The Plant Cell, Vol. 14, 2723–2743, November 2002, www.plantcell.org © 2002 American Society of Plant Biologists

A Unique Short-Chain Dehydrogenase/Reductase in Arabidopsis Glucose Signaling and Abscisic Acid Biosynthesis and Functions

Wan-Hsing Cheng,[a,b,1] Akira Endo,[c,1] Li Zhou,[a] Jessica Penney,[a] Huei-Chi Chen,[a] Analilia Arroyo,[d] Patricia Leon,[d] Eiji Nambara,[e] Tadao Asami,[f] Mitsunori Seo,[c,e] Tomokazu Koshiba,[c] and Jen Sheen[a,2]

[a] Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

[b] Institute of Botany, Academia Sinica, Taipei, Taiwan, Republic of China

[c] Department of Biological Sciences, Tokyo Metropolitan University, Hachioji-shi, Tokyo 192-0397, Japan

[d] Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de Mexico, Cuernavaca, Morelos 62271, Mexico

[e] Plant Science Center, RIKEN (The Institute of Physical and Chemical Research), Wako, Saitama 351-0198, Japan

[f] Plant Functions Laboratory, RIKEN (The Institute of Physical and Chemical Research), Wako, Saitama 351-0198, Japan

Glc has hormone-like functions and controls many vital processes through mostly unknown mechanisms in plants. We report here on the molecular cloning of *GLUCOSE INSENSITIVE1* (*GIN1*) and *ABSCISIC ACID DEFICIENT2* (*ABA2*) which encodes a unique Arabidopsis short-chain dehydrogenase/reductase (SDR1) that functions as a molecular link between nutrient signaling and plant hormone biosynthesis. SDR1 is related to SDR superfamily members involved in retinoid and steroid hormone biosynthesis in mammals and sex determination in maize. Glc antagonizes ethylene signaling by activating *ABA2*/*GIN1* and other abscisic acid (ABA) biosynthesis and signaling genes, which requires Glc and ABA synergistically. Analyses of *aba2*/*gin1* null mutants define dual functions of endogenous ABA in inhibiting the postgermination developmental switch modulated by distinct Glc and osmotic signals and in promoting organ and body size and fertility in the absence of severe stress. SDR1 is sufficient for the multistep conversion of plastid- and carotenoid-derived xanthoxin to abscisic aldehyde in the cytosol. The surprisingly restricted spatial and temporal expression of *SDR1* suggests the dynamic mobilization of ABA precursors and/or ABA.

INTRODUCTION

Plant growth and development is governed by signaling networks that connect inputs from environmental cues, hormone signals, and nutrient status. Recent studies have suggested a pivotal role of sugars as signaling molecules in plants that integrate external environmental conditions and other nutrients with intrinsic developmental programs modulated by multiple plant hormones (Smeekens, 2000; Coruzzi and Zhou, 2001; Finkelstein et al., 2002; Rolland et al., 2002). How sugar signals influence vital processes from germination, seedling development, root and leaf differentiation, and senescence to stress tolerance remains mostly unknown. Cellular and transgenic studies have indicated the involvement of hexokinase (HXK) as a sugar sensor with both signaling and metabolic functions in plants (Jang et al., 1997; Smeekens, 2000; Rolland et al., 2002). To further elucidate the molecular mechanisms underlying the plant sugar signaling network, a genetic approach was used to isolate sugar response mutants in Arabidopsis. Based on a bioassay in which a high level of Glc blocks the switch to postgermination development in Arabidopsis, both Glc-insensitive (*gin*) and Glc-oversensitive (*glo*) mutants were isolated. Phenotypic, genetic, and molecular characterization of these mutants has provided new insights into the regulatory mechanisms that link nutrient status to plant hormone synthesis and signaling (Zhou et al., 1998; Arenas-Huertero et al., 2000; Rolland et al., 2002).

Phenotypic analysis of the *gin1* mutant has suggested an interaction between the signaling pathways mediated by Glc and the plant stress hormone ethylene. Further characterization of the ethylene overproduction mutant *eto1*, the constitutive ethylene signaling mutant *ctr1*, and the ethylene-insensitive mutant *etr1* has led to the discovery that the Glc

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed. E-mail sheen@molbio.mgh.harvard.edu; fax 617-726-6893.

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.006494.

and ethylene signaling pathways antagonize each other and that GIN1 acts downstream of the Glc sensor HXK1 and the ethylene receptor ETR1 (Zhou et al., 1998). Studies of the *gin1* (allelic to *aba2*, *sañ3*, *sis4*, *isi4*, and *sre1*), *gin5* (allelic to *aba3 and los5*), and *gin6* (allelic to *abi4*, *sun6*, *sañ5*, *sis5*, and *isi3*) mutants have also revealed that another plant stress hormone, abscisic acid (ABA), is important for Glc responses (Leon-Kloosterziel et al., 1996; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Quesada et al., 2000; Rook et al., 2001; González-Guzmán et al., 2002). Interestingly, recent genetic and phenotypic analyses have revealed complex interactions between ABA and ethylene based on seedling assays (Beaudoin et al., 2000; Ghassemian et al., 2000). Both ABA and ethylene are well-known plant stress hormones with growth-inhibiting activities. How they exhibit opposite roles in Glc responses, and the molecular mechanisms underlying the Glc regulation of ethylene signaling and ABA biosynthesis, remain mostly unclear (Zhou et al., 1998; Arenas-Huertero et al., 2000; Finkelstein and Gibson, 2001; Gazzarrini and McCourt, 2001).

To elucidate the molecular mechanisms underlying the Glc regulation of ABA biosynthesis and ethylene signaling, molecular cloning of *ABA2*/*GIN1* was performed. *ABA2*/*GIN1* encodes the first described member of a large (56-member) short-chain dehydrogenase/reductase (SDR) gene family in Arabidopsis and plays a unique and specific role in the ABA biosynthesis pathway. We show that Glc activates *ABA2*/*GIN1* expression, which is required for antagonizing ethylene signaling. This result also prompted us to examine Glc regulation of the other ABA biosynthesis and signaling genes. These studies provide direct molecular evidence for extensive Glc control of the genes involved in ABA biosynthesis and signaling. Gene expression analyses in the *aba2*/*gin1* mutant also reveal that Glc acts synergistically with endogenous ABA and that Glc regulation is distinct from regulation by osmotic stress.

In addition to a role in Glc signaling, ABA has long been known as a plant hormone important for drought tolerance, by mediating guard cell signaling and stomatal closure to prevent extensive water loss, and for seed maturation, desiccation, dormancy, and germination (Zeevaart and Creelman, 1988; Finkelstein and Zeevaart, 1994; Leung and Giraudat, 1998; Koornneef et al., 2001; Schroeder et al., 2001; Finkelstein et al., 2002). In general, ABA is considered a stress hormone and a growth inhibitor. However, based on examination of the phenotypes of multiple null mutations in *ABA2*/*GIN1* specific to the ABA biosynthesis pathway and transgenic plants complemented with *ABA2*/*GIN1*, it is clear that endogenous ABA also plays crucial roles as a growth-promoting hormone in controlling fertility and vegetative growth and determining organ and body size. Thus, ABA possesses dual functions as a growth inhibitor and a growth promoter in the presence and absence of stresses, respectively.

Despite extensive analyses of ABA signaling at target sites, such as guard cells, mesophyll cells, maturing seeds, and germinating seedlings, the precise locations of ABA biosynthesis remain elusive. The SDR-related activities in ABA biosynthesis have been presumed to be constitutive and ubiquitous (Sindhu and Walton, 1987; Schwartz et al., 1997a). Examination of the spatial and temporal expression patterns of an *ABA2*/*GIN1* reporter gene has revealed surprising evidence for its highly restricted localization in specific vascular tissues. These results indicate interorgan and intercellular mobilization of ABA precursors and/or ABA into the target sites. Cellular and biochemical analyses demonstrate conclusively that the multistep conversion of the plastid-derived xanthoxin to abscisic aldehyde (ABAld) is catalyzed by the sole cytosolic SDR1 and is essential for the major and physiological pathway in plant ABA biosynthesis.

RESULTS

Interplays of Glc, ABA, and Ethylene Signaling

Genetic and phenotypic analyses of the Arabidopsis Glc, ethylene, and ABA mutants have suggested a connection between these signaling pathways based on a Glc bioassay in which a high level of Glc (6%) blocks the switch to post-germination development (Zhou et al., 1998; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001). Because three *gin1* mutants have a wilty phenotype and the mutations are mapped closely to the ABA-deficient locus *aba2* that also displays Glc insensitivity (Figure 1A), reciprocal crosses were performed between *gin1-3* and *aba2-1*. The progeny of both crosses showed Glc insensitivity (Figure 1B), establishing that *gin1* and *aba2* are allelic. To determine whether ABA deficiency is the cause of the *gin1* phenotype in the Glc-dependent bioassay, a noninhibitory level of ABA (100 nM) was added to the Glc medium. At 4% Glc, the addition of ABA restored the Glc sensitivity of *gin1* and another ABA-deficient mutant, *gin5*, but not that of the ABA-insensitive mutant *abi4*, which is allelic to *gin6* (Figure 1C). Although 4% mannitol reduced seedling development in the presence of ABA, the effects of Glc and mannitol were distinguishable (Figures 1C and 1D).

We have shown previously that the constitutive ethylene signaling mutant *ctr1* displays a Glc-insensitive phenotype similar to that of *gin1* (Zhou et al., 1998). However, after prolonged Glc treatment, *ctr1* remained smaller, with epinasty and light green leaves, conspicuous mid vein, and short roots (Figures 1A and 1E), suggesting a partial overlap of the *gin* and *ctr* phenotypes. To determine whether any other *gin* mutants are allelic to *ctr1*, we examined *gin2*, *gin3*, *gin4*, and *gin5* mutants on 6% Glc. Only *gin4-1* and *gin4-2* mutants resembled *ctr1* (Figure 1E); these were crossed to *ctr1* and analyzed for the constitutive triple response in etiolated seedlings grown in the dark (Stepanova and Ecker, 2000). The results showed that *gin4* is allelic to *ctr1* (Figure 1F), confirming an antagonistic interaction between the Glc and ethylene signaling pathways (Zhou et al., 1998). Consis-

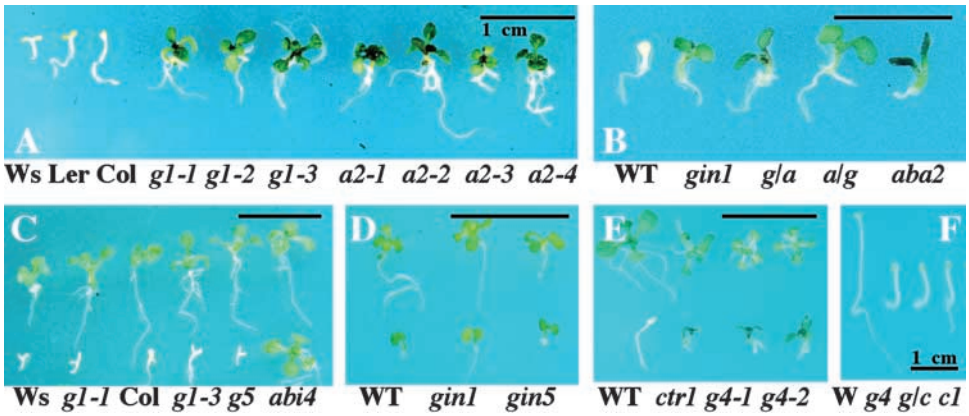

Figure 1. Genetic and Phenotypic Analyses of Arabidopsis *gin1* and *gin4* Mutants.

(A) Glc-insensitive mutants. Three wild-type (Col, L*er*, and Ws), three *gin1* (*gin1-1*, *gin1-2*, and *gin1-3*), and four *aba2* (*aba2-1*, *aba2-2*, *aba2-3*, and *aba2-4*) seedlings were grown on 6% Glc Murashige and Skoog (1962) (MS) plates under light for 10 days. All experiments were repeated twice with consistent results.

(B) *gin1-3* and *aba2-1* are allelic. Reciprocal crosses between *gin1-3* and *aba2-1* (*g*/*a* and *a*/*g*) were performed. Wild-type (WT) and F1 seedlings were grown on 6% Glc MS plates.

(C) ABA restores the Glc-sensitive phenotype. *gin1-1* (*g1-1*), *gin1-3* (*g1-3*), *gin5* (*g5*), and *abi4* seedlings were grown on 4% Glc MS plates without (top row) or with (bottom row) 100 nM ABA.

(D) Mannitol and Glc are distinct signals. Wild-type, *gin1-3* (*gin1*), and *gin5* seedlings were grown on 4% mannitol MS plates without (top row) or with (bottom row) 100 nM ABA.

(E) The phenotype of *gin4* resembles that of *ctr1*. Wild-type, *gin4-1* (*g4-1*), *gin4-2* (*g4-2*), and *ctr1* seedlings were grown on 2% (top row) and 6% Glc (bottom row) MS plates.

(F) *gin4* and *ctr1* are allelic. Wild-type (W), *gin4-1* (*g4*), F1 *gin4-1*/*ctr1-1* (*g*/*c*), and *ctr1-1* (*c1*) seedlings were grown on 2% Glc MS plates for 5 days in the dark. The scale bars in **(B)** to **(F)** = 1 cm.

tently, another sugar mutant screen also isolated a new *ctr1* allele as *sis1* (Gibson et al., 2001).

Map-Based Cloning of *ABA2/GIN1*

To understand the molecular basis and regulation of the *ABA2/GIN1* gene, a map-based cloning approach was used. We used PCR and available simple sequence length polymorphism (SSLP) markers (Bell and Ecker, 1994) to localize *GIN1* on chromosome 1 near the SSLP markers nga128 and nga280 (Zhou et al., 1998). For fine mapping, we generated 10 new SSLP markers for ecotypes Wassilewskija (Ws), Columbia (Col), and Landsberg *erecta* (L*er*) spanning >1700 kb covered by BAC clones (Figure 2A). After PCR analysis of 5627 DNA samples, the *GIN1* gene was localized to a region between BAC clones F11M15 and F6D8 (Figure 2A). One of the genes in the F19K6 BAC clone encodes a putative SDR (SDR1) that might perform the final oxidation steps in converting xanthoxin to ABA (Sindhu and Walton, 1987; Schwartz et al., 1997a) and was the best candidate for *ABA2/GIN1*. To determine whether there was any molecular defect in the candidate gene transcript in the *gin1* mutants, reverse transcriptase PCR (RT-PCR) was performed using primers in the predicted coding sequence of exon 2. Neither *gin1-1* nor *gin1-3* yielded the expected 762-bp RT-PCR product observed in the wild-type Ws and Col controls, whereas a similar RT-PCR product of the Suc synthase gene (*SUS*) encoded by the same BAC sequence was detected in both the wild type and the *gin1* mutants (Figure 2B). Map-based cloning using the *aba2-2* mutant allele also identified the same *SDR1* candidate gene (data not shown).

The full-length cDNA sequence (1179 bp) of *ABA2/GIN1* was obtained by 5′ and 3′ rapid amplification of cDNA ends (Figures 2C and 2D) and confirmed by RNA gel blot analysis (see Figure 6A). The deduced polypeptide is predicted to be 285 amino acids in length without an overt transmembrane domain (Figures 2D and 2E). To further validate the notion that *ABA2/GIN1* encodes the putative SDR1, the molecular bases of seven *aba2/gin1* alleles (*aba2-1*, *aba2-2*, *aba2-3*, *aba2-4*, *gin1-1*, *gin1-2*, and *gin1-3*) were analyzed and summarized (Figure 2E). At least three alleles, *gin1-1*, *gin1-3*, and *aba2-2*, appeared to be null mutations producing defective or no transcript (Figures 2B, 2E, and 6A). The *gin1-3* allele has a 53-bp deletion in exon 2, causing a frameshift after 22 amino acids and an early translation stop after 32 amino acids (Figure 2E). We observed no protein production

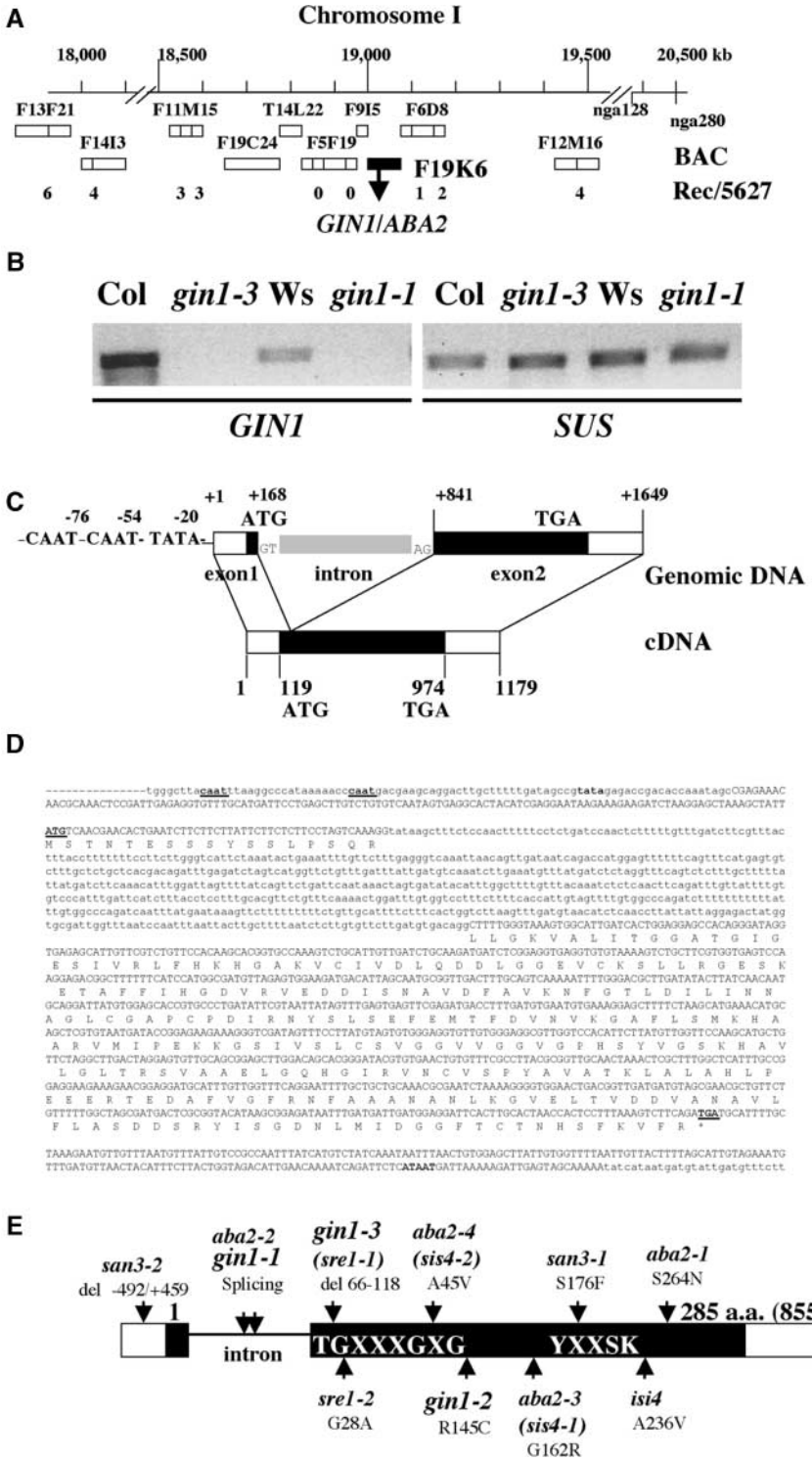

Figure 2. Map-Based Cloning of *ABA2*/*GIN1*.

(A) Physical map of chromosome I in the *ABA2*/*GIN1* region. Ten new SSLP markers are marked as lines in six of the BAC clone boxes. The number of recombinants (Rec) found in 5627 DNA samples is shown.

(B) The *gin1-1* and *gin1-3* alleles lack exon 2 sequences. RT-PCR was performed using primers derived from the exon 2 sequence for *GIN1*. A control experiment was performed with primers for the Suc synthase gene (*SUS*) transcript.

with a *gin1-3* and green fluorescent protein (GFP) fusion (data not shown). Both *gin1-1* and *aba2-2* have variable intron mutations that gave no RT-PCR product for *gin1-1* (Figures 2B and 2E) and no *aba2-2* transcript (see Figure 6A) when analyzed with 5′-end exon 2 sequences. Five additional *aba2* alleles also have been analyzed independently by other groups (Figure 2E) (Laby et al., 2000; Rook et al., 2001; González-Guzmán et al., 2002).

ABA2/*GIN1* Encodes a Unique Member of the SDR Superfamily

Based on a BLASTP search, *ABA2*/*GIN1* is a member of a large *SDR* gene family. All 56 of the putative Arabidopsis SDR members range between 250 and 300 amino acids in length and have a NAD/NADH binding motif (TGXXXGXG) and a catalytic site (YXXS/T/AK) that are characteristic of and conserved in SDR superfamily members in *Escherichia coli*, yeast, flies, worms, mice, humans, and plants (Figure 3) (Jörnvall et al., 1999; Oppermann et al., 2001). The *ABA2*/*GIN1* gene encoding Arabidopsis SDR1 is likely to have a unique function, because the most closely related member, SDR2 (with 62% similarity to SDR1), is a homolog of the maize TASSELSEED2 (ZmTS2, with 73% similarity to AtSDR2) that is important for sex determination (Figure 3) (DeLong et al., 1993). Judging from the severe phenotypes displayed in the null alleles of *aba2*/*gin1* mutants and the homology of AtSDR2 with ZmTS2, Arabidopsis SDR1 has nonredundant functions.

ABA2/*GIN1* as a Link between Glc and Ethylene Signaling

To determine the link between ethylene and ABA pathways in Glc responses, a double mutant of the strongest ethylene-insensitive mutant, *ein2* (Alonso et al., 1999), displaying Glc oversensitivity (Figure 4A), and the Glc-insensitive mutant *gin1* was generated. Because the Glc-oversensitive phenotype of *ein2* on 4% Glc was blocked in the *ein2 gin1* double mutant (Figure 4A), ethylene could contribute to the Glc-dependent phenotypes by antagonizing ABA biosynthesis, as suggested previously (Ghassemian et al., 2000). To determine whether ethylene signaling is antagonized by Glc through ABA, the expression of an ethylene-regulated gene, *PDF1.2* (Alonso et al., 1999), was examined. The presence of 2 and 6% Glc repressed *PDF1.2* expression that requires endogenous ethylene in the wild type but not in *aba2*/*gin1* (Figure 4B). Unlike 2% Glc, 2% mannitol as an osmotic control did not repress *PDF1.2* expression in either the wild type or *aba2*/*gin1*. Unlike 6% mannitol, 6% Glc strongly repressed *PDF1.2* expression in the wild type but activated its expression in *aba2*/*gin1* (Figure 4B). These results support the model that the antagonistic relationship between Glc and ethylene signaling is mediated in part through *ABA2*/*GIN1* involvement in ABA biosynthesis. Although there is an ABA-dependent osmotic effect at 6% mannitol, the Glc and mannitol signals are distinguishable.

ABA as a Growth-Promoting Regulator

Although ABA has long been considered a stress hormone that accumulates under stress conditions and a growth inhibitor when applied exogenously, the ABA-deficient mutant *aba2*/*gin1* exhibited severe growth retardation in cotyledons, rosettes, stems, roots, and siliques in the absence of exogenous sugars and stress conditions (Figures 4C and 4D). Thus, endogenous ABA also could be critical for the promotion of plant growth. This dual function of ABA is similar to that of Glc in promoting growth at low concentrations but inhibiting growth at high concentrations (Xiao et al., 2000). Examined with a dissecting microscope, it appeared that the smaller cotyledons were attributable to a lack of cell expansion and the smaller rosettes were attributable to a reduction in both cell number and size (data not shown). The root system of *aba2*/*gin1* was shorter, with fewer lateral roots (Figures 4C and 4D). A similar growth defect was found when *aba2*/*gin1* plants were grown under high humidity (Figure 4D). To further test the idea that the growth defect of *aba2*/*gin1* is not caused by chronic water stress, wild-type and *aba2*/*gin1* plants were grown in a container with nearly 100% RH. As shown in Figure 4E, the rosettes and root system of *aba2*/*gin1* remained significantly smaller, even though the leaf expansion of the wild type also was limited under this condition. The *aba2*/*gin1* mutant showed

Figure 2. (continued).

(C) Scheme of the genomic and cDNA structure of *ABA2*/*GIN1*. The deduced polypeptide is predicted to be 285 amino acids in length with a molecular mass of 30.2 kD, a pI of 5.85, and an α-β structure. Comparison of the cDNA and genomic DNA sequences (in the F19K6 BAC clone; 41428 to 43279 bp) indicated that *ABA2*/*GIN1* contains two exons of 168 and 808 bp and one intron of 672 bp. The black box represents the coding region.

(D) Genomic and cDNA sequences of *GIN1*/*ABA2*. The 5′ and 3′ ends of the cDNA (uppercase letters) were determined by rapid amplification of cDNA ends and sequencing.

(E) Molecular basis of *aba2*/*gin1* mutants. TGXXXGXG is the conserved NAD binding site, and YXXSK is the catalytic site. The mutation sites of 11 *aba2*/*gin1* alleles are shown. Names of the same alleles are shown in parentheses. a.a., amino acids.

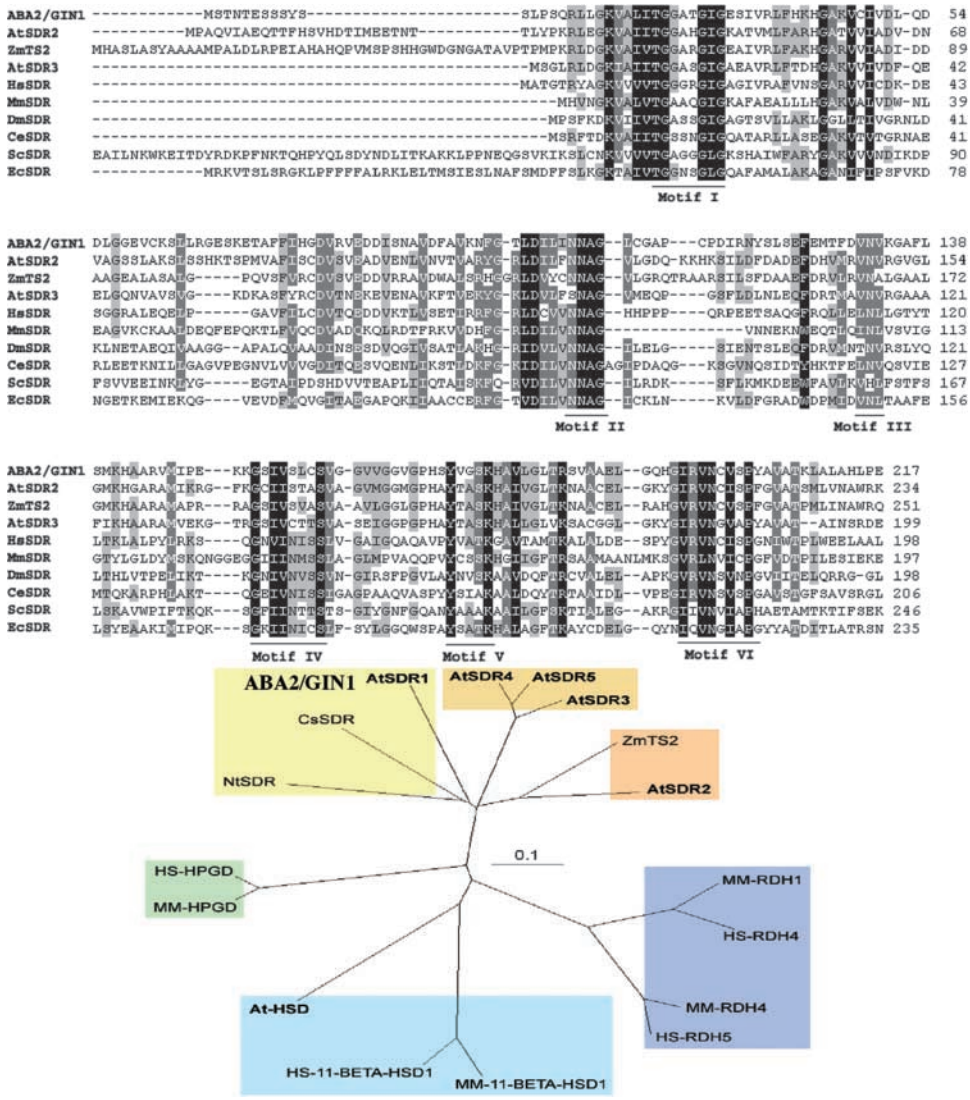

Figure 3. Arabidopsis *ABA2/GIN1* Encodes a Unique Member of the SDR Superfamily.

The *ABA2/GIN1* coding sequence (217 of 285 residues) covering the six conserved motifs was aligned with sequences of putative SDRs from seven other model organisms using the Clustal alignment program. Arabidopsis ABA2/GIN1 (AtSDR1) is grouped with tobacco (NtSDR) and cucumber (CsSDR) homologs in the phylogenetic tree at bottom. AtSDR2 is closely related to maize ZmTS2 and different from AtSDR3, AtSDR4, and AtSDR5. AtHSD (11-β-hydroxysteroid dehydrogenase) is closely related to the human (HS) and mouse (MM) HSDs. The human and mouse HPGDs (15-hydroxyprostaglandin dehydrogenase) and human and mouse RDHs (retenoid dehydrogenase) also are shown. The scale value of 0.1 indicates 0.1 amino acid substitutions per site.

a wilty phenotype within 5 min of exposure to lower humidity (Figure 4E), supporting the importance of basal ABA levels in the guard cell response to water stress. It has been suggested that the growth defect in tomato ABA-deficient mutants is the result of an inhibitory effect of ethylene (Sharp et al., 2000). By comparing the phenotypes of *aba2/gin1*, *ein2*, and *gin1 ein2* plants, it was obvious that the growth retardation of *aba2/gin1* shoots could be attributed to ABA deficiency even in the completely ethylene-insensitive *ein2* background (Alonso et al., 1999), regardless of the humidity (Figure 4F) (data not shown). However, the root phenotype of the *gin1 ein2* double mutant resembled that of *ein2* but not that of *aba2/gin1* (Figure 4F). These results support the antagonistic roles of ABA and ethylene in root development and a surprising uncoupling of the shoot and root phenotypes modulated by endogenous ABA.

Complementation

To ensure that *ABA2/GIN1* encodes SDR1 and that the *aba2/gin1* phenotypes are the consequence of a single gene mutation, complementation was performed by transforming the null *gin1-3* mutant with a 7.7-kb *ABA2/GIN1* genomic clone that includes the 3-kb promoter or with the full-length cDNA under the control of a constitutive 35S promoter (*35S*::*GIN1*) in a miniature binary vector (pCB302) through *Agrobacterium tumefaciens* (Xiang et al., 1999). Ten independent lines with clear complementation in the greenhouse were brought to homozygous T3 generation before detailed characterization. The *ABA2/GIN1* genomic clone and *35S*::*GIN1* restored the Glc sensitivity of *gin1* (Figure 5A and data not shown). The growth defects in cotyledons and rosettes (Figure 5B), lateral roots (Figure 5C), stems (Figure 5D), siliques (Figure 5E), and embryos (Figure 5F), as well as the wilty (Figure 5G) and lack of seed dormancy (Figure 5H) phenotypes of *gin1*, also were complemented. These results are consistent with the dual function of endogenous ABA as a growth-promoting regulator in addition to its roles in Glc signaling, stress tolerance, and seed dormancy.

Tissue, Stress, and ABA Regulation of ABA Biosynthesis Gene Expression

To determine the tissue-specific expression patterns of *ABA2/GIN1*, RNA gel blot analysis was performed. Surprisingly, *ABA2/GIN1* was expressed predominantly in roots

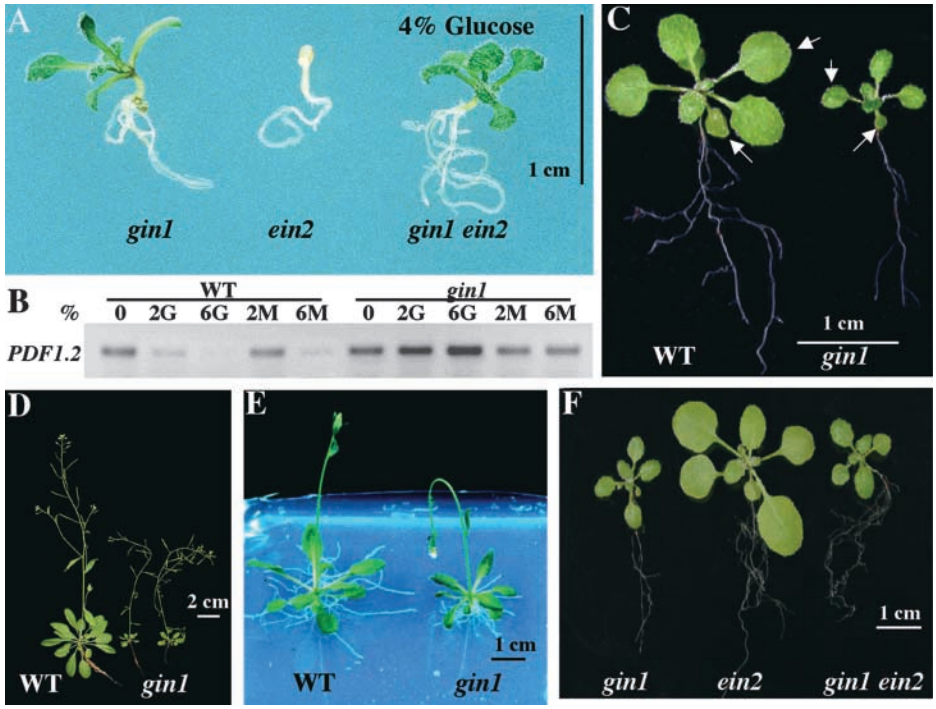

Figure 4. Interplay of Glc, ABA, and Ethylene Signaling Pathways.

(A) *gin1* is epistatic to *ein2*. Single-mutant (*gin1* and *ein2*) and double-mutant (*gin1 ein2*) seedlings were grown on 4% Glc MS plates under light for 12 days. All experiments were repeated twice with consistent results.

(B) Glc repression of *PDF1.2* expression requires ABA. Plants were grown on MS plates without (0) and with 2 and 6% Glc (2G and 6G) or 2 and 6% mannitol (2M and 6M) for 14 days. RT-PCR was repeated twice with consistent results. WT, wild type.

(C) Growth defects in *gin1*. Wild-type and *gin1* plants were grown on soil under a 12/12-h light/dark photoperiod for 14 days. The two top arrows point to cotyledons with different cell sizes. The two bottom arrows indicate developing true leaves with similar cell sizes but different cell numbers.

(D) Growth defects in *gin1*. Wild-type and *gin1* plants were grown on soil under a 12/12-h light/dark photoperiod for 50 days with 40% (*gin1*; middle plant) or 85% (*gin1*; right plant) RH.

(E) Wilty phenotype in *gin1*. Wild-type and *gin1* plants were grown on 2% Glc MS medium under a 16/8-h light/dark photoperiod and 100% RH for 24 days. The plastic cover was removed for 5 min.

(F) Uncoupling of shoot and root growth regulation in *gin1*. Single-mutant (*gin1* and *ein2*) and double-mutant (*gin1 ein2*) plants were grown on soil under a 12/12-h light/dark photoperiod for 16 days.

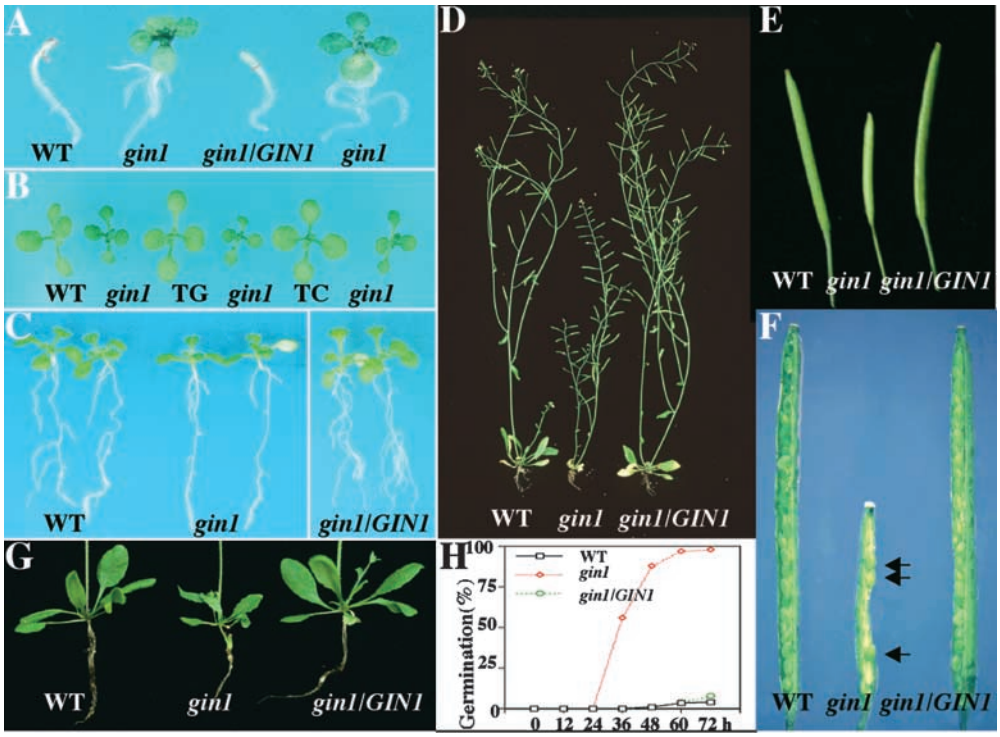

Figure 5. Complementation.

(A) A genomic *ABA2/GIN1* clone restores Glc sensitivity. The T2 segregation of *gin1/GIN1* and *gin1* is shown. Plants were grown on 6% MS plates under light for 10 days. WT, wild type.

(B) Genomic and cDNA clones restore cotyledon and leaf growth defects. The T2 segregation of TG (transgenic with the genomic *ABA2/GIN1* clone), TC (transgenic with the *35S*::*GIN1* cDNA clone), and *gin1* plants is shown. Plants were grown on 2% Glc MS plates under light for 14 days.

(C) A genomic *ABA2/GIN1* clone restores the *gin1* lateral root defects.

(D) A genomic *ABA2/GIN1* clone restores the *gin1* defects in leaves, stems, and siliques. Plants were grown under constant light and watering for 37 days.

(E) A genomic *ABA2/GIN1* clone restores the *gin1* defect in silique size.

(F) A genomic *ABA2/GIN1* clone restores the *gin1* defects in embryos and fertility. Arrows indicate three normal sized seeds in the *gin1* silique.

(G) A genomic *ABA2/GIN1* clone eliminates the wilty phenotype of *gin1* (28 days).

(H) A genomic *ABA2/GIN1* clone restores seed dormancy in *gin1*. Dry seeds (50 to 80) were allowed to imbibe in water for 12 h and then germinated on water-saturated filter paper (7 cm in diameter) under constant light. The values shown (percent germination) are averages from three independent experiments.

and stems but at much lower levels in rosettes and seeds, the established ABA target sites (xA). Analyses of the petiole and blade RNA samples isolated from "rosettes" further supported the preferential expression of *ABA2/GIN1* outside of the presumed ABA target sites, mesophyll, and guard cells. Unless the ABA2/GIN1 protein is mobile, interorgan and intercellular transport of ABA precursors and/or ABA likely occurs to coordinate ABA biosynthesis and response, because genes that encode other ABA biosynthesis enzymes, such as zeaxanthin epoxidase (ZEP), 9-*cis*-epoxycarotenoid dioxygenase (NCED3), and Arabidopsis aldehyde oxidase 3 (AAO3), are expressed differentially (Seo et al., 2000a; Xiong et al., 2002; M. Seo and T. Koshiba, unpublished data). Interestingly, *Commelina* stomatal closure is induced by xanthoxin when it is added through the transpiration stream but not by direct application on epidermal strips (Raschke et al., 1975).

It has been shown that genes important for ABA biosynthesis, such as Arabidopsis *ZEP* (*ABA1/LOS6*), *NCED3*, *AAO3*, and *ABA3* (*LOS5*), are induced by water stress (Qin and Zeevaart, 1999; Seo et al., 2000a; Iuchi et al., 2001; Xiong et al., 2001, 2002), although the enzymes that convert xanthoxin to ABAld and ABA are detected constitutively in leaves (Sindhu and Walton, 1987). In contrast to the *NCED3* transcript that was induced rapidly by dehydration and absent in water, the *ABA2/GIN1* transcript did not increase

during dehydration treatment (Figure 6B). Thus, *ABA2/GIN1* is important for the basal level of ABA but is not the rate-limiting factor for the stress-inducible accumulation of ABA (Qin and Zeevaart, 1999; Iuchi et al., 2000). The expression of *ZEP* (*ABA1/LOS6*), *NCED3*, and *AAO3* also was induced by ABA in shoots and roots, but the expression of *ABA2/GIN1* was not (Figure 6C). It appears that the expression of *ABA2/GIN1* is not coordinated with that of other ABA biosynthesis genes by stress and ABA.

Glc Regulation of ABA Biosynthesis and Signaling Gene Expression

To determine how Glc controls ABA accumulation, the expression of *ABA2/GIN1* was examined with or without exogenous Glc in wild-type and *aba2/gin1* seedlings. As shown in Figure 7, the *ABA2/GIN1* transcript was induced significantly by 2 and 6% Glc only in wild-type but not in *aba2/gin1* seedlings. Because *ABA2/GIN1* is not induced by ABA (Figure 6C) but ABA deficiency in *aba2/gin1* prevented its Glc induction, it is likely that both Glc and ABA are required synergistically for *ABA2/GIN1* regulation. Mannitol at 2 and 6% also induced *ABA2/GIN1* expression approximately twofold in an ABA-dependent manner (Figure 7). To investigate the extent of Glc regulation, we also examined other genes involved in ABA biosynthesis and ABA signaling by RT-PCR. The results showed that other ABA biosynthesis genes, such as *ABA1*, *AAO3*, and *ABA3*, were upregulated by 2 and 6% Glc, similar to the effect found for *ABA2/GIN1* (Figure 7). Glc activation was abolished in the *gin1-3* null mutant, suggesting a critical role of endogenous ABA in the Glc induction of ABA biosynthesis genes (Figure 7). Interestingly, unlike 6% Glc, which activated gene expression, 6% mannitol repressed these genes in both the wild type and *aba2/gin1* (Figure 7). The expression of *NCED3* was extremely low and not activated by Glc, distinguishing Glc signals from stress signals that activated *NCED3* (Figures 6B and 6C). Interestingly, *ABI3*, *ABI4*, and *ABI5* but not *ABI1* and *ABI2* transcripts showed dramatic induction by 6% Glc but not by 6% mannitol. The induction was eliminated completely in *aba2/gin1* (Figure 7). These results are consistent with the Glc-insensitive phenotypes shown in the *aba1*, *aba2*, *aba3*, *abi4*, and *abi5* mutants (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000). As controls, the expression of a Glc sensor encoded by *AtHXK1* and a ubiquitin gene (*UBQ5*) was not influenced by Glc or mannitol (Figure 7) (Jang et al., 1997).

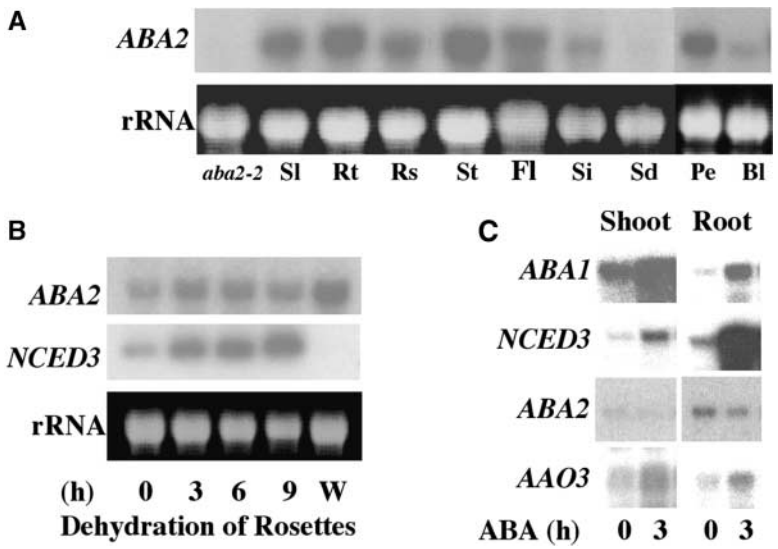

Figure 6. Tissue, Stress, and ABA Regulation of Gene Expression.

(A) Tissue expression pattern. *ABA2/GIN1* expression was examined in the *aba2-2* null mutant and in wild-type (Col) seedlings (Sl) and adult roots (Rt), rosettes (Rs), stems (St), flowers (Fl), siliques (Si), and dry seeds (Sd). The rosettes were separated further to petioles (Pe) and blades (Bl). Seedlings were grown on MS agar plates for 14 days, and adult plants were grown in pots for ~40 days. Each lane contained 10 μg of total RNA. A 366-bp fragment of *ABA2/GIN1* cDNA digested with AluI was used as a probe for hybridization. rRNA was used as a loading control. The experiment was repeated twice with consistent results.

(B) Gene expression under dehydration. The expression of *ABA2* and *NCED3* was determined at various time points after dehydration in rosettes of 40-day-old soil-grown plants. Samples with water (W) were used as a control. The experiment was repeated twice with consistent results.

(C) ABA regulation of ABA biosynthesis genes in shoots and roots. Wild-type plants (L*er*) grown on agar plates for 14 days were transferred to filter paper soaked with 100 μM ABA and incubated for 0 and 3 h. The experiment was repeated twice with consistent results.

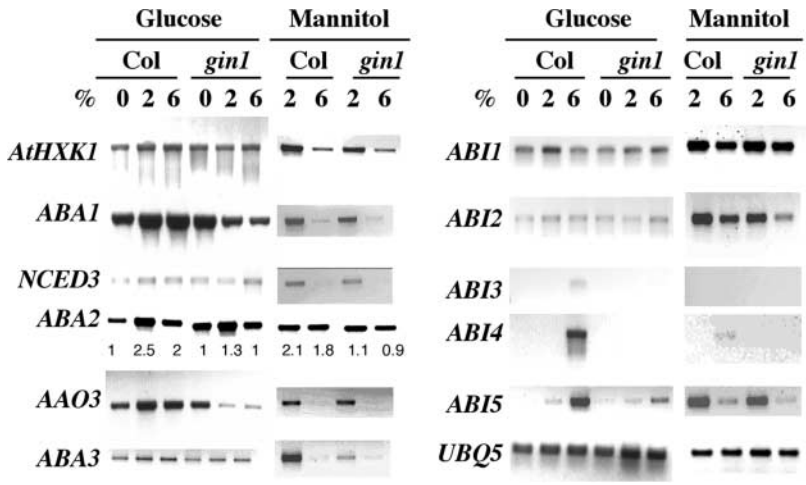

Figure 7. Glc Regulation of Gene Expression.

Glc regulation of ABA biosynthesis and signaling genes. Plants were grown on MS plates without (0) and with 2 and 6% Glc or 2 and 6% mannitol for 14 days under a 16/8-h photoperiod. Mannitol treatment was included as an osmotic control. RT-PCR analyses were performed four times with consistent results. The numbers below the *ABA2* gels indicate the band intensity (quantified with 1D Gel Analysis Software; Phoretix, Durham, NC) relative to the loading control, *UBQ5*, and are normalized to the "0" sample. The PCR product of *ABA2* in *gin1-3* is 53 bp shorter than that in the wild type as a result of a deletion in the mutant.

Restricted Spatial and Temporal Expression of *ABA2/GIN1*

To determine the spatial and temporal expression patterns of *ABA2/GIN1* more precisely, the *ABA2/GIN1* promoter (2906 bp upstream of the ATG start codon) was fused to a reporter gene encoding β-glucuronidase (GUS) to generate *GIN1*::*GUS*. GUS was chosen for its high sensitivity because *ABA2/GIN1* is not highly expressed. The promoter was used successfully for complementation in the 8-kb genomic clone. The *GIN1*::*GUS* fusion was introduced into the wild type and the *gin1-3* null mutant through Agrobacterium-mediated transformation (Jang et al., 2000). In general, *GIN1*::*GUS* had similar expression patterns in multiple independent transgenic lines of both the wild-type and *gin1* backgrounds under normal growth conditions, based on staining with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid. However, GUS staining usually was stronger in *aba2/gin1* than in the wild type, suggesting negative feedback control by ABA in the absence of high exogenous Glc (see below). GUS staining was readily visible in vascular bundles of hypocotyls (Figures 8A and 8B) and the branching points of lateral and primary roots (Figure 8C) of wild-type and *aba2/gin1* transgenic seedlings grown on soil for 10 days after germination. GUS staining in hypocotyls became weak and started to appear in petioles of cotyledons and true leaves at 20 days (Figures 8D and 8E). The staining signal in petioles of cotyledons and the first pair of true leaves became more pronounced at 30 days, especially in the *aba2/gin1* background (Figure 8F). The staining at branching points of primary and lateral roots remained visible at maturity.

In adult plants (after 30 days), pollen showed strong GUS staining in opening and opened flowers but not in flower buds and unopened flowers (Figure 8G). There was strong GUS staining at the junctions of pedicels and young developing siliques (Figure 8H) but not before petal, anther, and sepal abscission (Figure 8G), suggesting a role of ABA in early embryogenesis. At a later stage (57 days), GUS staining appeared in the vascular bundles of petioles and the mid veins of aged rosette leaves, stems, and the junctions of pedicels and siliques (data not shown). GUS staining was strong in septa and funiculi inside maturing siliques but was faint in seeds (Figure 8I) and developing embryos (data not shown). These GUS staining results are consistent with RNA gel blot analyses of *ABA2/GIN1* expression in specific tissues (Figure 6A). It appears that *ABA2/GIN1* has restricted expression patterns in hypocotyls, petioles, leaf veins, stems, flowers, pedicels, septa, funiculi, and roots but is not abundant in the ABA target sites, such as mesophyll cells, guard cells, and seeds.

Distinct Glc Regulation of the *ABA2/GIN1* Promoter

Although the expression of *ABA2/GIN1* does not seem to be affected significantly by drought or ABA, based on RNA gel blot analysis in the wild type, we examined GUS staining to determine whether the expression pattern changes as a result of salt stress (250 mM NaCl for 6 h) and cold stress (4°C

for 6 h) in the wild type and *aba2/gin1*. For salt or cold treatment of 10-day-old seedlings, GUS staining showed no detectable changes in the wild type (Figures 8A, 8B, 8J, and 8L). However, GUS staining showed a significant increase in vascular bundles of cotyledons in *aba2/gin1* (Figures 8K and 8M). Because *ABA2/GIN1* showed a slight downregulation by ABA in RNA gel blot analysis (Figure 6C), the enhanced GUS staining may reflect the lack of this negative ABA feedback control in *aba2/gin1*.

Because *ABA2/GIN1* expression was enhanced by Glc (Figure 7), we examined the GUS expression patterns in both the wild type and *aba2/gin1* in the presence of 2 and 6% Glc. Consistent with the results of RNA gel blot analysis (Figure 7), the presence of 2% Glc or mannitol enhanced GUS expression significantly, which is restricted to the vascular bundles of cotyledons in addition to hypocotyls and roots (Figures 8N, 8O, 8Q, and 8R). Although the *aba2/gin1* mutant transcript was not enhanced by 2% Glc (Figure 7), the *ABA2/GIN1* promoter still was activated by Glc in *gin1* (Figure 8O). This result suggests a direct regulation of the *ABA2/GIN1* promoter by Glc independent of ABA. The stability of the *ABA2/GIN1* transcript may be ABA dependent. Importantly, 6% Glc activated the *ABA2/GIN1* promoter more dramatically and caused developmental arrest in the wild type (Figure 8P). Both Glc and ABA were required for the effect of 6% Glc, because the intense GUS staining and developmental arrest were not observed in *aba2/gin1* (Figure 8P). Treatment with 6% mannitol to induce osmotic stress did not mimic the effect of 6% Glc in the wild type and *aba2/gin1*. However, 6% mannitol did result in more intense and broader GUS staining in the whole seedlings, including the root tips (Figures 8S and 8T). These results are consistent with the idea that Glc and mannitol exert distinguishable regulatory mechanisms in ABA biosynthesis and seedling development. A quantitative GUS assay further supported the distinct responses of the *ABA2/GIN1* promoter to 6% Glc and 6% mannitol (Figure 9). There appear to be multiple mechanisms for the regulation of the *ABA2/GIN1* promoter and *ABA2/GIN1* transcripts (Figures 6 to 9). The dynamic regulation of *ABA2/GIN1* and the distinct 6% Glc regulation of other ABA biosynthesis and signaling genes (Figure 7) seem to be important for the Glc-dependent developmental arrest (Figure 8P).

Subcellular Localization and Biochemical Analyses of ABA2/GIN1

It has been suggested that the complete ABA biosynthesis pathway could exist inside chloroplasts (Milborrow, 2001). However, biochemical analyses of ABA biosynthetic enzymes have indicated that the last steps of ABA biosynthesis may occur in the cytosol (Sindhu and Walton, 1987). To determine the subcellular localization of the ABA2/GIN1 protein in Arabidopsis, a GFP fusion protein, GIN1-GFP, was generated. Using the Arabidopsis protoplast transient expression assay and confocal microscopy, we showed that GIN1-GFP was localized exclusively in the cytosol, similar to a cytosolic GFP marker used as a control (Figure 10A) (Hwang and Sheen, 2001).

To determine whether the Glc-insensitive phenotype of *aba2/gin1* is attributable to a deficiency in the Glc induction of ABA accumulation, the endogenous levels of ABA were measured in wild-type, *aba2/gin1*, and transgenic seedlings complemented with the *ABA2/GIN1* genomic clone. As shown in Figure 10B, *aba2/gin1* seedlings had a low basal ABA level that was not induced by Glc, as in the wild type. The *ABA2/GIN1* genomic clone complemented the basal and Glc-induced levels of ABA in transgenic plants (Figure 10B), supporting a critical role of *ABA2/GIN1* in endogenous ABA biosynthesis and in Glc responses. The growth-promoting and growth-inhibiting dual functions of ABA may have different threshold levels.

Although it has been suggested that xanthoxin is converted to ABAld by SDR1 and then to ABA by AAO3 (Sindhu and Walton, 1987; Zeevaart and Creelman, 1988; Schwartz et al., 1997a; Seo et al., 2000a, 2000b), the possibility remained that xanthoxic acid but not xanthoxin is the substrate for SDR1 (Cowan, 2000; Seo and Koshiba, 2002). Moreover, because three chemical reactions are required to convert xanthoxin to ABAld, it is unclear whether enzymes other than SDR1 might be required (Cowan, 2000; González-Guzmán et al., 2002; Seo and Koshiba, 2002). To determine the SDR1 substrate specificity and whether SDR1 alone can catalyze multiple chemical reactions, recombinant SDR1 was expressed in the yeast *Pichia pastoris* for in vitro enzymatic analyses (Koiwai et al., 2000). His-tagged SDR1 was purified to homogeneity using ammonium sulfate fractionation, Ni^{2+} column chromatography, and DEAE column chromatography and analyzed by SDS-PAGE, as shown in Figure 10C. The purified recombinant SDR1 catalyzed xanthoxin (K_m of 20.2 μM) but not xanthoxic acid as a substrate and generated a product with the same retention time as that of ABAld, as detected by HPLC and gas chromatography–mass spectrometry with a mother fragment with a mass-to-charge ratio of 246 (Figure 10D). The reaction absolutely required a coenzyme NAD (data not shown). Consistent with these results, AAO3 did not use xanthoxin as a substrate, as determined by HPLC (data not shown). Thus, our data provide conclusive evidence that the final steps of the major endogenous ABA biosynthesis pathway involve two enzymes, SDR1 and AAO3, which convert xanthoxin to ABAld and then convert ABAld to ABA, respectively.

DISCUSSION

ABA in Glc Signaling

Genetic and phenotypic analyses of the Arabidopsis mutants displaying *gin* and *glo* phenotypes have revealed

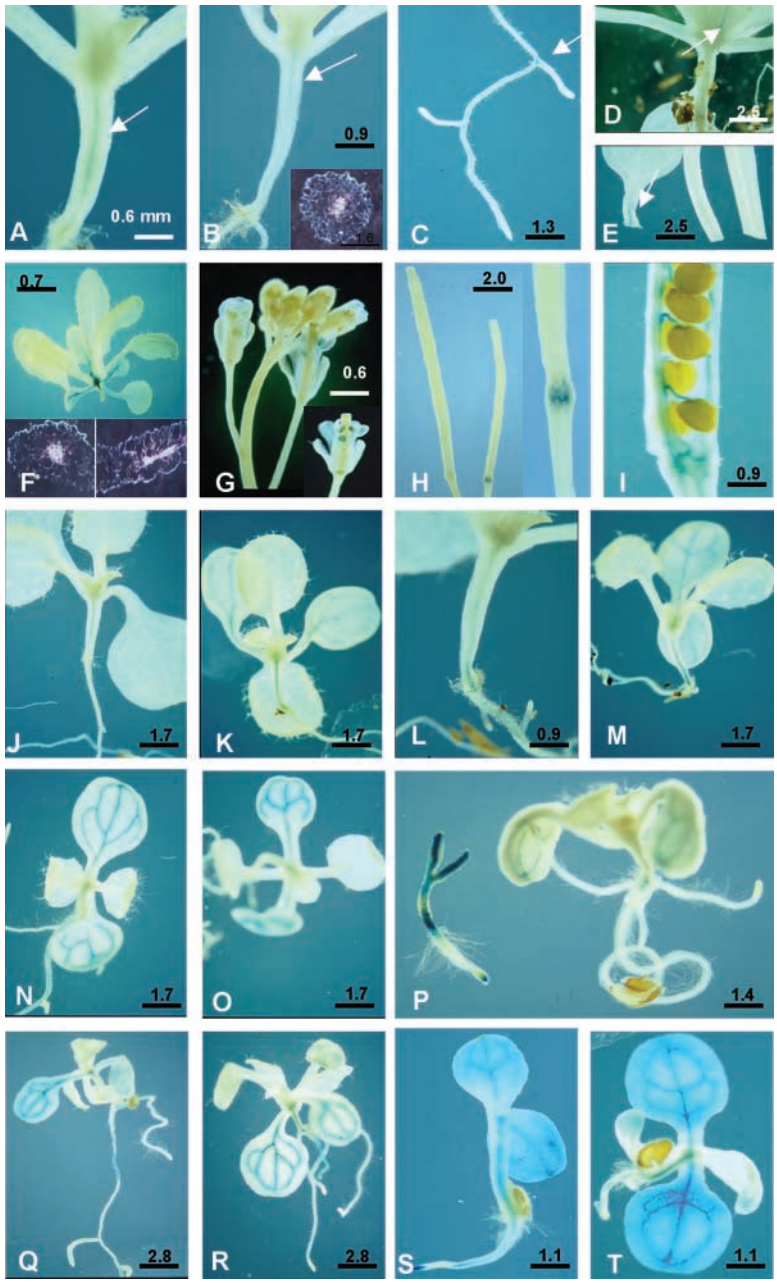

Figure 8. Spatial and Temporal Expression Patterns of *ABA2*/*GIN1*.

(A) GUS staining in the hypocotyl of a wild-type plant grown on soil for 10 days. Three independent transgenic lines displayed similar patterns. The experiment was repeated twice with consistent results.
(B) GUS staining in the hypocotyl of a *gin1* plant grown on soil for 10 days. A cross-section of a hypocotyl showing GUS staining in the vascular bundle is shown in the inset. The experiment was repeated twice with consistent results.
(C) GUS staining in a wild-type root showing strong signals at the branching point.
(D) GUS staining in a wild-type plant grown on soil for 20 days.
(E) GUS staining in the cotyledon and first and second true leaves (left to right) excised from the plant shown in **(D)**.
(F) GUS staining in a *gin1* plant grown on soil for 30 days. The cross-sections of a petiole (left inset) and a leaf vein (right inset) showing GUS staining in the vascular tissues are shown. Wild-type plants displayed similar GUS staining patterns.
(G) GUS staining in the pollen of opened flowers in wild-type plants. Young flowers and flower buds did not show GUS staining.
(H) GUS staining at the junctions of pedicels and young siliques in wild-type plants.

extensive interactions between the metabolic and hormonal signaling pathways (Zhou et al., 1998; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Gibson et al., 2001; Rook et al., 2001). Our studies provide molecular evidence for direct and extensive Glc control of the genes important for ABA biosynthesis and signaling. Interestingly, in the case of *ABA2*/*GIN1*, *ABI3*, and *ABI4*, Glc and ABA act synergistically. These genes are not activated by ABA alone, but their Glc activation requires endogenous ABA and is abolished in the *aba2*/*gin1* mutant (Figures 6 and 7) (Arenas-Huertero et al., 2000; Söderman et al., 2000). In most cases, plants display different responses to Glc and mannitol signals. Thus, our studies show that Glc signaling is mediated partly by the activation of ABA biosynthesis and signaling genes through two mechanisms—a synergistic effect of Glc and ABA, and an ABA positive feedback control loop—because *ABA1*/*ZEP*, *AAO3*, *ABA3*, and *ABI5* also are activated by ABA (Figures 6 and 7) (Leung et al., 1997; Lopez-Molina et al., 2001; Xiong et al., 2001, 2002). The synergistic interaction between Glc and ABA also controls seedling development. Increased ABA accumulation stimulated by a high level of Glc (Figure 10B) is required to block the post-germination developmental switch, demonstrating ABA's function as a growth inhibitor. However, this Glc induction of ABA levels and ABA biosynthesis gene expression is not the result of differences in developmental stage and morphology in seedlings. As shown previously, Glc induction of ABA levels occurs in *gin6*/*abi4* without a developmental arrest phenotype (Arenas-Huertero et al., 2000).

Our studies also reveal that Glc antagonizes the responses of endogenous ethylene through ABA. For example, the Glc hypersensitive phenotypes of *ein2* and the Glc repression of an ethylene maker gene, *PDF1.2*, are eliminated in the *aba2*/*gin1* mutant. In this condition, ethylene acts as a growth-promoting regulator (Zhou et al., 1998), as has been shown in hypocotyl elongation under light (Smalle et al., 1997; Alonso et al., 1999). In the absence of a high level of Glc and at basal ABA levels, the physiological roles of endogenous ABA and ethylene are reversed. In addition, ethylene also may repress ABA biosynthesis, as observed in the *ein2* mutant (Ghassemian et al., 2000), or ABA signaling (Beaudoin et al., 2000). Thus, Glc signals can profoundly influence plant hormone biosynthesis and actions and plant growth and development (Figure 11).

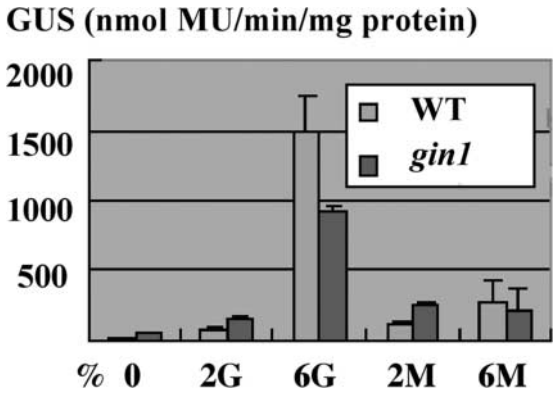

Figure 9. Glc Regulation of *ABA2*/*GIN1* Promoter Activity.

GUS activity was measured in 16-day-old wild-type and *gin1* mutant plants carrying *GIN1*::*GUS* and grown on MS plates without (0) or with 2 and 6% Glc (2G and 6G) or 2 and 6% mannitol (2M and 6M). Results from duplicate samples in two independent experiments are shown with error bars. MU, methylumbelliferone; WT, wild type.

Distinct Glc and Osmotic Signaling

The expression of *NCED* is highly activated by stress conditions and encodes a rate-limiting enzyme for the stress-inducible accumulation of ABA in many plant species (Tan et al., 1997; Qin and Zeevaart, 1999; Iuchi et al., 2000, 2001). Here, we show that Glc does not activate *NCED3* significantly in Arabidopsis. This finding suggests that stress- and Glc-induced ABA accumulation is mediated by distinct

Figure 8. (continued).

(I) GUS staining in a mature wild-type silique at 57 days after germination.
(J) GUS staining in a wild-type seedling (grown on soil for 10 days) after cold treatment (4°C) for 6 h.
(K) GUS staining in a *gin1* seedling (grown on soil for 10 days) after cold treatment (4°C) for 6 h.
(L) GUS staining in a wild-type seedling (grown on soil for 10 days) after NaCl treatment (250 mM) for 6 h.
(M) GUS staining in a *gin1* seedling (grown on soil for 10 days) after NaCl treatment (250 mM) for 6 h.
(N) GUS staining in a wild-type seedling grown on 2% Glc MS medium for 10 days.
(O) GUS staining in a *gin1* seedling grown on 2% Glc MS medium for 10 days.
(P) GUS staining in wild-type (left) and *gin1* (right) plants grown on 6% Glc MS medium for 14 days.
(Q) GUS staining in a wild-type seedling grown on 2% mannitol MS medium for 10 days.
(R) GUS staining in a *gin1* seedling grown on 2% mannitol MS medium for 10 days.
(S) GUS staining in a wild-type seedling grown on 6% mannitol MS medium for 10 days.
(T) GUS staining in a *gin1* seedling grown on 6% mannitol MS medium for 10 days.

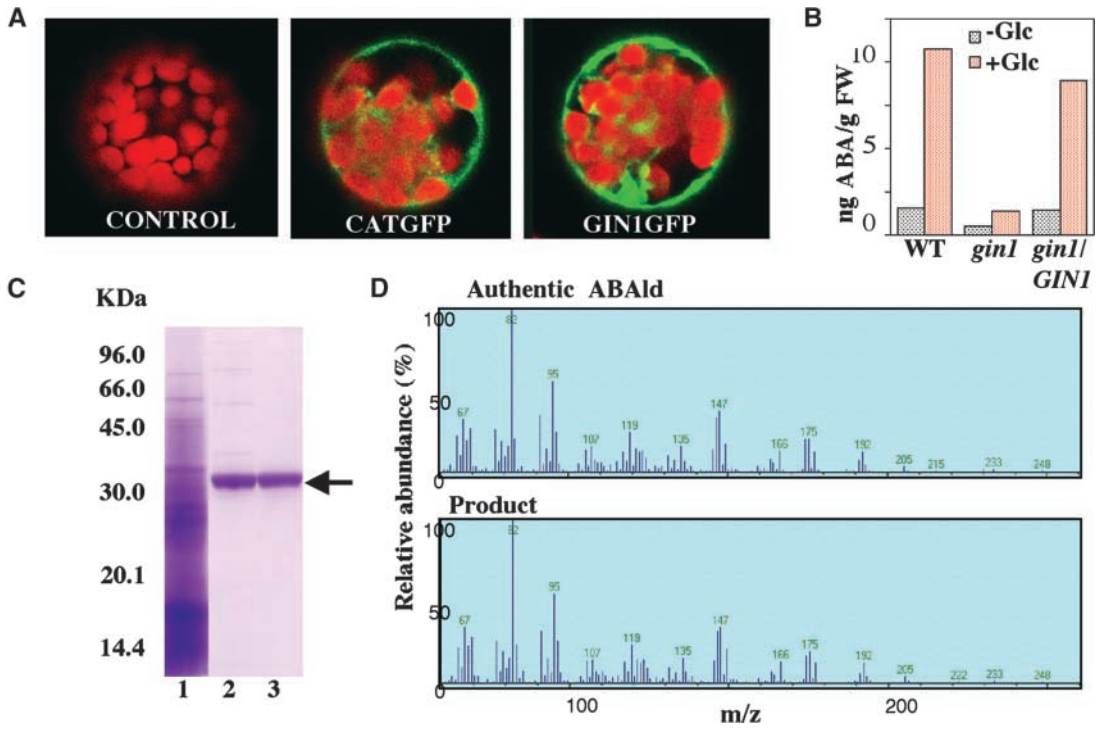

Figure 10. ABA2/GIN1 Subcellular Localization and Biochemical Activities.

(A) GIN1-GFP localization. Arabidopsis mesophyll protoplasts expressing a cytosolic GFP marker (CATGFP) or GIN1-GFP were examined by confocal microscopy. Chloroplasts show red autofluorescence. These mesophyll protoplasts have large vacuoles.

(B) Glc induces ABA accumulation. Wild-type (WT), *gin1*, and complemented (*gin1/GIN1*) seedlings were grown without (−Glc) or with (+Glc) 6% Glc for 10 days. Glc-induced ABA accumulation was abolished in *gin1*. Results shown are averages of two experiments. FW, fresh weight.

(C) Purification of the recombinant ABA2/GIN1/SDR1 protein. His-tagged ABA2/GIN1/SDR1 protein expressed in *P. pastoris* was purified by ammonium sulfate fractionation (0 to 45% saturation) (lane 1), Ni^{2+} column (stepwise) chromatography (lane 2), and DEAE column chromatography (lane 3). Protein fractions from each step were subjected to SDS-PAGE and stained with Coomassie blue. The arrow indicates the ABA2/GIN1/SDR1 protein.

(D) Mass spectra of authentic ABAld and the product from xanthoxin catalyzed by ABA2/GIN1/SDR1. GC-MS fragment patterns of ABAld (top spectrum) and the reaction product (relative abundance) of xanthoxin by ABA2/GIN1/SDR1 (bottom spectrum) are shown.

mechanisms. This notion is consistent with the differences in seedling morphology and *GIN1*::*GUS* expression patterns after 6% Glc and 6% mannitol treatments. *aba2/gin1* mutant alleles have been obtained from a variety of other screens for Suc mutants (*sis* and *isi*) (Laby et al., 2000; Rook et al., 2001) and salt resistance mutants (*sañ* and *sre*) (Quesada et al., 2000; González-Guzmán et al., 2002). The common feature of these mutations is the important role of ABA in sugar and salt/osmotic signaling, although Glc and stress signaling are distinct in their ABA regulation.

For the Glc bioassay under which *gin* and *glo* mutants were screened, the imbibition phase and the radicle protrusion of Arabidopsis seeds (Bewley, 1997; Koornneef et al., 2001) are not blocked by Glc (Jang et al., 1997; Zhou et al., 1998). However, excess Glc signals block the switch to postgermination development, including cotyledon greening and expansion, as well as apical shoot meristem and root differentiation (Jang et al., 1997; Zhou et al., 1998). This developmental arrest imposed by Glc presumably signals a feedback regulation in the source tissues, the seeds that provide carbon nutrients for seedling growth. Under natural conditions, an inappropriate environment could limit early seedling growth and sugar consumption after germination, resulting in Glc accumulation and developmental arrest. This safeguard process for early seedling development involves newly synthesized and increased ABA (Figure 10B) and transcription factors ABI3, ABI4, and ABI5 controlled by Glc (Figure 7). Glc regulation differs from osmotic, salt, and drought stress controls (Leon-Kloosterziel et al., 1996; Quesada et al., 2000; González-Guzmán et al., 2002) by modulating additional signaling processes of other plant hormones, including ethylene, auxin, and cytokinin (Zhou et al., 1998; Sheen et al., 1999; Rolland et al., 2002; L. Zhou and J. Sheen, unpublished data). Feedback control by Glc is well documented in other types of

source tissues, such as photosynthetic leaves, that provide carbon nutrients for the development of sink tissues, including young leaves, roots, stems, flowers, and seeds (Sheen, 1994; Koch, 1996; Stitt and Krapp, 1999; Wobus and Weber, 1999; Smeekens, 2000; Rolland et al., 2002). We propose that endogenous Glc signals could be indicators of the plant growth environment and are monitored in different cell types and organs to adjust source (carbon export) and sink (carbon import) interactions through the regulation of genes involved in plant hormone synthesis and actions and in metabolism, thus coordinating plant growth and development with the environment.

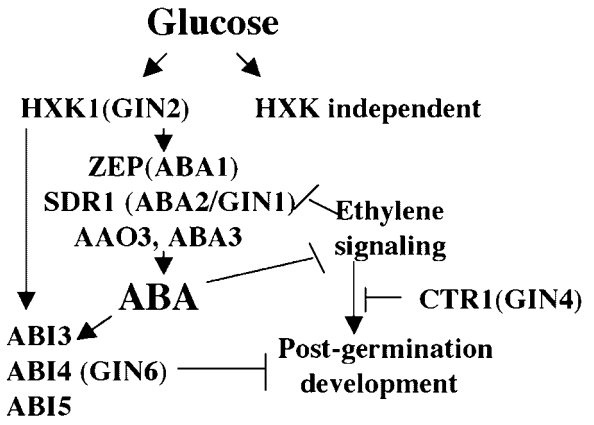

Figure 11. Model of the Interplay of the Glc, ABA, and Ethylene Signaling Pathways.

This model is based on postgermination development phenotypes of Arabidopsis seedlings.

ABA in Growth Promotion

ABA has long been defined as a stress hormone and growth inhibitor because of its roles in stress responses and its inhibitory growth effects when applied exogenously. However, the growth retardation phenotypes of ABA-deficient mutants have implicated an essential function of endogenous ABA in the promotion of leaf and stem development (Koornneef, 1986; Zeevaart and Creelman, 1988; Rock and Zeevaart, 1991; Finkelstein and Zeevaart, 1994; Nambara et al., 1998; Zhou et al., 1998; Sharp et al., 2000). It has been suggested that the pleiotropic phenotypes of ABA-deficient mutants are the result of auxin and ethylene production and/or an indirect effect of photoprotection or water relations (Koornneef, 1986; Sharp et al., 2000). The Arabidopsis ABA-insensitive mutants *abi1*, *abi2*, *abi3*, *abi4*, and *abi5* do not display overt morphological abnormality, probably as a result of tissue or pathway specificity and/or functional redundancy (Finkelstein and Zeevaart, 1994; Rock and Quatrano, 1995; Leung and Giraudat, 1998; Söderman et al., 2000). Analyses of Arabidopsis mutants in the ABA biosynthesis pathway, such as *aba1*, *aba3*, *aao3*, and the *NCED3* knockout mutant, have not offered a solution because the *ABA1* and *ABA3* gene products have functions other than ABA biosynthesis (Koornneef, 1986; Leon-Kloosterziel et al., 1996; Schwartz et al., 1997a), whereas the *aao3* mutant and a *NCED3* knockout mutant lack severe morphological phenotypes, presumably as a result of functional redundancy provided by other members of the multigene families (Seo et al., 2000a; Iuchi et al., 2001; Seo and Koshiba, 2002). Molecular cloning and BLAST searches have revealed that *ABA2/GIN1* encodes a unique SDR1 specific to the ABA biosynthesis pathway. At present, the *aba2* mutants are available only in Arabidopsis (Seo and Koshiba, 2002). Thus, analyses of the Arabidopsis *aba2/gin1* null mutants and complementation by *ABA2/GIN1* in transgenic plants provide an opportunity to reevaluate the physiological functions of ABA in growth promotion. It is clear that endogenous ABA is important for cotyledon, leaf, root, stem, and silique development and fertility (Figures 4, 5, and 12). Blocking ethylene signaling partially restores root growth and development in *aba2/gin1*, but other phenotypes are independent of ethylene, even when grown under high-humidity conditions.

The size and morphology of flowers and mature seeds in *aba2/gin1* are similar to those in the wild type (data not shown). However, the smaller *aba2/gin1* siliques have many unfertilized ovaries and/or aborted embryos at early stages and produce many fewer mature seeds than do wild-type siliques (Figures 5E and 5F). ABA may play a role in the early embryo differentiation found during the study of somatic embryogenesis (Zimmerman, 1993; Rock and Quatrano, 1995). The striking expression of *GIN1*::*GUS* at the junction of the pedicel and the young silique is consistent with such a role (Figure 8H). The precise stage and mechanisms of ABA function during early embryogenesis require further investigation. The expression of *GIN1*::*GUS* also is prominent in the septa and funiculi of mature siliques (Figure 8I), suggesting a maternal supply of ABA for seed maturation, dormancy, and/or desiccation (Figure 12), as shown by genetic analysis in Arabidopsis (Raz et al., 2001).

Analysis of the *aba2/gin1* mutants also supports the idea that preexisting ABA is critical to rapid guard cell signaling (within minutes) in response to water stress (Figure 12). Stress-induced ABA accumulation requires de novo transcription and translation of NCED as a rate-limiting enzyme and takes hours to occur (Qin and Zeevaart, 1999; Iuchi et al., 2000). Overexpression of *NCED3* in transgenic plants enhances drought tolerance over that of the wild type (Iuchi et al., 2001; Qin and Zeevaart, 2002), indicating that high ABA levels may be important for long-term survival under severe growth conditions. Although not inducible under stress conditions, *ABA2/GIN1* plays a key role in the support of many physiological functions of ABA that do not require induction by stress. There could be a physiological connection between Glc and ABA at lower endogenous levels that promotes rather than inhibits plant growth.

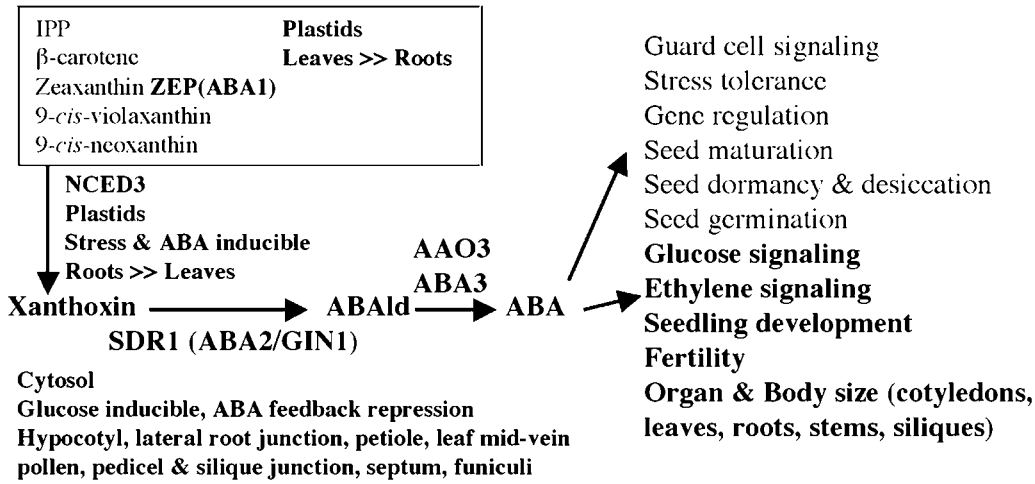

Figure 12. Proposed ABA Biosynthesis Pathway and Functions.

The ABA biosynthesis steps that are expressed preferentially in leaves are highlighted in the box. It is likely that early ABA precursors have distinct functions in photosynthesis and light stress protection. It is not clear whether these ABA precursors are transported from leaves to petioles, stems, and roots. The expression of NCED3 and SDR1 is regulated differently. Xanthoxin needs to be exported from plastids to be converted to ABAld by SDR1 in the cytosol. The active sites of the *GIN1* promoter are listed. New ABA functions presented in this study are listed in boldface.

ABA Biosynthesis Pathway and Regulation

ABA is a sesquiterpenoid (C_{15}) and is produced from an indirect pathway from the cleavage products of carotenoids (C_{40}) in the plastids (Figure 12) (Zeevaart and Creelman, 1988; Seo and Koshiba, 2002). The cloning and biochemical analysis of the first ABA pathway–specific enzyme, NCED, encoded by maize *VP14*, provided conclusive evidence that the ABA precursor xanthoxin is generated from the cleavage of 9-*cis*-epoxycarotenoids by NCED (Schwartz et al., 1997b; Tan et al., 1997). Cellular expression and chloroplast import studies show that NCED is localized in the chloroplasts (Figure 12) (Qin and Zeevaart, 1999; Iuchi et al., 2000; Tan et al., 2001). Recent genetic and biochemical characterizations of the Arabidopsis *aba2*, *aao3*, and *aba3* mutants and the tomato *sitiens* and *flacca* mutants have supported the conversion of xanthoxin to ABAld and ABA as the final steps (Figure 12) (Leon-Kloosterziel et al., 1996; Schwartz et al., 1997a; Sagi et al., 1999; Seo et al., 2000a, 2000b; Seo and Koshiba, 2002). However, the location at which, and the chemical reactions by which, xanthoxin is converted to ABA remain controversial. For example, the final steps of ABA biosynthesis have been observed to occur in both chloroplasts and cytosol (Sindhu and Walton, 1987; Milborrow, 2001). Abscisic alcohol and xanthoxic acid, instead of ABAld, have been proposed to be metabolic intermediates (Cutler and Krochko, 1999; Cowan, 2000; Milborrow, 2001; Seo and Koshiba, 2002). Our cellular analysis of GIN1-GFP localization and biochemical analyses of the purified enzyme support the conversion of xanthoxin to ABAld by SDR1 in the cytosol in plants (Figure 12). This physiological function of SDR1 is further confirmed by the fact that SDR1 cannot use xanthoxic acid as a substrate and AAO3 can use only ABAld but not xanthoxin as a substrate. However, other minor ABA biosynthesis pathways may exist in *aba2*/*gin1* null mutants, and these require further investigation (Cowan, 2000; Seo and Koshiba, 2002).

Many genes involved in the biosynthesis of plant growth hormones, such as gibberellins, brassinosteroids, and sterols, are expressed in actively growing cells and in the target sites (Silverstone et al., 1997; Jang et al., 2000; Bishop and Yokota, 2001). The enzymes that catalyze the conversion of xanthoxin to ABA were thought previously to be ubiquitous and expressed constitutively (Sindhu and Walton, 1987; Schwartz et al., 1997a; Sagi et al., 1999; Seo et al., 2000a). Unexpectedly, the spatial and temporal expression patterns of *ABA2*/*GIN1* are restricted to specific areas of vascular tissues and are distinct from the tissue expression patterns of other genes, such as *ZEP*, *NCED3*, *AAO3*, and *ABA3*, that are important for ABA biosynthesis (Audran et al., 1998; Qin and Zeevaart, 1999; Iuchi et al., 2000, 2001; Thompson et al., 2000; Xiong et al., 2001, 2002; M. Seo and T. Koshiba, unpublished data). These results indicate that interorgan, intercellular, and interorganelle transport of ABA precursors and/or ABA may be required for the completion of ABA synthesis and to reach target sites (Figure 12). Additional cellular analyses of promoter fusions and immunolocalization of the ABA biosynthesis enzymes and ABA will be required to understand the physical location and regulation of the ABA biosynthesis pathway.

Physiological Functions of the SDR Superfamily in Eukaryotes

The SDR superfamily represents a very large family of enzymes, most of which are known to be NAD- or NADP-dependent oxidoreductases from *E. coli* to humans. Because the first characterized member of this family was the novel alcohol dehydrogenase in *Drosophila*, this family used to be called "insect-type" or "short-chain" alcohol dehydrogenase. The discovery of 15-hydroxyprostaglandin dehydrogenase as the first mammalian SDR brought general interest to SDR functions (Jörnvall, 1999). The first cloned plant *SDR* gene was maize *TS2*, which is involved in sex determination by controlling cell death through unknown mechanisms (DeLong et al., 1993). It was suggested, based on its sequence homology with hydroxysteroid dehydrogenases (HSDs) in prokaryotes, that ZmTS2 might control plant steroid metabolism (DeLong et al., 1993). However, sequence alignment revealed that the putative Arabidopsis HSD is evolutionarily distant from ABA2/GIN1/SDR1, SDR2, and ZmTS2 (Figure 3). Although many plant genes involved in brassinosteroid and sterol biosynthesis have been isolated using mutants in Arabidopsis, none encodes SDR (Bishop and Yokota, 2001). The molecular cloning of *ABA2/GIN1* reveals the functional significance of plant SDR in ABA biosynthesis: it is critical for growth and development, stress responses, and Glc signaling (Figure 12). The tobacco and cucumber *SDR* genes are closely related to *ABA2/GIN1* and may be involved in ABA biosynthesis (Figure 3). It remains to be determined whether other distinct *SDR* genes may provide a low level of activity for ABA biosynthesis with different expression patterns. The Arabidopsis TS2 homolog (SDR2) is related more closely to SDR1 than to HSDs of 56 SDR members in the Arabidopsis genome. It will be interesting to determine the functions of Arabidopsis *SDR2* and *HSD* genes by isolating and characterizing knockout mutants and performing enzymatic assays. In mammals, SDRs play diverse and important roles, as in the metabolism of steroid and retinoid hormones and the interaction with amyloid B-peptide in human Alzheimer's disease (Oppermann et al., 1999, 2001). Functional analyses of plant SDRs may provide useful clues to SDR functions in hormone biosynthesis and the metabolic signaling that controls embryogenesis and postnatal growth and development in mammals.

METHODS

Mutant Isolation and Genetic Analyses

Three *gin1* mutants, *gin1-1* (Wassilewskija [Ws] ecotype), *gin1-2* (Landsberg *erecta* [L*er*]), and *gin1-3* (Columbia [Col] ecotype), and two *gin4* mutants, *gin4-1* (Col) and *gin4-2* (Col), were selected on 7% Glc Murashige and Skoog (1962) plates as described by Zhou et al. (1998). Allelism tests showed that none of the *gin1* or *gin4* mutants complemented each other in the Glc bioassay or in the greenhouse. Although selected from T-DNA–tagged pools, none was tagged by T-DNA. The null mutant *gin1-1* was used for cloning, and the null mutant *gin1-3* was used for all other studies except for those shown in Figure 6A, for which null *aba2-2* was used. All mutants were recessive and backcrossed to the wild type to ensure the cosegregation of phenotypes before extensive fine mapping was performed. For the analysis of *gin1-1* and *gin1-3* reverse transcriptase (RT) PCR products, the following exon 2 primers were used: 5′-AGTGGCATTGATCACTGGAG-3′ and 5′-GTGAATCCTCCATCAATCATC-3′. Note that the 5′ primer sequence was deleted in the *gin1-3* mutant except for the initial AGT.

Map-Based Cloning of *GIN1*

The mapping population was generated by crossing *gin1-1* (Ws) to the Col or L*er* wild type. F2 populations of *gin1-1* mutants (5627) were isolated on 7% Glc Murashige and Skoog (1962) plates. Genomic cDNA was extracted to perform PCR using simple sequence length polymorphism (SSLP) markers to identify recombinants as described previously (Bell and Ecker, 1994). Fine mapping was performed by designing new SSLP markers. The primers were synthesized based on BAC DNA sequences and tested by PCR using DNA isolated from three ecotypes. New SSLP primer sequences and PCR fragment sizes (Col/L*er*/Ws, in bp) for fine mapping are listed below: F6D8-33-A3, 5′-ACATGGCTAGAAGGTGGAGAC-3′, and F6D8-33-B3, 5′-ATATTATGCTCCAGTATTTAGG-3′ (183/194/183); F6D8-3-A1, 5′-TCTACATCTAGACGGGAAGG-3′, and F6D8-3-B1, 5′-CGCCATTGCTTCTCGACTTG-3′ (1140/900/1140); F5F19-19-A3, 5′-ACGTGTTCACAGCAACATCAAG-3′, and F5F19-19-B3, 5′-GTATTTTCAGACTAAGCTTTTGCC-3′ (96/93/90); F5F19-12-A1, 5′-CCAGTTGCTATCCATATCTATTG-3′, and F5F19-12-B1, 5′-ATTCCTCATATGAGATCCGAGG-3′ (180/194/178); F5F19-1-3A, 5′-CTGTGGTTAGACTGACAAGTG-3′, and F5F19-1-3B, 5′-ACCAAAGAGCCCCTGAAGAC-3′ (105/105/96); F11M15-24-2A, 5′-AATGGTTAGATTCATTAGGATG-3′, and F11M15-24-2B, 5′-TTCTCTGTTTGTGAAGCCAGC-3′ (123/156/123); F11M15-1-A2, 5′-TGGAGTTGCTGAACCTAGCC-3′, and F11M15-1-B2, 5′-ATGGATCGAAAATATGAGTATC-3′ (129/129/120); and F13F21-15-A3, 5′-GTTGAACGGTTTAGATTGAAGG-3′, and F13F21-15-B3, 5′-TACGGAGATGCTTAGCCGAGC-3′ (282/275/211). The full-length cDNA was isolated using 5′ and 3′ rapid amplification of cDNA ends based on the manufacturer's protocol (SMART RACE cDNA Amplification Kit; Clontech, Palo Alto, CA). The primers were located at 42451 to 42473 and 42783 to 42802 bp in the F19K6 BAC clone, with a 351-bp overlap.

Constructs and Transgenic *Arabidopsis thaliana*

The *ABA2/GIN1* genomic clone (7.7 kb), covering 3 and 2 kb of the 5′ and 3′ flanking regions, respectively, was amplified by PCR, confirmed by sequencing, and cloned into a miniature binary vector (Xiang et al., 1999). The *ABA2/GIN1* full-length cDNA was amplified by PCR from a cDNA library (Jang et al., 1997), confirmed by sequencing, and cloned into a binary vector (pSMAB704) under the control of the constitutive 35S promoter to generate *35S*::*GIN1*. The *GIN1*::β-glucuronidase (*GUS*) fusion gene was generated by fusing

the 3-kb 5′ promoter of *GIN1* to *GUS* at the ATG site. The promoter was obtained by PCR from the genomic clone using the following primers with underlined HindIII and SmaI sites, respectively: 5′-ATCTCACTACGGAAGCTTCTGG-3′ and 5′-GTGACCCCGGGAATAGCTTTAGCTCCTTAGATCTTC-3′, starting at nucleotides 38638 and 41545, respectively, in the F19K6 BAC clone. The promoter sequence was confirmed by DNA sequencing and cloned into binary vector pSMAB704. The binary vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. The null *gin1-3* mutant and wild-type plants were transformed by Agrobacterium using the floral-dip method (Hwang and Sheen, 2001). T1 transgenic plants showing herbicide resistance were confirmed further by PCR and DNA gel blot analyses. More than 100 transgenic plants were obtained for all constructs. Ten independent transgenic lines for each of the cDNA and genomic clones were analyzed further. GUS expression pattern analyses were performed with three randomly chosen transgenic lines in the wild type and *gin1-3*. T2 and homozygous T3 plants were used to perform complementation tests and GUS expression pattern analyses as described (Jang et al., 2000).

Subcellular Localization of the GIN1–Green Fluorescent Protein Fusion

The GIN1 coding region was amplified by RT-PCR and fused to green fluorescent protein (GFP) in a plant expression vector for protoplast transient expression analyses, as described previously (Hwang and Sheen, 2001). The following primers with a BamHI site at the 5′ end and a StuI site at the 3′ end (underlined) were used: 5′-AAGAAGATCTAAGGATCCAAAGC-3′ and 5′-ATGCAAGGCCTTCTGAAGACTTTAAAGGAGTGG-3′. RT-PCR amplification of mutant coding regions (*gin1-2*, *gin1-3*, and *aba2-1*) was performed to make GFP fusions. The expression of the *gin1-3*–GFP fusion was not observed in protoplasts, suggesting a null mutation (data not shown).

Protein Sequence Alignment and Phylogenetic Tree Construction

Arabidopsis short-chain dehydrogenase/reductase (SDR)–related sequences (46% identity and 60% similarity) were obtained using the BLAST program from the TAIR World Wide Web site (http://www.arabidopsis.org/Blast), and SDR, 11-β-hydroxysteroid dehydrogenase (HSD), retenoid dehydrogenase, and 15-hydroxyprostaglandin dehydrogenase sequences (30% identity and 50% similarity) from other model systems were obtained using the National Center for Biotechnology Information PSI-BLAST program based on the ABA2/GIN1 sequence (http://www.ncbi.nlm.nih.gov/BLAST). The phylogenic tree was generated using SDR sequences containing motifs I to VI, without the divergent N and C termini, with Clustal X 1.81 (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX) and was displayed with Treeview 1.6.5 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The scale of 0.1 in the unrooted tree (Figure 3) indicates 0.1 amino acid substitutions per site. Arabidopsis SDR2 (62%), SDR3 (62%), SDR4 (61%), and SDR5 (60%) share the highest sequence similarity to SDR1. Based on the Clustal alignment program, Arabidopsis ABA2/GIN1 (*AtSDR1*) is grouped with tobacco (*NtSDR*) and cucumber (*CsSDR*) homologs in the phylogenic tree. AtSDR2 is closely related to maize ZmTS2 and is different from AtSDR3, AtSDR4, and AtSDR5. Arabidopsis 11-β-hydroxysteroid dehydrogenases is closely related to human and mouse 11-β-hydroxysteroid dehydrogenases. The human and mouse 15-hydroxyprostaglandin dehydrogenases and human and mouse retenoid dehydrogenases also are shown in Figure 3.

Expression and Purification of SDR1

For SDR1 expression in *Pichia pastoris*, *ABA2/GIN1* cDNA was cloned into the pPICZC vector to produce a fusion protein with the MYC epitope and polyhistidine tags with an ACC sequence before the initiation ATG codon, according to the manufacturer's instructions (Easy Select Pichia Expression Kit; Invitrogen, Carlsbad, CA). Transformation and expression of recombinant protein were performed as described (Koiwai et al., 2000). The cells were harvested after methanol induction for 48 h. The crude enzyme extract was fractionated with ammonium sulfate (0 to 45% saturation) and dialyzed against 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM EDTA and 5 μM leupeptin. Ammonium sulfate–fractionated enzyme samples were purified with the ProBound protein purification kit (Invitrogen) according to the manufacturer's instructions, except that the elution was performed with 50 mM potassium phosphate buffer, pH 7.5, containing 30 mM EDTA. The eluate was dialyzed against 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM EDTA, 10 mM 2-mercaptoethanol, and 10% glycerol and applied to a DEAE column (DEAE-5PW; 7.5 mm i.d. × 75 mm; TOSOH, Tokyo, Japan). The adsorbed proteins were eluted with a linear gradient of KCl from 50 mM to 0.5 M in 20 mM potassium phosphate buffer, pH 8.0, containing 2.5 mM EDTA, 10 mM 2-mercaptoethanol, and 10% glycerol. SDR1 activity was recovered in the fraction that eluted at ~0.3 M KCl. The purity of recombinant SDR1 was examined by SDS-PAGE and protein staining (Figure 10C).

SDR1 Enzymatic Assay

The enzyme sample used for the activity assay was the fraction purified on the Ni^{2+} column. To determine the K_m value, reaction mixtures (100 μL) containing 5 μL of enzyme solution (~5 μg of protein), 0.01 to 0.1 mM xanthoxin, 50 mM potassium phosphate buffer, pH 7.5, and 5 mM NAD were incubated at 30°C for 5 to 20 min. After incubation, the reaction was stopped by adding 10 μL of 1 N HCl and 30 μL of 100% methanol, successively. After centrifugation, the supernatant (100 μL) was subjected to HPLC. Activity was assayed by determining the amount of abscisic aldehyde product, as described previously (Koshiba et al., 1996). The coenzyme dependence of the reaction was determined using 5 mM NAD, NADH, NADP, or NADPH. To determine the reaction product, the reaction was performed with 1 mL of reaction mixture using enzyme solutions purified on the DEAE column. The reaction product was partially purified by ether fractionation and HPLC and subjected to gas chromatography–mass spectrometry (GC-MS).

Abscisic Acid Determination

Seedlings (0.5 to 1 g) were homogenized in 80% (v/v) acetone containing 0.1 mg/mL 2,6-di-tert-butyl-4-methylphenol. After adding ^{13}C-abscisic acid (ABA) (Asami et al., 1999) as an internal standard, the homogenate was shaken for 1 h on ice in darkness and then centrifuged (1200*g* for 10 min) at 4°C. The precipitate was reextracted, and the combined supernatant was evaporated to remove acetone.

ABA was partially purified from the residual aqueous solution by partitioning using hexane and ether and then by HPLC. ABA was methylated with diazomethane and analyzed by GC–selected ion monitoring–MS by monitoring 192 (from ^{13}C-ABA) and 190 (from endogenous ABA) fragments.

GC-MS Analysis

GC-MS analysis was performed with a mass spectrometer (GCMS-QP5050; Shimadzu, Kyoto, Japan) coupled to a gas chromatograph (GC-17A; Shimadzu) with a DB-1 capillary column (0.25 mm i.d. × 30 m, film thickness of 0.25 μm; J&W Scientific, Folsom, CA). The injection temperature was set at 250°C, and the inlet pressure was 70 kPa. After injection, the oven temperature was maintained at 80°C for 1 min, increased to 290°C at a rate of 20°C/min, and then kept at 290°C for 5 min. Mass spectra were obtained under the following conditions: electron energy of 1.5 kV, ion source temperature of 250°C, capillary interface temperature of 250°C, and ion range of 50 to 450 (mass-to-charge ratio).

Preparation of Xanthoxic Acid

A *cis* and *trans* mixture of 4′-benzoyl xanthoxic acid ethyl ester was synthesized according to the method described previously (Yamamoto and Oritani, 1996). Treatment of this diester with 2.5 equivalent KOH in dry ethanol under reflux conditions gave a potassium salt of the acid. The usual workup gave a *cis* and *trans* mixture of xanthoxic acid in high yield. Each isomer was separated by HPLC to give xanthoxic acid.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

Accession Numbers

The accession number for *ABA2/GIN1* is At1g52340, and that for the F19K6 BAC clone is AC037424. Accession numbers for other sequences mentioned in this article are as follows: *ZmTS2*, L20621; *HsSDR*, NM_016246; *MmSDR*, NM_008278; *DmSDR*, AAF51984; *CeSDR*, Z72508; *ScSDR*, NC_001143; *EcSDR*, NC_000913; *AtSDR1*, AC037424; *NtSDR*, NTAJ3177; *CsSDR*, AF286651; AtSDR2 (AL132968, At3g51680); AtSDR3 (AC004411, At2g47130); AtSDR4 (AB026657, At3g29250); AtSDR5 (AB026657, At3g29260); AtHSD, NM_124448; human HSD, NM_005525; mouse HSD, S75207); human HPGD, NM_000860; mouse HPGD, NM_008278; human RDH, NM_003708, XM_006732; and mouse RDH, AY028928, AF013288.

ACKNOWLEDGMENTS

We thank P. Rodriguez and M. Bevan for sharing unpublished results, M. Koornneef for advice, J. Ecker for *ein2* seeds, and the ABRC (Columbus, OH) for all other ABA and ethylene mutant seeds. H. Ichikawa is thanked for the binary vector pSMAB704, C. Xiang for the miniature binary vector pCB302, and M.R. Willmann for help with the transgenic plants. We also thank Q. Hall for silique pictures, A. Schaeffner for protocols, D. Bongard for BAC DNA sequence annotation, P.-C. Lin for GUS assays, and B. Moore and S.-H. Cheng for help with the confocal microscope. I. Hwang provided a database search. R. Patharkar, G. Tena, and J. Plotnikova are thanked for harvesting and testing transgenic seeds, T. Oritani for kindly providing xanthoxin, and F. Rolland for critical reading of the manuscript. J.S. was supported by grants from the National Science Foundation (IBN-9723610) and the National Institutes of Health (GM60493). T.K. was supported in part by a Grant-in-Aid for Scientific Research (B) (13490024) from the Ministry of Education, Science, Sports, and Culture of Japan. M.S. was supported by a research fellowship from the Japan Society for the Promotion of Science for Young Scientists. P.L. was supported by a grant from the Consejo Nacional de Ciencia y Tecnología (31791) and by the Howard Hughes Medical Institute.

Received July 18, 2002; accepted August 6, 2002.

REFERENCES

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science **284,** 2148–2152.

Arenas-Huertero, F., Arroyo, A., Zhou, L., Sheen, J., and Leon, P. (2000). Analysis of Arabidopsis glucose insensitive mutants, *gin5* and *gin6*, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes Dev. **14,** 2085–2096.

Asami, T., Sekimata, K., Wang, J.M., Yoneyama, K., Takeuchi, Y., and Yoshida, S. (1999). Preparation of (±)-[1,2-$^{13}C_2$]abscisic acid for use as a stable and pure internal standard. J. Chem. Res. (Synop.) **11,** 658–659.

Audran, C., Borel, C., Frey, A., Sotta, B., Meyer, C., Simonneau, T., and Marion-Poll, A. (1998). Expression studies of the zeaxanthin epoxidase gene in *Nicotiana plumbaginifolia*. Plant Physiol. **118,** 1021–1028.

Beaudoin, N., Serizet, C., Gosti, F., and Giraudat, J. (2000). Interactions between abscisic acid and ethylene signaling cascades. Plant Cell **12,** 1103–1115.

Bell, C.J., and Ecker, J.R. (1994). Assignment of 30 microsatellite loci to the linkage map of Arabidopsis. Genomics **19,** 137–144.

Bewley, J.D. (1997). Seed germination and dormancy. Plant Cell **9,** 1055–1066.

Bishop, G.J., and Yokota, T. (2001). Plants steroid hormones, brassinosteroids: Current highlights of molecular aspects on their synthesis/metabolism, transport, perception and response. Plant Cell Physiol. **42,** 114–120.

Coruzzi, G.M., and Zhou, L. (2001). Carbon and nitrogen sensing and signaling in plants: Emerging 'matrix effects.' Curr. Opin. Plant Biol. **4,** 247–253.

Cowan, A.K. (2000). Is abscisic aldehyde really the immediate precursor to stress-induced ABA? Trends Plant Sci. **5,** 191–192.

Cutler, A.J., and Krochko, J.E. (1999). Formation and breakdown of ABA. Trends Plant Sci. **4,** 472–478.

DeLong, A., Calderon-Urrea, A., and Dellaporta, S.L. (1993). Sex

determination gene *TASSELSEED2* of maize encodes a short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. Cell **74,** 757–768.

Finkelstein, R.R., Gampala, S.S.L., and Rock, C.D. (2002). Abscisic acid signaling in seeds and seedlings. Plant Cell **14** (suppl.), S15–S45.

Finkelstein, R.R., and Gibson, S.I. (2001). ABA and sugar interactions regulating development: Cross-talk or voices in a crowd. Curr. Opin. Plant Biol. **5,** 26–32.

Finkelstein, R.R., and Zeevaart, J.A. (1994). Gibberellin and abscisic acid biosynthesis and response. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Gazzarrini, S., and McCourt, P. (2001). Genetic interactions between ABA, ethylene and sugar signaling pathways. Curr. Opin. Plant Biol. **4,** 387–391.

Ghassemian, M., Nambara, E., Cutler, S., Kawaide, H., Kamiya, Y., and McCourt, P. (2000). Regulation of abscisic acid signaling by the ethylene response pathway in Arabidopsis. Plant Cell **12,** 1117–1126.

Gibson, S.I., Laby, R.J., and Kim, D. (2001). The *sugar-insensitive1* (*sis1*) mutant of Arabidopsis is allelic to *ctr1*. Biochem. Biophys. Res. Commun. **280,** 196–203.

González-Guzmán, M., Apostolova, N., Belles, J.M., Barrero, J.M., Piqueras, P., Ponce, M.R., Micol, J.L., Serrano, R., and Rodríguez, P.L. (2002). The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin into abscisic aldehyde. Plant Cell **14,** 1833–1846.

Huijser, C., Kortstee, A., Pego, J., Weisbeek, P., Wisman, E., and Smeekens, S. (2000). The Arabidopsis *SUCROSE UNCOUPLED-6* gene is identical to *ABSCISIC ACID INSENSITIVE-4*: Involvement of abscisic acid in sugar responses. Plant J. **23,** 577–585.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature **413,** 383–389.

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2001). Regulation of drought tolerance by gene manipulation of 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. Plant J. **27,** 325–333.

Iuchi, S., Kobayashi, M., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2000). A stress-inducible gene for 9-*cis*-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. Plant Physiol. **123,** 553–562.

Jang, J.C., Fujioka, S., Tasaka, M., Seto, H., Takatsuto, S., Ishii, A., Aida, M., Yoshida, S., and Sheen, J. (2000). A critical role of sterols in embryonic patterning and meristem programming revealed by the *fakcel* mutants of *Arabidopsis thaliana*. Genes Dev. **14,** 1485–1497.

Jang, J.C., Leon, P., Zhou, L., and Sheen, J. (1997). Hexokinase as a sugar sensor in higher plants. Plant Cell **9,** 5–19.

Jörnvall, H. (1999). Multiplicity and complexity of SDR and MDR enzymes. Adv. Exp. Med. Biol. **463,** 359–364.

Jörnvall, H., Höög, J.O., and Persson, B. (1999). SDR and MDR: Completed genome sequences show these protein families to be large, of old origin, and of complex nature. FEBS Lett. **445,** 261–264.

Koch, K.E. (1996). Carbohydrate modulated gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 509–540.

Koiwai, H., Akaba, S., Seo, M., Komano, T., and Koshiba, T. (2000). Functional expression of two Arabidopsis aldehyde oxidases in the yeast *Pichia pastoris*. J. Biochem. **127,** 659–664.

Koornneef, M. (1986). Genetic aspects of abscisic acid. In A Genetic Approach to Plant Biochemistry, A.D. Blonstein and P.J. King, eds (Vienna: Springer Verlag), pp. 35–54.

Koornneef, M., Bentsink, L., and Hilhorst, H. (2001). Seed dormancy and germination. Curr. Opin. Plant Biol. **5,** 33–36.

Koshiba, T., Saito, E., Ono, N., Yamamoto, N., and Sato, M. (1996). Purification and properties of flavin- and molybdenum-containing aldehyde oxidase from coleoptiles of maize. Plant Physiol. **110,** 781–789.

Laby, R.J., Kincaid, M.S., Kim, D., and Gibson, S.I. (2000). The Arabidopsis sugar-insensitive mutants *sis4* and *sis5* are defective in abscisic acid synthesis and response. Plant J. **23,** 587–596.

Leon-Kloosterziel, K.M., Gil, M.A., Ruijs, G.J., Jacobsen, S.E., Olszewski, N.E., Schwartz, S.H., Zeevaart, J.A., and Koornneef, M. (1996). Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. Plant J. **10,** 655–661.

Leung, J., and Giraudat, J. (1998). Abscisic acid signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. **49,** 199–222.

Leung, J., Merlot, S., and Giraudat, J. (1997). The Arabidopsis *ABSCISIC ACID-INSENSITIVE2* (*ABI2*) and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell **9,** 759–771.

Lopez-Molina, L., Mongrand, S., and Chua, N.H. (2001). A post-germination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proc. Natl. Acad. Sci. USA **98,** 4782–4787.

Milborrow, B.V. (2001). The pathway of biosynthesis of abscisic acid in vascular plants: A review of the present state of knowledge of ABA biosynthesis. J. Exp. Bot. **52,** 1145–1164.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 473–497.

Nambara, E., Kawaide, H., Kamiya, Y., and Naito, S. (1998). Characterization of an *Arabidopsis thaliana* mutant that has a defect in ABA accumulation: ABA-dependent and ABA-independent accumulation of free amino acids during dehydration. Plant Cell Physiol. **39,** 853–858.

Oppermann, U., Salim, S., Hult, M., Eissner, G., and Jörnvall, H. (1999). Regulatory factors and motifs in SDR enzymes. Adv. Exp. Med. Biol. **463,** 365–371.

Oppermann, U.C., Filling, C., and Jörnvall, H. (2001). Forms and functions of human SDR enzymes. Chem. Biol. Interact. **130–132,** 699–705.

Qin, X., and Zeevaart, J.A. (1999). The 9-*cis*-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. Proc. Natl. Acad. Sci. USA **96,** 15354–15361.

Qin, X., and Zeevaart, J.A. (2002). Overexpression of a 9-*cis*-epoxycarotenoid dioxygenase gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. Plant Physiol. **128,** 544–551.

Quesada, V., Ponce, M.R., and Micol, J.L. (2000). Genetic analysis of salt-tolerant mutants in *Arabidopsis thaliana*. Genetics **154,** 421–436.

Raschke, K., Firn, R.D., and Pierce, M. (1975). Stomatal closure in response to xanthoxin and abscisic acid. Planta **125,** 149–160.

Raz, V., Bergervoet, J.H., and Koornneef, M. (2001). Sequential steps for developmental arrest in Arabidopsis seeds. Development **128,** 243–252.

Rock, C.D., and Quatrano, R. (1995). The role of hormones during seed development. In Plant Hormones: Physiology, Biochemistry and Molecular Biology, P.J. Davies, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 671–697.

Rock, C.D., and Zeevaart, J.A. (1991). The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. Proc. Natl. Acad. Sci. USA **88,** 7496–7499.

Rolland, F., Moore, B., and Sheen, J. (2002). Plant sugar sensing and signaling. Plant Cell **14** (suppl.), S185–S205.

Rook, F., Corke, F., Card, R., Munz, G., Smith, C., and Bevan, M.W. (2001). Impaired sucrose-induction mutants reveal the modulation of sugar-induced starch biosynthetic gene expression by abscisic acid signalling. Plant J. **26,** 421–433.

Sagi, M., Fluhr, R., and Lips, S.H. (1999). Aldehyde oxidase and xanthine dehydrogenase in a *flacca* tomato mutant with deficient abscisic acid and wilty phenotype. Plant Physiol. **120,** 571–578.

Schroeder, J.I., Kwak, J.M., and Allen, G.J. (2001). Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature **410,** 327–330.

Schwartz, S.H., Leon-Kloosterziel, K.M., Koornneef, M., and Zeevaart, J.A. (1997a). Biochemical characterization of the *aba2* and *aba3* mutants in *Arabidopsis thaliana*. Plant Physiol. **114,** 161–166.

Schwartz, S.H., Tan, B.C., Gage, D.A., Zeevaart, J.A., and McCarty, D.R. (1997b). Specific oxidative cleavage of carotenoids by VP14 of maize. Science **276,** 1872–1874.

Seo, M., Koiwai, H., Akaba, S., Komano, T., Oritani, T., Kamiya, Y., and Koshiba, T. (2000a). Abscisic aldehyde oxidase in leaves of *Arabidopsis thaliana*. Plant J. **23,** 481–488.

Seo, M., and Koshiba, T. (2002). The complex regulation of ABA biosynthesis in plants. Trends Plant Sci. **7,** 41–48.

Seo, M., Peeters, A.J., Koiwai, H., Oritani, T., Marion-Poll, A., Zeevaart, J.A., Koornneef, M., Kamiya, Y., and Koshiba, T. (2000b). The *Arabidopsis aldehyde oxidase 3* (*AAO3*) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proc. Natl. Acad. Sci. USA **97,** 12908–12913.

Sharp, R.E., LeNoble, M.E., Else, M.A., Thorne, E.T., and Gherardi, F. (2000). Endogenous ABA maintains shoot growth in tomato independently of effects on plant water balance: Evidence for an interaction with ethylene. J. Exp. Bot. **51,** 1575–1584.

Sheen, J. (1994). Feedback control of gene expression. Photosynth. Res. **39,** 427–438.

Sheen, J., Zhou, L., and Jang, J.C. (1999). Sugars as signaling molecules. Curr. Opin. Plant Biol. **2,** 410–418.

Silverstone, A.L., Chang, C., Krol, E., and Sun, T.P. (1997). Developmental regulation of the gibberellin biosynthetic gene *GA1* in *Arabidopsis thaliana*. Plant J. **12,** 9–19.

Sindhu, R.K., and Walton, D.C. (1987). Conversion of xanthoxin to ABA by cell-free preparations from bean leaves. Plant Physiol. **85,** 916–921.

Smalle, J., Haegman, M., Kurepa, J., Van Montagu, M., and Straeten, D.V. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc. Natl. Acad. Sci. USA **94,** 2756–2761.

Smeekens, S. (2000). Sugar-induced signal transduction in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **51,** 49–81.

Söderman, E.M., Brocard, I.M., Lynch, T.J., and Finkelstein, R.R. (2000). Regulation and function of the Arabidopsis *ABA-insensitive4* gene in seed and abscisic acid response signaling networks. Plant Physiol. **124,** 1752–1765.

Stepanova, A.N., and Ecker, J.R. (2000). Ethylene signaling: From mutants to molecules. Curr. Opin. Plant Biol. **3,** 353–360.

Stitt, M., and Krapp, A. (1999). The interaction between elevated carbon dioxide and nitrogen nutrition: The physiological and molecular background. Plant Cell Environ. **22,** 583–621.

Tan, B.C., Cline, K., and McCarty, D.R. (2001). Localization and targeting of the VP14 epoxy-carotenoid dioxygenase to chloroplast membranes. Plant J. **27,** 373–382.

Tan, B.C., Schwartz, S.H., Zeevaart, J.A., and McCarty, D.R. (1997). Genetic control of abscisic acid biosynthesis in maize. Proc. Natl. Acad. Sci. USA **94,** 12235–12240.

Thompson, A.J., Jackson, A.C., Parker, R.A., Morpeth, D.R., Burbidge, A., and Taylor, I.B. (2000). Abscisic acid biosynthesis in tomato: Regulation of zeaxanthin epoxidase and 9-*cis*-epoxycarotenoid dioxygenase mRNAs by light/dark cycles, water stress and abscisic acid. Plant Mol. Biol. **42,** 833–845.

Wobus, U., and Weber, H. (1999). Sugars as signal molecules in plant seed development. Biol. Chem. **380,** 937–944.

Xiang, C., Han, P., Lutziger, I., Wang, K., and Oliver, D.J. (1999). A mini binary vector series for plant transformation. Plant Mol. Biol. **40,** 711–717.

Xiao, W., Sheen, J., and Jang, J.C. (2000). The role of hexokinase in plant sugar signal transduction and growth and development. Plant Mol. Biol. **44,** 451–461.

Xiong, L., Ishitani, M., Lee, H., and Zhu, J.K. (2001). The Arabidopsis *LOS5/ABA3* locus encodes a molybdenum cofactor sulfurase and modulates cold stress– and osmotic stress–responsive gene expression. Plant Cell **13,** 2063–2083.

Xiong, L., Lee, H., Ishitani, M., and Zhu, J.K. (2002). Regulation of osmotic stress-responsive gene expression by the *Los6/ABA1* locus in Arabidopsis. J. Biol. Chem. **277,** 8588–8596.

Yamamoto, H., and Oritani, T. (1996). Stereoselectivity in the biosynthetic conversion of xanthoxin into abscisic acid. Planta **200,** 319–325.

Zeevaart, J.A., and Creelman, R.A. (1988). Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol. Plant Mol. Biol. **39,** 439–473.

Zhou, L., Jang, J.C., Jones, T.L., and Sheen, J. (1998). Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. Proc. Natl. Acad. Sci. USA **95,** 10294–10299.

Zimmerman, J.L. (1993). Somatic embryogenesis: A model for early development in higher plants. Plant Cell **5,** 1411–1423.