-1	100	100	*abi4-8*
E72-2	100	92	*abi4-9*
F4-4	100	100	*abi4-10*
F19-9	100	100	*abi4-11*
T45-3	100	100	*abi4-12*
E23-14	100	100	*abi5-5*
E42-1	100	100	*abi5-6*
E42-6	100	100	*abi5*(sib)
E74-1	84	100	*abi5-7*
G22-9	100	26	*cho2-1*
E3-20	100	0	*cho1-1*
E11-5	100	0	*cho1-2*
F2-7	100	0	*cho1-3*
F41-8	100	0	*abi3-11*
E31-18	87	0	*abi3-13*

which contains a missense mutation in the B3 domain, causes only a mild insensitivity to ABA and otherwise has relatively normal seed development (Bies-Etheve *et al.* 1999). All *abi4* and *abi5* alleles identified to date are ABA insensitive in seeds and have no other obvious phenotypes. To discern if all these new alleles are equivalent in terms of their response to ABA isomers we tested the germination rates on 3 μM (+)-ABA or 10 μM (−)-ABA. Although these were the concentrations of hormone that were used in the initial screen, because we are monitoring germination rates rather than a static time point, we could get a kinetic measurement of insensitivity. All 10 independent *abi4* mutants germinated equally fast or slightly faster on 10 μM (−)-ABA than on 3 μM (+)-ABA; the 3 independent *abi5* mutants germinated faster on 3 μM (+)-ABA than on 10 μM (−)-ABA (Figure 3; data not shown). It therefore appears that a defect in the *ABI5* gene causes a stronger insensitivity to (+)-ABA *vs.* (−)-ABA compared to *abi4* mutant alleles.

In contrast to *abi4* and *abi5* mutants, which fall into discrete classes with respect to their differential response to ABA stereoisomers, the pattern of germination response to (+)- and (−)-ABA is not consistent between various *abi3* alleles (Figure 3; data not shown). For example, although some *abi3* alleles such as *abi3-9* showed no significant difference in insensitivity to either ABA isomer, one *abi3* allele, *abi3-8*, was able to germinate much faster on 3 μM (+)-ABA *vs.* 10 μM (−)-ABA whereas *abi3-12* showed the opposite effect (Figure 3).

The *ABI3* gene has been identified and found to be highly homologous to the maize seed-specific transcription factor VP1. Moreover, many of the phenotypes seen in *abi3* null mutants are reflected in loss-of-function *viviparous1* (*vp1*) alleles (McCarty *et al.* 1989; Nambara *et al.* 1995). Molecular comparisons between VP1 and ABI3 have defined a number of conserved domains. Three basic regions, designated B1, B2, and B3, appear to be involved in protein-protein and DNA-protein interactions (Suzuki *et al.* 1997; Nakamura *et al.* 2001). The B3 domain in concert with B2 binds DNA *in vitro* (Suzuki *et al.* 1997); however, the B3 domain has been shown not to be necessary for VP1-dependent ABA response (Suzuki *et al.* 1997). Sequence analysis of our *abi3* alleles showed that *abi3-13* contains a missense mutation in an invariant aspartic acid residue in the B3 domain. In contrast *abi3-11* and *abi3-12* contain mutations that result in a premature stop codon prior to the B3 domain (Figure 4). The *abi3-9* and *abi3-10* alleles contain missense mutations within the B2 domain (Figure 4). The *abi3-8* mutation causes an amino acid substitution with conversion of leucine 298 to a phenylalanine within the B1 domain (Figure 4). All amino acid residues with missense mutations identified in this study are highly conserved in a variety of ABI3 orthologs. Although two *abi3* alleles were isolated in the weak ABA-insensitive class (Table 1), no weak *abi4* or *abi5* alleles were identified.

Differential responses of the *abi3* alleles to ABA in the presence of glucose: The varied responses of *abi3* alleles to (+)- and (−)-ABA suggested that subtle phenotypes of various alleles can be uncovered and perhaps these mutations can define the roles of different protein motifs of ABI3. To further pursue this idea we tested the germination and subsequent seedling growth of these *abi3* alleles on (+)-ABA in the presence and absence of glucose. Externally applied sugar can have a myriad of effects on Arabidopsis germination and growth (see Gibson 2000 for review). At low concentrations, sugar stimulates wild-type germination as measured by radicle emergence and inhibits the effects of exogenous ABA on inhibition of germination (Finkelstein and Lynch 2000a). Conversely, at higher concentrations, sugar represses cotyledon development and early seedling growth and these responses have been used to identify sugar-insensitive mutants in Arabidopsis (Pego *et al.* 1999; Arenas-Huertero *et al.* 2000; Huijser *et al.* 2000; Laby *et al.* 2000). Many of these sugar-insensitive mutants turn out to be new alleles of ABA auxotrophs or loss-of-function alleles of *ABI4*. Interestingly, *ABI1-1*, *ABI2-1*, and *abi3-1* mutants do not confer a sugar-insensitive phenotype, suggesting that only certain ABA response genes are involved in sugar sensing (Arenas-Huertero *et al.* 2000; Huijser *et al.* 2000; Laby *et al.*

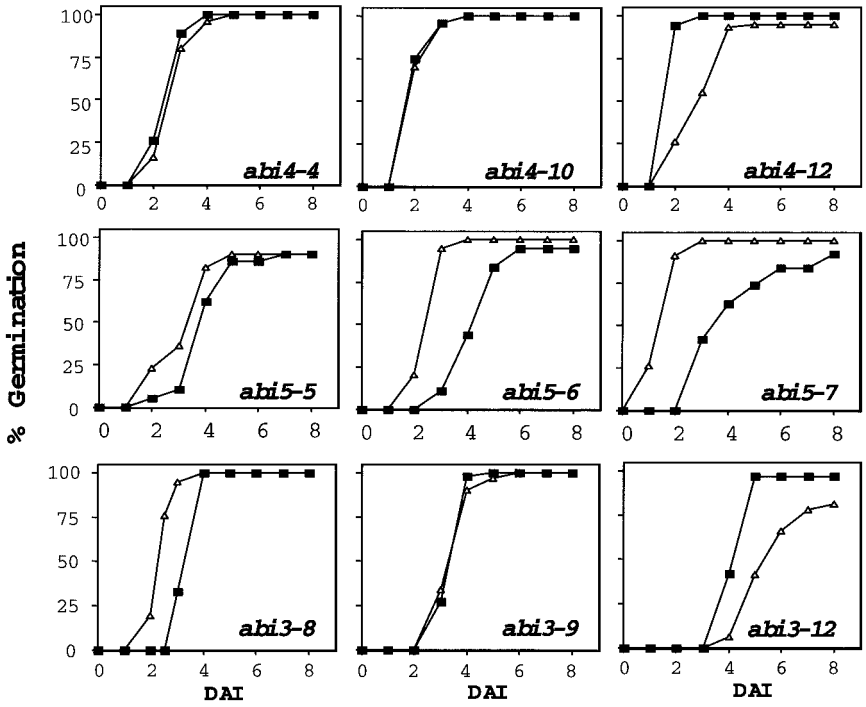

FIGURE 3.—Germination of the *abi3*, *abi4*, and *abi5* mutants on (+)- or (−)-ABA. Mutant seeds were sown on 10 μM (−)-ABA (solid squares) or on 3 μM (+)-ABA (open triangles), chilled for 4 days, and cultured under continuous light conditions at room temperature. Percentage of germination is plotted onto DAI.

2000). Because our collection of the *abi3* alleles appear to discriminate some of ABA signaling pathways, these might give us further insight to understand the interaction between ABA and sugar signaling. We therefore tested germination response of our *abi3* alleles on low concentrations of (+)-ABA in the presence of 2% glucose, a concentration that does not normally inhibit Arabidopsis germination or seedling growth. Radicle emergence of the wild-type seeds on 1 μM (+)-ABA was retarded, but this effect is rescued by coapplication of glucose (FINKELSTEIN and LYNCH 2000a; data not shown). However, the combination of glucose and ABA caused an inhibition of early wild-type seedling growth that resulted in underdeveloped plantlets that were chlorotic and anthocyanic (Figure 4). Although all *abi3* alleles were able to germinate and grow on ABA in the absence of glucose, the presence of glucose had varied effects on different alleles. Both *abi3-9* and *abi3-12* seedlings were similar to those of the wild type, whereas the remaining *abi3* alleles tested were insensitive, showing green cotyledons and root growth on glucose plus ABA (Figure 4). Unlike the *abi3-8* mutation that maps to the B1 domain, the two other glucose-insensitive *abi3* alleles, *abi3-10* and *abi3-11*, are the result of a base substitution in the B2 domain and a premature stop codon just outside the B2 domain, respectively (Figure 4). In contrast to the *abi3* mutants, *abi4* mutants identified in this study exhibited early seedling growth in the presence of 1 μM (+)-ABA plus 2% glucose (data not shown).

DISCUSSION

ABA signaling during seed germination: We isolated at least 17 strong ABA-insensitive mutants and 5 weak ABA-insensitive mutants using (−)-ABA. All the mutants exhibit, more or less, insensitivity to both (+)-ABA and (−)-ABA, but the degree of insensitivity is different among these lines. Although we used an artificial compound to screen mutants, all lines identified in this study also show altered response to (+)-ABA, suggesting that these loci are involved in (+)-ABA signaling *in vivo*.

Among the strongest group, 4 *abi3*, 10 *abi4*, and 3 *abi5* alleles were isolated. Mutations in these loci have been identified in other ABA response screens, which is consistent since reduction in these gene functions causes decreased sensitivity to both isomers (KOORNNEEF *et al.* 1984; FINKELSTEIN 1994; Table 1). Moreover, this study demonstrates that establishment of correct ABA responsiveness in the seed requires at least two additional genes, *CHO1* and *CHO2*. These mutants exhibit significant reduction in responsiveness to (−)-ABA and subtle reduction in responsiveness to (+)-ABA. Although the molecular identity of these genes is unknown, genetic mapping suggests that the (−)-ABA-insensitive screen does identify new genes that contribute to ABA signaling in the seed. Finally, the weakest (−)-ABA-insensitive mutants appear to uncover genes involved in other plant hormone responses such as auxin and ethylene. Consistent with this, *ctr1* has been identified as an enhancer of *ABI1-1* and this ethylene constitutive mutant confers an ABA insensitivity to the seed in the *ABI1-1* mutant background (BEAUDOIN *et al.* 2000; GHASSEMIAN *et al.* 2000). Furthermore, some already characterized auxin-resistant mutants and mutants defective in auxin transport exhibit a subtle insensitivity to (+)-ABA (data not shown).

In principle, mutants that show a differential response to the ABA stereoisomers could contain mutations in

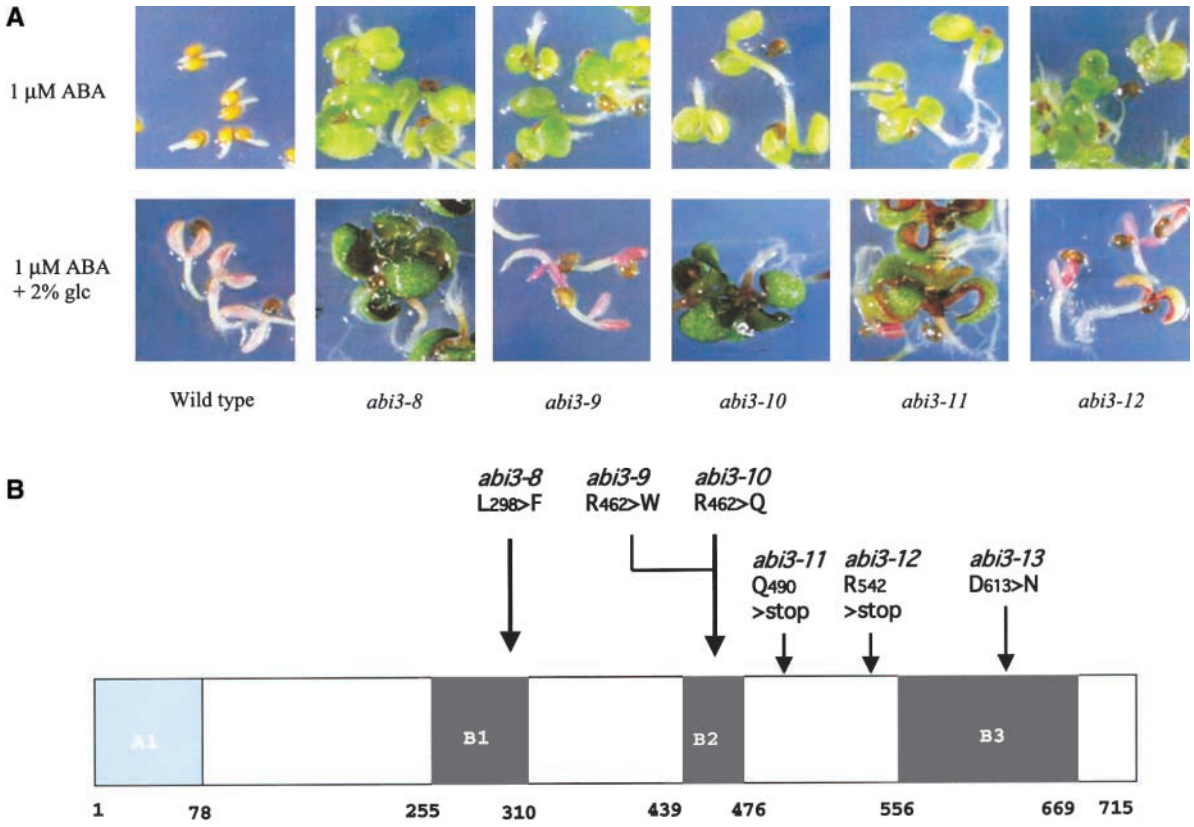

FIGURE 4.—Effects of various *abi3* mutations on sugar responsiveness. (A) Top: 7-day-old seedlings on 1 μM (+)-ABA. Bottom: 7-day-old seedlings on 1 μM (+)-ABA plus 2% glucose. (B) A schematic diagram of the ABI3 protein with the location of the various mutations identified in this study. The mutations are as follows based on GenBank accession no. X68141: *abi3-8* (C-to-T transition at position 1297), *abi3-9* (C-to-T transition at position 1789), *abi3-10* (G-to-A transition at position 1790), *abi3-11* (C-to-T transition at position 1873), *abi3-12* (C-to-T transition at position 2029), and *abi3-13* (G-to-A transition at position 2242). The four conserved domains have been described by GIRAUDAT *et al.* (1992).

genes involved in ABA reception, ABA degradation, or ABA transport. For example, in suspension-cultured barley cells, (−)-ABA is less effective than (+)-ABA in inhibiting saturable uptake of 3H-(+)-ABA and is itself not transported through the carrier as efficiently as natural (+)-ABA (PERRAS *et al.* 1994). Mutations that increase degradation or decrease transport of (−)-ABA might show a preferential decrease in sensitivity to this unnatural ABA isomer. The fact that (−)-ABA can induce some response in Arabidopsis protoplasts while having little or no effect on others also suggests that perception and/or downstream ABA signaling may be involved in the differential response between these two isomers (WINDSOR and ZEEVAART 1997). Possibly, an ABA receptor exists that has different affinities for the two isomers. This appears likely for receptors involved in ABA reception in stomatal closure in which there is a strict requirement for the natural isomer (SONDHEIMER *et al.* 1971). In such a model, downstream outputs are differentially activated by differences in signaling flux (Figure 5). Alternatively, multiple receptors that differentiate between the two stereoisomers may signal to different ABA response outputs.

***ABI3* appears to play a complex role in ABA signaling and sugar sensing:** Aside from identifying new ABA response genes, our screen has also allowed the finer dissection of known ABA response genes in terms of their roles in ABA signaling. For example, a collection of *abi3* missense and nonsense alleles has been useful in further understanding the role of protein motifs in *ABI3* functions. The *ABI3* gene is composed of four amino acid domains that are highly conserved between ABI3 orthologs. These are the A1 domain, a region in the acidic N terminus of the protein, and three COOH terminal basic domains, designated B1, B2, and B3 (GIRAUDAT *et al.* 1992). In the ABI3 ortholog of maize, *VP1*, the B3 domain has been shown to act cooperatively with the B2 domain to bind the Sph element, an enhancer sequence that is widely conserved in seed-specific promoters (SUZUKI *et al.* 1997). Several of our new *abi3* alleles contain nonsense mutations that should produce immature proteins lacking the B3 domain. Although these *abi3* nonsense alleles show reduced ABA sensitivity, their phenotypes represent only a subset of the phenotypes seen in more severe alleles of *ABI3*. For example, the *abi3-6* mutant, a deletion allele of *abi3*, cannot complete

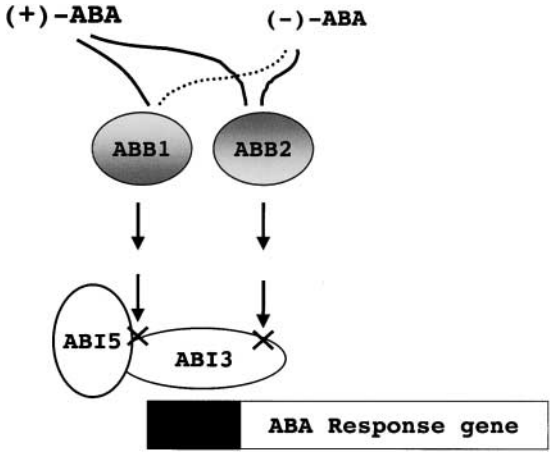

FIGURE 5.—A hypothetical model of parallel ABA signaling pathways. Two ABA binding proteins (ABP1 and ABP2) bind (+)-ABA or (−)-ABA to different degrees. Dotted line represents less activation than a solid line. The ABPs then activate separate parallel signaling pathways that intersect at the ABI3 transcription factor. ABI5 interacts with ABI3 to preferentially regulate the (+)-ABA response pathway.

late embryogenesis and its seeds are desiccation intolerant (NAMBARA *et al.* 1994). Together, these results suggest the B3 domain of the ABI3 is not essential for completion of late embryogenesis or the acquisition of desiccation tolerance. Similar conclusions have been drawn using a truncated mutant lacking the B3 domain of VP1 in maize (MCCARTY *et al.* 1989). Possibly other B3 domain-containing proteins in Arabidopsis might complement the truncation of *ABI3* B3 domain. A likely candidate is *FUS3*, which has been shown to act with *ABI3* in the numerous aspects of seed maturation (PARCY *et al.* 1997; NAMBARA *et al.* 2000).

Another *abi3* allele isolated in our screen, *abi3-8*, confers an increased insensitivity to (+)-ABA *vs.* (−)-ABA. By contrast, the other *abi3* alleles showed no difference or are more insensitive to (−)-ABA than to (+)-ABA. Furthermore, *abi3-8* has a similar differential response to ABA isomers that were observed in the *abi5* alleles. The similarity of phenotypes of these mutants suggests *ABI3* and *ABI5* may interact in the same ABA-dependent pathway. Recently, the B1 domain of ABI3 has been shown to interact with the *ABI5* protein in a yeast two-hybrid assay (NAKAMURA *et al.* 2001). Consistent with this, *abi3-8* contains a missense mutation at leucine 298 in the B1 domain. This leucine is invariant in ABI3 orthologs identified so far. Perhaps this mutation disrupts the *ABI3-ABI5* interaction, thus resulting in a preferential insensitivity to (+)-ABA. On this point, all *abi5* alleles identified showed an increased insensitivity to (+)-ABA *vs.* (−)-ABA, suggesting ABI5 may preferentially respond to a hypothetical (+)-ABA signal *vs.* (−)-ABA signal (Figure 5). This is in contrast to *abi4* mutants that either do not show a differential response or in some cases are more insensitive to (−)-ABA. Recently, ABI5 has been shown to be post-translationally modified in an ABA-dependent manner (LOPEZ-MOLINA *et al.* 2001). It is likely that phosphorylation of the ABI5 may be one of the components of the (+)-ABA signal. Further genetic, molecular, and biochemical analysis of these alleles should reveal how ABI5 and perhaps ABI4 in combination with ABI3 may differentiate between these two isomers and provide insights into the subtleties of ABA signaling.

Recently, a number of genes that determine the response of plants to the hormones ethylene and abscisic acid have also been shown to be involved in early seedling sugar sensing (see GAZZARRINI and MCCOURT 2001 for review). Although results suggest that ABA signaling and carbon homeostasis are tightly coupled, these interactions are complex since only a subset of ABA response mutants alter the response of plants to high sugar. Reductions in ABA biosynthesis and loss-of-function mutations in *ABI4* or *ABI5* confer a sugar-insensitive phenotype, but the *ABI1-1*, *ABI2-1*, and *abi3-1* mutations that reduce ABA sensitivity do not show an altered sugar sensitivity. In this study we observed that although all our *abi3* alleles have reduced sensitivity to exogenous ABA, specific alleles do show an altered sensitivity to glucose in the presence of ABA. It therefore appears that ABI3 has a role in sugar-ABA interactions, but this function appears to be allele specific. The lack of sugar insensitivity in some alleles may reflect the severity of the *abi3* allele. However, there is no clear correlation of ABA-insensitive phenotype and the sugar response between different alleles, suggesting that the allele specificity is complex. This is further verified at the molecular level. For example, when arginine 462 is converted to a glutamine the seedling becomes sugar insensitive in the presence of ABA, whereas if the same arginine is mutated to a tryptophan decreased sugar sensitivity is not observed (Figure 4). The lack of a clear clustering of mutations between alleles that are sugar insensitive *vs.* sugar sensitive in the presence of ABA suggests that ABI3 perceives the sugar signal not by a single conserved domain.

The (+)/(−)-ABA insensitivity screen: Although there are advantages to using stereoisomers to identify mutations in ABA responsiveness, there are also limitations. As noted earlier, the premise of the screen was to identify mutants that showed a differential response to the ABA isomers. However, we screened first for reduced sensitivity to (−)-ABA and then further tested the seed germination on (+)-ABA in the next generation. Therefore, identification of mutants more insensitive to (+) *vs.* (−) isomer would be biased against. This scenario is illustrated by the fact that only 3 independent alleles of *abi5* were identified, whereas 10 alleles of *abi4* were uncovered. Since the *ABI4* and *ABI5* genes are approximately the same size, this bias is most likely due to the fact that loss-of-function *abi5* mutants are more

insensitive to (+)- than to (−)-ABA. We are presently testing this hypothesis by screening first for (+)-ABA insensitivity mutants and then retesting them on (−)-ABA.

Still other genes may have been missed because we used reduced sensitivity to ABA as a screening criterion. Although the use of purified isomers improves the chances of uncovering redundant functions it is still possible that loss of one redundant component causes too mild a phenotype to score.

On this note, during this screen a number of lines (class III) that failed to germinate on 10 μM (−)-ABA, but were able to germinate much faster than wild type on 1 μM (+)-ABA, were identified. Some of these mutants exhibited ethylene constitutive triple response phenotypes in the dark similar to those observed for *ctr1* and *eto* mutants of Arabidopsis. The phenotypes of these putative mutants is consistent with previous reports that show mutations in ethylene responses alter ABA responsiveness in the *ABI1-1* mutant background during seed germination (Beaudoin *et al.* 2000; Ghassemian *et al.* 2000). The fact that putative ethylene-related mutants that are very weakly insensitive to ABA were identified in our screen is consistent with the view that weak ABA-insensitive mutants can be isolated. With this said, we did identify two new loci that cause a reduction in responsiveness to (−)-ABA with only a subtle reduction in responsiveness to (+)-ABA. This demonstrates the usefulness of our screening procedure in that a different spectrum of mutants was identified compared to previous ABA-insensitive screens. This also supports the contention that the difference of the response to the ABA isomers is likely because of the difference in biological properties of these stereoisomers. The cloning of *CHOTTO1* and *CHOTTO2* will further test this assumption.

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