Identification of Drought Tolerance Determinants by Genetic Analysis of Root Response to Drought Stress and Abscisic Acid[1]

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Drought stress is a common adverse environmental condition that seriously affects crop productivity worldwide. Due to the complexity of drought as a stress signal, deciphering drought tolerance mechanisms has remained a major challenge to plant biologists. To develop new approaches to study plant drought tolerance, we searched for phenotypes conferred by drought stress and identified the inhibition of lateral root development by drought stress as an adaptive response to the stress. This drought response is partly mediated by the phytohormone abscisic acid. Genetic screens using Arabidopsis (*Arabidopsis thaliana*) were devised, and *drought inhibition of lateral root growth* (*dig*) mutants with altered responses to drought or abscisic acid in lateral root development were isolated. Characterization of these *dig* mutants revealed that they also exhibit altered drought stress tolerance, indicating that this root response to drought stress is intimately linked to drought adaptation of the entire plant and can be used as a trait to access the elusive drought tolerance machinery. Our study also revealed that multiple mechanisms coexist and together contribute to whole-plant drought tolerance.

Drought stress is the most common adverse environmental condition that can seriously reduce crop productivity. Increasing crop resistance to drought stress would be the most economical approach to improve agricultural productivity and to reduce agricultural use of fresh water resources. As a result, understanding the mechanisms of drought tolerance and breeding for drought-resistant crop plants has been the major goal of plant biologists and crop breeders. However, drought tolerance is recalcitrant to molecular genetics study mainly due to our limited awareness of specific traits linked to drought tolerance. Furthermore, it is difficult to conduct drought stress treatments in a quantitative and reproducible way. These difficulties have significantly impeded research on plant drought tolerance. Consequently, the biological basis for drought tolerance is still largely unknown and few drought tolerance determinants have been identified (Ludlow and Muchow, 1990; Bohnert et al., 1995; Araus et al., 2002; Bruce et al., 2002). The slow pace in revealing drought tolerance mechanisms has hampered both traditional breeding efforts and use of modern genetics approaches in the improvement of drought tolerance of crop plants.

Despite the lack of understanding of drought tolerance mechanisms, physiological and molecular biological studies have documented several plant responses to drought stress (Bohnert et al., 1995; Blum, 1996; Ingram and Bartel, 1996; Bray, 1997; Schroeder et al., 2001; Luan, 2002). In particular, drought can result in the closure of stomata and increased biosynthesis of the stress hormone abscisic acid (ABA), as well as the induction of drought- and ABA-responsive genes. In the last decade, molecular and biochemical studies have identified many of these ABA- and stress-responsive genes and a few of the transcription factors responsible for their induction in model plants as well as crop plants (Ingram and Bartel, 1996; Hasegawa et al., 2000; Thomashow, 2001; Finkelstein et al., 2002; Oztur et al., 2002; Shinozaki et al., 2003; Yu and Setter, 2003; Buchanan et al., 2005; Poroyko et al., 2005). The products of certain stress-responsive genes could function in alleviating stress damage through still unclear mechanisms (Bray, 1997; Close, 1997; Hasegawa et al., 2000; Thomashow, 2001; Shinozaki et al., 2003). Many laboratory studies, as well as a couple of field trials, have shown that transgenic expression of some of these stress-regulated genes, either by overexpressing these target genes directly or by regulating their transcription factors, results in increased tolerance to drought and other stresses (e.g. Xu et al., 1996; Kasuga et al., 1999; Haake et al., 2002; Bahieldina et al., 2005). These transgenic

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approaches are currently the mainstream method to bioengineer drought tolerance in crop plants. Nonetheless, enhanced expression of these genes is frequently associated with retarded growth and thus may limit its practical applications. Clearly, breeding or bioengineering the next generation of drought-tolerant crop plants requires better understanding of the molecular and genetic basis of drought tolerance.

Genetics approaches are known to be useful in dissecting complex cellular processes. A number of studies have exploited plant responses to ABA and stresses in an attempt to understand stress signaling and stress tolerance mechanisms. Genetic study of ABA response in seed germination, gene expression, or guard cell movement has uncovered several components involved in ABA signaling (Finkelstein et al., 2002). Stress-inducible promoters were also used to identify components affecting stress gene expression (Ishitani et al., 1997; Foster and Chua, 1999). Because drought stress induces the closure of stomata and results in a higher leaf temperature, screens for mutants with altered leaf temperatures were also conducted (Raskin and Ladyman, 1988; Merlot et al., 2002). Plant roots have the ability to grow toward the direction of high water availability and away from that of high osmolarity (hydrotropism) and Arabidopsis (*Arabidopsis thaliana*) mutants defective in hydrotropism were isolated (Eapen et al., 2003; Takahashi et al., 2003). Although these screen approaches have been successful, few components directly involved in drought tolerance were identified. Therefore, innovative strategies are needed to directly identify drought tolerance determinants and the molecular mechanisms for drought tolerance.

As mentioned above, our limited awareness of plant phenotypes specifically conferred by drought stress has prevented researchers from using traditional (forward) genetics approaches to directly study drought stress tolerance. Meanwhile, using molecular mapping techniques, researchers have concluded that drought tolerance in crop plants is controlled by multiple quantitative trait loci (QTL), with each locus only accounting for a small percentage of the variations in drought tolerance (Sanchez et al., 2002; Diab et al., 2004; Lanceras et al., 2004; Nguyen et al., 2004; Yue et al., 2005). These QTL studies raise concerns as to whether drought tolerance can be efficiently studied by using the forward genetics approach, an approach that has been successfully used to isolate genes that affect plant response to other abiotic stresses, such as salt, heat, and cold stresses (Warren et al., 1996; Burke et al., 2000; Zhu, 2000; Hong et al., 2003).

To develop novel methods to study drought tolerance mechanisms, we began a few years ago to search for new phenotypes that are conferred by drought stress. In this study, we found that inhibition of lateral root development is a typical adaptive response of roots to drought stress. Genetic analysis with Arabidopsis was conducted and *drought inhibition of lateral root growth* (*DIG*) loci were defined. Our data suggest that this drought response is linked to drought tolerance of the entire plant and can be used to directly identify drought tolerance determinants. An example of these *dig* mutants, *dig3*, was characterized in this study. The *DIG3* locus is required for ABA inhibition of lateral root growth as well as drought tolerance and may define a novel pathway controlling plant drought tolerance.

RESULTS

Root Response to Drought and Osmotic Stress

Because roots are the very place where plants first encounter drought stress, it is likely that roots may be able to sense and respond to the stress condition. Significant progress has been made in understanding root growth under drought stress (Sharp et al., 2004). However, there has been no genetically defined drought-adaptive response in root development. Previously, a couple of reports had described certain responses of Arabidopsis roots to drought stress. It was reported that, in response to drought, root hairs become bulbous and shortening (Schnall and Quatrano, 1992) or short, tuberized, hairless roots form in soil-grown Arabidopsis (Vartanian et al., 1994). We tried to repeat these observations under our experimental conditions where seedlings were grown on agar plates with low water potential. The osmotic stress in the plates was imposed either by adding mannitol or by equilibrating the plates with polyethylene glycol solutions (van der Weele et al., 2000). However, under these experimental conditions, we were unable to observe consistent alterations in root morphology as those described in the literature. In addition, we found that many other factors (e.g. pH of the media, agar types, and light conditions) besides those well described (such as nutrient levels in the media) also significantly affect root hair development (L. Xiong, unpublished data). Thus, it may not be easy to use these root traits as sensitive phenotypes to conduct genetic studies of drought stress response.

While doing these assays, we noticed that, when the osmotic stress treatment went on for an extended period of time, there was a significant change in root architecture that had not clearly been described in the literature before we started our work. Whereas plants without mannitol treatment developed a number of lateral roots, those treated with mannitol (at 50 or 75 mM) did not develop or were delayed in lateral root development. Because we were also investigating root responses to nutrient deficiency, we compared the development of lateral roots under different nutrient status. We confirmed, as previously reported (López-Bucio et al., 2003), that there is a general increase in lateral root production under reduced nutrient levels. To facilitate the observation of lateral root development, we thus chose to use approximately one-third the strength of normal Murashige and Skoog (MS) nutrient medium as the basal nutrient medium for this study (see "Materials and Methods"). Under these conditions,

Arabidopsis seedlings can give rise to a significant amount of lateral roots within 1 week on the control plate, whereas the elongation of lateral roots is significantly inhibited by mannitol treatment (Fig. 1, A and B).

To investigate whether the observed root response to osmotic stress on the agar plate also exists for plants growing in soil under drought stress, Arabidopsis seedlings were grown in rhizoboxes where root development can be directly visualized without disturbing the soil (see "Materials and Methods"). The soil was maintained at two water regimes: well watered (80% of soil water-holding capacity) and drought stressed (20% of soil water-holding capacity). After 3 weeks of growth, it was found that seedlings under the drought stress treatment had a significantly smaller root mass (fewer lateral roots) than those growing under well-watered conditions (Fig. 1C). Therefore, drought stress also inhibits lateral root development of soil-grown plants.

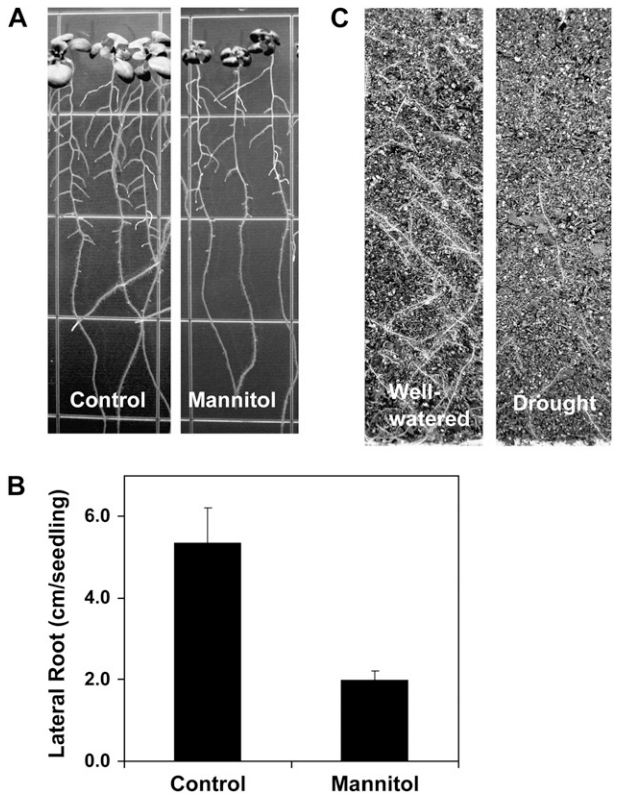

Figure 1. Drought stress inhibits lateral root growth. A, Root morphology of wild-type Arabidopsis seedlings (Col *gl1*) on an agar plate without (control) or with a supplement of 75 mM mannitol. Seeds were first germinated and grown on regular MS medium and then individually transferred to the shown plates. Pictures were taken on the seventh day after the transfer. B, Total length of lateral roots of the wild-type seedlings grown on the control or mannitol (75 mM) agar plates as shown in A. Data are means and SES from seven seedlings. C, Lateral root development for seedlings growing in soil under well-watered (80% water-holding capacity; left) or drought stress (20% water-holding capacity; right) conditions. Seedlings in the shown rhizoboxes were grown for 3 weeks before taking the pictures.

ABA Partly Mediates Drought Regulation of Root Development

Because many drought responses are regulated by ABA, it is likely that ABA may mediate drought inhibition of lateral root development described above. We supplemented agar medium with ABA at concentrations from 0.1 to 5 μM and compared root responses under these conditions. Indeed, even at 0.1 μM, ABA clearly inhibits lateral root development, whereas it has relatively little effect on the growth of primary roots at low concentrations (Fig. 2A; data not shown). We then used ABA biosynthetic mutants *aba1*, *aba2*, and *aba3*, as well as ABA response mutants *abi1*, *abi2*, *abi3*, *abi5*, and *era1*, to test their response to drought and ABA in root growth. It was found that *abi2*, *abi3*, and *abi5* appeared to have little change in root response to drought stress, whereas *era1* was defective in root growth under the control conditions (as also reported by Brady et al., 2003) and these mutants were not tested further. All ABA-deficient mutants have some defects in root development under the control conditions and the variations in lateral root growth were larger than those of the wild type. Nonetheless, these mutants generally tend to have more lateral roots under nonstressful control conditions. On agar plates supplemented with mannitol, the magnitude of inhibition of lateral root elongation was reduced in *aba* mutants compared to the wild type, although these mutants still responded to the treatment in reducing lateral root elongation. An example with the *aba2*-1 mutant (Leon-Kloosterziel et al., 1996) is shown in Figure 2A. This suggests that inhibition of lateral root elongation by mannitol is partly mediated by ABA. Interestingly, all examined *aba* mutants exhibited an enhanced response to ABA in reducing lateral root elongation (Fig. 2A; data not shown). Increased sensitivity of the ABA-deficient mutants *los5/aba3* and *los6/aba1* to exogenous ABA in up-regulating the expression of stress-responsive genes was observed in our previous studies (Xiong et al., 2001, 2002), suggesting that increasing the sensitivity of cellular processes to ABA may represent an adaptive response of ABA-deficient mutants.

The *abi1*-1 mutation affects many ABA-regulated processes. We investigated whether this mutation also affects the above observed root response to osmotic stress and ABA. It was noted that seedlings of the Landsberg *erecta* (L*er*) ecotype (*abi1*-1's background) had more lateral roots than those of the Columbia (Col-0) background under the control conditions. Nonetheless, both mannitol and ABA treatments still reduced the total length of visible lateral roots in both the wild type and *abi1*-1. Relative to its wild-type L*er*, however, *abi1*-1 seedlings were less responsive to these treatments in the inhibition of lateral root growth (Fig. 2A). The reduced sensitivity in lateral root growth of *abi1*-1 was particularly clear when the seedlings were kept on ABA medium for an extended period of time, where the *abi1*-1 mutant had significantly more lateral

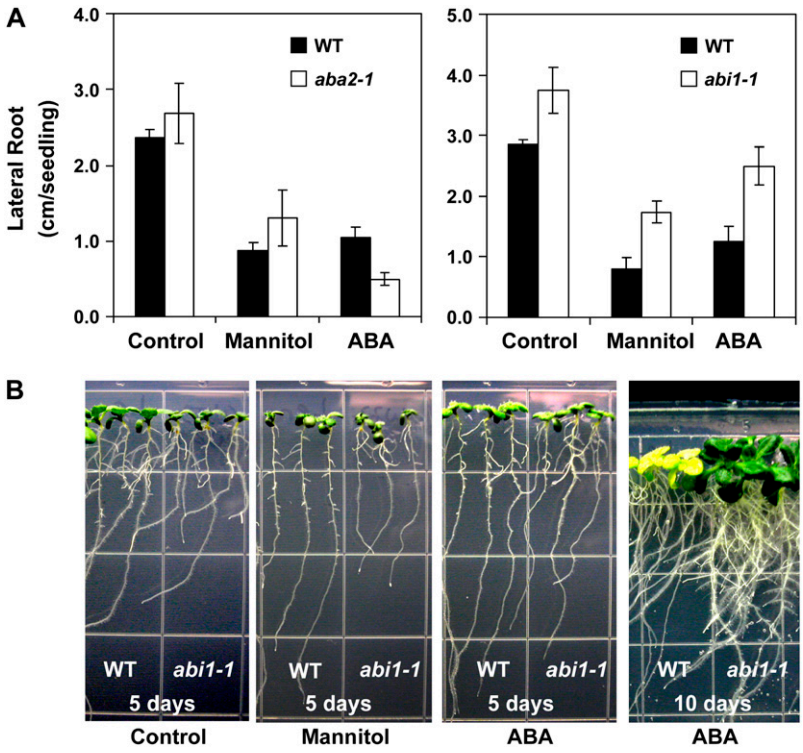

Figure 2. Osmotic stress inhibition of lateral root elongation is partly mediated by ABA. A, ABA has an effect similar to osmotic stress in inhibiting lateral root elongation, and osmotic stress inhibition of lateral root elongation is compromised in *aba2* and *abi1*-1. Five-day-old wild-type Col-0 and *aba2*-1 or L*er* and *abi1*-1 seedlings grown on regular MS agar plates were transferred to the treatment plates without (control) or with 75 mM mannitol or 1.0 μM ABA plates. Total lateral root length of each seedling was measured 4 d after the transfer. Data are the means and SES from seven seedlings. Black bars, Wild type; white bars, *aba2*-1 or *abi1*-1. B, Root morphology of the wild type (L*er*) and the *abi1*-1 mutants without (control) or with 75 mM mannitol or 1.0 μM ABA. Seedlings were first grown on regular MS plates for 5 d before being transferred to the shown treatment plates and were allowed to grow for either 5 or 10 d before taking the pictures.

roots than the wild type (Fig. 2B). The mutant seedlings also grew much better than the wild type, whose leaves turned yellowish as a result of the treatment (Fig. 2B). These data indicate that ABI1 may play a role in mediating osmotic stress and ABA inhibition of lateral root growth.

During the course of this work, reports on the influence of osmotic stress and ABA on Arabidopsis root development were recently published (De Smet et al., 2003; Deak and Malamy, 2005). These authors also found that ABA and osmotic stress inhibit lateral root development, although the experimental conditions used in these studies are very different from ours. In fact, osmotic stress or drought stress inhibition of lateral root growth was also documented in a few earlier reports (e.g. van der Weele et al., 2000), although its significance was previously unclear. Thus, our study and those of others demonstrate that osmotic stress and drought stress can regulate lateral root development. With these findings, we further hypothesized and subsequently confirmed (see below) that the characteristic inhibition of lateral root development by drought/osmotic stress may represent an adaptive response to drought stress.

Genetic Analysis of Root Response to Drought Stress: Isolation of *dig* Mutants

To address whether the inhibition of lateral root growth by drought stress is an adaptive response and can be used to discover drought tolerance mechanisms, we decided to investigate whether this response can be genetically studied. We mutagenized Arabidopsis seeds (ecotype Col-0) with ethyl methanesulfonate and screened the M_2 seedlings for mutants defective in the process. We initially screened seedlings for their response to mannitol (75 mM). To increase seedling survival rate (mannitol at this concentration moderately inhibits seedling growth; Fig. 1A) and to save on cost, we later mainly used ABA in the screen because ABA and mannitol have very similar effects on root development (Fig. 2). In the screen, 5-d-old M_2 seedlings grown on regular MS agar medium were individually transferred to new plates that were supplemented with either 0.1 or 1.0 μM ABA. Seedlings were then scored for their root development, growth response, and leaf coloration starting 5 d after the transfer. We noted that inhibition of lateral root growth was most obvious when the seedlings were grown on ABA plates for 7 to 10 d. Relative to the majority of the seedlings, those with either significantly more lateral roots on 1.0 μM ABA plates or fewer lateral roots on 0.1 μM ABA plates were noted and transferred to soil for seed setting. A diagram depicting the screen is shown in Figure 3.

By screening approximately 50,000 M_2 seeds, we obtained about 350 putative mutants with altered lateral root development. After excluding those clearly defective in auxin transport and responses (e.g. with epinastic leaves and diminished apical dominance) and those with general growth defects (seedlings of significantly different sizes cannot be compared for drought tolerance in our soil-based assays), we selected about 100 putative mutants to test their drought tolerance (see ''Materials and Methods''). In the

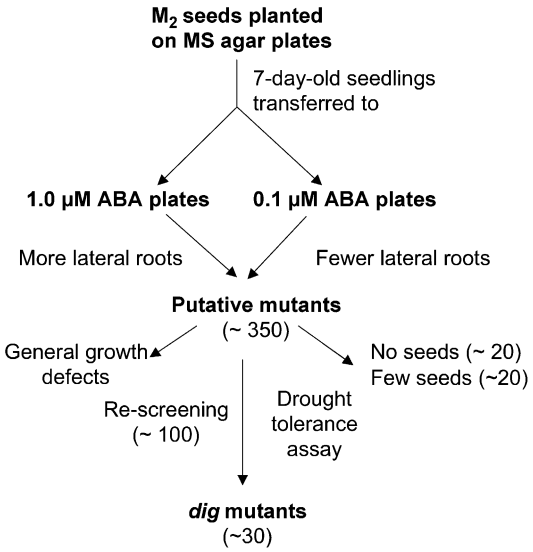

Figure 3. Diagram illustrates the screen scheme for isolation of *dig* mutants. Numbers in parentheses denote the number of lines isolated.

assays, about 30 mutants were found clearly altered in drought tolerance. Among them, three exhibited increased drought tolerance, whereas the rest were drought sensitive. It was found that those hypersensitive to ABA in lateral root growth are more tolerant to drought stress, whereas those insensitive to ABA are drought sensitive. Interestingly, we did not recover mutants with an opposite combination of these phenotypes (e.g. sensitive to ABA in lateral root growth but also sensitive to drought stress or vice versa). Our genetic data thus demonstrate that root response to drought is intimately linked to drought tolerance machinery in the whole plant and that drought inhibition of lateral root growth represents an adaptive response to drought stress. Therefore, we have now secured a strategy to directly identify drought tolerance determinants. To reflect the nature of the mutants isolated in this study, we named these loci *DIG*.

DIG3 Locus Mediates ABA Inhibition of Lateral Root Growth

Whereas a few *dig* mutants (such as *dig1* and *dig2*; L. Xiong, unpublished data) were found to be hypersensitive to ABA and drought in the inhibition of later root growth, most of the isolated mutants exhibited an insensitive response to the stress treatments. One such mutant is *dig3*. On agar medium without ABA, the *dig3* mutant roots grow like wild-type roots, albeit the length of their primary roots is about 28% shorter than that of the wild type (Fig. 4A). On plates with 0.5 or 1.0 μM ABA, although primary root elongation was not affected much, elongation of lateral roots of wild-type seedlings was inhibited by 44% and 65%, respectively. In contrast, the lateral root growth of the *dig3* seedlings was essentially not affected by ABA treatment (Fig. 4, B and C). These data indicate that the *dig3* mutant is insensitive to ABA inhibition of lateral root growth. Thus, the wild-type *DIG3* gene is required for plants to respond to ABA in inhibiting lateral root growth.

dig3 Mutant Is Drought Susceptible

If the restriction of lateral root growth represents an adaptation to drought stress, one would predict that plants with reduced inhibition of lateral root growth under drought stress would be more susceptible to drought stress under natural conditions. We thus tested whether *dig3* mutant plants are more sensitive to drought stress. Under well-watered conditions, *dig3* mutant seedlings were smaller than wild-type plants, suggesting that the *DIG3* gene may be involved in normal growth of the plants as well (Fig. 5, A and B).

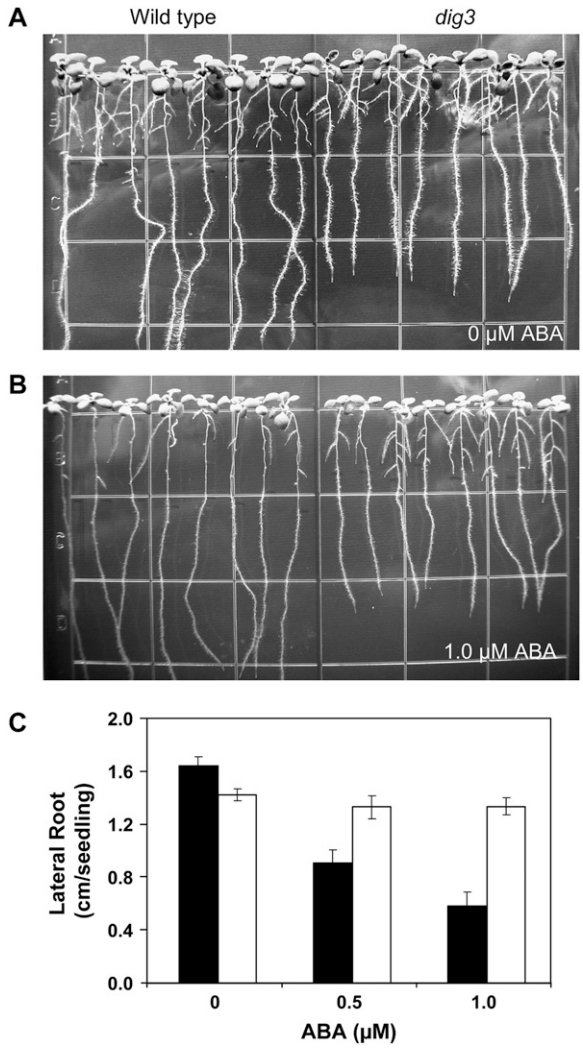

Figure 4. *dig3* mutant is insensitive to ABA inhibition of lateral root growth. A and B, Five-day-old wild-type and *dig3* mutant seedlings grown on regular MS agar plates were transferred onto the shown plate without (A) or with (B) 1.0 μM ABA supplement. Pictures were taken 7 d after the transfer. C, Total length of lateral roots of wild-type and *dig3* seedlings without or with 0.5 or 1.0 μM ABA supplement. Data are means and SEs of seven seedlings.

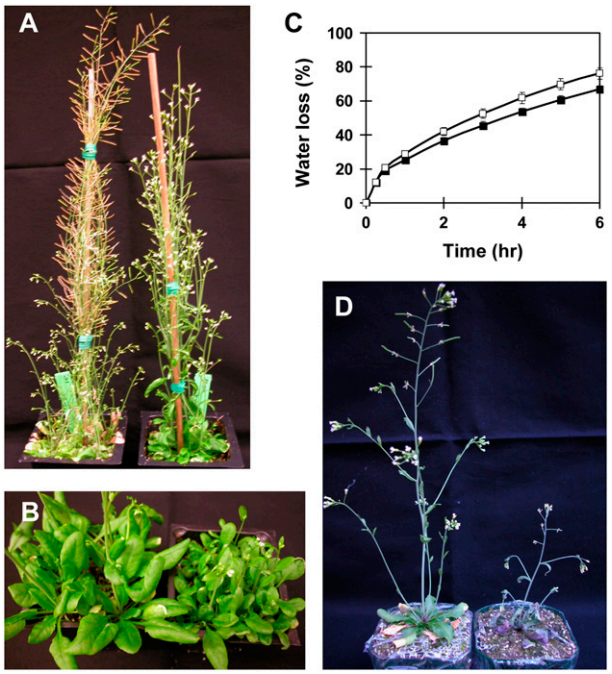

Figure 5. *dig3* mutant is drought sensitive. A and B, Morphology of wild-type and *dig3* mutant plants at the adult (A) or seedling (B) stage with adequate water supply. C, Higher transpirational water loss from *dig3* mutant leaves. Data are means and SES from three replicates. Black symbols, Wild type; white symbols, *dig3*. D, Morphology of wild-type and *dig3* mutant seedlings at 3 weeks after withholding water.

We first compared the transpirational water loss of the *dig3* mutant leaves. It was found that the detached leaves of the *dig3* mutant lost water faster than the wild-type plants. During the course of 6 h, the *dig3* leaves on average lost 30% more water than the wild type (Fig. 5C), suggesting that *dig3* mutant seedlings may not be able to efficiently conserve water in case of drought stress.

To test seedling drought sensitivity, wild-type and *dig3* mutant seeds were planted on MS agar petri dishes and 7-d-old seedlings of a similar size were then selected and transferred to soil. Pots with the seedlings were saturated with water so that their initial water content in the soil/pot was identical. These pots were then covered with plastic wrap to prevent evaporation. Drought treatments were started by withholding water. During the treatments, plants were constantly monitored for their changes in growth, leaf color, and turgor maintenance. Twenty days after withholding water, it was found that the *dig3* mutant seedlings were all withered, whereas the wild-type seedlings were still turgid (Fig. 5D). Continued drought stress for 4 more days eventually killed the *dig3* mutants, whereas the wild-type seedlings were able to survive and recover after rewatering (data not shown). It should be noted that the size of the *dig3* seedlings was smaller than that of the wild type (Fig. 5); smaller plants are expected to consume less water per plant. The fact that the *dig3* plants withered earlier than the wild type indicated that the *dig3* plants have higher transpiration rates despite their smaller stature, which is consistent with the higher transpirational water loss of the *dig3* leaves (Fig. 5C).

DIG3 May Define a Novel Pathway for Drought Response

As mentioned in the introduction, an important mechanism of stress tolerance is the activation of stress-responsive genes. We thus checked whether stress-responsive genes are regulated differentially in *dig3* mutants. Wild-type and *dig3* mutant seedlings were grown on the same MS agar petri dish. Ten-day-old seedlings were then treated with ABA (by spraying with 100 μM ABA) or NaCl (300 mM). Total RNA was extracted from the treated seedlings and subjected to RNA-blot analysis. We chose three genes (*RD29A*, *COR47*, and *RAB18*) as marker genes in the analysis. It was found that the transcript levels for these stress-responsive genes were not significantly different between the mutant and the wild type (Fig. 6). Therefore, DIG3 may regulate drought stress response through a novel pathway independent of the well-characterized CBF regulon (Thomashow, 2001; Shinozaki et al., 2003).

As a first step toward isolation of the *DIG3* gene, we generated mapping populations and started positional cloning. The *dig3* mutant was crossed with both L*er* and C24 wild-type plants and the F_2 populations were obtained. Initial mapping, using 367 individual plants from the F_2 population derived from the crossing with the L*er* plant, placed the *DIG3* locus on the lower arm of chromosome III. However, fine mapping with the L*er*-derived population became difficult due to the interference of the L*er* background with the lateral root phenotypes of the *dig3* mutant. We therefore used the population derived from the C24 crossing for fine mapping. By examining 1,116 recombinant chromosomes, the *DIG3* locus was mapped to a 108-kb interval flanked by the molecular markers F9D24-3 and F14P22-2. In this interval, all potential candidate genes

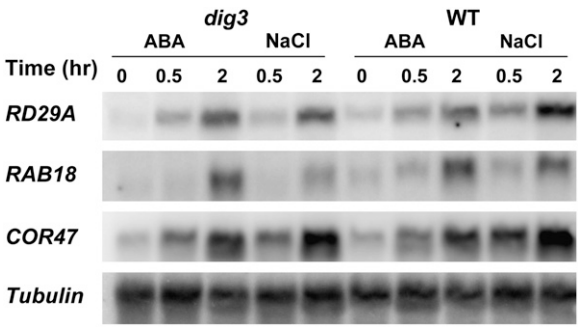

Figure 6. Transcript level of stress-responsive genes in wild-type and *dig3* plants. Ten-day-old wild-type and *dig3* seedlings growing in the same agar plates were treated with either ABA or NaCl for the indicated time and total RNA was extracted after the treatment. Ten micrograms of total RNA were loaded into each lane. A β-tubulin gene was used as a loading control.

that might be involved in drought stress and ABA response (based on current knowledge; Xiong and Ishitani, 2006) were amplified from the *dig3* mutant and sequenced. However, no mutation in these candidate genes was detected, which further suggests that the *DIG3* gene may encode a component in a novel pathway that mediates ABA and drought stress response. Our ongoing work will determine the molecular identity of the *DIG3* locus and elucidate the mechanisms for its regulation of drought tolerance and ABA response.

DISCUSSION

Inhibition of Lateral Root Growth Is an Adaptive Response to Drought Stress

In this study, we investigated root response to drought stress and identified the inhibition of lateral root elongation as a reliable response to the stress and ABA. Our preliminary studies indicated that this drought response resulted mainly from inhibition of elongation but not initiation of lateral roots because the number of lateral root primordia per roots was similar between the control and drought or ABA treatments under our experimental conditions (H. Chen and L. Xiong, unpublished data). Using this lateral root developmental phenotype in a moderate screen effort, we isolated more than 300 putative mutants defective in lateral root development. This suggests that there are far more genes controlling lateral root development than are currently known (Casimiro et al., 2003). What interests us the most are those mutants that are more wild-type-like under control conditions, yet specifically defective in drought inhibition of lateral root growth. We thus chose mutants with relatively normal development to further study their drought tolerance. We found that those *dig* mutants with enhanced response to ABA in inhibiting lateral root growth are also drought tolerant, whereas those with reduced response are drought sensitive. These genetic data strongly suggest that inhibition of lateral root growth is an adaptive response to drought stress. Significantly, this adaptive process also occurs in soil (Fig. 1C) and can be observed in crop plants as well (L. Xiong, unpublished data).

Now that inhibition of lateral root growth by drought stress is an adaptive response, what would its benefits be to the plants? Under drought or any other abiotic stresses, there is a significant decrease in photosynthesis and, consequently, a reduction in the amount of metabolites and energy. It is imperative for plants to use this reduced amount of resources to their maximal advantage—usually to survive stresses. Apparently, under drought stress conditions, an urgent need for plants would be to increase the uptake of water, which is usually more available deep down in the soil. Restriction of the horizontal proliferation of lateral roots in the topsoil and allocation of more resources to the growth of primary roots certainly would offer an advantage to the plants by expanding their domains of water supply. Thus, the adaptive response of the root system to water deficit by means of inhibiting the growth of lateral roots and promoting the growth of the primary root is in sharp contrast to its response to nutrient deficiency. Under nutrient starvation conditions, increased proliferation of lateral roots is commonly observed, which may help plants increase their exploitation of the topsoil where bioavailable nutrients are more enriched relative to the subsoil. It should be noted that, although drought stress is expected to enhance the growth of primary roots while simultaneously inhibiting lateral root growth, stimulation of primary root growth by drought stress or ABA is less commonly observed under our current experimental conditions (data not shown).

Drought Inhibition of Lateral Root Growth Is Partly Mediated by ABA

ABA mediates many drought responses, including guard cell closure and stress-gene regulation. Drought inhibition of lateral root growth also appears to be partly mediated by ABA. First, exogenous ABA has similar inhibitory effects on lateral root development as drought stress (Fig. 2A). The inhibitory effect of ABA on lateral root development was also recently reported (De Smet et al., 2003; Deak and Malamy, 2005). Thus, ABA may have a general regulatory role in controlling lateral root development. Second, the *abi1*-1 mutation impairs ABA repression of lateral root development (Fig. 2). However, the response of ABA-deficient mutants and the *abi1*-1 mutant to drought stress is complex. In both *aba* mutants and the *abi1* mutant, osmotic stress still represses lateral root development, although the magnitudes are significantly reduced compared to wild-type plants. This suggests that there are either ABA-independent effects of drought stress on lateral root growth or these ABA-deficient and ABA-insensitive mutants are leaky. That these ABA mutants are not completely defective in this drought response in fact offers an advantage of using this response to uncover novel drought tolerance determinants. It was noted in the thermo-imaging screen for abnormal leaf temperatures that at least six of the eight loci defined are ABA biosynthetic or response genes previously identified (Riera et al., 2005), suggesting that it might not be easy to uncover novel drought tolerance determinants by screening for guard cell response to drought stress.

Identification of Drought Tolerance Determinants by Analyzing Root Response to Drought Stress

After establishing the inhibition of lateral root growth as a general response to drought stress, it is of great interest to see whether this response can be exploited to isolate drought tolerance determinants and to elucidate drought tolerance mechanisms. With the mutants we isolated for their altered lateral root growth in

response to drought stress, we found that many of these mutants have indeed altered drought sensitivity as predicted. One example of these mutants is *dig3*. Lateral root growth of the *dig3* mutant is virtually insensitive to ABA inhibition (Fig. 4). Mutant plants are also very susceptible to drought stress, which may be partly due to their higher transpiration rates (Fig. 5). Our genetic studies thus suggest that this drought response is closely linked to other drought tolerance mechanisms and that plants may use these coordinated responses to optimize their adaptation to drought stress. Therefore, by analyzing root response to drought stress, one may be able to isolate drought tolerance determinants and reveal the elusive mechanisms of drought tolerance.

Complexity of Drought Tolerance Mechanisms

In this study, drought tolerance is loosely defined as the ability of plants to withstand water deficit while maintaining appropriate physiological activities. Nonetheless, it should be noted that plant drought tolerance is a very complex trait and that plants may have as many ways to respond to the signal as the number of attributes embedded in drought stress (Xiong and Ishitani, 2006). To distinguish different plant responses to drought stress, researchers sometimes divide drought adaptation into several categories (Levitt, 1980), such as drought escape (shortening life cycle), drought avoidance (growing deeper roots, depositing leaf wax, and closing stomata), and drought tolerance (production of osmolytes, antioxidants, and other stress-relieving agents). QTL analyses have been able to localize the chromosomal regions controlling some of these diverse drought response traits (Lilley et al., 1996; Price et al., 2002; Robin et al., 2003; Yue et al., 2005), although the actual contribution of these traits to drought tolerance is unknown. In this study, genetic analyses not only established that the inhibition of lateral root growth is an adaptive response to drought stress, but also demonstrate that there are multiple mechanisms controlling drought tolerance. Analysis of the *dig3* mutant indicates that, although the *dig3* mutant is hypersensitive to drought stress, it does not have significantly reduced expression of the stress-regulated genes belonging to the CBF/DREB regulon (Fig. 6). Our characterization of several other *DIG* loci also found that these other loci might define drought tolerance mechanisms differently from those defined by the *DIG3* locus (L. Xiong, unpublished data). Thus, consistent with previous QTL analyses, our current genetic study of root response to drought stress suggests that many different mechanisms may indeed coexist that together contribute to whole-plant adaptation and tolerance to drought stress.

MATERIALS AND METHODS

Plant Materials, Growth Media, and Mutant Screen

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 carrying the *glabrous1* mutation was used to conduct mutagenesis with ethyl methanesulfonate. Unless otherwise stated, seeds were surface sterilized and planted on 1 × regular MS medium (1.2% agar and 3% Suc) as described previously (Xiong et al., 2001). The plates were then incubated at 4°C for 3 d before being placed vertically under constant white light at 23°C for germination and seedling growth. For root growth assays, 5-d-old seedlings were individually transferred with a pair of forceps to the treatment medium consisting of the following basal salts along with 4% Suc solidified with 1.2% agar (catalog no. A-1296; Sigma): 1.0 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.4 mM KH_2PO_4, 6.0 mM KNO_3, and 7.0 mM NH_4NO_3. Micronutrients were added at full strength (1 × that used in the MS medium) and the pH was adjusted to 5.7 with KOH. Mannitol at 75 mM or ABA at 0.1 or 1.0 μM was added to the medium before (for mannitol) or after (for ABA) autoclaving, respectively. For mutant screens, seedlings with fewer lateral roots on 0.1 μM ABA or more lateral roots on 1.0 μM medium were noted and transferred to soil. Seeds from these plants were harvested and tested in the rescreen. Selected mutants were used in the drought tolerance assays and were back-crossed to wild-type plants. Progeny were used in physiological assays.

Measurement of Lateral Root Length

After growing for the indicated time (usually 5 to approximately 12 d) on the treatment medium, seedlings were photographed with a digital camera. The images were downloaded into a computer and analyzed using National Institutes of Health (NIH) image software (http://rsb.info.nih.gov/nih-image). The length of the primary roots and the number and length of lateral roots were measured using the software. The total length of lateral roots of each individual plant was calculated and the means for each line was used as an index to measure lateral root growth. Lateral root initiation versus elongation was examined using a differential interference contrast microscope as described (Chen and Xiong, 2005).

Rhizobox Observation of Root Development

The rhizobox was made with two transparent Plexiglas acrylic sheets (5 mm thick) of 10 cm × 15 cm (width × length) and spaced on both sides with 1 cm × 15 cm (width × length) bars cut from the same kind of sheet (5 mm thick). The bottom was sealed with tape (pierced to allow water to flow through) and the top left open. Soil (Fafard superfine germinating mix; ACW) was packed into the rhizobox and water content was monitored using an electronic balance. There are two water regimes (80% and 20% water-holding capacity; see below). Germinated seeds were planted on the top with two seeds planted in each rhizobox. The rhizoboxes were wrapped with aluminum foil and incubated in the growth chamber at 22°C with a 16-h light period. There are three replicates for each water regime. Upon completion of the treatment, the aluminum foil was removed and pictures of the roots were taken using a digital camera.

To measure the water-holding capacity of the soil, dry soil was packed into the rhizobox and weighed. The rhizobox was then half submerged in water and allowed to equilibrate overnight. Free water was let to drain off for 6 h and water retained in the rhizobox was weighed and the soil water-holding capacity calculated.

Transpirational Water Loss and Drought Tolerance Assay

For transpirational water loss assay, leaves of the mutant and wild-type seedlings at the rosette stage were detached and placed in a weighing boat, and changes in fresh weight over time were monitored using an electronic balance. Rate of water loss was calculated from the loss in fresh weight of the samples. For drought tolerance assays, 5-d-old seedlings of the mutant and wild type growing on the petri dish were transferred to soil (one seedling in each pot). After seedling establishment, the soil was saturated with water and surface wrapped with plastic wrap to prevent evaporation. The pots were then kept in a greenhouse (22°C, 16-h light period) and no longer received water. The growth of the seedlings was monitored over time and pictures were taken.

RNA-Blot Analysis

For RNA analysis, seedlings of the wild type and *dig3* mutants were grown in the same MS agar plates (0.6% agar and 3% Suc) for 10 d. ABA treatments were conducted by spraying 100 μM ABA and incubating the seedlings under white light for either 30 min or 2 h before harvesting for RNA extraction. Salt

treatment was conducted by transferring the seedlings onto filter paper saturated with 300 mM NaCl and incubating under white light for 30 min or 2 h before harvesting the samples for RNA extraction. Total RNA was extracted using TRIzol reagent (Molecular Research Center) according to the manufacturer's protocol. RNA-blot analysis and the probes were as described (Xiong et al., 2001).

Genetic Mapping

The *dig3* mutant was crossed with the L*er* and the C24 wild type, respectively. The resulting F_1 plants were allowed to self-pollinate to generate F_2 populations for mapping as described previously (Xiong et al., 2001). Fine mapping was performed with the C24 mapping population. The primer sequences for the simple sequence length polymorphism markers F9D24-3 were 5′-CCTTCATCATCAGAAACAGG-3′ and 5′-TACTGATGCATCTGAAGAGG-3′. For F14P22-2, the sequences were 5′-CGGAGATTTATAAGAAGAAC-3′ and 5′-CTCAACTCCAAATAAGTCTC-3′.

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