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 take 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
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.

**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-I and abi5-I 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°C). 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-
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 fell 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 64029,000 M1 fast neutron-, and 13,500 M1 gamma ray-irradiation mutagenized seeds were 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.

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which contains a missense mutation in the B3 domain, causes only a mild insensitivity to ABA and otherwise has relatively normal seed development (Bres-Étheve 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 \( \mu M \) (+)-ABA or 10 \( \mu M \) (−)-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 \( \mu M \) (−)-ABA than on 3 \( \mu M \) (+)-ABA; the 3 independent \( abi5 \) mutants germinated faster on 3 \( \mu M \) (+)-ABA than on 10 \( \mu M \) (−)-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 \( \mu M \) (+)-ABA vs. 10 \( \mu M \) (−)-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 virescens1 (\( vp1 \)) 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 asparatic 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 \( ABH4 \). 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. 2000).
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 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 (G-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 (G-to-T transition at position 1873), abi3-12 (G-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).

AB3 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 nonsense and nonsense alleles has been useful in further understanding the role of protein motifs in ABI3 function. 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...
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 ctri1 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-I mutant background during seed germination (Beaudoin et al. 2000; Ghassmann 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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LITERATURE CITED

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ABA Response Mutants in Arabidopsis


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