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Modulation of Abscisic Acid Signal Transduction and Biosynthesis by an Sm-like Protein in *Arabidopsis*

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Summary

The phytohormone abscisic acid (ABA) regulates plant growth and development as well as stress tolerance. The *Arabidopsis sad1* (supersensitive to ABA and drought) mutation increases plant sensitivity to drought stress and ABA in seed germination, root growth, and the expression of some stress-responsive genes. *sad1* plants are also defective in the positive feedback regulation of ABA biosynthesis genes by ABA and are impaired in drought stress induction of ABA biosynthesis. *SAD1* encodes a polypeptide similar to multifunctional Sm-like snRNP proteins that are required for mRNA splicing, export, and degradation. These results suggest a critical role for mRNA metabolism in the control of ABA signaling as well as in the regulation of ABA homeostasis.

Introduction

The plant hormone abscisic acid (ABA) has a wide range of important roles in plant growth and development, including embryogenesis, seed maturation and dormancy, root and shoot growth, transpiration, and stress tolerance. Biochemical, molecular, and genetic approaches have been applied to understand the mechanisms by which ABA regulates the above cellular processes (Koornneef et al., 1998; Leung and Giraudat, 1998; McCourt, 1999). Based on the inhibitory effect of ABA on seed germination, a number of plant mutants defective in ABA biosynthesis or responsiveness have been isolated. Among mutants with altered ABA responsiveness, *Arabidopsis* ABA-insensitive (*abi*) mutants can be identified by virtue of their germination tolerance to exogenous ABA (Koornneef et al., 1984). The dominant *abi1-1* and *abi2-1* mutations reduce ABA responsiveness of root growth, stomatal closing, and gene induction by osmotic stress (Koornneef et al., 1998; Leung and Giraudat, 1998). The *ABI1* and *ABI2* genes encode homologous serine/threonine protein phosphatase 2Cs (Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997). Loss-of-function alleles of *ABI1* showed supersensitivity to ABA, providing evidence for *ABI1* as a negative regulator of ABA responses (Gosti et al., 1999). Another group of mutants, termed *era* (enhanced response to ABA), are hypersensitive to ABA inhibition of seed germination (Cutler et al., 1996). *era1* plants also exhibit enhanced ABA sensitivity within certain vegetative tissues. For example, guard cells of *era1* plants are more sensitive to ABA, leading to reduced transpirational water loss and increased drought tolerance (Pei et al., 1998). The *ERA1* gene encodes a protein farnesyl transferase (Cutler et al., 1996), suggesting that a key protein in ABA signal transduction requires farnesylation.

We report here a novel *Arabidopsis* mutant that shows enhanced sensitivity to ABA and osmotic stress but is deficient in ABA. The mutant, designated *sad1* (supersensitive to ABA and drought), was identified because the stress-responsive *RD29A* gene in the mutant is superinduced by ABA and osmotic stress but not by cold stress. The *SAD1* gene was isolated by positional cloning. It encodes a polypeptide similar to multifunctional Sm-like U6 small nuclear ribonucleoproteins (snRNP) in animals and yeast that are required for mRNA metabolism and implicated in regulating nuclear receptor activities. These results imply a critical role for mRNA metabolism in the control of plant sensitivity to ABA and drought. They also suggest the possibility that a conserved mechanism might exist in plants similar to animal intracellular hormone receptors, whereby ABA directly modulates gene transcription and ABA homeostasis through a SAD1-containing nuclear signaling complex.

Results

Identification of the *SAD1* Locus and Enhanced ABA- and Osmotic Stress-Responsive Gene Expression in *sad1* Mutant Plants

To screen for mutants with altered ABA and/or stress responses, transgenic *Arabidopsis* plants with ABA and stress-inducible bioluminescence were constructed by introducing a chimeric gene (*RD29A-LUC*) consisting of the *RD29A* promoter fused to the firefly luciferase reporter (Ishitani et al., 1997). The *RD29A* promoter contains the DRE/CRT and ABRE elements that confer responsiveness to cold/osmotic stress and ABA, respectively (Yamaguchi-Shinozaki and Shinozaki, 1994). Mutants that respond abnormally to ABA, cold, and/or osmotic stress were selected from the progeny of ethyl methanesulfonate-mutagenized *RD29A-LUC* seeds (Ishitani et al., 1997). One of the mutants, designated *sad1* (for supersensitive to ABA and drought), was chosen for detailed characterization.

In response to ABA or hyperosmotic stress but not cold, *sad1* plants emitted dramatically enhanced bioluminescence as compared to wild-type (Figures 1A–1E). In addition to the increased amplitude of the ABA and hyperosmotic stress response, the *sad1* mutation also reduced the activation threshold of *RD29A-LUC* transcription by these treatments (Figures 1F–1G). The higher levels of luciferase (*LUC*) mRNA in *sad1* mutant plants under ABA or osmotic stress treatments were

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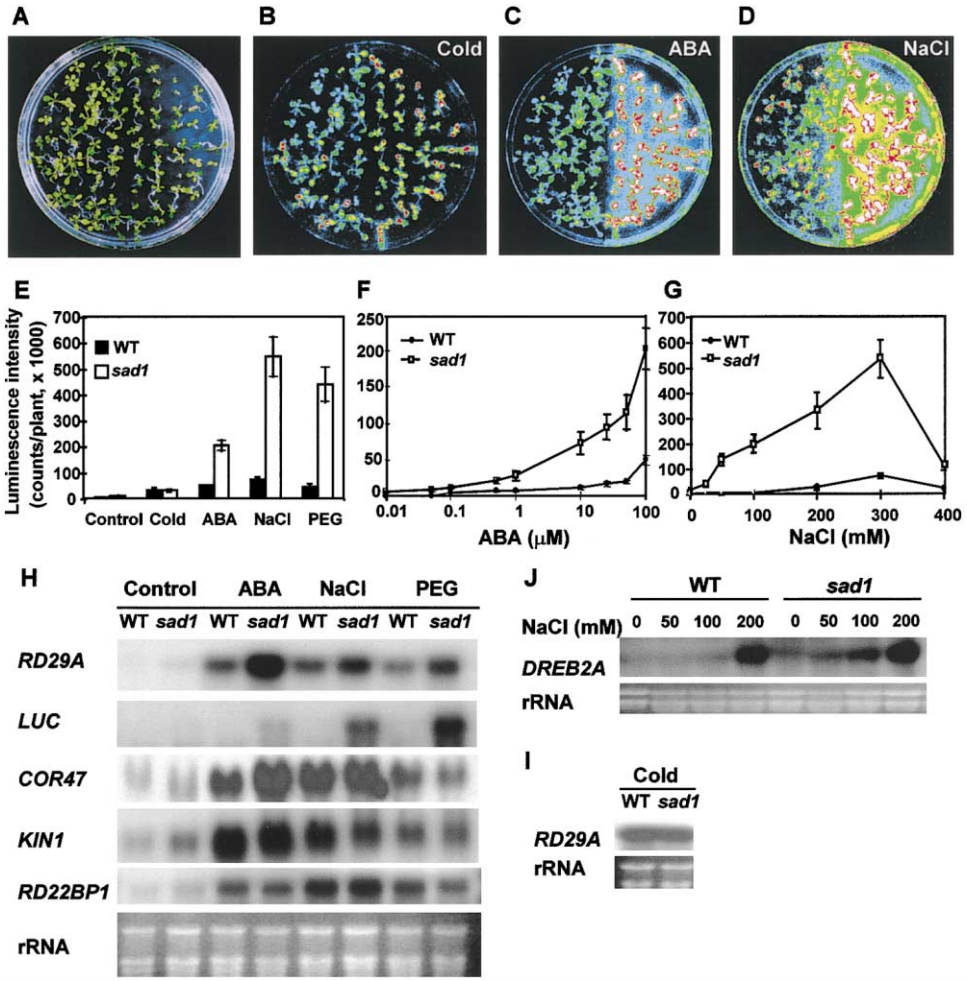

Figure 1. The *sad1* Mutation Enhances *RD29A-LUC* and Endogenous *RD29A* Expression in Response to ABA and Osmotic Stress Treatments

RD29A-LUC expression was measured as luminescence intensities.

(A) Wild-type (left) and *sad1* (right) seedlings growing in an agar plate.

(B) Luminescence after cold treatment at 0°C for 24 hr.

(C) Luminescence after treatment with 100 μM ABA for 3 hr.

(D) Luminescence after flooding the plate with 300 mM NaCl for 3 hr. The luminescence intensity correlates with the brightness of the false color.

(E) Quantitation of the luminescence intensities of wild-type (WT) and *sad1* seedlings in (B) (cold), (C) (ABA), and (D) (NaCl). Also shown are control (without stress treatment) and PEG treatment (30% polyethylene glycol, for 5 hr).

(F and G) Dosage-response curves showing luminescence intensities of *sad1* and wild-type plants treated for 3 hr with different concentrations of ABA and NaCl, respectively. Data in (E)–(G) represent means of 20 individual seedlings. Error bars are standard deviations.

(H–J) Transcript levels of stress-responsive genes in *sad1* and wild-type plants.

(H) Plants were untreated (control) or treated 100 μM ABA for 3 hr, 300 mM NaCl for 3 hr, or 30% PEG for 5 hr.

(I) *RD29A* expression in *sad1* and wild-type plants treated at 0°C for 24 hr.

(J) Expression of *DREB2A* in *sad1* and wild-type plants treated with NaCl at the indicated concentrations for 1 hr. Twenty micrograms of total RNA was loaded in each lane. Ribosomal RNA (ethidium bromide staining) was used as a loading control.

confirmed using RNA blot analysis (Figure 1H). The steady-state level of the endogenous *RD29A* transcript was also higher in *sad1* mutant plants under ABA or osmotic stress treatments but not under cold stress (Figures 1H and 1I). Transcripts of another stress-responsive gene (*COR47*) were also found at higher levels following ABA treatment of *sad1* plants as compared to the ABA-treated wild-type (Figure 1H). Interestingly, *COR47* transcript levels under osmotic stress (NaCl or PEG) were not higher in *sad1* plants (Figure 1H). Transcripts of the stress-responsive gene *KIN1* were found at similar levels in the wild-type and *sad1* plants (Figure 1H).

DREB2A is an osmotic stress-inducible transcription factor that binds to the DRE element in the *RD29A* promoter and likely controls osmotic stress induction of *RD29A* transcription (Liu et al., 1998). In wild-type plants, *DREB2A* transcript levels were greatly increased by 200 mM NaCl treatment, but not by treatment with 50 or 100 mM NaCl (Figure 1J). In contrast, substantial increases in *DREB2A* transcript levels were observed following

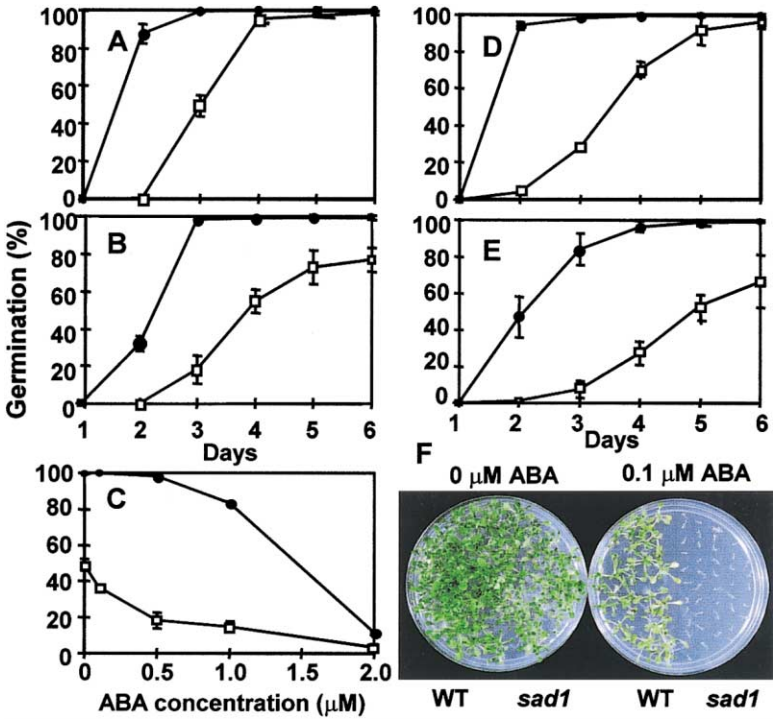

Figure 2. Germination of *sad1* Seeds Is Hypersensitive to ABA and Salt Stress

(A), (B), (D), and (E) show percentage of germination of wild-type (closed circles) and *sad1* mutant (open squares) seeds under different treatments over time (days after imbibition): (A) water control, (B) 0.5 μM ABA, (D) 25 mM NaCl, and (E) 50 mM NaCl.

(C) Germination of wild-type (closed circles) and *sad1* (open squares) in the presence of different concentrations of ABA at day 3 after imbibition. Results are the average of three replicates ± SD.

(F) Growth of wild-type and *sad1* plants germinated and grown in agar media supplemented with 0 μM ABA (plate on the left) or 0.1 μM ABA (plate on the right). The picture was taken 2 weeks after seed imbibition.

treatment of *sad1* mutant plants with 50, 100, or 200 mM NaCl (Figure 1J). This indicates that *DREB2A* expression is more sensitive to osmotic stress in the *sad1* mutant and implies that the *sad1* mutation acts upstream of DREB2A. The effect of *sad1* on *DREB2A* expression appears specific, since the transcript levels of another transcription factor gene, *RD22BP1* (Abe et al., 1997), are not more sensitive to ABA or osmotic stress in *sad1* mutant plants (Figure 1H).

The *sad1* Mutation Enhances ABA and Osmotic Stress Sensitivity in Seeds and Vegetative Tissues

To determine whether the *sad1* mutation affects seed germination in response to ABA or osmotic stress treatments, wild-type and *sad1* seeds were plated on filter papers saturated with water, ABA, or NaCl solutions. In the absence of exogenous ABA or NaCl, *sad1* seeds germinated later than the wild-type (Figure 2A). This delayed germination represents increased dormancy in *sad1* seeds, presumably due to their increased sensitivity to endogenous ABA. In the presence of 0.1 or 0.5 μM ABA, the germination of *sad1* seeds was further delayed (Figures 2B and 2C). At day 3, virtually all wild-type seeds germinated. In contrast, only 37% and 18% of *sad1* seeds germinated in the presence of 0.1 or 0.5 μM ABA, respectively (Figures 2B and 2C). In the presence of 0.5 μM ABA, more than 20% of *sad1* seeds failed to germinate, even after prolonged incubation (Figure 2B). The sensitivity of *sad1* seeds in germination to ABA was also seen at other ABA concentrations tested (Figure 2C).

On agar medium, supplementation of 0.1 μM ABA had a striking effect on *sad1* seed germination and seedling growth. The germination of *sad1* seeds was again delayed by treatment with ABA (data not shown). However, even after *sad1* seeds had germinated (i.e., the radicals had emerged), the growth of the seedlings was arrested by ABA (Figure 2F). In contrast, ABA at a concentration of 0.1 μM did not significantly delay the germination of wild-type seeds and did not arrest wild-type seedling growth.

The germination of *sad1* seeds was also substantially more sensitive than the wild-type to NaCl stress (Figures 2D and 2E). The presence of 25 mM NaCl had no effect on wild-type seed germination but delayed germination of *sad1* seed (Figure 2D). In the presence of 50 mM NaCl, the germination of wild-type seeds was also delayed. However, for *sad1* seeds the effect of 50 mM NaCl was much more pronounced; approximately 40% of *sad1* seeds failed to germinate even after prolonged incubation (Figure 2E).

To test the effect of *sad1* on root sensitivity to ABA, wild-type and *sad1* seedlings grown in vertical agar plates lacking ABA were transferred to vertical agar plates supplemented with various concentrations of ABA. New root growth was measured 6 days after transfer. As compared to the wild-type, *sad1* root elongation was substantially more sensitive to ABA (Figure 3A). The increased sensitivity to ABA inhibition was particularly dramatic at low concentrations of ABA. At 1.0 μM ABA, *sad1* root elongation was inhibited by approximately 40% whereas wild-type root growth was stimulated (Figure 3A). At higher ABA concentrations, wild-type root elongation was also inhibited, but the degree of inhibition was consistently lower than that of *sad1* roots. Compared to that of the wild-type, root elongation of *sad1* mutant plants was also inhibited to a greater extent by NaCl stress (Figure 3B).

Hyperosmotic stress causes damage and even death to plants. *sad1* mutant plants were more sensitive to

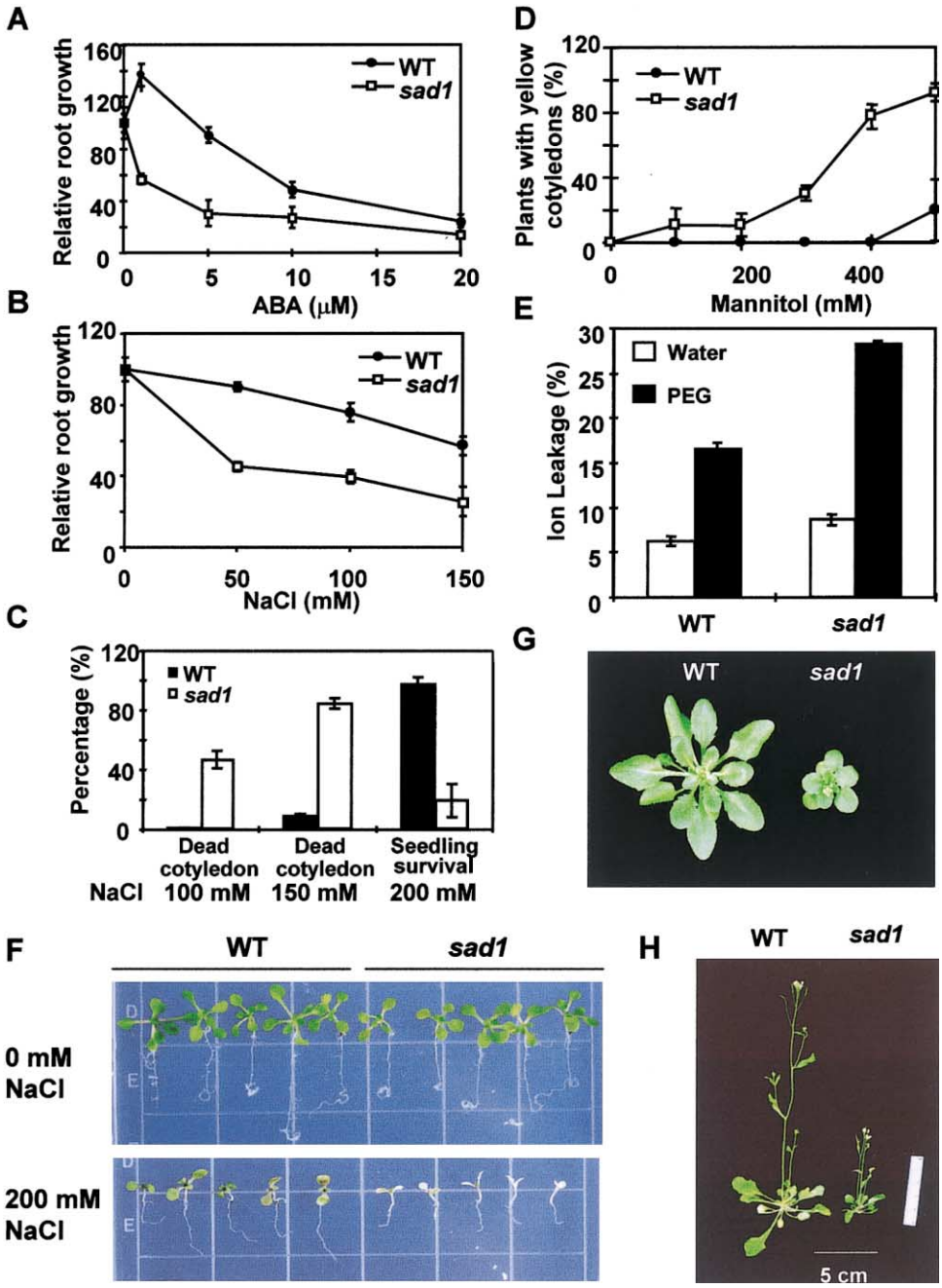

Figure 3. Stress Sensitivity and Morphology of *sad1* Mutant Plants

(A and B) Relative root elongation of *sad1* and wild-type seedlings on vertical agar plates supplemented with different concentrations of (A) ABA or (B) NaCl (n = 20).

(C and D) Seedling damage on agar media supplemented with different concentrations of (C) NaCl or (D) mannitol. The damage was scored at tenth day after the treatment. In (A), (B), and (D), closed circles are wild-type and open squares are *sad1* mutants.

(E) Electrolyte leakage from *sad1* and wild-type seedlings treated with 30% PEG for 5 hr. Shown are averages of four replicates.

(F) Photography of wild-type and *sad1* plants on MS agar plate (0 mM NaCl) or MS agar plate supplemented with 200 mM NaCl. Six-day-old seedlings grown vertically on MS agar plates were transferred to the plates shown and photographed 8 days after the transfer.

(G) Morphology of wild-type (left) and *sad1* (right) seedlings at the rosette stage. (H) Adult wild-type (left) and *sad1* (right) plants. White bar represents 5 cm. In (A)–(E), error bars represent SD.

damage by high concentrations of NaCl or mannitol (Figures 3C and 3D). Exposure to 100 mM NaCl did not kill the cotyledons of wild-type seedlings but killed the cotyledons in nearly 50% of *sad1* seedlings (Figure 3C). More than 85% of *sad1* cotyledons were killed by 150 mM NaCl, compared to less than 10% of wild-type cotyledons (Figure 3C). Exposure to 200 mM NaCl for 7 days killed most *sad1* seedlings whereas virtually all wild-

type seedlings survived (Figures 3C and 3F). Similarly, high concentrations of mannitol also caused significantly more damage to *sad1* seedlings as indicated by the greater proportion of *sad1* plants than wild-type having yellow (i.e., damaged) cotyledons (Figure 3D).

To test sensitivity to desiccation, *sad1* and wild-type seedlings were treated with 30% (w/v) polyethylene glycol (PEG) to mimic drought stress, and leakage of electrolytes was determined as a measure of tissue damage. Seedlings of *sad1* showed substantially greater ion leakage, indicating that the mutant is more sensitive to drought stress (Figure 3E). Compared to wild-type plants, adult *sad1* mutant plants are approximately 60% smaller in size, with dark green, round leaves (Figures 3G and 3H).

The sensitivity of *sad1* mutant plants to other hormones was also investigated. With respect to root growth or hypocotyl elongation, no differences were observed between *sad1* and the wild-type in response to treatments with *epi*-brassinosteroid, 2,4-D, gibberellic acid (GA_3) and the ethylene precursor aminocyclopropane-1-carboxylic acid (ACC) (Figure 4 and data not shown). Application of GA_3 to soil-grown *sad1* seedlings until bolting (which involved spraying with 10 μM GA_3, twice weekly) did not significantly rescue the short stature of *sad1* (data not shown). Unlike for the wild-type, root elongation in *sad1* plants was not stimulated by 1% glucose, yet the response to higher concentrations of glucose was similar to that of the wild-type (Figure 5A).

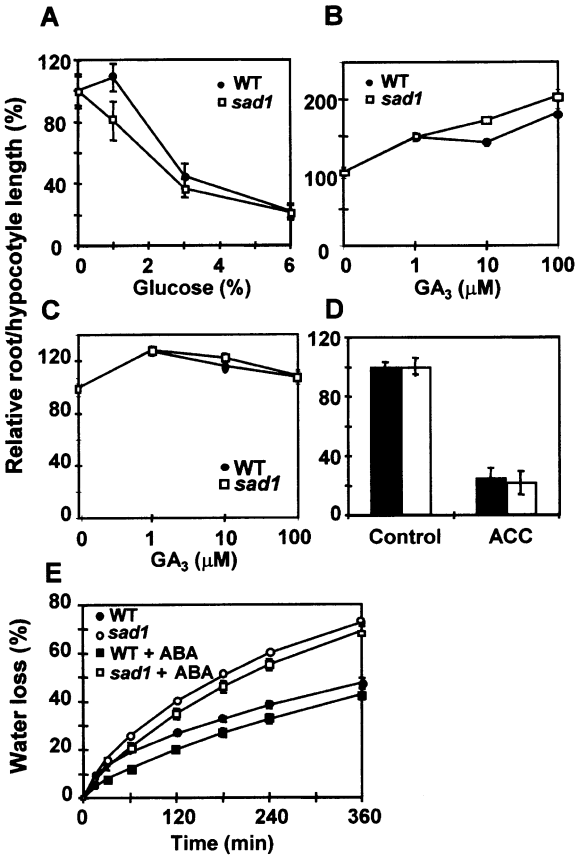

Figure 4. Responses of *sad1* Mutants to Other Growth Regulators and Glucose and the Rate of Transpiration Water Loss in *sad1*

(A–D) Shown are relative new root growth (A, C, and D) or hypocotyl length (B) of wild-type and *sad1* seedlings measured at the ninth day after being transferred from MS medium to MS media supplemented with the indicated concentrations of (A) glucose, (B and C) gibberellic acid (GA_3), or (D) 2 μM ACC. Closed symbols, wild-type; open symbols, *sad1*. Data are means ± SEM (n = 20).

(E) Rate of transpirational water loss from *sad1* and wild-type plants. Rosette plants were detached from soil surface, placed under 30% relative humidity and weighed at the designated time points. Transpirational water loss was also measured 4 hr after 100 μM ABA treatment. Percent loss of fresh weight was calculated based on the initial weight of the plants. Error bars represent SEM (n = 3).

sad1 Plants Are Defective in Drought-Induced ABA Biosynthesis

In the ABA-hypersensitive mutants, *era1* and *abh1*, increased ABA sensitivity is accompanied by a reduced transpirational water loss (Pei et al., 1998; Hugouvieux et al., 2001). In contrast, the *sad1* mutant plants showed increased transpirational water loss (Figure 4E), suggesting that the mutant plants may be defective in stomatal regulation. To determine whether the defect was due to an ABA deficiency or to ABA insensitivity, both of which would lead to enhanced transpiration, ABA was applied and transpirational water loss was determined. Treatment with exogenous ABA reduced water loss rates in *sad1* plants at magnitudes similar to those observed for the wild-type, suggesting that, although *sad1* mutant plants do not appear to be hypersensitive to ABA in stomatal regulation under this particular treatment condition, they are not insensitive to ABA.

The abilities of *sad1* and wild-type plants to synthesize ABA were quantified by gas chromatography mass spectrometry (GC-MS) analysis of total and heavy oxygen-labeled ABA and ABA catabolites using plants subjected to drought stress within an $^{18}O_2$ atmosphere (Rock and Ng, 1999). As compared to the wild-type, *sad1* plants synthesized significantly less ABA (Table 1). A 4-fold increase in ABA content was observed in wild-type plants in response to drought stress. In contrast, there was only a slight increase in ABA content in *sad1* plants in response to drought stress. This increase was significantly lower than that seen for the wild-type, whether measured as total ABA or as $^{18}O_2$-enriched (i.e., de novo synthesized) ABA (Table 1). In addition, the immediate catabolite of ABA, phaseic acid (PA), was observed to accumulate to a lower level in *sad1* plants than in the wild-type (Table 1). The levels of ABA-glucose esters (*cis*-ABA·GE and *trans*-ABA·GE) did not differ significantly between *sad1* and wild-type plants (Table 1).

Positional Cloning of the *SAD1* Locus

The mutant phenotypes described above imply a crucial role for *SAD1* in regulating ABA and osmotic stress sensitivity and ABA homeostasis. Genetic analysis suggested that *sad1* is a recessive mutation in a single nuclear gene (data not shown). In order to clone *SAD1*, the *sad1* mutation was first mapped to a 130 kb region on chromosome 5 (Figure 5A). Then, two overlapping transformation-compatible bacterial-artificial-chromosome (TAC; Liu et al., 1999) clones (K24G6 and K19E20) covering this region, as well as a neighboring clone (K20J1), were separately introduced into the *sad1* mutant plants by *Agrobacterium*-mediated transformation.

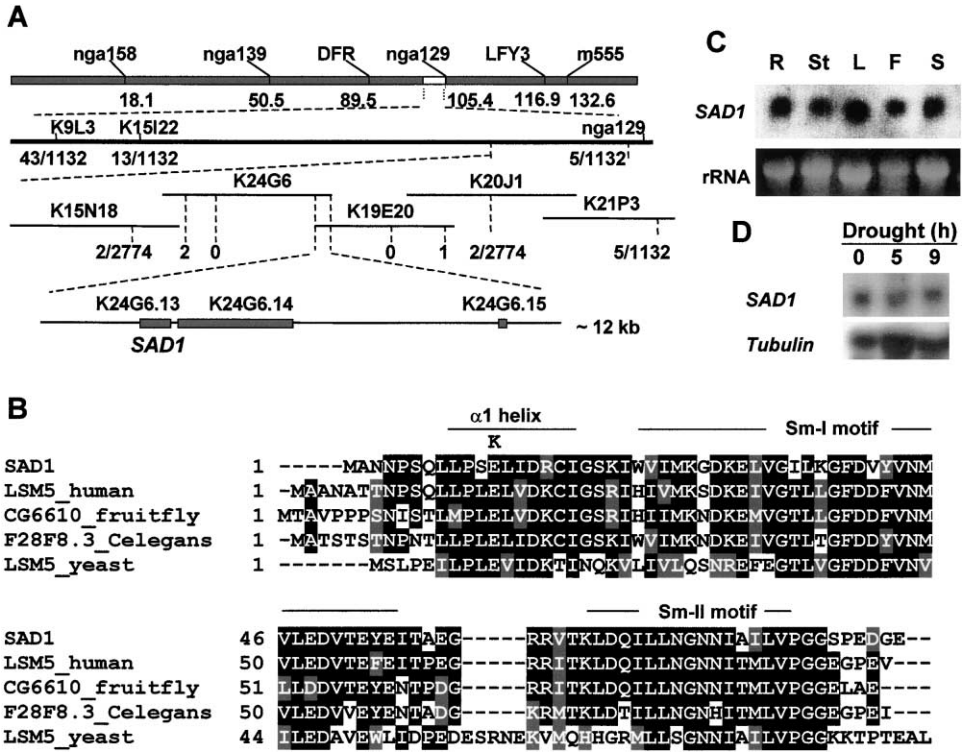

Figure 5. Positional Cloning and the Expression of *SAD1* Gene

(A) On the basis of the analysis of 2774 recombinant chromosomes, the *SAD1* locus was mapped to the lower arm of chromosome V covered by TAC clones K24G6 and K19E20. Genetic complementation delimited *SAD1* to a 12 kb overlapping region between K24G6 and K19E20. Sequence analysis revealed a mutation in the predicted open reading frame of K24G6.13.

(B) Amino acid alignments of the *Arabidopsis* SAD1 (accession AY034896) with homologs from human (LSM5-human, accession CAB45868), *Drosophila* (CG6610, accession AAF50703), *C. elegans* (F28F8.3, accession CAB03013), and *S. cerevisae* (LSM5-yeast, accession NP-011073). The conserved glutamic acid (E) at position 12 is changed to lysine (K) in the *sad1* mutant. Amino acids are shaded in black to indicate identity and in gray to indicate similarity. Dotted lines indicate gaps that are introduced to maximize alignment. The α helix, Sm-I, and Sm-II motifs are indicated.

(C and D) RNA blot analysis of *SAD1* expression in different plant parts (R, root; St, stem; F, flower; L, leaves; S, siliques) (C) and in seedlings treated with drought stress (D). Drought treatment was conducted by removing whole seedlings from the soil and dehydrating them in the air for the indicated time period (hr). rRNA (ethidium bromide staining) and β-tubulin were used as controls for equal loading of the samples.

Analysis of the progeny of transformed *sad1* showed that transgenic expression of either K24G6 or K19E20 complemented the mutant phenotype (data not shown). In contrast, transgenic expression of clone K20J1 was unable to complement *sad1*. These results implied that the *SAD1* gene resides in the 12 kb region of overlap between K24G6 and K19E20.

Genomic DNA corresponding to this overlapping region was amplified and sequenced from wild-type and *sad1* plants. A single G to A nucleotide change was found in *sad1* mutant plants within the predicted K24G6.13 open reading frame. This gene was cloned from wild-type genomic DNA and introduced into *sad1* mutant plants by *Agrobacterium*-mediated transformation. Thirty transgenic plants were analyzed for plant size and the *RD29A-LUC* expression phenotype. All were found to exhibit wild-type phenotypes (data not shown), confirming that the K24G6.13 gene indeed is *SAD1*.

SAD1 Encodes an Sm-like Protein

The *SAD1* open reading frame was obtained by comparing *SAD1* genomic sequence with cDNAs obtained by reverse transcription-PCR. *SAD1* is predicted to encode a 9.7 kDa polypeptide of 88 amino acids (accession AY034896). Database searches revealed that the *SAD1* gene product is most similar to Lsm5 proteins (Achsel et al., 1999; Tharun et al., 2000) from diverse organisms ranging from yeast to human (Figure 5B). SAD1 shows 70% identity and 89% similarity in amino acid sequence to human Lsm5, and 31% identity and 62% similarity in amino acid sequence to yeast Lsm5.

The *sad1* mutation results in a conversion of a highly conserved negatively charged glutamic acid residue to a positively charged lysine in the α1 helix outside of the Sm domain (Figure 5B). Whereas the Sm domain is known to mediate Sm protein-protein interaction (Herman et al., 1995; Kambach et al., 1999) and Sm protein-RNA interactions (Urlaub et al., 2001), the function of the α1 helix, which lies on the surface of adjacent β-sheets (Kambach et al., 1999), is unclear. The fact that the *sad1* mutation lies within the α1 helix suggests that α1 helix is required for full function of Lsm proteins and likely for Sm proteins as well.

The *SAD1* gene transcript was found at low levels

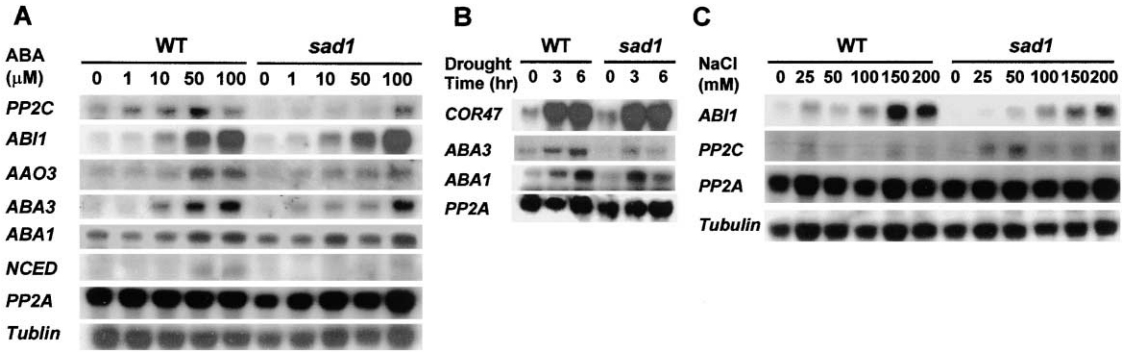

Figure 6. Expression of ABA Signaling and ABA Biosynthesis Genes in *sad1* under Drought, ABA, and Salt Treatments

(A) Gene expression under ABA treatment. Seedlings growing in agar plates were treated with indicated concentrations of ABA for 1 hr. A β-tubulin gene was used as a loading control.

(B) Gene expression under drought treatment. Rosette plants detached from the soil were dehydrated to lose 20% fresh weight, and allowed to incubate at 100% relative humidity for 3 or 6 hr before RNA extraction. Time 0 represents a control without dehydration. A PP2A gene (accession U08047) was used as a loading control.

(C) Gene expression under salt treatment. One-week-old seedlings growing in agar plates were transferred on to filter paper saturated with the indicated concentrations of NaCl and treated for 1 hr. A tubulin gene was used as a loading control.

within all plant tissues (Figure 5C). Transcript levels were not altered by drought stress (Figure 5D) or by treatment with exogenous ABA (data not shown).

Specific Regulation of ABA Signaling Genes by SAD1

As the only Lsm5 ortholog present within the *Arabidopsis* genome, SAD1 may regulate several aspects of mRNA metabolism. Therefore, one might expect that the *sad1* mutation might have a general impact on metabolism of all mRNAs. To assess the global impact of the *sad1* mutation on gene expression, a cDNA microarray assay was conducted using *sad1* and wild-type seedlings that either were untreated or treated with 100 μM ABA for 1 hr. It was found that the expression of only a very few genes was changed in the mutant relative to wild-type plants (data not shown). Among these, a protein phosphatase 2C (PP2C)-like gene (accession 4159705) was found to have a lower level of expression in the mutant. RNA gel blot analysis confirmed that transcripts of this PP2C-like gene were at lower levels in *sad1* plants than in the wild-type following ABA treatments (Figure 6A), whereas transcripts for a PP2A gene (accession U08047) were found at similar levels within *sad1* and wild-type plants. Since this PP2C shows 50% similarity to ABI1 (also a PP2C but not included in the microarray), we probed the same blot with *ABI1*. Interestingly, the *ABI1* transcript levels in wild-type and *sad1* plants were similar, suggesting that ABA induction of *ABI1* is not impaired in *sad1*.

Transcript accumulation of the PP2C-like gene was not induced by salt treatment and the low basal level in *sad1* did not differ much from that of the wild-type plants (Figure 6C). Interestingly, the induction of *ABI1* by salt in *sad1* was substantially reduced relative to that in wild-type (Figure 6C). For example, *ABI1* transcript abundance under 150 mM NaCl treatment in the wild-type was 1.8 times higher than that in *sad1* (Figure 6C).

SAD1 Regulates the Expression of ABA Biosynthesis Genes through a Feedback Loop

Because *sad1* appeared affected in ABA homeostasis, we examined transcript levels for genes that encode enzymes involved in ABA biosynthesis. These included *ABA1* (encoding zeaxanthin epoxidase) (accession 4757396), *LOS5/ABA3* (encoding the molybdenum cofactor sulfurase) (Xiong et al., 2001b), *AAO3* (encoding ABA aldehyde oxidase) (Seo et al., 2000), and *NCED* (encoding 9-*cis*-epoxycarotenoid dioxygenase) (accession 5041970). For both the wild-type and *sad1* plants, transcript levels of *AAO3* and *ABA3* were clearly enhanced by treatment with exogenous ABA (Figure 6A). In contrast, transcript levels for *NCED* was not induced by ABA treatment, and the transcript levels of *ABA1* were only slightly enhanced and then only by higher ABA concentrations. However, in *sad1* plants, the degree of ABA induction of the *AAO3* and *ABA3* genes was substantially lower than that observed for the wild-type (Figure 6A). Additionally, the induced elevation of *ABA3* transcripts under drought stress was lower in *sad1* than in the wild-type (Figure 6B). Alterations in the transcript levels from the *AAO3* gene was too low to be seen clearly with our stress treatment (data not shown), which was less severe than that employed in another study (Seo et al., 2000). These data suggest that ABA positively feedback regulates the expression of some ABA biosynthesis genes and that *sad1* mutation impairs this regulation.

Since the *sad1* mutation reduces drought- and ABA-regulated *LOS5/ABA3* and *AAO3* gene expression and the products of these genes function in the last step of ABA biosynthesis, namely the conversion of ABA-aldehyde to ABA, we examined whether this step might be impaired in *sad1* mutant plants. Rosette leaves of *sad1* plants converted exogenous ABA aldehyde to ABA at only one-third the levels seen for leaves of the corresponding wild-type (Table 1). Levels of ABA catabolites

Table 1. The *sad1* Mutant Has Lower ABA Biosynthesis Than Wild-Type in Response to Drought Stress and Is Impaired in the Conversion of ABA-aldehyde to ABA

Genotype/Treatment	ABA	*cis*-ABA·GE	*trans*-ABA·GE	PA	^{18}O-ABA Enrichment (percent of total)
	(pg/mg fresh weight of tissue)				
Experiment I					
WT unstressed	90 ± 20	1 ± 1	1 ± 1	6 ± 3	—
WT drought-stressed	430[a] ± 130	2 ± 1	10 ± 6	29 ± 13	33 ± 6
sad1 unstressed	130 ± 40	1 ± 0.1	1 ± 0.5	4 ± 2	—
sad1 drought-stressed	170[†] ± 60	1 ± 0.4	1 ± 0.2	4 ± 2	19[c] ± 5
Experiment II					
WT unstressed	220 ± 90	9 ± 2	5 ± 1	NA	–
WT drought-stressed	810[a] ± 150	13 ± 3	19 ± 7	NA	34 ± 5
sad1 unstressed	150 ± 70	7 ± 1	14 ± 9	NA	—
sad1 drought-stressed	320[b] ± 40	12 ± 4	6 ± 4	NA	22[c] ± 6
ABA-aldehyde Conversion					
WT	1044 ± 178	12 ± 0.3	11 ± 7	67 ± 12	—
sad1	338 ± 55	4 ± 1	5 ± 1	6 ± 4	—

Drought stressed plants were incubated under $^{18}O_2$:N_2 atmosphere for 8 (Experiment II) or 24 (Experiment I) hr, and unlabeled and ^{18}O-labeled ABA and catabolites [ABA-glucose ester (GE) isomers, phaseic acid (PA)] were quantified by GC-MS (Roch and Ng, 1999). For Experiment I, data are from two or three independent experiments. Results are the mean ± SEM of 3–8 samples.

[a] Significantly higher than unstressed wild-type plants (WT), $p < 0.02$.

[b] Significantly lower than wild-type drought-treated plants, $p < 0.03$.

[c] Significantly lower than wild-type ^{18}O enrichment, $p < 0.098$ (one-sided Student's *t* test, equal variance assumed).

NA, not analyzed. For ABA-aldehyde feeding, wild-type and *sad1* mutant rosettes were infiltrated with a 2.5 μg/mL solution of ABA-aldehyde, incubated for 24 hr, and ABA and catabolites quantified by GC-MS. Data are mean ± SEM (n = 3).

(i.e., *cis*-ABA·GE, *trans*-ABA·GE, and PA) in *sad1* plants were also much lower than those found in the wild-type (Table 1). This suggests that the lower ABA content in *sad1* is due to a decreased rate of conversion of ABA-aldehyde to ABA, rather than an increased rate of ABA turnover. These data clearly demonstrate that *sad1* is impaired in the conversion of ABA aldehyde to ABA.

Discussion

Compared to other mutants that are impaired in either ABA biosynthesis or sensitivity, *sad1* is unique in that the mutation affects both ABA sensitivity and drought-induced ABA biosynthesis. Since *SAD1* does not encode an enzyme involved in ABA biosynthesis, the impaired ABA biosynthesis under drought stress in *sad1* plants may be through a regulatory component(s) that modulates ABA biosynthesis.

In the present study, we found that the transcript levels of several ABA biosynthesis genes are enhanced by ABA treatment. This is particularly evident for *AAO3* and *LOS5/ABA3*. Previous studies showed that transcript levels for these genes are upregulated by drought stress (Seo et al., 2000; Xiong et al., 2001b). Our finding that *AAO3* and *LOS5/ABA3* genes are additionally regulated by ABA suggests a feedback regulatory mechanism whereby an initial increase in ABA level under drought stress may result in an immediate speedup of the last step of ABA biosynthesis. This may contribute to quick and sustained physiological responses for plants under drought stress. It is likely that this ABA regulation is fine tuned by ABA sensitivity, i.e., a higher sensitivity may downregulate this positive feedback, whereas a reduced sensitivity may increase the positive feedback and result in increased ABA accumulation. This is suggested by the observation that *sad1* mutant shows a lower degree of transcript accumulation for ABA biosynthetic genes under drought or ABA treatment. It is intriguing that not all ABA-responsive mutants have altered ABA biosynthesis. The *era1* (Cutler et al., 1996), *fry1* (Xiong et al., 2001a), and *abh1* (Hugouvieux et al., 2001) are not impaired in ABA biosynthesis. The specific defect of *sad1* in ABA biosynthesis indicates that only SAD1 plays a critical role in regulating this feedback loop. The physiological significance of the *sad1* defect in the feedback regulation by ABA of the ABA biosynthesis genes *AAO3* and *LOS5/ABA3* is further underscored by the results of the ABA-aldehyde feeding experiment, which indicate that the *sad1* mutant is defective in the final step of ABA biosynthesis—the conversion of ABA-aldehyde to ABA. Because both ABA3 and AAO3 function in the last step of ABA biosynthesis, reduced expression of both genes would be expected to cause a more drastic reduction in ABA biosynthesis than either one alone. Consistent with this idea, Seo et al. (2000) found that *aba3aao3* double mutant had the strongest ABA deficiency phenotypes.

The wide range of ABA and osmotic stress-related mutant phenotypes in *sad1* suggests that the *SAD1* locus encodes an important negative regulator in an early step of ABA signaling. To our surprise, the *SAD1* sequence implies that it encodes an Sm-like (Lsm, for Like-Sm) snRNP protein. Lsm proteins were identified as a conserved family of proteins that are structurally related to the Sm proteins. Sm proteins are a family of small proteins that assemble the core component of spliceosomal snRNPs and have been found in all eukaryotes and in prokaryotes (Salgado-Garrido et al., 1999). Lsm proteins are multifunctional molecules that modulate RNA metabolism such as splicing, export, and degradation (He and Parker, 2000). Recent studies show that the yeast ring-shaped Lsm1-Lsm7 and Lsm2-Lsm8 heptameric complexes function in mRNA decapping and

decay and in U6 snRNP biogenesis for pre-mRNA splicing, respectively (Achsel et al., 1999; Bouveret et al., 2000). Therefore, SAD1 may be involved in mRNA metabolism in plants.

One possibility is that the *sad1* mutation affects the decay rate of mRNA for an early component(s) in ABA signaling. The fact that both the *LUC* (luciferase gene) and endogenous *RD29A* transcript levels are higher in *sad1* than those in the wild-type suggests that *sad1* mutation does not specifically affect the *RD29A* or *LUC* transcript turnover since *LUC* and *RD29A* are unrelated in nucleotide sequence. Thus, SAD1 must directly or indirectly regulate the upstream signaling events that control gene transcription. Because low temperature gene regulation and responses to other hormones such as ethylene, cytokinin, and brassinolides are not significantly altered in the mutant, *sad1* appears either specifically to affect ABA and osmotic stress responses or to have a relatively more significant impact on ABA and osmotic stress signaling.

Consistent with the ABA- and osmotic stress-specific phenotypes of *sad1* mutant plants, gene expression profiling using a cDNA microarray suggests that *sad1* mutation does not affect general gene expression. Out of the 600 genes examined, only a few showed altered expression in *sad1* relative to the wild-type. Among these few are the protein phosphatase 2C-like gene (accession 4159705) and *ABI1* (Leung et al., 1994; Meyer et al. 1994). Since *PP2Cs* encode negative regulators of ABA signaling (e.g., Sheen, 1998; Gosti et al., 1999), the reduced ABA induction of *PP2C* may contribute to the enhanced ABA responsiveness in *sad1*. Similarly, the reduced salt stress induction of *ABI1* could contribute to the enhanced osmotic stress responses in the mutant. Interestingly, a lower basal transcript level for another PP2C gene has been reported in the ABA hypersensitive mutant *abh1* (Hugouvieux et al., 2001). These observations imply that the transcript of an early regulator(s) of these *PP2C*s may be the target of the SAD1/ABH1 machinery.

It is noteworthy that *abh1* also potentially affects RNA metabolism since *ABH1* encodes a nuclear mRNA cap binding protein (Hugouvieux et al., 2001). Among the insertion mutants we generated by T-DNA mutagenesis in the wild-type *RD29A-LUC* background (http://stress-genomics.org/stress.fls/tools/mutants/arabid/T_DNA_mutants/table1.html), we also identified a T-DNA insertion in the putative nuclear cap binding protein CBP80 (accession At2g13540). This *cbp80* mutant showed a diminished *RD29A-LUC* regulation in response to ABA and also exhibited altered responses to ABA at the germination and seedling stages (J.-K.Z., unpublished data). In yeast, the nuclear Lsm2 to Lsm8 heptameric ring complex, which contains the SAD1 homolog Lsm5, functions together with the cap binding complex (CBC) to mediate pre-mRNA splicing and export (for review, see Will and Luhrmann, 2001). It is not yet known whether CBC and the Lsm ring complex interact with each other. In yeast two-hybrid assays, neither ABH1/CBP80 nor CBP20 interacted with SAD1 (L.X., Y.G., V.H., J. Schroeder, and J.-K.Z., unpublished data). A second Lsm5-containing complex in yeast is the Lsm1-Lsm7 heptameric complex in the cytoplasm. This ring complex binds to the decapping protein Dcp1 and functions in the degradation of mRNA in the cytoplasm (Bouveret et al., 2000; Tharun et al., 2000). With these important roles, Lsm proteins are indispensable and null mutants of yeast Lsm5 and other Lsm proteins are lethal (Tharun et al., 2000). In the present study, the failure to isolate more *sad1* alleles despite the strong luminescence phenotype may be related to a severe consequence of null mutations, in addition to the small size of the *SAD1* gene. The fact that *sad1* mutation is not lethal may be a consequence of the nature of the mutation, i.e., a single residue change in the α1 helix, which may not affect the general cellular function of the Lsm complex but may impair the degradation/metabolism of certain specific or labile transcripts, for example, those that encode early ABA signaling components if these transcripts have a very high turnover rate (Figure 7). It is well documented that protein turnover through ubiquitination plays a pivotal role in controlling auxin sensitivity (del Pozo and Estelle et al., 1999). Our findings on SAD1 together with those on ABH1 (Hugouvieux et al., 2001) point to a critical role of RNA metabolism in controlling ABA signaling.

It is also possible that SAD1 may have alternative functions, such as a role in nuclear ABA perception and gene transcription. Recent studies in yeast and animals

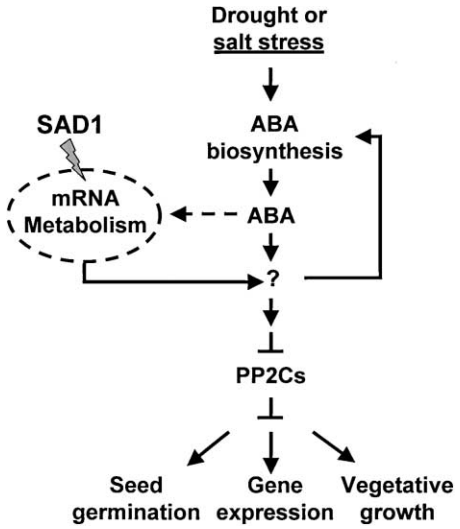

Figure 7. A Hypothetic Model for SAD1 Modulation of ABA Signaling and Plant Sensitivity to Osmotic Stress

SAD1 is part of the LSM ring-shaped complex affecting mRNA splicing, export, and degradation. The transcript(s) for an unidentified early signaling component(s) (shown with a question marker) may have a high turnover rate. The *sad1* mutation impairs the full function of the LSM complex in mediating the degradation of transcript for this component. This component may negatively regulate genes coding for negative regulators of ABA signaling such as 2C-type protein phosphatases (PP2Cs). Reduced PP2C expression may contribute to the enhanced ABA sensitivity of seed germination, gene expression, and vegetative growth in *sad1* mutant plants. In addition, drought and salt stress induce ABA biosynthesis. ABA further stimulates the expression of some of the biosynthesis genes through positive feedback regulation. The unknown component may also regulate this feedback loop, thereby leading to ABA-deficiency in *sad1* mutant plants. In the text, an alternative model is presented, which hypothesizes that SAD1 may directly participate in nuclear ABA perception and gene activation.

have suggested that nuclear (i.e., CBC) or cytoplasmic (i.e., eIF4E) RNA cap binding complex and other RNA processing machinery can mediate growth factor-regulated and stress-regulated gene expression (for review, see Wilson and Cerione, 2000). Lsm proteins interact with Dcp2/Psu1 (Fromont-Racine et al., 2000), a protein required for not only mRNA turnover but also regulation of nuclear receptors. Dcp2/Psu1 interacts in a ligand-dependent manner with the ligand binding domain of several nuclear receptors including thyroid hormone, estrogen, retinoic acid, retinoid X, and vitamin D3 receptors, and is required for transactivation of gene expression by the AF-1 activation domain of nuclear receptors (Gaudon et al., 1999). Therefore, Lsm proteins may regulate the activity of nuclear receptors through Dcp2/Psu1-like proteins. As observed for retinoic acid biosynthesis in animals, abscisic acid is synthesized in plants by oxidative cleavage of carotenoids (Koornneef et al., 1998). The overall similarity in the structures of ABA and retinoic acid suggests conserved mechanisms might exist for plant and animal hormone signaling in the nucleus. Although there is no protein encoded in the *Arabidopsis* genome that shows significant homology to animal nuclear receptors, the possibility that plant intracellular hormone receptors might have diverged from animal ones at the level of primary sequence cannot be ruled out. Future studies may reveal whether there exists an intracellular receptor for ABA that is modulated by SAD1 through Dcp2/Psu1 homologs.

Experimental Procedures

Plant Materials, Mutant Screens, and Stress Treatments

Transgenic *Arabidopsis thaliana* of the C24 ecotype expressing the *RD29A-LUC* transgene (referred to as the wild-type) were mutagenized with ethyl methanesulfonate. Mutant isolation and stress treatments were as described (Ishitani et al., 1997). For luminescence imaging, seedlings growing in MS agar plates (Ishitani et al., 1997) were sprayed uniformly with 1 mM luciferin in 0.01% Triton X-100 and kept in the dark for 5 min before taking images. All images were acquired with a 5 min exposure time, using a thermoelectrically cooled CCD camera (Princeton Instruments, Trenton, NJ) as described (Ishitani et al., 1997). The luminescence intensity of individual seedlings was quantified with the WinView software provided by the camera manufacturer.

Germination and Stress Tolerance Assays

For germination assays, approximately 100 seeds each from *sad1* and the wild-type were planted in triplicate on filter papers soaked with distilled water or with different concentrations of ABA or NaCl. Germination (emergence of radicals) was scored daily for 10 days. Three separate experiments were conducted and similar results were obtained. For the assay of salt and drought sensitivities, wild-type and *sad1* seeds were germinated on MS agar plate and transferred to MS agar plates supplemented with different concentrations of NaCl or mannitol. Seedling damage (number of seedlings with yellow or dead cotyledons) was scored daily up to 2 weeks. On the medium containing 200 mM NaCl, only seedlings that developed true leaves that were green were considered to have survived the stress. For the PEG-induced ion leakage assay, 10-day-old seedlings were placed on filter paper saturated with 30% polyethylene glycerol (molecular weight, 6,000). After 5 hr incubation under light, the seedlings were briefly rinsed in deionized water and immediately placed in glass test tubes containing 2 ml deionized water. The samples were shaken overnight and conductivity was measured (Xiong et al., 2001a). The tubes containing the samples were then autoclaved and shaken overnight and the conductivity measurement repeated. The ratio of the conductivities measured before and after autoclaving corresponded to the ion leakage and was expressed as a percentage.

RNA Analysis

Ten-day-old seedlings grown on MS agar plates were treated with low temperature, ABA, or PEG. The respective treatment conditions were as stated in the text. Total RNA from control or treated plants was extracted and analyzed as previously described (Lee et al., 2001).

ABA Measurements and ABA-aldehyde Feeding Assays

For ABA measurements, three to five shoots of 4-week-old wild-type and mutant plants were frozen immediately for unstressed controls or for drought-stress treatments blown with a cool air stream until 15% of the fresh weight was lost, followed by 8 or 24 hr under a 20%:80% $^{18}O_2$:N_2 atmosphere. ABA and catabolites were purified and quantified by GC-MS as previously described (Rock and Ng, 1999).

For ABA-aldehyde feeding experiments, intact rosettes were infiltrated with a 2.5 μg mL^{-1} aqueous solution of *cis*-ABA-aldehyde containing 0.05% (v/v) Tween-20 and incubated for 24 hr. As a negative control, autoclaved wild-type rosettes were also incubated with ABA-aldehyde. The rosettes were immediately frozen in liquid nitrogen for quantification of ABA and catabolites. No conversion of ABA-aldehyde was observed in the autoclaved control. ABA-aldehyde was the kind gift of Dr J.A.D. Zeevaart, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI.

Genetic Mapping and Gene Cloning

For genetic mapping of *sad1* mutation, *sad1* was crossed with wild-type plants of the Columbia ecotype. The resulting F_1 plants were allowed to self and the F_2 seeds were collected. Homozygous *sad1* mutants in the segregating F_2 population were selected based on their ABA-hypersensitive luminescence. Mapping of the mutation was carried out as described previously (Lee et al., 2001). The transformation-compatible artificial chromosome (TAC; Liu et al., 1999) clones K24G6, K19E20, and K20J1 were transferred into *sad1* mutant plants by *Agrobacterium*-mediated infiltration. T_1 and T_2 plants were analyzed for luminescence expression and scored for morphological phenotypes. Genomic DNA in the region overlapped by both clones was amplified from *sad1* and the wild-type plants by PCR and sequenced. Complementary DNA of *SAD1* was obtained by RT-PCR and cloned into pBluescript and sequenced. Genomic DNA including the *SAD1* coding region and 1.5 kb of 5′-upstream sequence was PCR amplified and cloned into the binary vector pCAMBIA1200 and transferred into *sad1* plants by the *Agrobacterium*-mediated flower dipping method. Plants of the T_2 generation were analyzed for luminescence and visible phenotypes.

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Accession Numbers

The accession number for the *Arabidopsis* SAD1 reported here is AY034896.