Ectopic Expression of ABSCISIC ACID 2/GLUCOSE INSENSITIVE 1 in Arabidopsis Promotes Seed Dormancy and Stress Tolerance

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Abscisic acid (ABA) is an important phytohormone that plays a critical role in seed development, dormancy, and stress tolerance. 9-cis-Epoxy-carotenoid dioxygenase is the key enzyme controlling ABA biosynthesis and stress tolerance. In this study, we investigated the effect of ectopic expression of another ABA biosynthesis gene, ABA2 (or GLUCOSE INSENSITIVE 1 [GIN1]), encoding a short-chain dehydrogenase/reductase in Arabidopsis (Arabidopsis thaliana). We show that ABA2-overexpressing transgenic plants with elevated ABA levels exhibited seed germination delay and more tolerance to salinity than wild type when grown on agar plates and/or in soil. However, the germination delay was abolished in transgenic plants showing ABA levels over 2-fold higher than that of wild type grown on 250 mM NaCl. The data suggest that there are distinct mechanisms underlying ABA-mediated inhibition of seed germination under diverse stress. The ABA-deficient mutant aba2, with a shorter primary root, can be restored to normal root growth by exogenous application of ABA, whereas transgenic plants overexpressing ABA2 showed normal root growth. The data reflect that the basal levels of ABA are essential for maintaining normal primary root elongation. Furthermore, analysis of ABA2 promoter activity with ABA2::β-glucuronidase transgenic plants revealed that the promoter activity was enhanced by multiple prolonged stresses, such as drought, salinity, cold, and flooding, but not by short-term stress treatments. Coincidently, prolonged drought stress treatment led to the up-regulation of ABA biosynthetic and sugar-related genes. Thus, the data support ABA2 as a late expression gene that might have a fine-tuning function in mediating ABA biosynthesis through primary metabolic changes in response to stress.

Plant growth and development are well regulated by the integration of external environmental cues and internal signals. The latter are involved in the production and action of phytohormones. Of these, abscisic acid (ABA) plays an important role in controlling many aspects of plant growth and development. For instance, during seed development and maturation, ABA stimulates the accumulation of protein and lipid storage reserves (Finkelstein and Somerville, 1988; Rock and Quatrano, 1995) and desiccation tolerance and dormancy (Karssen et al., 1983; Koornneef et al., 1989). It also mediates stomatal closure (Leung and Giraudat, 1998) to control water loss or transpiration. Endogenous ABA levels may regulate vegetative growth (for review, see Finkelstein et al., 2002) and help plants tolerate environmental stresses (drought, salt, and, to a lesser extent, low temperature; Leung and Giraudat, 1998; Qin and Zeevaart, 2002; Xiong et al., 2002). Recent studies also reveal that ABA may alter plant susceptibility to pathogen infection (Audenaert et al., 2002) and inhibit flowering (Razem et al., 2006).

Despite their physiological significance, expression and regulation of ABA biosynthetic genes at molecular levels were not well understood until recent years. Molecular genetic and biochemical studies have made great strides toward better understanding ABA biosynthesis and regulation in the past decade (for reviews, see Finkelstein et al., 2002; Seo and Koshiba, 2002; Schwartz et al., 2003; Xiong and Zhu, 2003). The combination of genetic and biochemical screens, especially in Arabidopsis (Arabidopsis thaliana), identified several mutants in the ABA biosynthetic pathway (for review, see Gibson, 2000; Finkelstein et al., 2002; Rolland et al., 2002; Xiong and Zhu, 2003). Most of these loci leading to mutant phenotypes have been cloned and characterized. For instance, AtABA1 (AtLOS6; homologous to tobacco [Nicotiana tabacum] ABA2) encodes a zeaxanthin epoxidase (ZEP) that catalyzes the epoxidation of zeaxanthin and antheraxanthin to violaxanthin in plastids (Marin et al., 1996; Xiong et al., 2002). After structural modification, violaxanthin is...
converted to 9-cis-epoxycarotenoid. The epoxycarotenoids 9'-cis-neoxanthin and/or 9'-cis-violaxanthin are then oxidized by 9-cis-epoxycarotenoid dioxygenase (NCED) to generate a C15 intermediate, xanthoxin (Schwartz et al., 1997). The product xanthoxin is subsequently transported to the cytosol and further converted to abscisic aldehyde by a short-chain dehydrogenase/reductase 1 encoded by ABA2 in Arabidopsis (Léon-Kloosterziel et al., 1996; Rook et al., 2001; Cheng et al., 2002; González-Guzmán et al., 2002). Arabidopsis aldehyde oxidase 3 (AAO3) catalyzes the last step in the conversion of abscisic aldehyde to ABA (Seo et al., 2000) and needs a molybdenum cofactor sulfatase encoded by AtABA3 (AtLOS5) for its activity (Léon-Kloosterziel et al., 1996; Bittner et al., 2001; Xiong et al., 2001).

ABA biosynthesis in higher plants occurs through an indirect pathway (Schwartz et al., 2003), which uses the NCED-cleaved neoxanthin (C40) product, xanthoxin (C15), as the ABA biosynthetic precursor. In addition, NCED expression in response to environmental stresses is so rapid and dramatic that it has been considered a key enzyme in the ABA biosynthetic pathway. As such, NCED has been studied extensively and identified in many plant species, such as maize (Zea mays; Tan et al., 1997), tomato (Lycopersicon esculentum; Burbidge et al., 1999), bean (Phaseolus vulgaris; Qin and Zeevaart, 1999), avocado (Persea americana; Chernys and Zeevaart, 2000), cowpea (Vigna unguiculata; Iuchi et al., 2000), and Arabidopsis (Iuchi et al., 2001). More recently, detailed study of tissue-specific expression of the AtNCED gene family revealed differential expression of AtNCED members in different tissues and subcellular localization in different plastid compartments (Tan et al., 2003). The differential membrane-binding capacity of AtNCEDs further implies posttranslational regulation of NCED activity. It is surprising that the spatial and temporal expression of ABA2 is primarily restricted to vascular bundles in roots, hypocotyls, and aged leaves, but not in ABA target sites, such as guard cells or seeds (Cheng et al., 2002). Further localization of AAO3, which catalyzes the last step of ABA biosynthesis and can be considered the ABA production site, also shows the protein to be present in vascular bundles in roots, hypocotyls and inflorescence stems, and leaf veins; additionally, AAO3 is detectable in guard cells (Koiwai et al., 2004). Taken together, these data suggest the dynamic mobility of ABA precursors and/or ABA to the target sites despite the guard cells themselves being capable of synthesizing ABA.

ABA has been considered a plant stress hormone because it is highly induced in vegetative tissues under stress conditions; the induction is associated with the up-regulation of ABA biosynthetic genes (such as ZEP, NCED, AAO3, and ABA3). However, these transcripts or ABA levels are reduced or abolished in most mutant alleles, which reflects the positive feedback regulatory circuit of ABA biosynthesis (for review, see Xiong and Zhu, 2003). Furthermore, tobacco ZEP (Audran et al., 1998) and tomato ZEP and NCED (Thompson et al., 2000a) show a diurnal expression pattern. Overexpression of NpZEP (Frey et al., 1999), tomato LeNCED1, and bean PeNCED1 in tobacco (Thompson et al., 2000b; Qin and Zeevaart, 2002) and AtNCED3 in Arabidopsis (Iuchi et al., 2001) displays an increased ABA level in seeds and extended seed dormancy. Transgenic tobacco plants expressing PeNCED1 driven by 35S or a dexamethasone-inducible promoter and transgenic Arabidopsis plants overexpressing AtNCED3 exhibit drought tolerance (Iuchi et al., 2001; Qin and Zeevaart, 2002). A similar result for increased seed dormancy is also obtained by the overexpression of ABSCISIC ACID INSENSITIVE (ABI) genes in Arabidopsis (for review, see Finkelstein et al., 2002). These data show an alternative way to generate deeper seed dormancy and stress-tolerant plants through genetic manipulation of ABA biosynthetic and/or responsive gene expression.

Our previous data showed that ABA2 is a unique gene whose regulation is distinct from other ABA biosynthetic genes in some aspects (Cheng et al., 2002). Most of the ABA biosynthetic genes, such as ABA1, NCED3, AAO3, and ABA3 in Arabidopsis, are up-regulated under stressful environments, whereas ABA2 shows no obvious induction under short-term drought or ABA treatment (Cheng et al., 2002; González-Guzmán et al., 2002). It also remains unknown whether transgenic plants overexpressing ABA2 show increased ABA levels, extended seed dormancy, and stress tolerance. In this study, we provide genetic evidence that overexpressing ABA2 in Arabidopsis transgenic plants leads to seed germination delay, maintenance of primary root growth, and an elevated level of ABA. Results from this study also demonstrate that ABA2 transgenic plants exhibit increased salinity tolerance relative to wild type. Additionally, the increase of ABA2 promoter activity and up-regulation of ABA2 and sugar-related genes under prolonged stress treatments further suggest that ABA2 is a late stress-responsive gene with a fine-tuning function in controlling ABA biosynthesis in response to stress.

RESULTS

Isolation and Characterization of ABA2 Overexpression Lines

Most of the ABA-deficient mutants display a wilty phenotype and, to some extent, a small plant size relative to their wild type. To further demonstrate whether constitutive expression of ABA2 (At1g52340) may increase ABA2 expression and lead to larger plant size or other unexpected phenotypes, ABA2 overexpression transgenic plants were generated. The ABA2 transgene in transgenic plants was driven by a 35S promoter and contained the BAR gene for Basta herbicide resistance as a selectable marker. More than 10 independent transgenic lines were isolated on the basis of herbicide resistance. Three homozygous transgenic

Effect of ABA on Primary Root Growth

Although ABA2 transgenic plants showed no visible differences from wild type at aerial parts (Fig. 1A), our previous data showed strong ABA2 expression in root tissue (Cheng et al., 2002). This led us to examine the effect of ABA on underground tissue in wild-type, aba2, and transgenic plants. As shown in Figure 3, with 11-d growth on 1% Suc, the aba2 mutant had a shorter primary root length; however, the primary root length in three transgenic lines had no significant difference from wild type (Fig. 3, A and B). Quantitative analysis demonstrated that the average length of newly grown seedlings grown in soil for 21 d. Two independent experiments were performed, each with a duplicate and with consistent results. WT, Wild type; UBQ, ubiquitin. [See online article for color version of this figure.]

Figure 1. Isolation and characterization of ABA2 overexpression lines. A, Phenotypic comparison of the wild type, aba2, and overexpression line (4-3) grown in soil for 21 d. B, RT-PCR results of wild type, aba2, and overexpression lines (4-3, 4-4, and 5-1). Total RNA was isolated from seedlings grown in soil for 21 d. Two independent experiments were performed, each with a duplicate and with consistent results. WT, Wild type; UBQ, ubiquitin. [See online article for color version of this figure.]

Transgenic Plants Overexpressing ABA2 Delay Seed Germination

Because the level of ABA content controls seed dormancy, we tested whether ABA2 overexpression lines might increase the ABA level and result in deeper seed dormancy. As shown in Figure 2, germination of the three transgenic lines was delayed relative to that of the wild type without cold pretreatment. For instance, after 2 d, wild type had 93.7% ± 5.9% seed germination, whereas transgenic lines 4-3, 4-4, and 5-1 had only 55.4% ± 8%, 51.8% ± 2.7%, and 36.5% ± 2.3% germination, respectively (Fig. 2A). With cold pre-treatment for 3 d at 4°C, the germination delay for transgenic lines was impaired after 2-d growth on agar plates with 1% Suc (Fig. 2B).

A similar seed germination delay in overexpression lines was also shown on 2% and, to a lesser extent, on 6% Glc agar plates (Fig. 2, C and D). As expected, the ABA-deficient mutant aba2 always germinated earlier than the wild type and the overexpression lines throughout the germination period tested. The data suggest that the delayed germination pattern shown in the overexpression lines is reduced at the higher sugar concentration. Compared to seeds grown on 1% Suc, those grown on 2% Glc had a lower germination rate. Low sugar concentration promotes, but high sugar concentration inhibits, seed germination (Zhou et al., 1998; Arenas-Huertero et al., 2000; Finkelstein and Lynch, 2000). Delayed germination was observed in all genotypes on 6% Glc as compared to 2% Glc. For instance, 6-d germination rates on 6% Glc for aba2, wild type, and transgenic overexpression lines 4-3, 4-4, and 5-1 were 88.9% ± 5.9%, 32% ± 2.8%, 19.8% ± 4.6%, 18% ± 4.2%, and 24.3% ± 7.4%, respectively; however, germination rates over 90% were observed in all genotypes on 2% Glc. It is noteworthy that wild type and overexpression lines showed seed germination followed by developmental arrest on 6% Glc (Fig. 2E). This observation can be explained by Glc-induced ABA accumulation that further suppresses seedling growth and development. However, the ABA-deficient mutant aba2 lacks such inhibition and grows steadily and develops true leaves (Cheng et al., 2002). This notion was further confirmed by the addition of fluridone, an ABA biosynthesis inhibitor, to the medium; wild type and ABA2 overexpression lines showed an aba2 phenotype, with root elongation, cotyledon expansion, and true leaf development on fluridone-containing medium (Fig. 2E). Thus, the developmental arrest displayed on 6% Glc is most likely due to de novo ABA biosynthesis and accumulation. Fluridone is a selective inhibitor of carotenoid synthesis, which interferes with desaturation steps of carotenoid biogenesis and further blocks the accumulation of carotenoids. Thus, fluridone treatment inhibits carotenoid biosynthesis and leads to an albino-like seedling phenotype due to photooxidation of chlorophyll (Henson, 1984).

Transgenic Plants Overexpressing ABA2 Delay Seed Germination

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primary roots in wild type, aba2, and three transgenic lines (4-3, 4-4, and 5-1) was 6.0 ± 0.17, 4.74 ± 0.19, 5.92 ± 0.19, 6.02 ± 0.28, and 5.93 ± 0.19 cm, respectively (Fig. 3B). Further ABA immunoassays demonstrated that transgenic plants grown on 1% Suc for 11 d had elevated levels of ABA, on average approximately 44.7% higher than wild type, whereas the aba2 mutant had the lowest level of ABA, approximately 27% of the wild-type level (Fig. 3C). The average length of newly grown primary roots for the wild type and mutant was 7.68 ± 0.40 and 6.33 ± 0.38 cm, respectively. A similar result was also observed between gin1-2 and its corresponding wild type, Landsberg erecta (data not shown). Again, exogenous application of ABA (25–500 nM) restored the

Figure 2. ABA2 overexpression lines delay seed germination. A, Germination of wild type and overexpression lines without cold treatment. Seeds were grown on 1% Suc agar plates for the times shown. Radicle emergence over 1 mm is referred to as germination. B, Germination of wild type and overexpression lines with cold pretreatment for 3 d at 4°C. The seed growth condition was the same as in A. C, Germination of wild type, aba2, and overexpression lines on 2% Glc agar plates. D, Germination of wild type, aba2, and overexpression lines on 6% Glc agar plates. E, Phenotypes of wild type, aba2, and overexpression lines grown on 6% Glc agar plates with or without 1 μM fluridone for 14 d. Germination rates from A to D were counted as germinated seeds/total germinated seeds at day 10. The results shown were the means ± SD of three independent experiments using different seed batches, with consistent results, each with 200 to 250 seeds. The seeds in B, C, D, and E were subjected to cold pretreatment at 4°C for 3 d before planting; in A, without cold treatment. WT, Wild type. [See online article for color version of this figure.]
gin1-1 primary root length to near that of its wild type. Taken together, the results indicate that the reduction of primary root length shown in the mutant is primarily due to ABA deficiency, regardless of ecotype. In addition, ABA2 overexpression lines with elevated ABA levels did not show a longer primary root length, which reflects that basal levels of ABA are essential for maintaining primary root elongation.

Effect of Osmoticum on Seedling Growth and Development

It has been reported that overexpression of NCED3 causes elevated levels of ABA and enhances plant tolerance to drought in Arabidopsis (Iuchi et al., 2001) and tobacco (Qin and Zeevaart, 2002). To test whether ABA2/GIN1 overexpression lines with increased levels of ABA also show tolerance to drought, we grew seedlings on a polyethylene glycol (PEG)-infused medium that created osmotic stress mimicking drought conditions. Several PEG concentrations were tested (12.5%, 25.0%, 40.0%, 55.0%, and 70.0%). Seedlings grown on 40% PEG-infused medium (−0.7 MPa Ψw, according to Verslues and Bray, 2004) consistently displayed differential growth rates among wild type, aba2, and the ABA2-overexpressing lines. Seedling growth was strongly inhibited on medium with greater than 40% PEG (data not shown). Seedling growth in one set of experiments is shown in Figure 4A. Wild type had a higher bleached cotyledon ratio (41.1% ± 6.9%) than overexpression line 4-4 (27.4% ± 3.9%; Fig. 4B), suggesting that transgenic plants may be more tolerant to low water potential than wild type.

It is surprising that the aba2 mutant had the lowest damage (11.7% ± 3.8%) under the same growth conditions. Similar results were also observed in another ABA-deficient mutant, sto1/nced3, versus its corresponding wild type (C24; data not shown). The reason for this discrepancy remains to be illustrated. One of the possibilities might be related to an increase in...
stomatal aperture under such conditions. Ruggiero et al. (2004) reported that the sto1/nced3 mutant’s stomatal aperture at day and night is larger than that of wild type under high humidity conditions. This finding might explain why the aba2 mutant had a higher survival rate because of its efficient absorption of water vapor from the air within the high-humidity petri dish. We also observed that wild-type and transgenic plants showed anthocyanin accumulation in newly growing true leaves with a slower growth rate, whereas the aba2 mutant retained light greening with a higher growth rate or had a larger plant size on average under low-water-potential conditions (Fig. 4A).

**Effect of Salinity on Germination and Growth of ABA2 Overexpression Lines**

ABA has long been believed to be a stress-related phytohormone whose biosynthesis or level can be induced by various stresses. To further determine whether the seed germination delay shown on sugar medium also occurs under salinity stress, we grew seeds on 1% Suc agar plates with or without NaCl. As shown with sugar treatments, the aba2 mutant showed early germination regardless of the presence or absence of NaCl (compare Figs. 2 and 5). For instance, the aba2 mutant began germination within 24 h with 125 mM NaCl and 48 h with 250 mM NaCl, whereas germination of wild type and transgenic lines was delayed until 24 h with 125 mM NaCl (Fig. 5A) and 72 h with 250 mM NaCl (Fig. 5B). The higher NaCl concentration delayed seed germination in all genotypes. Transgenic plants showed a slight germination delay relative to wild type at 125 mM NaCl (Fig. 5A), whereas transgenic lines germinated in a manner similar to the wild type at 250 mM NaCl (Fig. 5B). At 125 mM NaCl, germination rates within the first 2 d for aba2, wild type, 4-3, 4-4, and 5-1 were 90.8% ± 1.8%, 76.8% ± 3.9%, 65.6% ± 8.1%, 57.7% ± 6.8%, and 54.2% ± 2.4%, respectively. At 250 mM NaCl, the aba2 mutant displayed expanded cotyledons with light greening, but no true leaf development after 7 d. In contrast, wild type and overexpression lines germinated and then showed developmental arrest (Fig. 5C). The light greening cotyledons in aba2 became bleached after 18 d of germination; the bleached seedlings were not viable after being transferred to fresh 1% Suc medium without NaCl, whereas the arrested seedlings of wild type and overexpression lines restored their green cotyledons and normal true leaf growth thereafter (data not shown). This observation indicates that wild type and overexpression lines have a self-protecting ability modulated by ABA and inhibited further growth and development under high NaCl conditions. NaCl-induced developmental arrest can be overcome with 1 μM fluridone: Wild type and overexpression lines showed the aba2 phenocopy with expanded cotyledons (Fig. 5C). The result indicates that NaCl-induced developmental arrest is most likely due to de novo ABA biosynthesis and accumulation.

Furthermore, immunoassays were used to determine ABA levels in seedlings from each genotype treated with 125 and 250 mM NaCl, respectively. The results exhibited that the aba2 mutant had only 45.3% (Fig. 5D) and 45.7% (Fig. 5E) of the wild-type ABA levels at 125 and 250 mM NaCl, respectively. However, transgenic lines were, on average, approximately 1.74- and 2.11-fold (Fig. 5, D and E, respectively) higher in ABA levels than that of wild type at 125 and 250 mM NaCl, respectively. The average increase of ABA level in these three transgenic lines is statistically significant, with $P < 0.05$ ($P = 0.024$ at 125 mM NaCl and $P = 0.025$ at 250 mM NaCl), Student’s $t$ test. Surprisingly, transgenic seedlings with high ABA levels did not show a germination delay when grown on 250 mM NaCl. Thus, the data suggest that there are distinct mechanisms that may impair or overcome ABA-mediated inhibition of seed germination under severe salinity stress.
Phenotypic comparison revealed that the aba2 seedlings at 200 mM NaCl had lighter greening and glassier leaves than wild type and overexpression line seedlings (Fig. 5F). When grown on 1% Suc agar plates containing various NaCl concentrations (125–200 mM), seeds may germinate and develop true leaves; upon prolonged culture, however, the seedlings became bleached and died. As shown in Figure 5, F and G, although the aba2 mutant revealed early germination on NaCl-containing medium, more aba2 bleached seedlings were observed after prolonged culture. For instance, with 200 mM NaCl, wild type, aba2, and ABA2 overexpression lines had bleached seedling frequencies of 55.8% ± 3%, 81.6% ± 5.7%, and 39.3% ± 9.6%, respectively. In soil, after 21-d-old seedlings were watered with increasing concentrations of NaCl (50, 100, 150, and 200 mM), each for 4 d for a total of 16 d. Seeds tested in this study were cold pretreated before planting on agar plates or in soil.

Figure 5. Effect of salinity on germination and plant growth. A and B, Germination of wild type, aba2, and overexpression lines on 1% Suc agar plates supplemented with 125 (A) or 250 mM (B) NaCl. Germination rates were the means ± SD of three independent experiments using different seed batches, each with 150 to 200 seeds. WT, Wild type. C, Phenotypic comparison of wild type, aba2, and overexpression lines grown on 1% Suc agar plates plus 250 mM NaCl with or without 1 µM fluridone for 14 d. D and E, ABA levels of wild type, aba2, and transgenic lines. Seedlings were grown on 1% Suc agar plates for 5 d, then transferred to the same fresh medium supplemented with 125 mM NaCl for another 7 d (D) or 250 mM NaCl for another 2 d (E). The values are the means ± SD of three independent experiments using different seed batches, each performed in duplicate. F, Phenotypic comparison of wild type, aba2, and transgenic line 4-4. Seedlings were grown on 1% Suc plates supplemented with 200 mM NaCl for 28 d. G, Quantification of bleached plants of wild type, aba2, and transgenic line 4-4 in F. The values are the means ± SD of three independent experiments using different seed batches and with consistent results, each with 200 to 250 seeds. H, Phenotypic comparison of wild type, aba2, and overexpression line 4-4 in response to salinity. A total of 75 plants from each genotype, grown in soil for 21 d, were tested by watering with four increasing concentrations of NaCl (50, 100, 150, and 200 mM), each for 4 d for a total of 16 d. Seeds tested in this study were cold pretreated before planting on agar plates or in soil.
ABA2 Promoter Activity in Response to Stresses

Our previous data showed that ABA2 is a unique gene because its regulation is distinct from other ABA biosynthetic genes in response to stresses. For instance, ABA1, NCED3, and AAO3 transcripts are rapidly induced by dehydration and ABA treatment for 3 h, but ABA2 displays no conceivable change (Cheng et al., 2002). To test whether ABA2 expression is upregulated by stresses, we used a more sensitive test (i.e. β-glucuronidase [GUS] activity) to determine ABA2 promoter activity. Two transgenic lines harboring the ABA2 promoter and GUS reporter gene (ABA2::GUS), each in wild-type and aba2 backgrounds, were used to assay ABA2 promoter activity in response to stress. Southern-blot analysis indicated that transgenic line 4-12 (wild-type background) had at least two transgene insertions and the remaining lines had at least three transgene copies (data not shown). The ABA2 promoter region was approximately 2.9 kb upstream of the ATG start codon and had been used to complement the aba2 mutant to restore the wild-type phenotype (Cheng et al., 2002). Thus, this promoter region contains all or at least most of the cis-acting elements that are essential for controlling ABA2 gene expression.

To determine ABA2 promoter activity under rapid dehydration, aerial parts of tissues from transgenic plants grown in soil for 21 d were removed and placed in an electronic dry box with 40% relative humidity for 1.5 and 3 h. As shown in Figure 6A, 1.5-h dehydration only slightly reduced ABA2 promoter activity as compared to control 1.5 h. These reductions were relatively small, about 28%, 38%, 19%, and 43% of the control activity for lines 1-11 (wild-type background), 4-12 (wild-type background), 3-6 (aba2 background), and 3-12 (aba2 background), respectively. However, 3-h dehydration showed no significant change of ABA2 promoter activity in each line as compared to that of the 3-h control. ABA2 promoter activity was considerably higher after 4 d of withholding water than that of the control; for instance, the transgenic line 1-11 (wild-type background) under 4-d drought had 16-fold higher activity than that of the control; strikingly, activity in the transgenic plant line 3-12 (aba2 background) under 4-d drought was 38.6-fold higher than that of the control. Three-day cold treatment also induced promoter activity higher than that of the control, but in transgenic lines 1-11 (wild-type background) and 3-6 (aba2 background) the activity was

Figure 6. ABA2 promoter activity in response to diverse stress. A, Determination of GUS activity under drought condition. Aerial parts of tissues were removed from 21-d-old plants grown in soil and immediately placed on an electronic dry box with 40% relative humidity for 1.5 and 3 h. B, Determination of GUS activity under diverse stress. Plants were grown in soil for 21 d and subsequently treated with cold (4°C; 3 d), drought (4 d), flooding (1 d), and salt stresses (125 and 250 mM NaCl, each for 1 d), respectively. Control samples were harvested at 21 dap, at the beginning of stress treatments. C, Determination of GUS activity under Suc and NaCl treatments. Seedlings were grown on 1% Suc agar plates with or without 125 or 250 mM NaCl for 14 d. Results in A, B, and C are the means ± 3 SD of three independent experiments, each experiment with a duplicate. Transgenic plants harboring the ABA2::GUS transgene were under wild-type (lines 1-11 and 4-12) and aba2 mutant (lines 3-6 and 3-12) backgrounds.
reduced by approximately 60% and 26%, respectively, as compared to that of 4-d drought. Although the control samples shown here (Fig. 6B) were harvested at 21 days after planting (dap), at the beginning of stress treatments, we also harvested the control samples at the same time points as the stress-treated samples. The results turned out to have very small variation among those control samples. For example, the control samples at day 1 (22 dap), day 3 (24 dap), and day 4 (25 dap) had, respectively, GUS activity change all below 0.49-, 0.43-, and 1.0-fold for every transgenic line tested relative to that of the control samples at day 0 (21 dap; data not shown). Because these variations were very small compared to the variations between stress-treated and control samples at day 0 (Fig. 6B), we believe that there was no significant developmental effect on ABA2 promoter activity among these control samples during this 4-d development (i.e. 21–25 dap), at least in this case.

For NaCl treatments, transgenic plants grown in soil for 3 weeks were soaked with water, 125 or 250 mM NaCl for 24 h; water was sufficient to induce ABA2 promoter activity in transgenic plants (both wild-type and aba2 background), albeit with less efficiency than with NaCl and drought treatment (Fig. 6B). As water flooding may cause a submergence or hypoxia effect, we observed that the ABA2 promoter was submergence inducible (data not shown). Transgenic plants (wild-type background) with NaCl treatment had a relative induction of ABA2 promoter activity with a value higher than with cold (4°C) treatment, but slightly lower than with 4-d drought treatment. After 14 d on agar plates, ABA2 promoter activity in both the wild-type and aba2 backgrounds was induced by NaCl treatment. Such induction was more pronounced with the addition of 125 mM NaCl in transgenic plants with aba2 background (Fig. 6C). As compared with 1% Suc treatment, the addition of NaCl increased promoter activity 3.2-fold at 125 mM and 6.9-fold at 250 mM NaCl in line 1-11 (wild-type background); activity in line 3-12 (aba2 background) was increased 11-fold at 125 mM NaCl but was alleviated to only 6.3-fold at 250 mM NaCl. Such reductions in lines 3-6 and 3-12 (aba2 background) are most likely due to low metabolism at 250 mM NaCl treatment for 14 d. At that time point, some transgenic plants in the mutant background started showing a bleached phenotype, whereas transgenic lines in the wild-type background remained with developmental arrest. Taken together, these findings indicate that ABA2 promoter activity was up-regulated by NaCl stress.

In general, ABA2 promoter activity is remarkably induced by prolonged cold, drought, flooding, and salinity, but not short-term dehydration. Induction is profound in transgenic lines with an aba2 background. It indicates that the feedback inhibition of ABA2 promoter activity by ABA is likely missing in the ABA-deficient mutant aba2. Thus, mutants may build up considerable levels of ABA2 promoter activity relative to transgenic lines under a wild-type background. Up-Regulation of ABA- and Sugar-Related Genes in Response to Drought

As mentioned above, because ABA2 promoter activity was considerably changed under prolonged stress conditions but not short rapid stress treatment, ABA2 expression is controlled differently from other ABA biosynthetic genes that show early stress response. One of the possibilities is likely due to the primary metabolic change during prolonged stress treatments, which in turn regulate ABA2 expression. Because sugars play a central role in plant growth and development and ABA biosynthetic genes are up-regulated by sugars, we tested for alterations in sugar-related gene expression under prolonged stress conditions at the molecular level. As shown in Figure 7, the ABA2 transcript was overexpressed in transgenic plants grown in soil for 3 weeks followed by drought treatment for 4 d or without drought treatment. The levels of the ABA2 transcript were elevated in the wild type after 4-d drought treatment, in agreement with the induction of ABA2 promoter activity (Fig. 6B). However, because the aba2 transcript has a 53-bp deletion, its stability might be labile so that the accumulation of the aba2 transcript was not as high as its promoter activity in response to 4-d drought. Similarly, the NCED3 (At3g14440) transcript was also induced under the same growth conditions. It is interesting that the aba2 mutant accumulated a higher amount of NCED3 transcript than that of wild-type and transgenic plants. The reason for this accumulation remains unknown. One of the possibilities is that NCED3 expression is inducible by ABA and drought may increase ABA biosynthesis and accumulation; however, the aba2 mutant lacks ABA under drought conditions and might also result in a loss of negative feedback regulation of NCED3 expression.

For sugar-related genes, AtHXK1 (At4g29130), a sugar sensor, and AtSusy1 (At5g20830), a Suc-cleaving enzyme, were both up-regulated under stress conditions, whereas AtSusy2 (At5g49190) showed low to undetectable levels (data not shown). The up-regulation patterns of AtHXK1 and AtSusy1 expression in ABA2 transgenic plants under drought conditions were impaired or unchanged as compared to the wild type and aba2 mutant. Thus, these results provide evidence that late expression of ABA2 in response to drought, at least in part, is associated with up-regulated expression of sugar-related genes.

DISCUSSION

Overexpression of ABA2 in Arabidopsis Increases ABA Content and Promotes Seed Germination Delay and NaCl Tolerance

It has been reported that overexpression of ABA biosynthetic genes results in an elevated ABA level and extended seed dormancy under nonstress conditions (Frey et al., 1999; Thompson et al., 2000b; Iuchi et al.,
Expression of ABA2 in Response to Stress

It is believed that plants have multiple stress perceptions and signal transduction pathways to generate specific and common responses to stresses. Although the common response may have cross talk at various steps in the pathways, the specific response may reflect the unique pathway induced only by specific stresses (for review, see Chinnusamy et al., 2004). Thus, germinating seeds may generate a unique signal pathway in response to high Glc and NaCl stresses, which results in different seed germination patterns and seedling phenotypes. We also observed that the addition of the ABA biosynthesis inhibitor fluridone into the medium enhanced seed germination, but did not change the germination patterns under 6% Glc and 250 mM NaCl (data not shown), which indicates that the different germination patterns seen with Glc and NaCl stresses are most likely determined prior to de novo ABA biosynthesis and/or accumulation during seed germination. It is interesting that, despite their different effect on seed germination, both Glc and NaCl stresses may induce postgermination developmental arrest in wild type and overexpression lines, but not the aba2 mutant that displayed near-normal growth and expanded cotyledons with no true leaf development on 6% Glc and 250 mM NaCl, respectively. Developmental arrest can be overcome by the application of the ABA biosynthesis inhibitor fluridone, the treated seedlings displaying the aba2 mutant phenocopy (Figs. 2E and 5C). Thus, postgermination developmental arrest is predominantly due to de novo ABA biosynthesis and accumulation. In addition to elevated ABA levels and extended seed dormancy, transgenic plants overexpressing ABA2 also showed more tolerance to NaCl stress than wild type when grown on agar plates and/or in soil. Thus, this study provides extensive evidence that manipulation of ABA2 expression in Arabidopsis increases the ABA biosynthesis and/or accumulation. In addition to its function as a stress hormone, ABA has long been considered a plant growth inhibitor because the elevated endogenous ABA content induced by stress or exogenous ABA application normally suppresses vegetative tissue growth. However, most ABA-deficient mutants have a smaller size than the wild type, which reflects that the basal level of ABA is essential for normal plant growth and development. It is interesting that all overexpression lines of ABA biosynthetic genes reported to date, including those in this study, show a phenotype not different from that of wild type in aerial parts under normal growth conditions. However, because ABA2 is a unique

### Figure 7. Expression of ABA- and sugar-related genes in response to drought. Plants were grown in soil for 3 weeks, and then subjected to withholding water for 4 d (drought). Control samples were harvested at the same time as drought-treated samples. Three independent experiments were performed, except for control with two independent experiments, each with a duplicate and with consistent results. WT, Wild type; HXK, hexose kinase; Susy, Suc synthase; UBQ, ubiquitin.

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gene, its regulation and response to stresses are different from other ABA biosynthetic genes in some aspects. In addition, ABA2 tissue-specific expression is predominantly restricted to vascular bundles in many tissues, including roots. The latter compelled us to examine the effect of ABA on root growth.

Our data demonstrate that the aba2 mutant had a shorter primary root length than that of wild-type and transgenic plants (Fig. 3, A and B). The reduction is primarily due to ABA deficiency because exogenous application of ABA into the medium restored the root growth in the mutant to normal. We observed that ABA sensitivity differs in distinct ecotypes, to some extent, but the short primary root length caused by the lack of ABA was ecotype independent (Fig. 3, B and D). It is noteworthy that primary root elongation was not inhibited by low amounts of exogenous ABA (less than 500 nM); with more than 500 nM ABA, plants normally show retarded growth and have inhibited primary root elongation and decreased lateral root numbers. ABA inhibits radicle emergence, but not seedling growth; however, such an inhibitory effect can be suppressed with the presence of low concentrations of sugar (Finkelstein and Lynch, 2000). De Smet et al. (2003) also reported that ABA induces lateral root developmental arrest at a specific stage and subsequently blocks lateral root meristem formation. Consistent with this notion, all exogenous ABA concentrations tested in this study, except for 25 to 50 nM, inhibited lateral root growth and development (data not shown). A genetic approach also reveals that ABA promotes primary root elongation in maize under low-water-potential conditions (Sharp et al., 1994). Here, we extensively studied ABA2 overexpression lines. The results showed that the primary root length in ABA2 overexpression lines was similar to wild type with or without ABA treatments. These data reflected the function of basal levels of ABA in maintaining normal primary root growth. Thus, our current data further confirm the dual function of ABA in plant growth and development (Cheng et al., 2002); that is, the basal level or biophysiological concentrations of ABA may function as a plant growth promoter under normal growth conditions and as a plant growth inhibitor under stressful conditions, which may also be an adaptive response beneficial to the plant during recovery.

Up-Regulation of ABA2 Expression through Long-Term, But Not Short-Term, Stress Treatments

Our previous data demonstrated that ABA2 is a unique gene whose regulation is different from other ABA biosynthetic genes, such as AtABA1, AtNCED3, AtABA3, and AAO3, in response to stresses. The latter genes are transcriptionally up-regulated under short-term drought conditions (≤3 h), whereas ABA2 is not. The current study further confirms that ABA2 promoter activity had little change after a short period of drought treatment (≤3 h; Fig. 6A), which is consistent with no conceivable change in the level of ABA2 transcripts under the same conditions (Cheng et al., 2002; González-Guzmán et al., 2002). However, prolonged stressful treatments led to a relatively stronger induction of ABA2 promoter activity (Fig. 6, B and C) and detectable induction of the ABA2 transcript (Fig. 7).

The reason for the discrepant regulation between ABA2 and other ABA biosynthetic genes is unknown. Analysis of the ABA2 promoter within 2.8 kb upstream of the translational start site (ATG) using the Arabidopsis Gene Regulatory Information Server (agris; Ohio State University; http://Arabidopsis.med.ohio-state.edu/AtcisDB) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare; Lescot et al., 2002) revealed that the ABA2 (At1g52340, chromosome 1 location: 19493468–19495317) promoter possesses three ABA response element (ABRE)-like elements (TACGTGTC, TACGTG, and GCCACGTGT at 636, 708, and 1,418 bp upstream of ATG, respectively). In contrast, the AtNCED3 (At3g14440, chromosome 3 location: 4831295–4833606) promoter contains one dehydration response element (DRE; TACCCGACAT, at 1,632 bp upstream of ATG), two ABRE (CACCACGTGC at 207 and 2,372 bp upstream of ATG), and three ABRE-like elements (TACGTG, TACGTG, and GACCGTA at 807, 1,945, and 2,163 bp upstream of ATG, respectively). Thus, AtNCED3 with a DRE and more copies of ABRE and ABRE-like elements than that of ABA2 may respond to drought and other stresses more rapidly and efficiently. Another possibility is that the prolonged stressful conditions might change the primary metabolic features, such as elevated soluble sugars (Déjardin et al., 1999), which in turn regulate ABA2 expression. Evidence that supported this hypothesis was the elevated expression of sugar-related genes such as AtHXK1 and AtSusy1 under prolonged stress (Fig. 7; Déjardin et al., 1999). These two enzymes may generate the soluble sugars hexose-P, and UDP-Glc and Fru, respectively, to adapt plants to the stress environment. Our previous data also demonstrate that increased exogenous sugar concentration stimulates ABA2 gene expression and causes an elevated ABA level. The induction, however, is impaired or abolished in the aba2 mutant (Cheng et al., 2002). Thus, ABA2 is most likely a late expression gene in response to stresses, as compared with other ABA biosynthetic genes, and it might have a fine-tuning function in maintaining ABA levels in response to stresses through regulation of primary metabolic changes. A high Glc concentration also induces expression of ABI genes, such as ABI3, ABI4, and ABI5 (Cheng et al., 2002). Thus, sugar regulation of plant growth and development may, at least in part, act through modulation of ABA biosynthesis and responsive gene expression.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Plant materials used in this study were Arabidopsis (Arabidopsis thaliana) ecotype Columbia. The aba2 mutant is a gin1-3 allele with a 53-bp deletion at
the start of the second exon (Cheng et al., 2002). This mutant is also allelic to sal3, ski4, ski1, and ztl1 isolated by other groups (Laby et al., 2000; Quesada et al., 2000; Rook et al., 2001; González-Guzmán et al., 2002).

Unless stated otherwise, seeds in this study were sterilized and subjected to cold pretreatment at 4°C for 3 d in the dark, and then grown on agar plates or in soil as the first day of seed germination or planting. Seed germination conditions were at 24°C under long-day conditions with a 16-h light/8-h dark cycle with light intensity approximately 80 μmol s⁻¹ m⁻². For aseptic growth, seeds were sterilized with 70% ethanol for 1 min and one-half-strength commercial bleach for 12 min, followed by five washes with sterilized water, and placed at 4°C for 3 d in the dark for cold treatment. Subsequently, seeds were transferred to modified Murashige and Skoog (Murashige and Skoog, 1962) medium composed of one-half-strength Murashige and Skoog basal salts, B5 organic compounds ( Gamborg et al., 1968), and 0.05% MES. For experimental treatments, the medium was supplemented with Glc, Suc, NaCl, herbicide (glufosinate ammonium; Riedel-deHae¨n, catalog no. 45520), ABA (Sigma; catalog no. SF-A1049), or fluridone (Wako; catalog no. 086-03081) at concentrations listed in the “Results” section.

Transgene Constructs and Transgenic Plant Isolation

For overexpression, ABA2 (Gini) full-length cDNA was amplified by RT-PCR and cloned into pgEM-T Easy vector (Promega), followed by subcloning into the binary vector pSMAB704 driven by a constitutive 35S promoter (35S::ABA2). For tissue-specific expression and GUS assay, the ABA2 promoter, approximately 2.9 kb upstream of the ATG start codon, was amplified by PCR and fused to a GUS coding region to generate ABA2::GUS in the pSMAB704 binary vector. Transgene constructs were confirmed by sequencing and subsequently transformed into wild type or the aba2 mutant (T0) by use of the floral-dip method (Clough and Bent, 1998). T1 seeds with cold pretreatment were screened on 1% Suc agar plates containing 25 mg/L herbicide, glufosinate ammonium. At least 10 homozygous lines resistant to herbicide were obtained at the T3 or T4 generation. Three homozygous lines were randomly chosen for further study.

Germination and Root Elongation Tests

For germination tests, seeds harvested from the same batches were cold pretreated and then grown on modified Murashige and Skoog medium supplemented with Glc, Suc, NaCl, ABA, glufosinate ammonium, or fluridone at various concentrations listed in the “Results.” The medium was autoclaved and cooled to 50°C to 60°C prior to the addition of filter-sterilized ABA, glufosinate ammonium, or fluridone. For root elongation experiments, cold-pretreated seeds were first grown on agar plates with 1% Suc for 4 or 5 d, then uniform seedlings of similar size and primary root length were transferred to appropriate fresh medium and grown vertically for another 6 or 7 d.

Preparation of Low-Water-Potential Medium Plates

One-half-strength modified Murashige and Skoog medium (pH 5.7) with 0.7% Phyto agar (Duchefa Biochemie B.V.) was autoclaved. The sterilized medium was cooled to 50°C to 60°C and then aliquoted into petri dishes (100–20-mm depth), 20 mL each, for solidification. PEG-infused plates were made by dissolving PEG-8000 (Sigma) powder into one-half-strength Murashige and Skoog solution (pH 5.7) with the above-mentioned components, except phyto agar, and then filter sterilized; this PEG solution was then overlaid on the agar-solidified medium at a ratio of 3:2 (v/v) and equilibrated overnight (≥12 h). The excess PEG solution was then removed. The procedure essentially followed the protocol of Verslues and Bray (2004).

NaCl and Dehydration Treatments

Wild type, aba2, and ABA2 overexpression seeds with cold pretreatment were grown on agar plates with various NaCl concentrations for 18 or 28 d. The ratios of bleached to total plants were counted to define tolerance to salinity. Seeds with cold pretreatment were also grown in soil for 3 weeks; then plants were subjected to watering with solutions containing four gradually increased concentrations of NaCl (50, 100, 150, and 200 mM), each for 4 d for a total of 16 d (Shi et al., 2003). For dehydration, seeds with cold pretreatment were grown in soil for 21 d and then plants were excised. The aerial parts of tissues were placed on plastic weighing boats and kept in an electronic dry box (model-DX-76; Taiwan Dry Tech Corp.) with approximately 40% relative humidity. The fresh weights of tissues were measured at 0, 1.5-, and 3-h time points.

GUS Activity Assay

Cold-pretreated transgenic seeds harboring the ABA2::GUS transgene were grown on agar plates or in soil for different time periods, depending on experiments, and then subjected to various stresses (cold, drought, or salinity). The treated plants were harvested and ground with extraction buffer (50 mM NaHPO4 [pH 7.0], 10 mM β-mercaptoethanol, 10 mM NaEDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100). After centrifugation at 13K rpm for 10 min at 4°C, the supernatants were removed for further GUS assay, essentially according to the protocol of Jefferson et al. (1987), using a DyNA Quant 200 fluorometer (Amersham-Pharmacia Biotech).

RT-PCR

Total RNA was extracted from wild type, aba2, and ABA2 overexpression lines using TRIzol reagent (Invitrogen). Six micrograms of total RNA of each genotype with 1 μg oligo(dT) primer (Invitrogen) were heated at 70°C for 5 min and then chilled on ice immediately. RNA was then subjected to RT with reverse-transcriptase Avena myosilblossis virus Roche) at 42°C for 1 h according to the manufacturer’s protocol. Synthesized cDNA was used as a template for PCR.

ABA Assay

For ABA extraction, seedlings harvested at appropriate stages or after stress treatments were treated with extraction buffer (80% methanol and 2% glacial acetic acid) for 24 h under darkness, followed by centrifugation for 10 min at 2,000g. Supernatants were then extracted with chloroform and then evaporated to dryness under vacuum. The residue was reconstituted with 100 μL of 50 mM NaHPO4 [pH 7.0], 10 mM β-mercaptoethanol, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100 and 0.1% Tween 20. After centrifugation at 13K rpm for 10 min at 4°C, the supernatants were removed for further GUS assay, essentially according to the protocol of Jefferson et al. (1987), using a DyNA Quant 200 fluorometer (Amersham-Pharmacia Biotech).

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Expression of AtABA2/GIN1 in Response to Stress


Thompson AJ, Jackson AC, Parker RA, Morpeth DR, Burridge A, Taylor IB (2000a) Abscisic acid biosynthesis in tomato: regulation of zeaxanthin...