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The Arabidopsis *LOS5/ABA3* Locus Encodes a Molybdenum Cofactor Sulfurase and Modulates Cold Stress– and Osmotic Stress–Responsive Gene Expression

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To understand low temperature and osmotic stress signaling in plants, we isolated and characterized two allelic Arabidopsis mutants, *los5-1* and *los5-2*, which are impaired in gene induction by cold and osmotic stresses. Expression of *RD29A-LUC* (the firefly luciferase reporter gene under the control of the stress-responsive *RD29A* promoter) in response to cold and salt/drought is reduced in the *los5* mutants, but the response to abscisic acid (ABA) remains unaltered. RNA gel blot analysis indicates that the *los5* mutation reduces the induction of several stress-responsive genes by cold and severely diminishes or even completely blocks the induction of *RD29A*, *COR15*, *COR47*, *RD22*, and *P5CS* by osmotic stresses. *los5* mutant plants are compromised in their tolerance to freezing, salt, or drought stress. *los5* plants are ABA deficient, as indicated by increased transpirational water loss and reduced accumulation of ABA under drought stress in the mutant. A comparison with another ABA-deficient mutant, *aba1*, reveals that the impaired low-temperature gene regulation is specific to the *los5* mutation. Genetic tests suggest that *los5* is allelic to *aba3*. Map-based cloning reveals that *LOS5/ABA3* encodes a molybdenum cofactor (MoCo) sulfurase. MoCo sulfurase catalyzes the generation of the sulfurylated form of MoCo, a cofactor required by aldehyde oxidase that functions in the last step of ABA biosynthesis in plants. The *LOS5/ABA3* gene is expressed ubiquitously in different plant parts, and the expression level increases in response to drought, salt, or ABA treatment. Our results show that LOS5/ABA3 is a key regulator of ABA biosynthesis, stress-responsive gene expression, and stress tolerance.

INTRODUCTION

Plants respond to environmental challenges in part by altering their gene expression profiles, which ultimately leads to various adaptive responses at the cell and whole-plant levels (Bray, 1993; Ingram and Bartel, 1996; Zhu et al., 1997; Thomashow, 1999; Hasegawa et al., 2000). One important regulator of plant responses to abiotic stress environments is the phytohormone abscisic acid (ABA). ABA is involved in plant responses to abiotic stresses such as low temperature, drought, and salinity as well as the regulation of plant growth and development, including embryogenesis, seed dormancy, shoot and root growth, and leaf transpiration (Koornneef et al., 1998; Leung and Giraudat, 1998; McCourt, 1999; Rock, 2000). Evidence for a role of ABA in stress-responsive gene regulation in plants has been twofold. First, under cold, drought, or salt stress conditions, plants accumulate increased amounts of ABA, with drought stress having the most prominent effect on ABA accumulation. Second, the expression of many stress-responsive genes is induced by exogenous ABA, and their stress inducibility is decreased in mutant plants defective in ABA biosynthesis or responsiveness.

Genetic analysis based on the inhibitory effect of ABA on seed germination has yielded mutants with reduced ABA biosynthesis or altered ABA responsiveness (Koornneef et al., 1998; Leung and Giraudat, 1998; McCourt, 1999; Rock, 2000). The former group of mutants in Arabidopsis includes *aba1*, *aba2*, and *aba3*. The *ABA1* gene encodes a zeaxanthin epoxidase that functions in an early step of ABA biosynthesis by converting zeaxanthin to violaxanthin. Molecular cloning of *ABA2* or *ABA3* has not been reported thus far. Common phenotypes of these *aba* mutants include loss of seed dormancy, germination resistance to NaCl stress, and withering when transferred from high humidity to low humidity conditions. The use of ABA-deficient mutants along with ABA-responsive mutants in stress gene regulation studies led to the notion that stress-responsive gene expression in plants is mediated by both ABA-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki, 1997; Leung and Giraudat, 1998; Thomashow, 1999; Rock, 2000). Although the molecular mechanisms underlying the

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differences between ABA-dependent and ABA-independent gene regulation are unclear, analysis of the promoters of stress-responsive genes and the isolation of transcription factors that activate these genes support the notion that there are distinct regulatory mechanisms for the different pathways. The *ABRE* (ABA-responsive element) complex in these promoters mediates gene induction by ABA (Guiltinan et al., 1990; Yamaguchi-Shinozaki and Shinozaki, 1994; Shen and Ho, 1995; Vasil et al., 1995), whereas the *DRE*/*CRT* (dehydration-responsive element) mediates cold and osmotic stress responsiveness independently of ABA (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). Despite these differences in transcriptional activation, genetic analysis has indicated that the ABA-dependent and ABA-independent pathways have extensive interactions in controlling gene expression under abiotic stress (Ishitani et al., 1997; Xiong et al., 1999a).

We have been using a reporter gene approach to genetically determine ABA and stress signal transduction in Arabidopsis. ABA- and stress-responsive bioluminescent plants were constructed by introducing *RD29A-LUC*, the firefly luciferase reporter gene under the control of the *RD29A* promoter (containing both *ABRE* and *DRE*/*CRT* elements; Yamaguchi-Shinozaki and Shinozaki, 1994) into Arabidopsis. The *RD29A-LUC* plants were mutagenized, and mutants with abnormal bioluminescence in response to cold, drought, salt, or ABA were isolated (Ishitani et al., 1997). One group of mutants exhibited reduced luminescence responses to NaCl stress. Here we present the characterization and cloning of two allelic mutants from this group.

These two mutants, designated *los5-1* and *los5-2* (*l*ow expression of *o*smotically responsive genes), show reduced expression of stress-responsive genes under both cold and osmotic stress conditions. Although the role of LOS5 in the osmotic stress regulation of gene expression is mediated by ABA, the regulation of cold responsiveness by LOS5 is not dependent on ABA. The function of LOS5 in cold stress–and osmotic stress–responsive gene expression is independent of CBF/DREB1 or DREB2A transcription factors. *los5* mutant plants are more susceptible to damage by freezing, salt, and drought stresses, suggesting that LOS5 is critical for plant stress tolerance. The mutant plants also show enhanced transpirational water loss and accumulate less ABA in response to drought stress. Allelic tests show that *los5* is allelic to the *aba3* mutation. Map-based cloning of *LOS5*/*ABA3* reveals that it encodes a putative molybdenum cofactor (MoCo) sulfurase that catalyzes the sulfuration of the desulfo form of MoCo, which is consistent with previous findings that the *aba3* is impaired in the introduction of sulfur into MoCo (Schwartz et al., 1997a). Sulfurylated MoCo is a cofactor of ABA–aldehyde oxidase that functions in the last step of ABA biosynthesis. Expression of the *LOS5*/*ABA3* gene is upregulated by ABA, salt, and drought stresses. These data provide important insights into ABA biosynthesis and significantly further our understanding of stress gene regulation and stress tolerance.

RESULTS

Isolation of Arabidopsis Mutants with Reduced *RD29A-LUC* Induction by Salt Stress

Seed from Arabidopsis plants expressing the *RD29A-LUC* transgene (referred to as wild type) were mutagenized with ethyl methanesulfonate, and seedlings of the M_2 generation were screened for mutants with altered regulation of the transgene (Ishitani et al., 1997). One group of mutants was isolated that exhibit a clear reduction in NaCl-induced luminescence. Two allelic mutants, designated *los5* (*los1* to *los4* are mutants with specific defects in low temperature signaling; J.-K. Zhu, unpublished data), that show reduced luminescence induction in response to both NaCl and cold, were chosen for detailed characterization.

As shown in Figure 1, the luminescence intensities in *los5-1* mutant seedlings were considerably lower than those in the wild type when treated with cold (0°C) for 48 hr (Figure 1B) or with 300 mM NaCl for 5 hr (Figure 1F). In contrast, the luminescence expression in response to ABA (100 μM for 5 hr) was not lower than that in the wild type (Figure 1D). Without stress treatment, there was virtually no luminescence expression in either the wild type or the *los5* mutant. Quantification of the luminescence intensities in Figure 1 indicates that the levels of *RD29A-LUC* expression in *los5-1* seedlings were only 8 and 2% of the wild-type levels for cold and NaCl treatments, respectively, whereas the expression levels under ABA treatment were virtually the same for the mutant and wild type (Figure 1G).

To determine whether the *los5* mutant has an altered threshold response to either cold or salt stress, different low temperatures or NaCl dosages were applied and the luminescence expression in *los5* and wild-type plants was quantified. The results show that *los5* mutant plants exhibited a consistently lower luminescence expression under the temperatures tested (Figure 1H), and increased NaCl concentrations also did not result in the recovery of expression to wild-type levels (Figure 1I). These findings indicate that the reduced responsiveness to cold or osmotic stress in *los5* is not attributable to altered induction thresholds of these stresses.

los5-1 mutant plants were backcrossed to the wild-type plants. Analysis of luminescence expression of the F_1 seedlings and the selfed F_2 population indicated that *los5* is a recessive mutation in a nuclear gene (Table 1). Pairwise crosses with other mutants that also show reduced luminescence responses to NaCl treatment identified a second allele, *los5-2* (Table 1). The *los5-2* mutant has identical phenotypes as *los5-1* (data not shown).

Reduced Gene Regulation by Salt, Drought, and Cold in *los5* Mutants

To assess whether the *los5* mutation has a similar effect on expression of the endogenous *RD29A* as it has on the

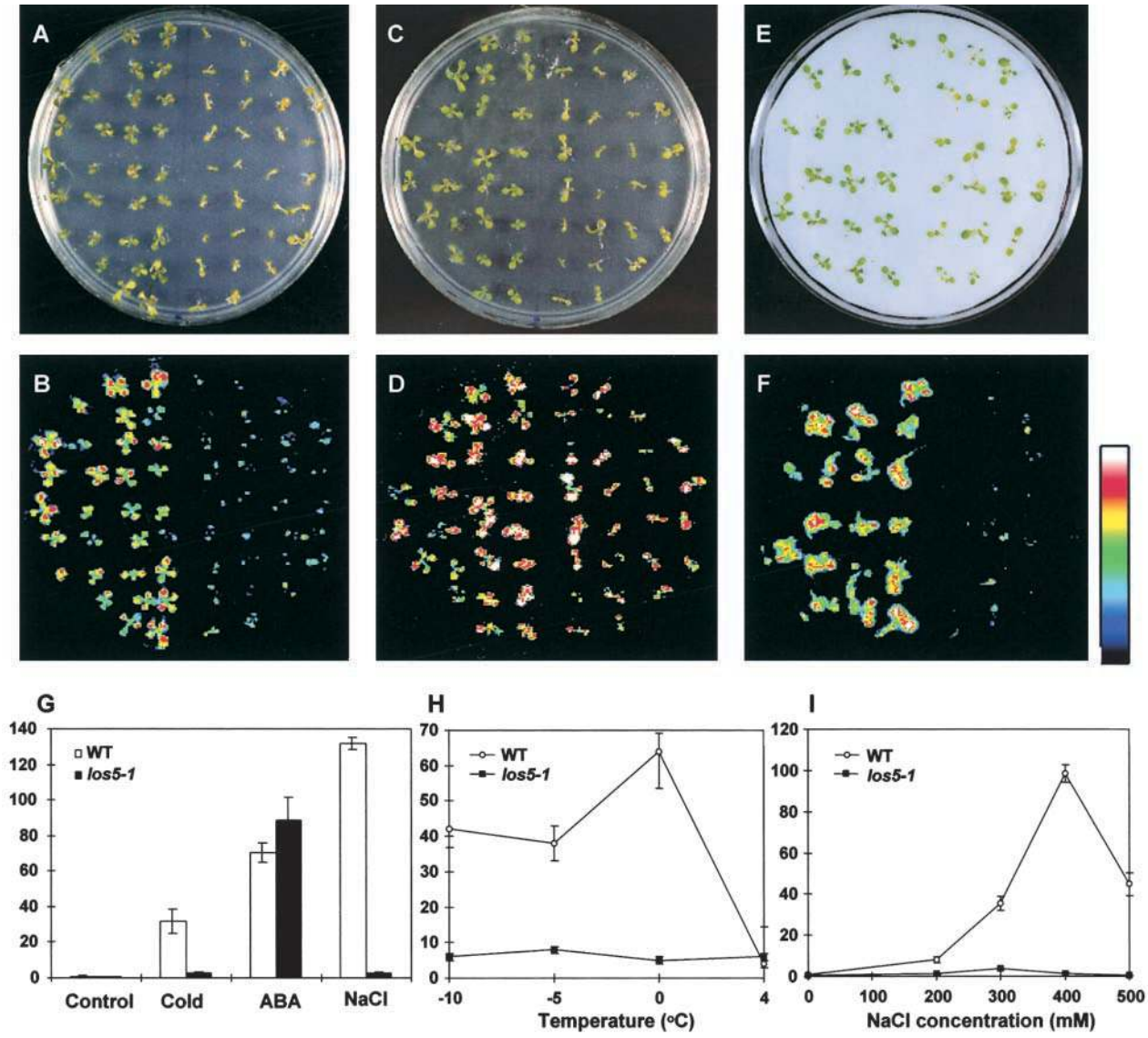

Figure 1. Luminescence Phenotypes of *los5* Mutant Plants.

(A) Morphology of wild-type (left) and *los5-1* (right) seedlings on an agar plate.
(B) Luminescence of **(A)** after low-temperature treatment at 0°C for 48 hr.
(C) Morphology of wild-type (left) and *los5-1* (right) seedlings on an agar plate.
(D) Luminescence of **(C)** after treatment with 100 μM ABA for 4 hr.
(E) Morphology of wild-type (left) and *los5-1* (right) seedlings on filter paper saturated with 300 mM NaCl.
(F) Luminescence of **(E)** after 5 hr of 300 mM NaCl treatment.
(G) Quantitation of the luminescence intensities of wild-type and *los5-1* plants in response to cold (0°C for 48 hr), ABA (100 μM for 4 hr), or NaCl (300 mM for 5 hr) treatment as shown in **(B)**, **(D)**, and **(F)**. Also shown are data for untreated plants (control).
(H) Low temperature dosage–response curves. Treatments at −5 or −10°C lasted for 3 hr, followed by incubation at room temperature for 2 hr. Treatment at other temperatures lasted for 48 hr before imaging.
(I) NaCl dosage–response curve. Treatment time was 3 hr.
The color scale at right shows the luminescence intensity from dark blue (lowest) to white (highest). Data in **(G)** to **(I)** represent means and ±SEs (n = 20). Open symbols, wild type (WT); closed symbols, *los5-1*.

Table 1. Genetic Analysis of the *los5* Mutation[a]

Crosses (female × male)	F_1		F_2	
	Wild Type	Mutant	Wild Type	Mutant
Wild type × *los5-1*	58	0	1023	336
Wild type × *los5-2*	21	0	440	135
los5-1 × *los5-2*	0	14		
los5-1 × *aba1-1*	6	0		
aba2-1 × *los5-1*	20	0		
los5-1 × *aba3-1*	0	29		

[a]Values shown indicate number of plants that showed wild-type or mutant luminescence phenotypes in response to NaCl treatment.

RD29A-LUC transgene, RNA gel blot analysis was performed with total RNA isolated from *los5-1* and wild-type seedlings that were not stress treated (control) or that were treated with cold (0°C) for 24 hr, 100 μM ABA for 2 hr, 300 mM NaCl for 3 hr, or 30% polyethylene glycol (PEG) for 5 hr. The results show that whereas ABA induction of the endogenous *RD29A* was not affected substantially, NaCl induction was blocked almost completely by the *los5* mutation (Figure 2). The mutation also clearly reduced *RD29A* expression in response to cold treatment. To determine whether the effect of NaCl was caused by ionic or osmotic stress, PEG (average molecular weight, 6000) was used. The steady state transcript level of *RD29A* was greatly reduced in *los5* in response to PEG treatment (Figure 2). This indicates that the *los5* mutation reduces gene expression under osmotic stress.

The *los5* mutation also has dramatic effects on the expression of other stress-responsive genes under osmotic or cold stress treatment. Several stress-responsive genes, including *COR15*A (Lin and Thomashow, 1992), *KIN1* (Kurkela and Franck, 1990), *COR47* (Gilmour et al., 1992), *RD22* (Yamaguchi-Shinozaki et al., 1992), and *P5CS*, were analyzed. The *los5* mutation almost completely blocked the osmotic stress induction of *COR15A*, *KIN1*, and *P5CS* (Figure 2). It also substantially reduced the induction of *RD22* and *COR47* by NaCl and PEG. Interestingly, whereas ABA induction of *COR47* was enhanced by the *los5* mutation, the induction of *COR15A* and *P5CS* by ABA was reduced by the mutation (Figure 2). As a control, the transcript level of an actin gene was determined, and the results show that its expression was not changed by stress treatments and was not substantially different between the mutant and the wild type under their respective treatments (Figure 2).

los5 Mutant Plants Are More Sensitive to Freezing Stress

The reduced expression of *RD29A* and other stress-responsive genes in *los5* might have an impact on the stress tolerance of the mutant plants. To test the sensitivity of *los5* mutant plants to low temperature, *los5-1* and wild-type plants were incubated at 4°C for up to 4 weeks. No significant difference in growth was found between the mutant and the wild type, indicating that *LOS5* is not critical for chilling resistance. To determine whether *los5* mutant plants are defective in cold acclimation, wild-type and *los5-1* rosette plants growing in soil (Figure 3A) were cold acclimated (4°C under light) for 48 hr. The plants were then treated at −7°C for 5 hr. After incubation in the growth chamber for 1 day, a clear difference was observed: whereas 97% of the wild-type plants survived −7°C freezing, all *los5-1* mutant plants were killed (Figure 3B and data not shown). These results show that *los5* mutant plants have reduced freezing tolerance.

los5 Mutant Plants Are More Sensitive to Drought and Salt Stress Damage

Steady state RNA levels, as shown in Figure 2, revealed a remarkable reduction in *los5-1* mutant plants in the expression of stress-responsive genes in response to salt or drought (i.e., PEG) treatments. Except for the *P5CS* (Δ^1-pyrroline-5-carboxylate synthase) gene, the function of most of the genes examined is not clear. *P5CS* catalyzes the rate-limiting step in the biosynthesis of proline, a major osmolyte important for plant tolerance to freezing, drought, and salt stresses (Xin and Browse, 1998; Roosens et al., 1999; Hong et al., 2000). Proline contents in wild-type and *los5-1* plants treated with ABA or salt stress were measured. In the absence of stress treatment (i.e., control), *los5-1* mutant plants had a slightly higher proline content than wild-type plants. The proline content in both *los5-1* and the wild-type plants increased in response to 150 mM NaCl treatment. However, the increase in *los5-1* plants was less than that in wild-type plants. The *los5-1* mutant and the wild-type plants had similar proline contents when treated with 50 μM ABA (Figure 4A).

To determine the drought sensitivity of *los5* mutant plants, *los5-1* and wild-type seedlings were treated with 30% PEG, and electrolyte leakage was measured as an indicator of drought-induced cellular damage. Although *los5-1* seedlings had higher electrolyte leakage than the wild type even without stress treatment, PEG treatment resulted in an electrolyte leakage in the mutant that was twice as great as that in wild-type plants (Figure 4B), indicating that *los5* mutant plants are more sensitive to drought stress.

Although *los5-1* mutant seeds were more tolerant of NaCl stress at germination, mutant root growth did not differ from that of the wild type in terms of NaCl sensitivity (data not shown). Despite similar levels of inhibition of root growth by NaCl stress, *los5-1* mutant plants showed increased sensitivity to NaCl in the shoot. At NaCl concentrations of 75 mM or greater, *los5-1* mutant seedlings became yellowish and were killed by prolonged exposure to the stress, whereas wild-type plants survived (Figure 4C and data not shown).

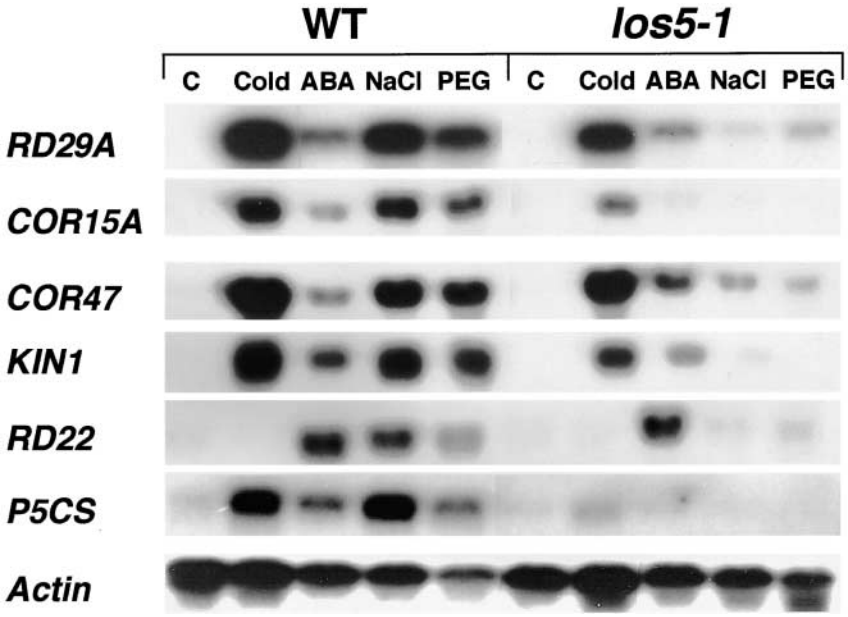

Figure 2. Transcript Levels of Stress-Responsive Genes in *los5-1* and Wild-Type Plants.

Seedlings were either not treated (C; control) or treated with low temperature (Cold; 0°C for 24 hr), 100 μM ABA for 2 hr, 300 mM NaCl for 3 hr, or 30% PEG for 5 hr. Actin was used as a loading control. WT, wild type.

los5 Mutant Plants Are Deficient in Stress-Induced ABA Accumulation

In addition to changes in stress sensitivity, *los5* mutant plants also were altered in development. Under our long day growth conditions, *los5-1* plants flowered ~5 days earlier than wild-type plants. Besides having a more dark green color, *los5-1* mutant leaves were narrower and slightly serrated at the edge compared with the more round leaves of wild-type plants (Figures 5A and 5B). In fact, *los5* mutant plants can be distinguished from the wild type based on these leaf characteristics. These visible phenotypes are shared by the *los5-2* mutant plants, cosegregate with the *los5* luminescence phenotypes, and are present in *los5-1* plants that had been backcrossed with the wild type four times.

When the aboveground parts were detached from the roots at the rosette stage (Figure 5C), the younger leaves of *los5-1* plants withered within 10 min under our room conditions (22 ± 2°C, ~30% RH). In contrast, wild-type leaves remained turgid under the same conditions (Figure 5D). The inflorescence of adult *los5* plants also readily became wilty when the plants were removed from growth chambers (22 ± 2°C, 90% RH) to our room conditions, whereas the wild-type inflorescence remained turgid (Figure 5E). These observations suggest that *los5* mutant plants may have a higher transpiration rate. Measurement of transpirational water loss showed that *los5* mutant plants lost water much faster than wild-type plants (Figure 5F), indicating potential defects in stomatal regulation, which is a typical phenotype of ABA-deficient and ABA-insensitive mutants.

To determine whether *los5* mutant plants are deficient in ABA or insensitive to ABA, wild-type and *los5-1* plants at the rosette stage were sprayed with 100 μM ABA 3 hr before detaching the aerial parts for water loss measurements. Figure 5F shows that whereas the ABA treatment did not significantly affect transpirational water loss from wild-type plants, the treatment reduced substantially the rate of water loss from *los5-1* plants. This observation is consistent with ABA deficiency in *los5-1* mutant plants and suggests that *los5-1* is not ABA insensitive.

ABA contents in *los5-1* and wild-type plants were measured using an immunoassay. In the absence of stress treatment, ABA contents in the wild-type and *los5-1* leaves were essentially the same (Table 2). When detached leaves were allowed to lose 30% of their fresh weight, ABA contents in both the wild type and the *los5-1* mutant increased. However, the magnitudes of the increases were quite different. Whereas ABA content in the wild type increased by more than 300% in response to the water stress, the increase in *los5* leaves was only 80%, with the wild type having nearly 250% as much ABA as in the mutant (Table 2).

These data clearly show that *los5-1* is an ABA-deficient mutant. To determine whether *los5* is allelic to known ABA-deficient mutants, *los5-1* was initially crossed with *aba1* and

aba2. Analysis of the F_1 plants indicated that *los5-1* is not allelic to either one (Table 1 and data not shown). When the *aba3-1* mutant became available later, we crossed it to *los5-1* and analyzed the phenotypes of F_1 and F_2 seedlings by luciferase imaging and water loss measurements. Luminescence images showed that the resulting F_1 seedlings had a low expression of luminescence when treated with 300 mM NaCl, indicating that *los5* is likely allelic to *aba3*. However, measurement of transpirational water loss in the F_1 seedlings was less conclusive in part because, when crossed to the wild-type *RD29A-LUC* plants (C24 background), the *aba3 ABA3* heterozygous plants showed an incomplete recessive phenotype (i.e., the F_1 plants lost water slower than *aba3* but faster than the *RD29A-LUC* wild type). Although the F_1 plants (*los5/aba3*) from a cross between *los5-1* and *aba3-1* lost water faster than the F_1 plants from a cross between *RD29A-LUC* wild type and *aba3-1*, the water loss rate of *los5-1/aba3-1* heterozygous plants was still lower than that in either *los5-1* or *aba3-1* (data not shown). The intermediate phenotypes probably are the result of the genetic differences between the two ecotypes (C24 versus Columbia).

LOS5 Regulates Cold Stress– and Osmotic Stress–Responsive Gene Expression through Distinct Mechanisms

Gene expression analysis in *los5-1* mutant plants suggests a critical role for *LOS5* in the regulation of stress-responsive genes by salt and drought and, to a lesser extent, by cold (Figures 1 and 2). In our mutant screen, a second genetic locus, *LOS6*, was defined by *los6-1* mutant plants that show

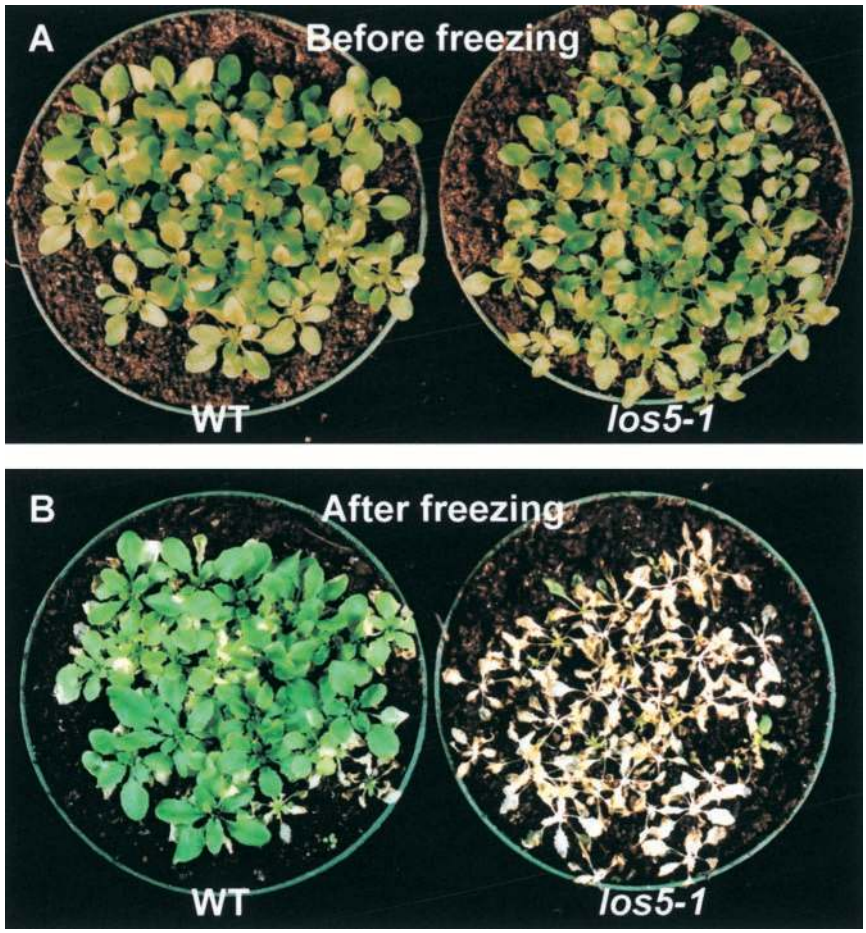

Figure 3. Freezing Sensitivity of *los5-1* Plants.

(A) Plants before freezing treatment.
(B) Plants after freezing treatment (−7°C for 5 hr). The photograph was taken 7 days after freezing treatment.
WT, wild type.

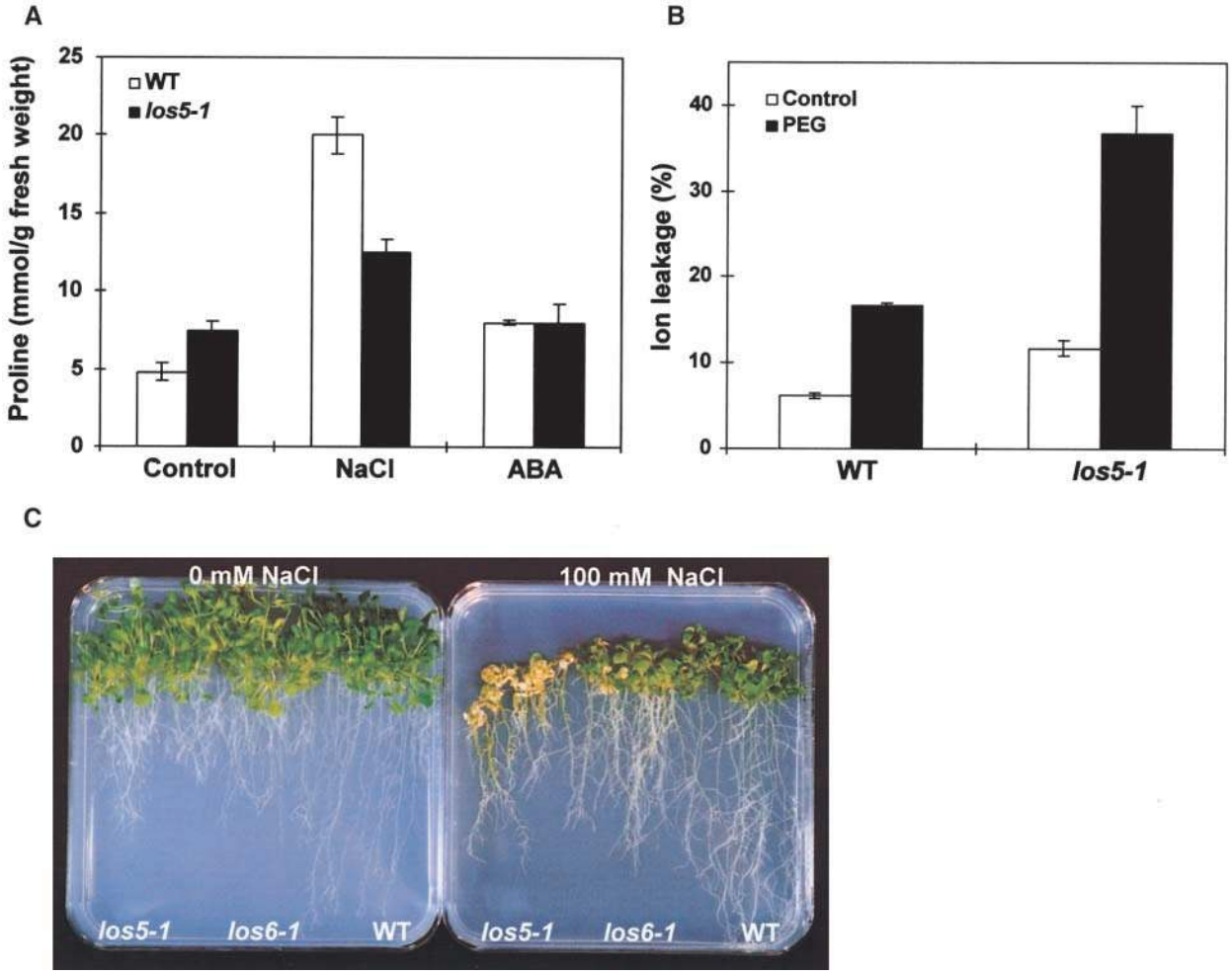

Figure 4. Proline Accumulation and Osmotic Stress Sensitivity of *los5-1* Mutant Plants.

(A) Proline accumulation in *los5-1* and wild-type plants that were untreated (Control) or treated with 150 mM NaCl or 50 μM ABA. Data represent means and ±SEs ($n = 3$).

(B) Drought sensitivity as measured by electrolyte leakage in wild-type and *los5-1* plants treated with 30% PEG. Data represent means and ±SEs ($n = 4$).

(C) *los5-1* plants are more sensitive to NaCl stress. One-week-old *los5-1*, *los6/aba1*, and wild-type seedlings were transferred from Murashige and Skoog (1962) nutrient agar medium to Murashige and Skoog agar plates without NaCl (0 mM NaCl) or with 100 mM NaCl. Note that *los5-1* mutant leaves were bleached as a result of NaCl stress. The photograph was taken 3 weeks after the seedlings were transferred to the treatment plates.

WT, wild type.

reduced gene induction by salt/drought treatments (J.-K. Zhu, unpublished observation). Interestingly, genetic analysis showed that *los6* is allelic to *aba1*. This provides an excellent opportunity to study the role of ABA in the cold and osmotic stress regulation of gene expression by using the very sensitive and reliable *RD29A-LUC* reporter and comparing two different ABA-deficient mutants, *los5* and *los6*. *RD29A-LUC* induction by salt stress was reduced significantly in *los5* and *los6* (Figure 6A). Interestingly, when ABA was administered simultaneously with salt stress, *RD29A-LUC* expression in both *los5* and *los6/aba1* was restored to the wild-type level or higher, indicating that exogenous ABA complements the reduced salt induction phenotype. This finding suggests that reduced gene induction by salt and drought in both *los5-1* and *los6/aba1* mutants can be accounted for solely by ABA deficiency.

Gene induction by cold also was analyzed using these two mutants. Without stress treatment, *los6*, like *los5* and

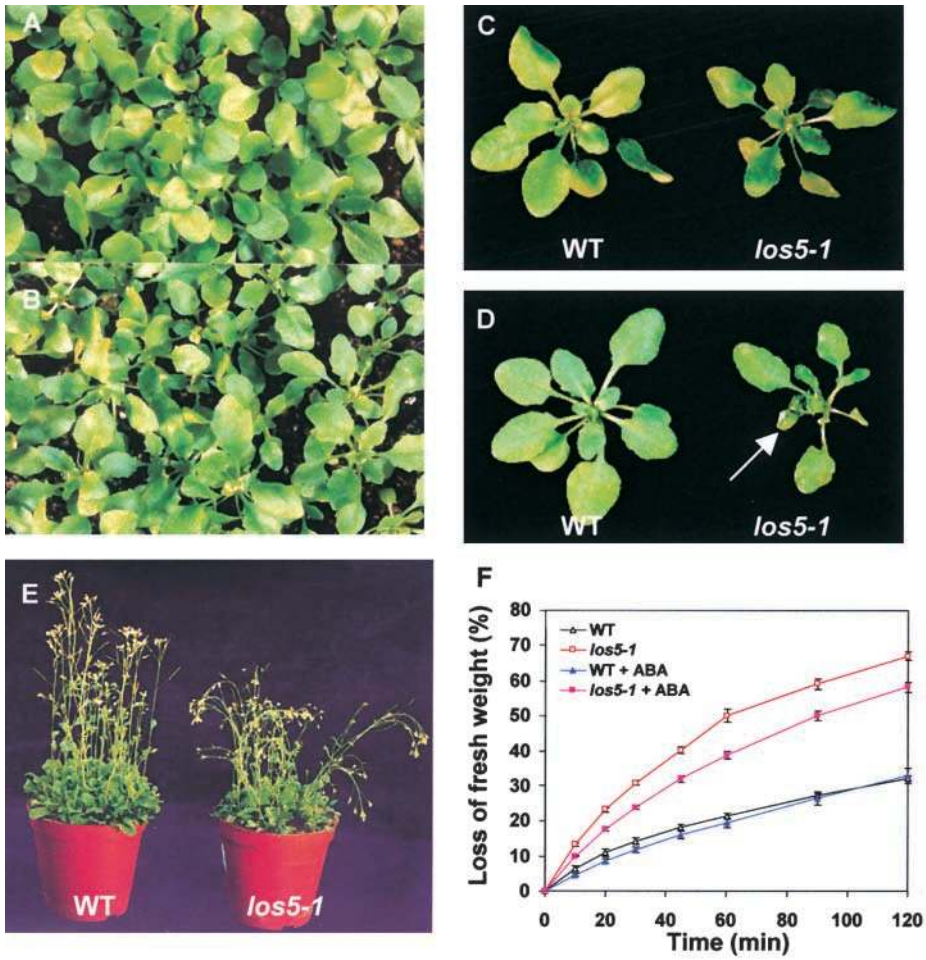

Figure 5. Leaf Morphology and Wilty Phenotypes of *los5-1* Mutant Plants.

(A) Wild-type plants in soil.
(B) *los5-1* mutant plants in soil.
(C) Wild-type and *los5-1* rosette plants are turgid immediately after root detachment.
(D) *los5-1* plants are wilty 10 min after root detachment. The arrow points to a wilty leaf in *los5-1*.
(E) Morphology of wild-type (left) and *los5-1* inflorescences 10 min after being moved from 90% to ~30% RH. Note that *los5-1* plants are wilty.
(F) Accumulative transpirational water loss in detached *los5-1* and wild-type shoots with or without 100 μM ABA treatment. Data are means and ±SEs ($n = 4$).
WT, wild type.

the wild type, showed no luminescence expression (data not shown). To our surprise, whereas the cold induction of *RD29A-LUC* in *los5-1* was reduced significantly, the expression in *los6/aba1* was increased significantly (Figure 6B). This increased luminescence expression in *los6* was observed consistently in numerous independent experiments. To test the role of ABA in cold gene regulation, ABA was administered together with cold treatment. Measurement of *RD29A-LUC* expression showed that ABA treatment restored the luminescence expression in *los6 aba1* close to the wild-type level (Figure 6B), indicating that the increased cold induction in *los6/aba1* may be an indirect consequence of ABA deficiency. In contrast, application of ABA to *los5* mutant seedlings failed to restore *RD29A-LUC* expression to the wild-type level. In fact, *los5* plants did not appear to respond to this ABA treatment compared with cold treatment alone (Figure 6B), suggesting that the reduced gene induction by cold in *los5* mutants is not a result of ABA deficiency.

Map-Based Cloning of the *LOS5* Gene

To clone the *LOS5* gene, homozygous *los5-1* mutant plants in the C24 ecotype were crossed with wild-type plants in the Columbia ecotype. The resulting F_1 plants were allowed to self. Initially, the F_2 seeds were plated on Murashige and Skoog (1962) agar plates, and seedlings were analyzed for luminescence expression. Putative mutants were selected by their reduced luminescence expression under cold treatment as well as salt treatment. Selected seedlings then were transferred to soil, and adult plants were tested for the wilty phenotype under reduced humidity conditions. Simple sequence length polymorphism markers distributed throughout the five Arabidopsis chromosomes that exhibit a size polymorphism between C24 and Columbia ecotypes were used for genetic mapping. The genetic mapping placed the *LOS5* locus on the upper arm of chromosome I, between the simple sequence length polymorphism markers *AtEAT1* and *nga248* (Figure 7A). This region corresponds roughly to where the *ABA3* locus was mapped (Léon-Kloosterziel et al., 1996). Because most of the mutant seedlings selected after the NaCl treatment failed to survive, we later used the salt tolerance phenotype of *los5* mutant seed during germination to select mutants for mapping. Specifically, the segregating F_2 seeds were plated on agar medium supplemented with 200 mM NaCl, a salt concentration that inhibits the germination of wild-type but not *los5* mutant seed. Putative *los5* seedlings then were transferred to soil and later were confirmed by examination of their wilty phenotype.

While the fine mapping of *LOS5* was in progress, genomic DNA sequence corresponding to the *LOS5* region was released. Given the ABA-deficiency phenotype, the availability of genomic sequence makes it possible to select candidate genes to find the *los5* mutation. A detailed examination of the genes on bacterial artificial chromosome clones in this region (http://www.arabidopsis.org/cgi-bin/maps/5chrom) identified several candidates that could function in ABA biosynthesis. Among them, the F19K19.13 gene on bacterial artificial chromosome clone F19K19 appears to be a good candidate (Figure 7A). BLAST searches suggest that the predicated gene product has high similarity to MoCo sulfurase from other organisms. Previous studies have indicated that the genetic lesion in *aba3* affects the introduction of sulfur into the MoCo (Schwartz et al., 1997a) and that sulfurylated MoCo is required by aldehyde oxidase (Schwartz et al., 1997a; Akaba et al., 1998; Sagi et al., 1999), which functions in the last step of ABA biosynthesis (Schwartz et al., 1997a).

Genomic DNA corresponding to F19K19.13 was amplified by polymerase chain reaction (PCR) from wild-type and *los5-1* mutant plants and sequenced. Comparison of the sequences revealed a G-to-A change 1083 bp downstream of the predicted translation initiation codon in *los5-1*. The F19K19.13 gene then was amplified from *los5-2* and sequenced. Sequence analysis identified a G-to-A change in *los5-2* 1040 bp downstream of the predicted translation initiation codon. These results strongly suggest that the F19K19.13 gene is *LOS5*.

Table 2. ABA Content in *los5* and Wild-Type Plants Treated with Drought Stress (μg/g Fresh Weight)[a]

Treatment[b]	Wild Type	*los5-1*
Unstressed	0.74 ± 0.04	0.70 ± 0.08
Stressed	3.06 ± 0.24	1.27 ± 0.10

[a] Data are means ±SE ($n = 3$).

[b] For drought stress treatment, detached rosette leaves were allowed to lose 30% of their fresh weight and then incubated at 100% RH for 5 hr. See Methods for details.

The F19K19.13 gene product is predicted to function in ABA biosynthesis at a step corresponding to ABA3. Our genetic analysis also suggested that *los5-1* likely is allelic to *aba3-1* (Table 2). Together, these results strongly suggested that F19K19.13 is the *LOS5*/*ABA3* gene. To test this hypothesis, we sequenced the F19K19.13 gene from *aba3-1* and *aba3-2* alleles, and the DNA sequences were compared with those from Columbia (*aba3-1* background) and Landsberg (*aba3-2* background) wild-type plants, respectively. Results showed that in *aba3-1*, a G-to-A change occurred at position 3707, whereas in the *aba3-2* allele, there are three mutations in a row with a single nonmutated nucleotide spacing them: a G-to-A change at position 3176, a T-to-A change at position 3178, and a deletion of T at position 3180. The nature of the mutations is consistent with the type of mutagens used: *aba3-1* was caused by ethyl methanesulfonate, whereas *aba3-2* was caused by γ-ray irradiation (Léon-Kloosterziel et al., 1996).

All of the changes in F19K19.13 DNA sequence in the four *los5*/*aba3* mutant alleles are predicted to cause changes in the predicted open reading frame. Together, these data unequivocally demonstrate that the *LOS5* locus is identical to *ABA3* and that the *LOS5*/*ABA3* gene is F19K19.13. After we identified the *los5* mutation, a third mutant allele of *ABA3*, *frs1*/*aba3-3*, was reported (Llorente et al., 2000). The freezing-sensitive phenotype and the degree of ABA deficiency in *frs1*/*aba3-3* (Llorente et al., 2000) are very similar to those in *los5*. For consistency, we propose to rename *los5-1* as *aba3-4* and *los5-2* as *aba3-5*.

LOS5/*ABA3* Encodes a Molybdopterin Cofactor Sulfurase

To obtain the cDNA sequence of *LOS5*, reverse transcriptase PCR was performed with mRNA extracted from wild-type Columbia plants. The reverse transcriptase PCR product was cloned and sequenced (accession number AY034895). After the *LOS5* cDNA was cloned, an identical sequence (accession number AF325457; submitted by F. Bittner and R.R. Mendel) was released in GenBank. Com-

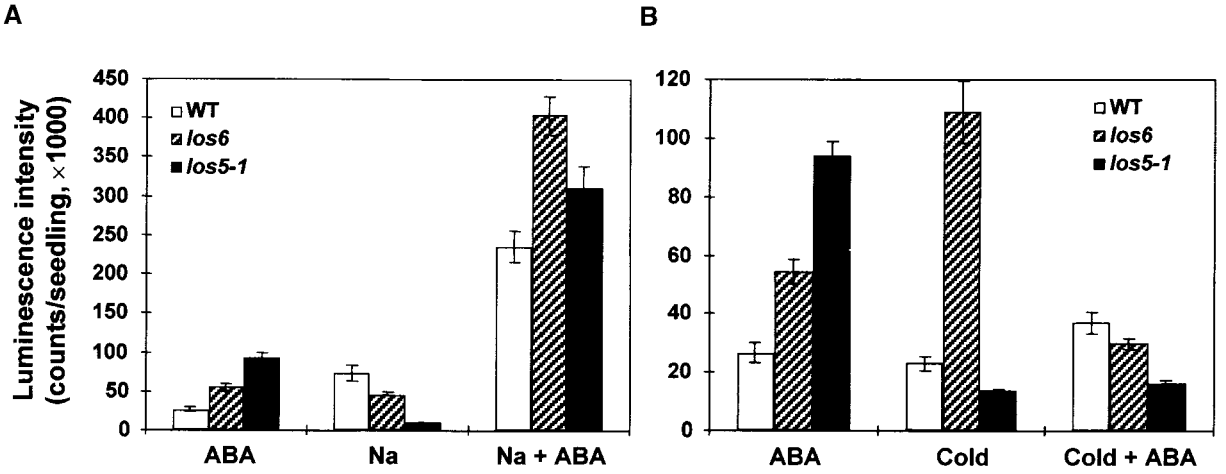

Figure 6. Cold Stress or Salt Stress Regulation of *RD29A-LUC* Expression in Wild-Type, *los5-1*, and *los6/aba1* Seedlings as Affected by Exogenous ABA.

(A) Salt stress responsiveness in *los5-1* and *los6-1* mutants is rescued by application of ABA.
(B) Cold responsiveness in *los5-1* mutant is not rescued by application of ABA.
Data are means and ±SEs ($n = 20$). Cold, 0°C for 48 hr; ABA, 100 μM ABA for 4 hr; Na, 300 mM NaCl for 4 hr; WT, wild type.

parison with genomic DNA sequence revealed that the *LOS5* gene consists of 21 exons and 20 introns (Figure 7B). The open reading frame consists of 2457 nucleotides and is predicted to encode a protein of 819 amino acids with an estimated molecular mass of 91.8 kD.

The *los5-1* mutation occurs at the 4th exon and changes a tryptophan residue at amino acid position 120 to a stop codon and thereby truncates the protein. The *los5-2* mutation also occurs at the 4th exon and changes a small glycine residue at position 106 to a larger, negatively charged glutamic acid residue. The *aba3-1* mutation occurs at the 13th exon and changes a glycine at position 469 to a glutamic acid residue. The *aba3-2* mutation occurs at the junction between 10th and 11th exons. The *aba3-2* mutation changes the leucine residue at position 387 to a stop codon and thereby truncates the protein from the 11th exon (Figures 7B and 8).

Database searches showed that LOS5/ABA3 has high sequence homology with the molybdopterin cofactor sulfurase (MCSU) recently identified from cattle (Watanabe et al., 2000), which belongs to a highly conserved protein family found from bacteria to humans (Figure 8). Overall, the LOS5 protein has 35% amino acid sequence identity and 53% similarity to the human homolog, 34% identity and 49% similarity to the Mal protein of *Drosophila melanogaster*, 35% identity and 51% similarity to MCSU of cattle (Watanabe et al., 2000), and 31% identity and 48% similarity to the HxB protein of *Aspergillus nidulans* (Amrani et al., 1999). The entire sequence of LOS5/ABA3 appears to consist of three domains. The N-terminal domain shows high sequence homology with the class V pyridoxal 5′ phosphate–dependent aminotransferases of type I fold, represented by the isopenicillin N epimerase, phosphoserine aminotransferase, aspartate decarboxylase, the small subunit of cyanobacterial soluble hydrogenase, and the NifS proteins from *Azotobacter vinelandii*. Recently, the structures of several NifS-like proteins were solved (Fujii et al., 2000; Kaiser et al., 2000), thus making it possible to identify conserved motifs in the NifS-like domain of LOS5/ABA3 (Figure 7C). This includes a putative pyridoxal phosphate (PLP) binding motif and a conserved cysteine motif (Figure 7C). The key residues in these motifs are marked in Figure 8. It is noteworthy that several putative proteins in the Arabidopsis genome show significant sequence similarity to this NifS-like domain (data not shown). The second domain, the junction that connects the NifS-like domain to the C-terminal domain, shows little sequence homology with other proteins except among members of this MCSU family. The C-terminal domain shows significant sequence similarity with a group of unknown proteins found in both Arabidopsis and other organisms. However, the Arabidopsis genome does not contain any other protein with significant overall sequence homology with the full-length LOS5/ABA3 protein, implying that *LOS5/ABA3* is a single-copy gene in the genome.

LOS5/ABA3 Is Expressed Ubiquitously, and Its Expression Is Enhanced by ABA and Drought Stress

To analyze the expression pattern of the *LOS5/ABA3* gene, full-length *LOS5* cDNA was used as a probe in RNA gel blot analysis, using total RNA extracted from different parts of

unstressed wild-type plants. The results indicate that *LOS5* is constitutively expressed at a relatively low level in all plant parts examined, including roots, stems, leaves, flowers, and siliques (Figure 9A). Interestingly, the transcript level of *LOS5* increased significantly in response to drought (Figure 9B), ABA, NaCl, and PEG treatments (Figure 9C). Cold treatment had no significant effect on *LOS5* expression (Figures 9C and 9D). The steady state *LOS5* transcript levels in *los5-1* seedlings were considerably lower than those in the wild type under the treatment conditions (Figure 9D), suggesting that the mutant transcript with a premature stop codon may trigger RNA surveillance mechanisms that remove abnormal transcripts (Hilleren and Parker, 1999).

The expression of *LOS5* also was analyzed in another ABA-deficient mutant, *aba1-1* (Koornneef et al., 1982), and in an ABA-insensitive mutant, *abi1-1* (Koornneef et al., 1984). The results indicate that the induction of *LOS5* by osmotic stress in *aba1* is not substantially different from that in the wild type. In the *abi1-1* mutant, the induction of *LOS5* appeared slightly lower compared with that in the wild type (Figure 9D).

Comparison between the Effects of *los5*/*aba3*, *aba1*, and *abi1* Mutations on Stress Gene Regulation

In contrast to *los5-1*, the *aba1-1* mutation did not decrease the induction of the *RD29A* transcript by cold or ABA (Figure 9D). In fact, cold induction of *RD29A* appeared higher in *aba1-1* compared with that in the wild type. The *aba1-1* mutation did decrease the induction of *RD29A* by NaCl or PEG stress, although the effect was not as dramatic as that of *los5-1*. The different effects on *RD29A* transcript induction caused by *los5-1* and *aba1-1* were in general agreement with the findings on the effects of *los5-1* and *los6-1* (a different *aba1* allele) on *RD29A-LUC* expression (Figure 6). *aba1-1* enhanced *ADH* (*ALCOHOL DEHYDROGENASE*) induction by cold and ABA but decreased the induction by NaCl or PEG stress (Figure 9D). Again, this is in sharp contrast to the findings with the *los5-1* mutation, which decreased the *ADH* induction by cold, ABA, NaCl, or PEG (Figure 9D). Both *aba1-1* and *los5-1* nearly completely blocked *RAB18* induction by osmotic stresses (Figure 9D). However, *los5-1* but not *aba1-1* also blocked *RAB18* induction by ABA (Figure

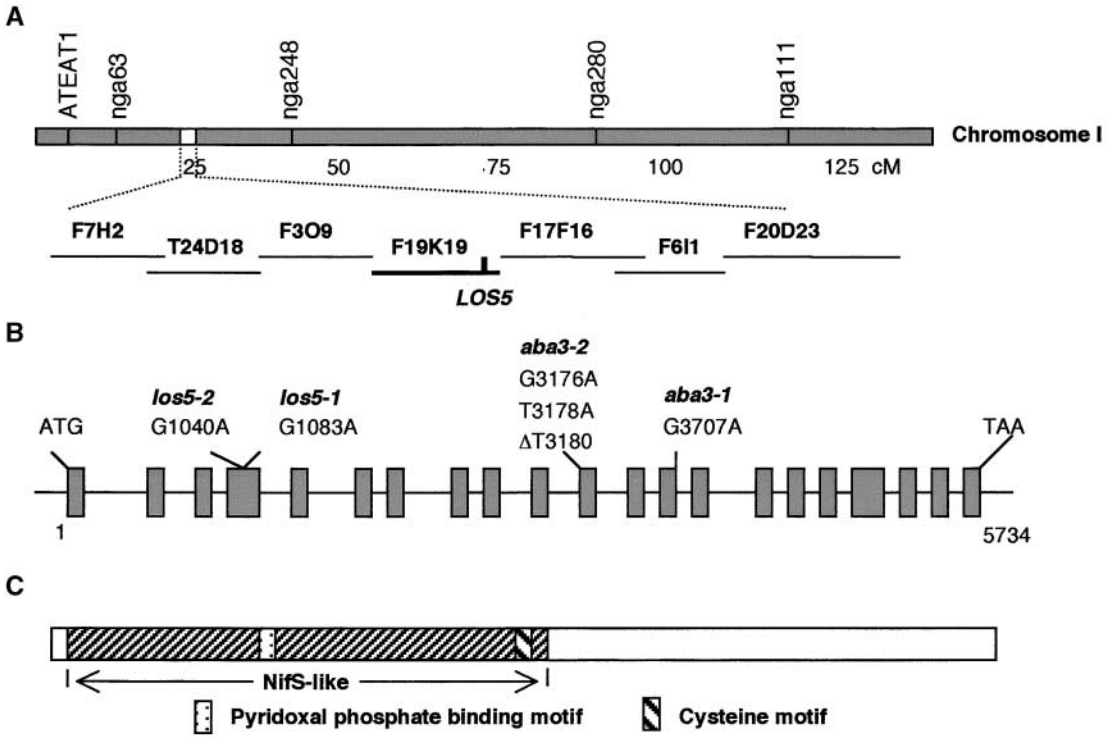

Figure 7. Positional Cloning of *LOS5* and the Organization of the *LOS5* Gene and Gene Product.

(A) *LOS5* was mapped to the upper arm of chromosome I and located on bacterial artificial chromosome clone F19K19. cM, centimorgan.
(B) Structure of the *LOS5* gene and positions of *los5*/*aba3* mutations. Positions are relative to the translation initiation codon. Closed boxes indicate the open reading frame, and lines between boxes indicate introns.
(C) Overall structure of the LOS5 protein.

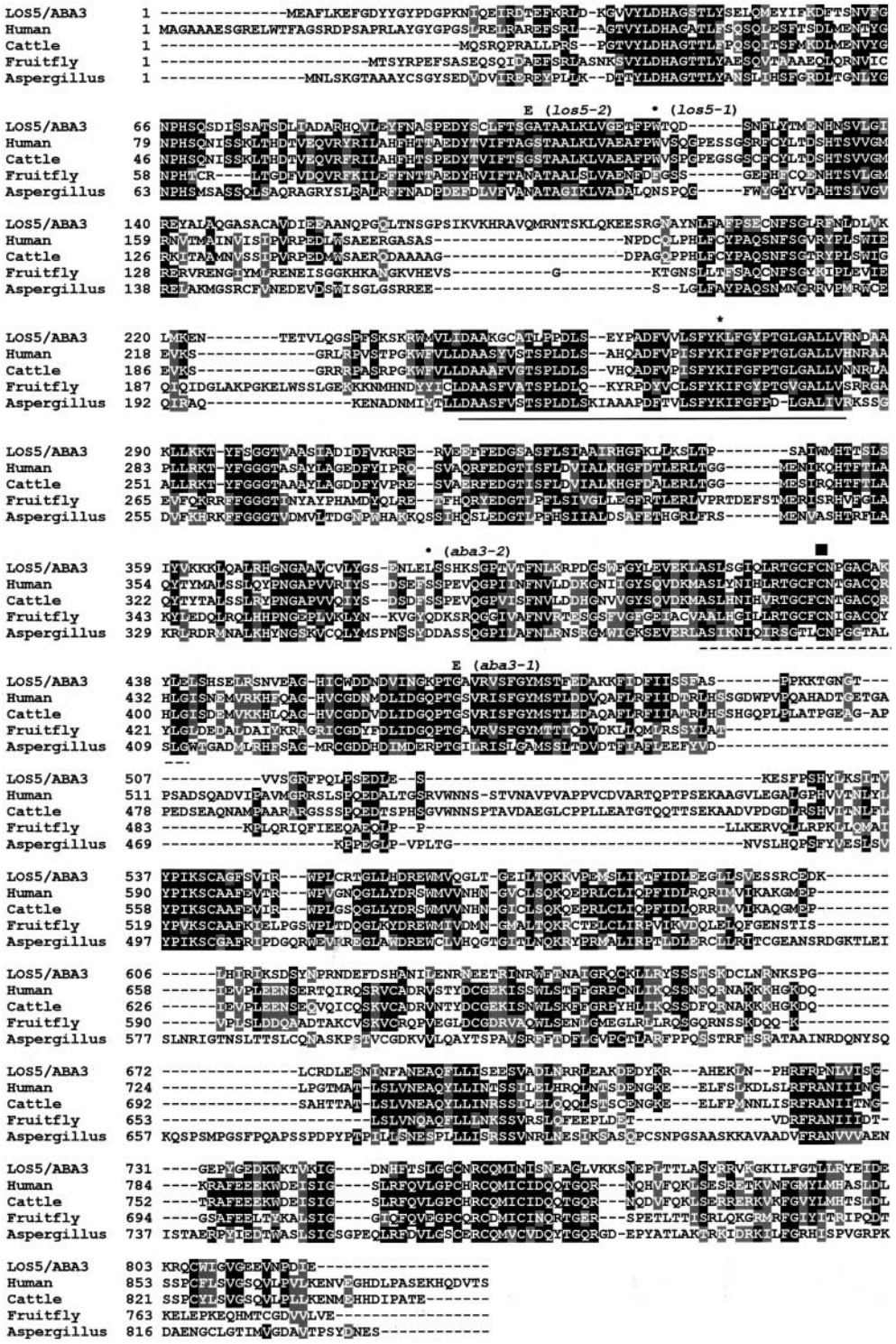

Figure 8. Sequence Alignment of LOS5 and Its Homologs from Other Organisms.

Residues shaded in black indicate identity, and those shaded in gray indicate similarity. Dotted lines indicate gaps that were introduced to maximize the alignment. The putative PLP binding motif is solid underlined, and the conserved cysteine motif is dash underlined. The conserved critical lysine residue in the PLP domain is indicated with an asterisk, and the conserved cysteine residue is indicated with a square. Also shown are the positions of *los5*/*aba3* mutations (closed circles indicate introduced stop codons). Sequence accession numbers for LOS5/ABA3 and its homologs are as follows: LOS5/ABA3, AY034895; human, BAA91354; fruitfly (Mal protein of *D. melanogaster*), AAF50901; cattle (MCSU of *Bos taurus*), BAA98133; and Aspergillus (HxB of *A. nidulans*), AAF22564.

9D). Compared with *aba1-1* or *los5-1*, the *abi1-1* mutation had little effect on *RD29A* or *ADH* induction by osmotic stresses (Figure 9D). Nevertheless, the induction of *RAB18* by ABA, NaCl, or PEG was reduced in the *abi1-1* mutant (Figure 9D).

Because the CBF (Stockinger et al., 1997) and DREB2 (Liu et al., 1998) family of transcription factors is known to bind to the *DRE* element present in the promoters of *RD29A* and several other stress-responsive genes, we were interested in determining whether the stress induction of these transcription factors is affected by the ABA-deficient or ABA-insensitive mutations. The results show that the expression of the cold-specific *CBF2*/*DREB1C* is not affected significantly *by los5-1*/*aba3-4* or *aba3-1* but is enhanced in the *aba2-1* mutant (Figure 9E). It was reported that *DREB2A* is induced specifically by osmotic stress (Liu et al., 1998). Under our treatment conditions, *DREB2A* expression also was upregulated by cold stress. Interestingly, whereas none of the ABA-deficient mutations significantly affected the osmotic stress induction of *DREB2A*, *los5* showed increased cold induction of *DREB2A* compared with the wild type (Figure 9E).

DISCUSSION

The phytohormone ABA plays many significant roles in plant growth and development and in plant responses to environmental stresses. Thus, understanding ABA biosynthesis pathways in plants is of critical importance. ABA biosynthesis mutants serve as excellent tools for understanding ABA biosynthesis and for studying gene regulation in response to stressful environments. In Arabidopsis and other plants such as maize, tobacco, and tomato, genetic analysis based on ABA promotion of seed dormancy has yielded a series of mutants that are defective in ABA biosynthesis (for recent reviews, see Koornneef et al., 1998; Cutler and Krochko, 1999; Liotenberg et al., 1999). Characterization of these mutants along with biochemical studies have revealed that in plants, ABA is synthesized from an "indirect" pathway via the cleavage of a carotenoid precursor. The Arabidopsis *aba1* mutant (and tobacco *aba2*) is defective in the epoxidation of zeaxanthin and antheraxanthin to violaxanthin (Rock and Zeevaart, 1991), and the affected gene encodes a zeaxanthin epoxidase (Marin et al., 1996). Oxidative cleavage of the 9-*cis*-neoxanthin by the VP14 protein yields xanthoxin. The *VP14* gene was isolated using the maize *vp14* mutant (Tan et al., 1997) and encodes a 9-*cis*-epoxycarotenoid dioxygenase (Schwartz et al., 1997b). It is thought that xanthoxin is converted to ABA by a two-step reaction via ABA-aldehyde. The Arabidopsis *aba2* mutant is impaired in the first step of this reaction and thus unable to convert xanthoxin into ABA-aldehyde. The Arabidopsis *aba3* mutant is defective in the last step of ABA biosynthesis, the conversion of ABA-aldehyde to ABA (Schwartz et al., 1997a), which is catalyzed by ABA-aldehyde oxidase. Mutations in either the aldehyde oxidase apoprotein (Seo et al., 2000b) or MoCo synthase impair ABA biosynthesis and lead to ABA deficiency in plants.

MoCo consists of a single molybdenum atom coordinated to the sulfur atoms of an organic moiety, molybdopterin. MoCo is highly conserved in cellular organisms and is used for the transfer of an oxygen atom in redox reactions involved in the metabolism of nitrogen, sulfur, and carbon (for review, see Kisher et al., 1997). Defects in MoCo biosynthesis have been reported to be associated with many diseases in humans and other animals (for review, see Reiss, 2000). In plants, three groups of MoCo-containing enzymes have been described (for review, see Mendel and Schwarz, 1999): nitrate reductase (NR), xanthine dehydrogenase (XDH), and aldehyde oxidase (AO). A fourth group, sulfite oxidase, also exists in the completely sequenced Arabidopsis genome. Different from NR and sulfite oxidase, which use the dioxyo form of MoCo, both XDR and AO require that the MoCo be modified in the last step of biosynthesis with the insertion of a sulfur atom to replace one of the two terminal oxygen atoms (Figure 10). This sulfuration reaction is catalyzed by MCSU. Mutants defective in this step have been identified from *D. melanogaster* (*mal*) (Wahl et al., 1982), *A. nidulans* (*hxB*) (Scazzocchio, 1973; Amrani et al., 1999), and cattle (Watanabe et al., 2000). Plant mutants defective in this sulfuration step also have been identified: tobacco *aba1* (Leydecker et al., 1995), tomato *flacca* (Marin and Marion-Poll, 1997), and Arabidopsis *aba3* (Schwartz et al., 1997a). Biochemical characterizations suggest that these plant mutants all are defective in the last step of ABA biosynthesis. As expected, the defects are specific to AO and XDH but not to NR (Leydecker et al., 1995; Marin and Marion-Poll, 1997; Schwartz et al., 1997a). As in the *D. melanogaster mal* mutant, resulfuration of the plant mutant extracts with Na_2S restores XDH and AO activities in Arabidopsis *aba3* (Schwartz et al., 1997a), tobacco *aba1* (Akaba et al., 1998), and tomato *flacca* (Sagi et al., 1999). These studies suggest that the wild-type genes corresponding to the respective mutations likely encode MoCo sulfurases that function in the last step of MoCo modification, which is required specifically by AOs and XDHs for their catalytic activities.

The identification of *los5* mutants as impaired in ABA biosynthesis and the cloning of the *LOS5*/*ABA3* gene demonstrate that *LOS5*/*ABA3* encodes a putative MCSU. Because free MoCo is extremely unstable, to date there has been no report on the sulfuration enzyme activity of MCSU using either MoCo or other substrates. Nonetheless, abundant evidence from both plant and animal studies that resulfuration of mutant extracts restores AO and XDH or other enzyme activities strongly suggests that MCSU have in vivo sulfuration activity against the desulfo form of MoCo. Additionally, sequence comparisons with other proteins also support the potential catalytic property of MCSU.

Like other MCSU proteins, the *LOS5*/*ABA3* gene product has extensive sequence similarity to the NifS-like protein in its N-terminal region (Figure 7C). The NifS protein of *A. vinelandii* is required for the activity of nitrogenase, the only

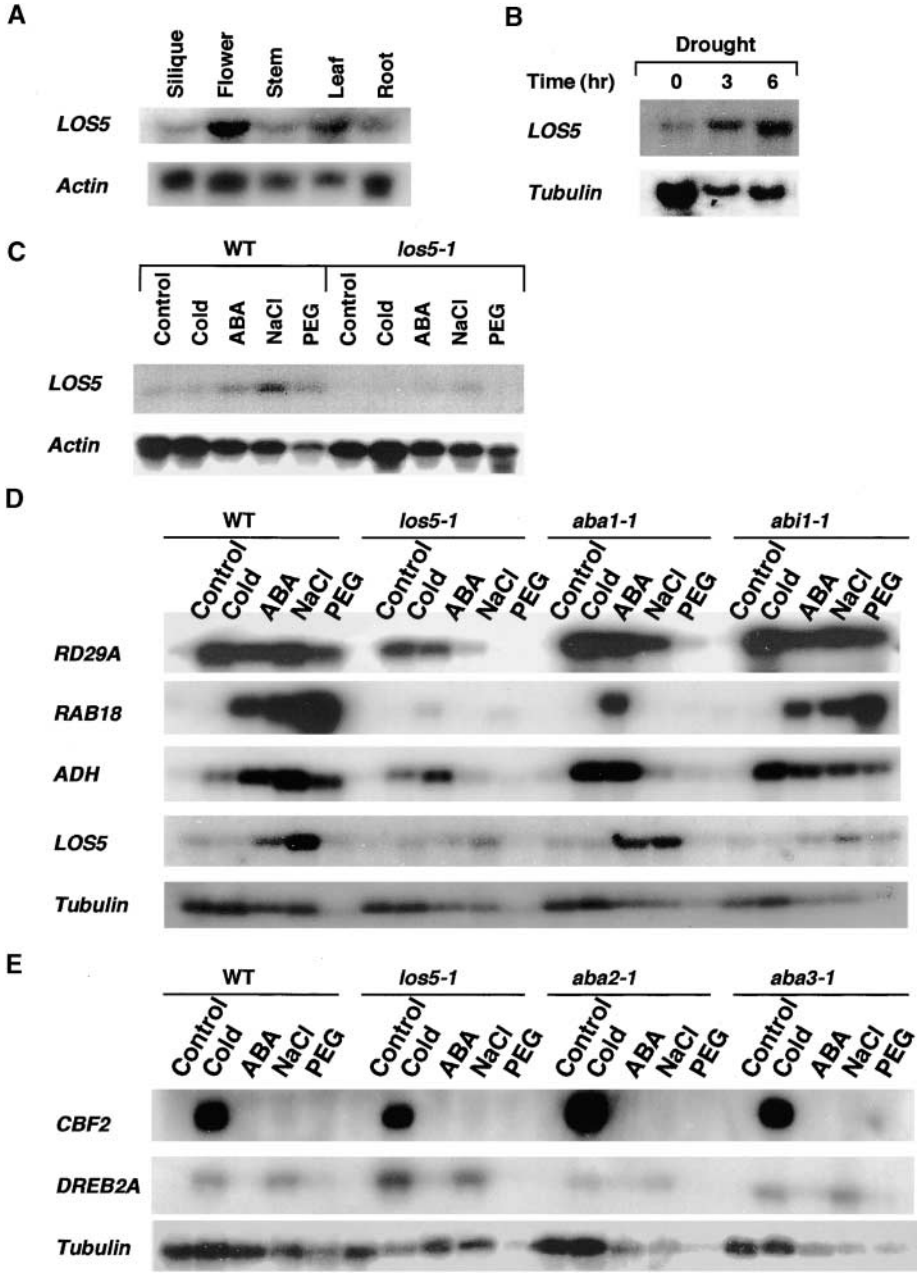

Figure 9. *LOS5/ABA3* Gene Expression and Stress Gene Regulation in ABA-Deficient or ABA-Insensitive Mutants.

(A) *LOS5* expression in different parts of plants.
(B) Upregulation of *LOS5* expression by drought in wild-type seedlings.
(C) *LOS5* expression under different stress treatments in wild-type and *los5-1* plants.
(D) Expression of *LOS5* and other stress-responsive genes in *los5-1*, *aba1-1*, and *abi1-1* plants.
(E) *CBF2* and *DREB2A* expression in ABA-deficient mutants.
Control, untreated; cold, 4°C for 12 hr; ABA, 100 μM ABA for 4 hr; NaCl, 300 mM NaCl for 5 hr; PEG, 30% PEG for 5 hr. *Tubulin* and *actin* are shown as loading controls.

molybdenum-containing enzyme that does not use molybdopterin cofactor but instead requires an iron-molybdenum-sulfur cluster for electron transfer. Although the exact function of the NifS protein in nitrogen fixation is not known, NifS was shown in vitro to be able to use L-cysteine as a substrate to form alanine and sulfide. Thus, NifS appears to act as a cysteine desulfurase in the biogenesis of the metallocluster by mobilizing an inorganic sulfide originating from the substrate L-cysteine (Zheng et al., 1993). This reaction is quite similar to the chemical reactions expected to be catalyzed by LOS5/ABA3 in the sulfuration of the desulfo-MoCo. In fact, both the PLP binding motif and the conserved cysteine motif required for NifS are well conserved in LOS5/ABA3 and its homologs in other organisms (Figures 7C and 8). Both by analogy with members of the class V aminotransferase-like proteins and by experimental analysis (Zheng et al., 1993), PLP was found to be a cofactor for the NifS protein. Given the high similarity with NifS and other PLP-dependent proteins in this class, it is likely that PLP also is a cofactor for LOS5/ABA3. By comparing LOS5/ABA3 with other NifS proteins whose structures were solved recently (Fujii et al., 2000; Kaiser et al., 2000), the conserved lysine at position 271 (Figure 8) probably is the residue at which PLP is covalently attached to make a Schiff base (Fujii et al., 2000). Likewise, the conserved cysteine residue at position 430 (Figure 8) is a likely sulfur donor for the *trans*-sulfuration reaction (Figure 10). Both conserved motifs are found in all LOS5/ABA3 homologs (Figure 8). It is expected that both the PLP and the cysteine motifs are required for the catalytic activity of LOS5/ABA3. Interestingly, the *aba3-1* mutation occurs just outside of the NifS-like domain, but it is still in a highly conserved region, suggesting that this region also is required for the enzyme function.

The overall structure of LOS5/ABA3 is similar to that of a chimeric protein that has evolved by fusing two separate proteins. The C-terminal domain does not have a known function yet, but it is likely also important, considering the high similarity in this domain among the MCSU proteins from diverse organisms and its high similarity to several unknown proteins in the Arabidopsis genome.

One interesting note related to the apparently multiple functional domain structure is that both the *los5-1* and *los5-2* mutants have a unique leaf morphology (Figure 5B), whereas in *aba3-1* and *aba3-2*, there is no such clear alteration in leaf form (data not shown). It is worth noting that both the *los5-1* and *los5-2* mutations occurred at the N-terminal part, whereas the *aba3-1* and *aba3-2* mutations occurred in the middle of the protein. It is tempting, therefore, to speculate that the N-terminal domain in the *aba3-1* and *aba3-2* mutant proteins may retain some activity that is required for maintenance of the wild-type leaf morphology. On the other hand, the *los5-1* and *los5-2* mutant forms may have lost this activity, resulting in an altered leaf form.

Because *LOS5/ABA3* is a single-copy gene in the Arabidopsis genome, it is not surprising that it is expressed ubiquitously (Figure 9A). This is in contrast to the Arabidopsis aldehyde oxidase (AAO) gene family, in which each member has a different expression pattern (Seo et al., 2000b). However, it is interesting that the tomato *flacca* mutant was reported to lose AO and XDH activities in the shoot but to retain measurable activities in the roots, where a notable amount of ABA accumulates (Sagi et al., 1999). This raises the possibility that there may exist more than one *LOS5/ABA3*-like MCSU gene in tomato and that a root-specific isoform(s) may remain active in the *flacca* mutant. In addition to impaired ABA biosynthesis in *flacca* mutant shoots, it was shown that the mutation might reduce the transport of ABA from the root to the shoot as well (Sagi et al., 1999).

In the present study, we showed that the expression of the *LOS5/ABA3* gene is upregulated when plants are treated with drought, salt, or ABA (Figure 9). We note that the promoter region of *LOS5/ABA3* contains putative *ABREs* (e.g., ACGTGG at −253 upstream from the translation initiation codon) and *DRE/CRT*-like elements, suggesting that the *LOS5/ABA3* gene may be regulated by ABA and drought/salt stress in a manner similar to other stress-responsive genes. In the ABA biosynthesis pathway, it is generally thought that the rate-limiting step is in the oxidative cleavage of the 9-*cis*-neoxanthin catalyzed by the VP14 protein (Schwartz et al., 1997b; Tan et al., 1997; Liotenberg et al., 1999; Iuchi et al., 2000; Taylor et al., 2000; Thompson et al., 2000). Given the relative low abundance of *LOS5/ABA3* transcript and the fact that it is the only gene encoding MCSU in Arabidopsis, it is likely that *LOS5/ABA3* also may regulate ABA biosynthesis. Low temperature appears to have little effect on the expression of *LOS5/ABA3* (Figures 9C and 9D), consistent with its limited effect on endogenous ABA biosynthesis (Thomashow, 1999). Qin and Zeevaart (1999) also found that low temperature did not induce the expression of *PvNECD1* (a *VP14* homolog in bean). Drought treatments (20% fresh weight loss and incubated for 3 or 6 hr) significantly increased the expression of *LOS5/ABA3* (Figure 9B), yet the same treatments failed to upregulate *AAO3* gene expression (data not shown). This lack of *AAO3* induction differs from the observation by Seo et al. (2000a) that dehydration (i.e., in an air flow hood for 3 hr) significantly induced *AAO3* expression in Arabidopsis shoot. The reason for this discrepancy is probably that our stress condition (dehydration in still air for ~40 min to lose 20% fresh weight, followed by incubation at 100% humidity for 3 or 6 hr) was not as severe. Our result implies that LOS5/ABA3 may be the key regulator in this last step of ABA biosynthesis. Consistent with this speculation, Sagi et al. (1999) found that sulfuration with Na_2S "superinduced" the activity of AO and XDH in wild-type tomato crude extracts, suggesting that MoCo sulfuration limits ABA-AO activity. ABA upregulation of *LOS5/ABA3* expression (Figures 9C and 9D) is very intriguing and may suggest a positive feedback regulation of ABA biosynthesis by ABA.

The availability of plant mutants defective in ABA biosynthesis has provided an excellent opportunity to study gene regulation by ABA under various abiotic stress conditions. In

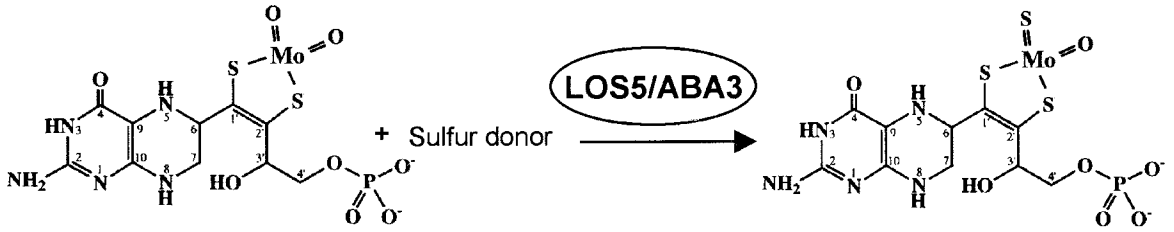

Figure 10. Reaction Catalyzed by LOS5/ABA3.

The desulfo/dioxyo form of MoCo (left) needs to be sulfurylated at one of the two terminal oxyo groups by LOS5/ABA3 MoCo sulfurase to generate the sulfide form of MoCo (right). The sulfide form of MoCo is a cofactor of AO and XDH, whereas the dioxyo form of MoCo is the cofactor for NR and sulfite oxidase. AO catalyzes the last step of ABA biosynthesis. The immediate sulfur donor could be a cysteine residue originating from LOS5/ABA3 or other sources. The structure of pterin and its numbering scheme are according to Rajagoplant (1991).

doing so, most researchers have used *aba1* or *abi1* and *abi2* along with their respective wild types. The Arabidopsis ABA-deficient mutant *aba1* was the first mutant defective in ABA biosynthesis isolated in Arabidopsis (Koornneef et al., 1982). Recently, additional ABA-deficient Arabidopsis mutants (*aba2* and *aba3*) became available (Léon-Kloosterziel et al., 1996). However, stress gene regulation in these recently isolated mutants has not been reported.

Extensive studies with *aba1* or *abi1/2* mutants have yielded considerable, yet sometimes conflicting, information. For example, Savoure et al. (1997) reported that the expression of *P5CS* genes is independent of ABA, because they observed that the expression level is similar in the wild type and in *aba1* under cold or drought treatments. They suggested that ABA might affect proline biosynthesis post-transcriptionally (Savoure et al., 1997). On the other hand, Yoshiba et al. (1999) reported that *AtP5CS1* induction by drought and salt stress is regulated by both ABA-dependent and ABA-independent pathways. Furthermore, Strizhov et al. (1997) found that stress-induced *P5CS1* gene expression absolutely requires ABA, which is consistent with our findings here (Figure 2). To help resolve the confusion, several reviews have been published and some consensus has been reached (Shinozaki and Yamaguchi-Shinozaki, 1997; Leung and Giraudat, 1998; Thomashow, 1999; Rock, 2000). Although low temperature treatment can trigger a transient increase in ABA and application of ABA can induce the expression of cold-responsive genes at warm temperatures and increase plant freezing tolerance, a general consensus is that ABA does not have an important role in regulating the expression of the *DRE/CRT* class of genes (Thomashow, 1999).

In the present study, we found that *los5-1* seedlings show a dramatic reduction in the expression of the *RD29A-LUC* transgene under low temperature treatment (Figures 1B and 1G). RNA gel blot analysis showed that the induction of *COR15*, *KIN1*, *COR47*, *RD29A*, *RAB18*, and *ADH* by low temperature also is reduced significantly in *los5-1* mutant plants (Figures 2 and 9D). The reduction in cold-regulated gene expression, as seen in *los5-1*, was not observed in *aba1-1* (Figure 9D). In fact, cold induction of both *ADH* and *RD29A* was enhanced in *aba1-1* (Figure 9D). Similarly, cold induction of *RD29A-LUC* expression was enhanced in *los6/aba1* mutant seedlings (Figure 6B). Our results with *los6/aba1* are consistent with previous studies on cold-regulated gene expression performed with *aba1-1* (reviewed by Thomashow, 1999). The different effects of *los5* and *aba1* mutations raise the question of whether the significant role played in cold-regulated gene expression by *LOS5/ABA3* is a result of ABA deficiency. This question was addressed partially by our experiment shown in Figure 6B; whereas treatment with ABA complemented the *los6/aba1* defect in cold-regulated *RD29A-LUC* expression, the same treatment failed to rescue *los5/aba3* (Figure 6B). This suggests that, in addition to its role in ABA biosynthesis, LOS5/ABA3 may have additional roles in cold regulation. Consistent with this notion is the finding that although exogenous ABA achieves similar or slightly higher expression of *COR47*, *RD22*, *RD29A*, and *ADH* (Figures 2, 6, and 9D) in *los5* relative to that in the wild type, ABA fails to induce the expression of *COR15* and *P5CS* and has a reduced induction of *KIN1* (Figure 2). These results strongly suggest that cold signaling requires a function of LOS5/ABA3 that is not related directly to ABA biosynthesis. At present, it is unclear how LOS5/ABA3 is involved in the cold or ABA regulation of some genes.

In contrast to low temperature, drought stress can dramatically stimulate de novo ABA biosynthesis; thus, ABA is more closely involved in drought/salt stress responses (Bray, 1993; Ingram and Bartel, 1996). Gene regulation by drought has been thought to involve both ABA-dependent and ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki, 1997). Because genes such as *RD29A*, *KIN1*, and *COR47* have both the *ABRE* complex and the *DRE/CRT* elements, presumably they can be activated by abiotic stress alone in the absence of ABA. Analysis using *aba1* or *abi* mutants showed that this is likely the case (Shinozaki and Yamaguchi-Shinozaki, 1997; Thomashow, 1999). However, our results with *los5* mutants clearly present a quite different situation. Under osmotic stress, the expression of *RD29A-LUC* is al-

most abolished in *los5* (Figures 1F and 1G). RNA gel blot analyses also found that the *los5* mutation virtually blocks the induction of *COR15*, *KIN1*, *RD22*, *P5CS*, and *RAB18* by salt and drought (PEG) stress (Figures 2 and 9D) and that it severely impairs the induction of *RD29A*, *COR47*, and *ADH* by these stresses (Figures 2 and 9D). To ascertain whether the effect of *los5* on osmotic stress–regulated gene expression is accounted for by ABA deficiency, we applied ABA together with salt stress and analyzed *RD29A-LUC* expression. The results indicate that ABA restores *RD29A-LUC* expression to wild-type levels in both *los5-1* and *los6/aba1* (Figure 6A). Our previous RNA gel blot analysis with the wild-type plants showed that under this treatment condition, the *RD29A-LUC* luminescence expression faithfully mirrors the pattern of endogenous *RD29A* expression (Xiong et al., 1999b). These results suggest that the observed defects in salt/drought-regulated gene expression in *los5* mutant plants most likely are a consequence of ABA deficiency. Currently, the alternative possibility that LOS5 may have yet unknown roles that are not related to ABA biosynthesis in regulating "ABA-independent" osmotic stress signaling cannot be ruled out completely. This is because the reduced magnitude of gene induction by drought or salt in *los6/aba1* was not as dramatic as in *los5* (Figure 6A), although these mutants show a similar extent of ABA deficiency by bulk quantitative measurement.

The *los5* mutation seems to have little effect on salt stress–regulated *DREB2A* expression (Figure 9E). This raises the possibility that DREB2A function may require ABA-dependent factor(s) to activate downstream gene expression. Previously, our genetic analysis using *RD29A-LUC* as a molecular marker has shown that ABA-dependent and ABA-independent signaling pathways may not function independently of each other. Rather, there exist extensive connections between them (Ishitani et al., 1997; Xiong et al., 1999a). The present molecular characterization of *los5* mutants casts further doubt on the ABA independence of "ABA-independent" stress signal transduction pathways, at least as far as the *DRE*/*CRT* genes are concerned. Furthermore, it has been shown that ectopic expression of *DREB2A* alone does not activate downstream gene expression (Liu et al., 1998). As has been suggested by Liu et al. (1998), DREB2A activity in activating stress-responsive genes may require post-transcriptional modifications. Thus, it is possible that phosphorylation/dephosphorylation of DREB2A or the functions of its cofactors may be dependent on ABA-regulated molecules such as ABI1, ABI2, Ca^{2+}-dependent protein kinases, or numerous other ABA-responsive regulatory factors (Leung et al., 1997; Leung and Giraudat, 1998; Finkelstein and Lynch, 2000; Rock, 2000; Merlot et al., 2001). This interdependence of ABA and stress signaling may underlie the mechanisms for the synergistic effect of ABA and drought/salt stress on the regulation of stress-responsive genes, as observed in the present study (Figure 6A) and elsewhere (Bostock and Quatrano, 1992; Xiong et al., 1999b).

METHODS

Isolation of *los5* Mutants

Arabidopsis thaliana (C24 ecotype) expressing the *RD29A-LUC* transgene (referred to as the wild type) were obtained by *Agrobacterium tumefaciens*–mediated transformation (Ishitani et al., 1997). Seed of wild-type *RD29A-LUC* were mutagenized by ethyl methanesulfonate (Ishitani et al., 1997). M2 seed were planted on 0.6% agar plates containing full-strength Murashige and Skoog (1962) salt base (MS salt base; JRH Biosciences, Lenex, KS) and germinated and grown at 22 ± 2°C under continuous white light. One-week-old seedlings were screened for mutants with altered *RD29A-LUC* (i.e., luminescence) expression in response to low temperature, abscisic acid (ABA), or osmotic stress using a thermoelectrically cooled charge-coupled device camera. For luminescence imaging, plants were sprayed with 1 mM luciferin in 0.01% Triton X-100 and then kept in the dark for 5 min before imaging. All images were acquired with a 5-min exposure time. The luminescence intensity of each seedling was quantified with WinView software (Princeton Instruments, Trenton, NJ).

Stress and ABA Treatment

For ABA treatment, 100 μM (±)-*cis*,*trans*-ABA in water was sprayed uniformly on leaves of seedlings, and the plants were incubated at room temperature under cool-white light for 4 hr (for luminescence imaging) or 2 or 4 hr (for RNA analysis). For NaCl or polyethylene glycol (PEG) treatment, seedlings on MS plates were transferred to filter paper saturated with MS solution supplemented with 300 mM NaCl or 30% PEG (molecular weight, 6000) and incubated for 5 hr. Unless stated otherwise, cold treatment for image analysis and RNA analysis was done by incubating seedlings growing on plates at 0°C in the dark for 48 hr (for imaging) or 12 hr (for RNA analysis). Because longer treatment at −5 or −10°C will result in freezing of the agar medium, these freezing temperature treatments lasted only 3 hr. After the treatment, the plates were placed at room temperature for 2 hr to thaw before luminescence image acquisition. For ABA plus NaCl treatment, seedlings were transferred onto a filter paper saturated with 300 mM NaCl in MS solution and sprayed immediately with 100 μM ABA. The plants then were incubated under cool-white light for 4 hr before luciferase imaging. For low temperature plus ABA treatment, seedlings on agar plates were incubated briefly at 0 ± 1°C for 10 min and then sprayed with 100 μM ABA and incubated in the dark at 0 ± 1°C for 48 hr before image analysis.

Genetic Analysis of *los5* Mutants and Map-Based Cloning of *LOS5*

For genetic analysis, the *los5* mutants were crossed to the wild type and to other mutants we isolated with similar luminescence phenotypes. The F_1 and F_2 seedlings were subjected to luminescence analysis and scored for *los5* luminescence phenotypes. *los5-1* also was crossed with *aba1*, *aba2*, and *aba3* mutants (obtained from the Arabidopsis Biological Resource Center, Columbus, OH). Some of the resulting F_1 and F_2 seedlings were treated with 300 mM NaCl for luminescence image analysis, and some were planted directly in soil for scoring of wilty phenotypes under reduced humidity. For genetic mapping of the *los5* mutation, the *los5-1* mutant in the C24 ecotype was crossed with wild-type plants of the Columbia ecotype. The

resulting F1 plants were allowed to self, and homozygous *los5-1* mutants in the segregating F2 population were selected based on their reduced luminescence when cold treated. A control treatment with ABA was used to rule out plants that did not have the *RD29A-LUC* transgene. Some of the seedlings also were tested for their reduced luminescence under NaCl treatment. Mapping of *LOS5* was performed as described previously (Lee et al., 2001). Simple sequence length polymorphism markers were developed by surveying released genomic DNA sequences for simple repeats using the RepeatMasker program (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). Primer pairs flanking these simple repeats that generate polymerase chain reaction products with size polymorphisms on 4% agarose gels between the C24 and Columbia ecotypes were used as molecular markers for mapping.

RNA Analysis

Ten-day-old seedlings on MS agar plates were treated with cold, ABA, NaCl, or PEG as described above. For drought treatment, seedlings at the rosette stage in potted soil were detached from the soil surface and allowed to lose 20% of their fresh weight. The dehydrated materials then were incubated in a container with 100% RH for 3 or 6 hr before being frozen in liquid N_2 for RNA extraction. Total RNA from control or treated plants was extracted and analyzed as described (Ishitani et al., 1998). Gene-specific probes were as described (Ishitani et al., 1998; Lee et al., 2001).

Stress Tolerance Assays

For the freezing sensitivity assay, wild-type and *los5* seedlings growing in potted soil in a growth chamber (22 ± 2°C, 16 hr of light and 8 hr of dark) for 3 weeks were first incubated at 4°C in the light for 48 hr to cold acclimate. After this cold acclimation, the plants were subjected to freezing at −7°C for 5 hr. Upon finishing the treatment, plants were transferred immediately to 4°C under white light and incubated overnight. The next morning, the plants were placed into a growth chamber. Seedling damage was scored at the times indicated in Results.

For NaCl tolerance tests, 7-day-old seedlings of *los5-1* and wild type growing on MS plates (solidified with 1.2% agar) were transferred to MS agar plates supplemented with different concentrations of NaCl. The plates then were placed vertically at 22 ± 2°C under white light, and root elongation was measured daily for up to 10 days. To measure ion leakage in seedlings induced by PEG treatment, 1-week-old wild-type and *los5-1* seedlings growing on MS agar plates were carefully removed from the plate, rinsed briefly in distilled water, and placed in solutions containing 30% PEG for 5 hr. After the treatment, seedlings were rinsed briefly in distilled water and placed immediately in a tube with 5 mL of water. The tube was agitated gently for 3 hr before the electrolyte content was measured. Four repetitions of each treatment were conducted.

Water Loss Measurements

For water loss measurements, plants at the rosette stage were detached from the soil surface and weighed immediately in a plastic weighing boat. The boat with the plants then was placed on a laboratory bench (RH ~30%) and weighed at designated time intervals. There were four replicates for each line. The percentage loss of fresh weight was calculated based on the initial weight of the plants.

Proline Assays

One-week-old seedlings of *los5-1* and wild type grown on MS agar plates were sprayed with 50 μM ABA or transferred to filter paper in a Petri dish saturated with 150 mM NaCl and incubated at 22 ± 2°C under white light for 24 hr. After the treatment, the samples were frozen in liquid nitrogen and kept at −80°C for proline assay. Proline concentration was determined as described by Bates (1973).

ABA Measurement

Rosette leaves were excised from 3-week-old mutant and wild-type plants grown in soil and placed on a laboratory bench. After the leaves lost 30% of their initial fresh weight (during a period of ~2 hr), they were placed in a sealed plastic bag with wet paper towels for an additional 5 hr. Unstressed control leaves were placed directly in a high-humidity sealed plastic bag without losing fresh weight. The tissues then were frozen in liquid nitrogen and ground into powder. One gram of the tissue was suspended in 15 mL of extraction solution containing 80% methanol, 100 mg/L butylated hydroxytoluene, and 0.5 g/L citric acid monohydrate. The suspension was stirred overnight at 4°C and centrifuged at 1000*g* for 20 min. The supernatant was transferred to a new tube and dried under vacuum. The dry residue was dissolved with 100 μL of methanol plus 900 μL of Tris-buffered saline (50 mM Tris, 0.1 mM $MgCl_2 \cdot 6H_2O$, and 0.15 M NaCl, pH 7.8). ABA concentration in the solution then was determined using the Phytodetek ABA immunoassay kit (Idetek, Inc., Sunnyvale, CA).

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