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Regulation of Osmotic Stress-responsive Gene Expression by the *LOS6/ABA1* Locus in *Arabidopsis**

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Drought and high salinity induce the expression of many plant genes. To understand the signal transduction mechanisms underlying the activation of these genes, we carried out a genetic screen to isolate *Arabidopsis* mutants defective in osmotic stress-regulated gene induction. Here we report the isolation, characterization, and cloning of a mutation, *los6*, which diminished osmotic stress activation of a reporter gene. RNA blot analysis indicates that under osmotic stress the transcript levels for stress-responsive genes such as *RD29A*, *COR15A*, *KIN1*, *COR47*, *RD19*, and *ADH* are lower in *los6* plants than in wild type plants. *los6* plants were found to have reduced phytohormone abscisic acid (ABA) accumulation and to be allelic to the ABA-deficient mutant, *aba1. LOS6/ABA1* encodes a zeaxanthin epoxidase that functions in ABA biosynthesis. Its expression is enhanced by osmotic stress. Furthermore, we found that there exists a positive feedback regulation by ABA on the expression of *LOS6/ABA1*, which may underscore a quick adaptation strategy for plants under osmotic stress. Similar positive regulation by ABA also exists for other ABA biosynthesis genes *AAO3* and *LOS5/ABA3* and in certain genetic backgrounds, *NCED3*. This feedback regulation by ABA is impaired in the ABA-insensitive mutant *abi1* but not in *abi2*. Moreover, the up-regulation of *LOS6/ABA1*, *LOS5/ABA3*, *AAO3*, and *NCED3* by osmotic stress is reduced substantially in ABA-deficient mutants. Transgenic plants overexpressing *LOS6/ABA1* showed an increased *RD29A-LUC* expression under osmotic stress. These results suggest that the level of gene induction by osmotic stress is dependent on the dosage of the zeaxanthin epoxidase enzyme.

Osmotic stress resulting from either high salinity or water deficit induces the expression of numerous stress-responsive genes in plants (1–5). Understanding the mechanisms that regulate the expression of these genes is a fundamental issue in basic plant biology and is instrumental for future genetic improvement of plant productivity under abiotic stresses. Considerable information has been accumulated as a result of molecular studies of gene regulation under osmotic stress (1–5). In contrast, genetic analysis of osmotic signal transduction has been very limited. Because the phytohormone abscisic acid (ABA)[1] is known to be involved in plant responses to various environmental stresses, the availability of ABA-deficient mutants (*aba*) or ABA-insensitive mutants (*abi*) in *Arabidopsis* has provided invaluable opportunities to investigate the role of ABA in plant stress responses. Using these mutants, changes in transcript levels of a few stress-responsive genes were analyzed under cold, drought, or salt stress (for reviews, see Refs. 3, 4, and 6). A general consensus resulting from these studies is that low temperature signaling is less influenced by ABA, whereas drought and salt stress signal transduction has both ABA-dependent and ABA-independent pathways (4, 6).

We have been using a reporter gene approach to dissect osmotic stress signal transduction networks. In this approach, *Arabidopsis* plants expressing the firefly luciferase gene under the control of the stress- and ABA-responsive *RD29A* promoter were used to screen for mutants with altered gene regulation by osmotic stress, cold, and/or ABA. In this report, we describe the isolation, cloning, and characterization of a mutation isolated in this screen. This mutation was designated as *los6* (for low expression of osmotic stress-responsive genes 6). Compared with wild type plants, *los6* mutant plants exhibit reduced luminescence induction by osmotic stress. *los6* plants were found to be ABA-deficient. Genetic analysis and cloning indicated that *LOS6* is allelic to *ABA1* and encodes a zeaxanthin epoxidase (ZEP). Analysis of *LOS6/ABA1* gene expression reveals a very intriguing phenomenon (*i.e.* the expression of *LOS6/ABA1* is significantly enhanced by exogenous ABA). Importantly, in *los6* mutant plants, osmotic stress up-regulation of *LOS6/ABA1* is reduced severalfold, whereas the expression level under ABA treatment is similar to that of the wild type. We found similar regulation of two other ABA biosynthesis genes *LOS5/ABA3* and *AAO3* by ABA. These observations uncover a positive feedback loop in the regulation of expression of ABA biosynthesis genes. In addition, we found that ABA regulation of ABA biosynthesis genes is partially impaired in the ABA-insensitive mutant *abi1* but not in *abi2*. In addition, we show that overexpression of *LOS6* leads to increased gene induction by osmotic stress.

EXPERIMENTAL PROCEDURES

Plant Materials and Stress Treatments—Arabidopsis plants (ecotype C24) expressing the *RD29A-LUC* transgene were obtained as previously described (7). This transgenic line, referred to as wild type, was mutagenized and mutants with altered luminescence in response to cold, salt, and ABA were screened as described (7). Soil-grown plants were kept in a growth chamber at 22 ± 2 °C with 16 h of light and 8 h of dark and a relative humidity of 90%. All image assays were done with seedlings growing in MS agar plates. For cold stress treatment, wild type and mutant seedlings growing in agar plates were incubated at 0 °C for 48 h. For NaCl treatment, wild type and mutant seedlings were transferred onto a filter paper saturated with 300 mM NaCl, and the plants were incubated under light for the indicated time. For ABA treatments, 100 μM ABA was sprayed directly on the seedlings growing

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[1] The abbreviations used are: ABA, abscisic acid; ZEP, zeaxanthin epoxidase; PEG, polyethylene glycol; NCED, 9-*cis*-epoxycarotenoid dioxygenase(s); PEG, polyethylene glycol; WT, wild type.

TABLE I
Genetic analysis of the los6 mutation

Values shown are number of seedlings that showed wild type or mutant luminescence phenotypes when treated with 300 mM NaCl.

Crosses (female × male)	F_1		F_2	
	WT	Mutant	WT	Mutant
Wild type × *los6*	22	0	295	86
los5–1 × *los6*	5	0		
aba2–1 × *los6*	11	0		
los6 × *aba1–1*	0	18		

in MS agar plates and incubated in the light for 3 h before being imaged for luminescence expression. For imaging, the treated seedlings were sprayed with 10 μM luciferin and incubated in the dark for 5 min, and the luminescence images were then taken with a CCD camera (Princeton Instruments, Trenton, NJ). The exposure time is 5 min. Luminescence intensity was quantified with the WinView software provided by the manufacturer.

RNA Analysis—Total RNA was isolated from 10-day-old *los6* and wild type plants growing in the same MS agar plates either untreated (unstressed controls) or treated with cold (0 °C for the indicated time), ABA (sprayed with 100 μM ABA and incubated under light for 5 h), or NaCl/polyethylene glycol (PEG) (transferred onto filter paper saturated with 300 mM NaCl or 30% polyethylene glycol with a molecular weight of 6000 and treated for 5 h). For drought stress treatment, seedlings growing in the soil were harvested and dehydrated to lose 20% of the fresh weight and incubated at 100% relative humidity for either 3 or 6 h before harvesting the samples. RNA blot analysis was conducted as described (8).

Transpirational Water Loss and ABA Measurement—Transpirational water loss was measured as described (9). For ABA measurement, rosette leaves were allowed to dehydrate on a laboratory bench to lose 30% of the initial fresh weight (over a period of 2 h). Afterward, the samples were placed in a sealed plastic bag with wet paper towels for an additional 5 h. Unstressed control leaves were placed directly in a high humidity sealed plastic bag without losing fresh weight. ABA was measured with an immunoassay method as described (9).

Genetic Analysis, Cloning, and Overexpression Assay—The *los6* mutants were back-crossed to the wild type *RD29A-LUC* plants and the F_1 seedlings were tested for luminescence expression in response to stress or ABA treatment as described above. The F_2 progeny of the self-pollinated F_1 were also scored for their luminescence expression after being treated with 300 mM NaCl. Genomic DNA was amplified from *los6* and wild type plants by polymerase chain reaction and sequenced. The *LOS6* cDNA was obtained by using reverse transcriptase-PCR and cloned into pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA) and sequenced. The cDNA was digested with *Eco*RI and was inserted into the pCAMBIA1200 under the control of cauliflower mosaic virus 35S promoter. The resulting plasmid was transferred into wild type *RD29A-LUC* plants via *Agrobacterium*-mediated flower dipping. T_3 generation of transgenic lines were used to test the luminescence responsiveness.

RESULTS

Isolation of los6 Mutant and Reduced Osmotic Stress-responsive RD29A-LUC Expression in los6 Mutant Plants—In our previous study, we constructed *Arabidopsis* plants expressing the firefly luciferase gene under control of the stress-responsive promoter *RD29A* (7). Mutants with altered expression of the *RD29A-LUC* transgene (*i.e.* bioluminescence expression) were isolated. One group of mutants that showed reduced luminescence expression relative to the wild type under salt stress were identified. Allelic tests suggested that these mutants defined two complementation groups. One locus defined by two alleles is *LOS5* (9). A second locus defined by one mutant is *LOS6* that is the subject of this study. Genetic analysis showed that *los6* mutation is recessive, and the mutation is in a single nuclear gene (Table I).

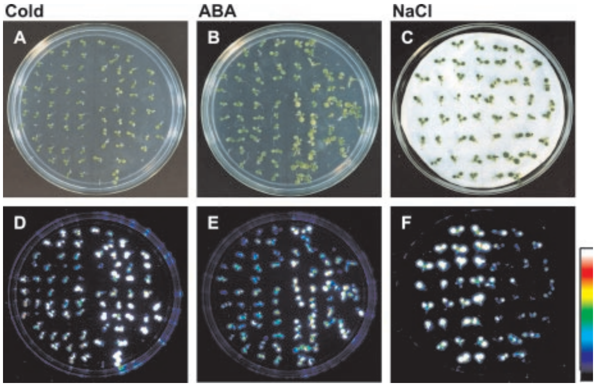

FIG. 1. ***RD29A-LUC* luminescence phenotypes of *los6* plants.** *A–C*, appearance of 1-week-old seedlings growing in agar plates for cold, ABA, and NaCl treatments, respectively. *D*, luminescence image of seedlings in *A* after incubation at 0 °C for 48 h. *E*, luminescence image of seedlings in *B* after treatment with 100 μM ABA for 3 h. *F*, luminescence image of seedlings in *C* after treatment with 300 mM NaCl for 3 h. The *color scale* on the *right* shows the luminescence intensity from *dark blue* (*lowest*) to *white* (*highest*).

Fig. 1 presents the luminescence phenotypes of the *los6* mutant. Whereas under unstressed condition, neither the wild type *RD29A-LUC* plants nor *los6* mutant plants exhibited any luminescence expression (data not shown), clear luminescence was seen in wild type plants under cold, salt, or ABA treatments (Fig. 1). Under cold treatment (0 °C for 48 h), *los6* exhibited a higher level of luminescence than the wild type (Fig. 1*D*). When treated with ABA (100 μM ABA, 3 h), *los6* also showed an increased luminescence expression relative to the wild type plants (Fig. 1*E*). In contrast, upon salt stress treatment (300 mM NaCl, 3 h), *los6* mutant plants showed reduced luminescence expression compared with the wild type (Fig. 1*F*). Quantitation of the luminescence intensities indicated that the luminescence level in *los6* plants is only 17% of the wild type level under salt stress (Fig. 2).

Regulation of Stress-responsive Gene Expression by LOS6—RNA blot analysis was conducted to determine whether the expression of the endogenous *RD29A* gene was affected in *los6* plants. It was found that the expression levels of *RD29A* in *los6* under cold, ABA, salt, and PEG treatment were all altered relative to those in wild type plants (Fig. 3). Consistent with the luminescence phenotype, the expression level of *RD29A* under ABA treatment was higher in *los6* plants. On the contrary, the *RD29A* transcript level under cold treatment was lower in *los6* than in the wild type (Fig. 3). Similar to what was observed with luminescence expression (Fig. 1), *RD29A* transcript level in *los6* plants was also significantly lower than that in the wild type when treated with NaCl (Fig. 3). To determine whether the reduced gene expression is specific to ionic stress or to general osmotic stress, the plants were treated with PEG and RNA blot analysis was conducted. Fig. 3 showed that the *RD29A* transcript level in *los6* was also considerably lower than that in the wild type under PEG treatment (Fig. 3), suggesting that *los6* impairs the activation of *RD29A* expression by general osmotic stress. Similar expression pattern was seen in several independent experiments (data not shown).

To determine whether the expression of other stress-responsive genes is affected by the *los6* mutation, the same RNA blot was probed with other stress-responsive genes. Cold regulation of all the low temperature-responsive genes was reduced in *los6* relative to that in the wild type, whereas ABA regulation of all of the genes except *RD22* was enhanced in *los6*. Impressively, the expression of nearly all the stress-responsive genes examined (*RD29B*, *COR15A*, *KIN1*, *COR47*, *RD19*, *RD22*, *ADH*, and *P5CS*) was reduced in *los6* mutant plants compared with the wild type under NaCl and PEG treatments (Fig. 2). The only exception was *RD19*, which did not show a reduced induction by NaCl in *los6*. The results demonstrate that *LOS6* has a very general role in regulating gene expression under osmotic stress, cold, and ABA treatment.

los6 Mutant Is Deficient in ABA and is Allelic to aba1—*los6*

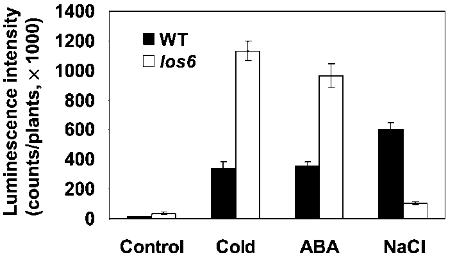

FIG. 2. **Quantitation of luminescence intensities in *los6* and wild type plants under stress or ABA treatment as shown in Fig. 1.** Data are means ± S.E. (n = 20).

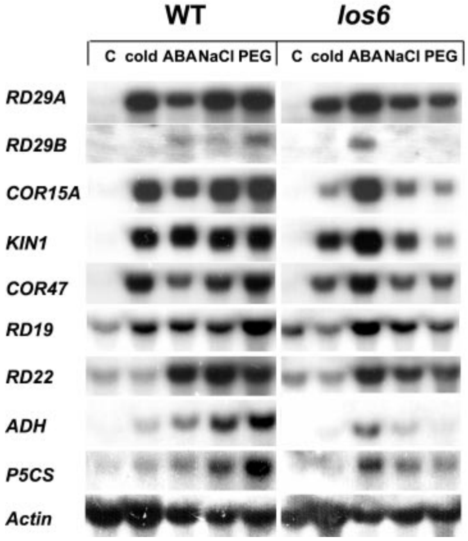

FIG. 3. **Expression of stress-responsive genes in *los6*.** Total RNA was extracted from *los6* and wild type plants without stress treatment (control) or subjected to cold (0 °C, 24 h), ABA (100 µM ABA, 3 h), NaCl (300 mM NaCl, 5 h), or PEG (30% PEG, 5 h) treatment. The actin gene was used as a leading control.

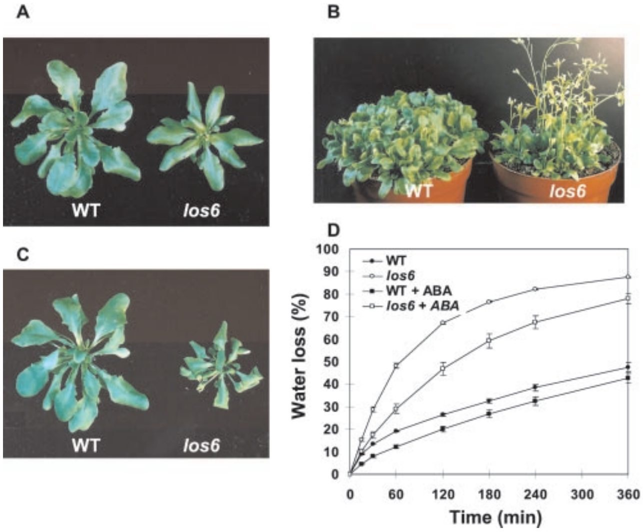

FIG. 4. **Morphology of *los6* plants and ABA deficiency phenotypes.** *A*, wild type and *los6* plants just detached from soil. *B*, *los6* plants flowered earlier. *C*, seedlings in *A* 15 min after the detachment. Note that *los6* seedlings became wilty. *D*, transpirational water loss in *los6* and wild type plants at the indicated time after detachment with or without pretreatment with ABA (100 µM ABA, 4 h). Data are means ± S.E. (n = 3).

TABLE II

ABA contents in wild type and los6 plants under unstressed or drought stressed treatments (µg/g fresh weight)

Data are the average of two independent assays. Stress treatment was conducted by dehydrating the leaves to lose 30% of the fresh weight following by a 5-h incubation at 100% relative humidity conditions.

Treatments	Wild type	*los6*
Unstressed	0.007	0.008
Stressed	0.10	0.01

plants were slightly smaller in stature and flowered about 1 week earlier than wild type plants under our growth conditions (Fig. 4*B*). The leaf morphology also appeared to be different from that of the wild type. Leaves of *los6* plants tended to be narrower (Fig. 4*A*), and the color was dark green when the plants were grown in soil but appeared slightly yellowish in agar plates (Fig. 1). The morphology was observed in *los6* mutants that had been back-crossed to the wild type plants three times. An interesting phenotype of *los6* plants is that they withered readily when removed from growth chamber (relative humidity ~90%) to room conditions (relative humidity 30–60%) (Fig. 4*C*). Measurements of transpirational water loss indicated that *los6* plants lost water faster than did the wild type plants (Fig. 4*D*). This suggests that *los6* plants could be ABA-deficient or ABA-insensitive, since both types of mutants could show a wilty phenotype. To see whether *los6* plants respond to ABA, ABA was sprayed onto the leaves of both wild type and *los6* plants, and transpirational water loss was measured 4 h after the treatment. The data showed that the rate of water loss decreased significantly in *los6* plants after ABA treatment, and the magnitude of decrease was higher than that in the wild type plants (Fig. 4*D*), further demonstrating that the *los6* plants may be ABA-deficient.

ABA contents in *los6* and wild type leaves were measured using an immunoassay kit. Without stress treatment, the basal ABA contents in the wild type and in *los6* were similarly at low levels (Table II). When the plants were drought-stressed (dehydration to lose 30% of fresh weight), ABA content in the wild type increased, whereas that in the *los6* plants did not change much (Table II), which resulted in the ABA content in the wild type being 10 times that in *los6*. These data show that *los6* plants are indeed ABA-deficient.

To test whether *los6* is allelic to known ABA-deficient mutants, *los6* was crossed to *aba1* (10), *aba2*, and *aba3* mutants (11), and luminescence expression as well as transpirational water loss rate were determined in the F_1 progenies. The result indicated that *los6* is allelic to *aba1* (Table I and data not shown).

Cloning and Expression Study of LOS6/ABA1—Previously, it was demonstrated that the tobacco *ABA2* locus encodes a ZEP (12). Biochemical studies showed that the *Arabidopsis aba1* mutant is defective in the reaction catalyzed by a ZEP (13, 14). In fact, tobacco *ABA2* gene could complement the *Arabidopsis aba1* mutant (12). When the *Arabidopsis* genome was partially sequenced, we searched the data base using tobacco *ABA2* and identified a gene (K8A10.10; GenBank™ accession number At5 g67030) on chromosome V (Fig. 5*A*) that has the highest similarity to tobacco *ABA2* and was annotated as a *ZEP* gene. In fact, this gene represents the only *ZEP* gene in the *Arabidopsis* genome. The genomic DNA corresponding to this gene was amplified from *los6* and wild type plants and sequenced. Sequencing results revealed that in *los6* mutant, a G at position 1588 downstream of the translation initiation codon was mutated to A, which would result in a missense mutation in the predicted protein. To determine whether the *aba1* mutant also has a mutation in this gene, we sequenced the genomic DNA of *aba1-1*. In *aba1-1*, a G at position 2129 was mutated to A, and this would introduce a premature stop codon in the transcript. Together, these results demonstrate that the K8A10.10 gene is *LOS6/ABA1*.

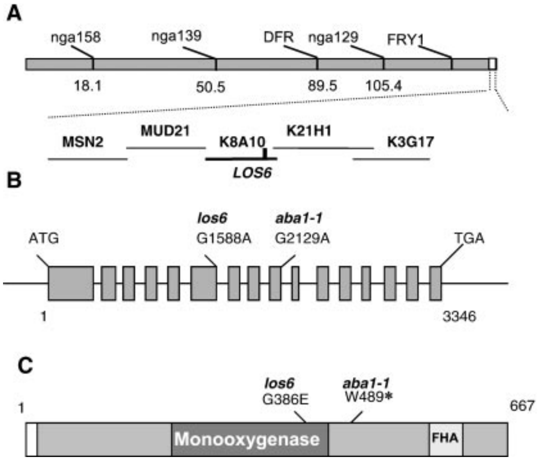

FIG. 5. **Structure of the *LOS6/ABA1* gene.** *A*, *LOS6* is localized on chromosome V, on the BAC clone K8A10. *B*, structure of *LOS6/ABA1* gene and position of *los6* and *aba1-1* mutations. *C*, structure of the predicted LOS6/ABA1 protein. The N-terminal chloroplast transit peptide, the monooxygenase domain, and the FHA motif are shown.

The cDNA corresponding to K8A10.10 was isolated by reverse transcriptase-PCR. Comparison of the cDNA with the genomic sequence indicated that the *LOS6/ABA1* gene consists of 16 exons and 15 introns (Fig. 5*B*). The gene was predicted to encode a polypeptide of 667 amino acids. Analysis of the predicted protein indicated that it has an N-terminal chloroplast transit peptide, a monooxygenase domain, and a FHA motif with unknown functions (Fig. 5*C*). The *los6* mutation that occurred in the monooxygenase domain (Fig. 5*C*) changed a small glycine residue at amino acid position 386 to a large and negatively charged glutamate. The *aba1-1* mutation changed the tryptophan residue at position 489 to a stop codon and thus truncated the protein. These mutations suggest that both the monooxygenase domain and the C-terminal part are important for full function of the protein.

LOS6/ABA1 shows very high sequence homologies to ZEP proteins from other plant species (Fig. 6). At the amino acid level, LOS6/ABA1 is 62–71% identical to ZEP from rice, tobacco, tomato, cowpea, pepper, and apricot. LOS6/ABA1 has less than 30% amino acid identities to monooxygenases in other organisms, indicating that ZEP may have evolved specifically in plants.

The *LOS6/ABA1* gene was expressed in every plant part examined, yet the expression levels varied among them (Fig. 7*A*). In the vegetative parts, *LOS6/ABA1* was expressed more in leaves and stems than in roots (Figs. 7, *A* and *B*). In reproductive organs, flowers had a much higher expression than siliques (Fig. 7*A*). Additionally, although *LOS6/ABA1* was constitutively expressed, the expression levels in whole seedlings and in leaves were enhanced by drought, salt, or PEG treatment (Fig. 7, *B–D*). Both in the shoot and root, *LOS6/ABA1* was up-regulated by NaCl treatment (Fig. 7*B*), although the induction in the shoot was more obvious. This expression pattern is different from that of the tobacco *ABA2* gene, which was reported to be enhanced by drought stress only in the root (15). In the *aba1-1* mutant, the *LOS6/ABA1* transcript level was very low (Fig. 7*D*). This is probably because transcripts with a premature stop codon are known to induce an mRNA surveillance mechanism that degrades the abnormal transcripts (16).

Regulation of LOS6/ABA1 Expression by ABA—Since the expression of many stress-responsive genes is regulated by ABA, it was of interest to see whether the stress-responsive ABA biosynthesis genes are also regulated by ABA. The ABA biosynthesis genes encoding 9-*cis*-epoxycarotenoid dioxygenases (NCED) in cowpea and in tobacco were shown not to be regulated by ABA (17, 18). We recently found that the expression of the *LOS5/ABA3* gene encoding a molybdenum cofactor sulfurase in ABA biosynthesis was enhanced by ABA (9). In the present work, the expression of *LOS6/ABA1* in the wild type, *los6*, and *aba1–1* mutant under ABA treatment was examined. The expression of *LOS6/ABA1* was clearly enhanced by exogenous ABA (Figs. 7*D* and 8*A*). In the *aba1-1* mutant, although the transcript level was very low, *LOS6/ABA1* gene induction could be seen under the ABA treatment (Fig. 7*D*).

Remarkably, in the *los6* mutant, unlike in the wild type, the expression of *LOS6/ABA1* was not enhanced by either NaCl or PEG. However, the expression of *LOS6/ABA1* in *los6* was clearly enhanced by ABA (Fig. 8*A*). Under ABA treatment, the level of *LOS6/ABA1* expression in *los6* was similar to that in the wild type.

To investigate whether other ABA biosynthesis genes are similarly regulated by ABA, we analyzed the expression of *NCED3*, *AAO3* (encoding an ABA-aldehyde oxidase (19)), and *LOS5/ABA3* in wild type and *los6* plants in response to stress or ABA treatment. In wild type plants, as expected, these genes were induced by NaCl and PEG (Fig. 8). Interestingly, ABA also induced the expression of *AAO3* and *LOS5/ABA3* but failed to induce the expression of *NCED3* (Fig. 8*A*). In *los6* mutant background, NaCl and PEG inductions of all of these three genes were greatly impaired as compared with those in wild type plants (Fig. 8*A*). In *los6* plants, ABA induced *AAO3* expression to a higher level than in wild type plants. Intriguingly, although ABA failed to induce *NCED3* expression in wild type plants, it induced the expression of *NCED3* in *los6* (Fig. 8*A*).

To ascertain whether the defects in the induction of ABA biosynthesis genes by stress and ABA as observed in *los6* is specific to *los6* mutation or are general defects of all ABA-deficient mutants, the expression of these genes under the same stress or ABA treatments was analyzed in other ABA-deficient mutants. Fig. 8*B* showed that the expression of *LOS6* and *NCED3* in *los5/aba3* under NaCl or PEG treatment was also diminished compared with that in the wild type. Moreover, *NCED3* was also induced by ABA treatment in *los5* mutant plants, demonstrating that the observed defects in ABA biosynthesis gene regulation in these mutants are due to ABA deficiency. Similar results were observed in other ABA-deficient mutants such as *aba1-1*, *aba2-1*, and *aba3-1* (data not shown).

ABA Regulation of ABA Biosynthesis Genes in abi1, abi2, and era1 Mutants—To gain insights into the mechanisms by which ABA biosynthesis genes are regulated, we studied the expression of these genes in the ABA-insensitive mutants *abi1* and *abi2*. Since both of these mutants are in the ecotype Landsberg, Landsberg wild type (*i.e.* Landsberg *erecta*) was used for control treatments. Whereas the induction patterns of *LOS6* and *AAO3* by stress and ABA treatments in these mutants are similar to those observed in wild type C24 plants (Fig. 8*C*), the expression pattern for *NCED3* in response to cold and ABA is different from that in the wild type C24 plants. The *NCED3* gene was not induced by cold or ABA in C24 wild type (Fig. 8, *A* and *B*) but was clearly induced in Landsberg wild type plants, albeit at relatively low levels (Fig. 8*C*).

The induction of these ABA biosynthesis genes by NaCl or PEG in *abi1* was lower compared with that in the wild type under the same treatment conditions (Fig. 8*C*). Moreover, the induction of *LOS6* and *AAO3* by ABA in *abi1* is significantly lower than that in the wild type plants (Fig. 8*C*). ABA induction of *NCED3* is also abolished in *abi1* (Fig. 8*C*). In contrast, the induction of these genes in the *abi2* mutant is virtually unchanged compared with that in the wild type (Fig. 8*C*).

Previous studies suggested that part of ABA signal transduction is affected in the enhanced response to ABA mutant

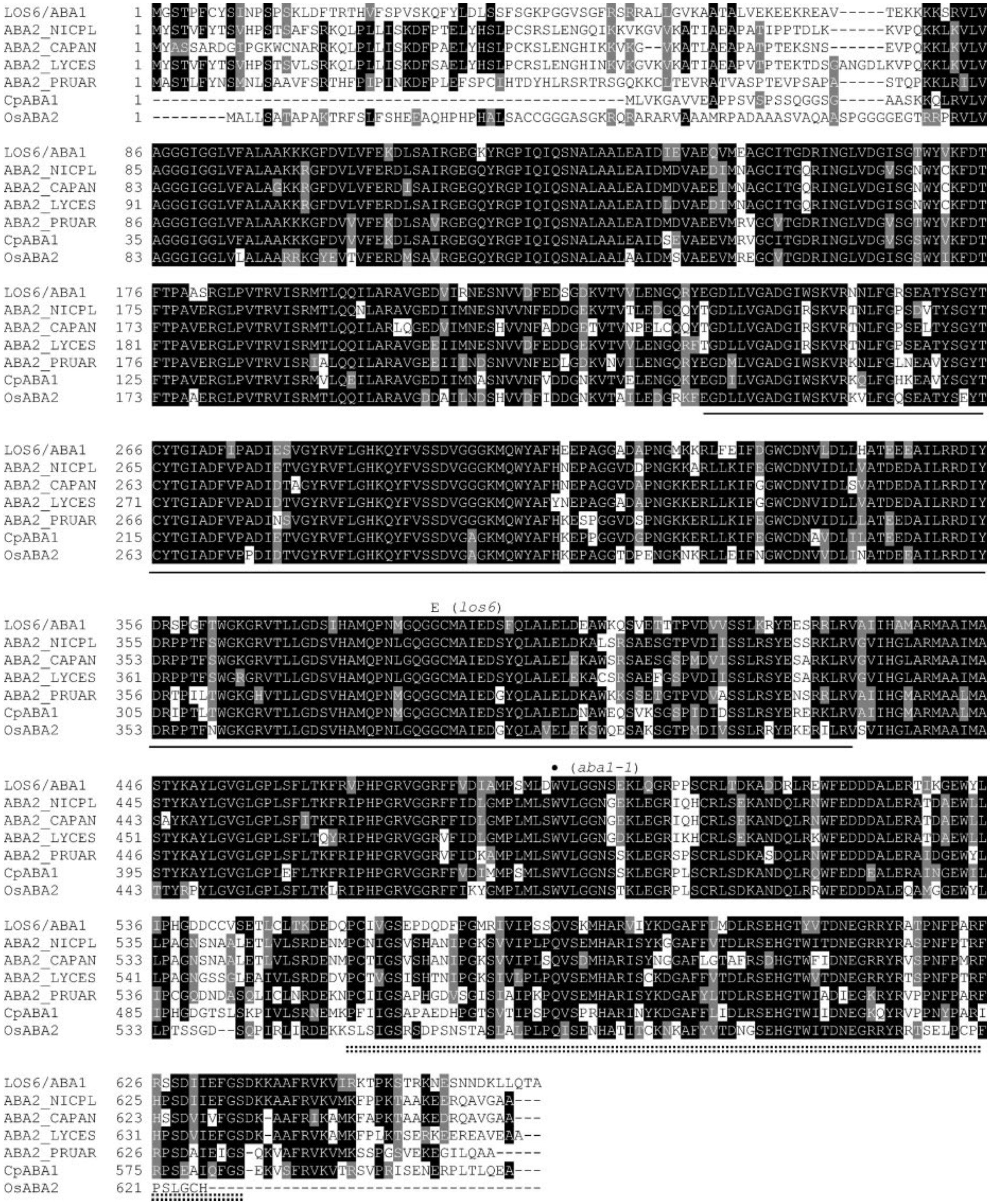

FIG. 6. **Homology of LOS6/ABA1 with ZEP proteins from other plant species.** Amino acids *shaded in black* indicate identity, and those *shaded in gray* indicate similarity. *Dotted lines* indicate gaps that are introduced to maximize alignment. The putative monooxygenase domain is *solid-underlined* and FHA motif is *double dash-underlined*. Also shown are the positions of *los6* and *aba1-1* mutations. Sequence accession numbers are as follows: LOS6/ABA1, At5 g67030; ABA2_NICPL, Q40412; CpABA1, BAB11934; ABA2_LYCES, P93236; ABA2_CAPAN, Q96375; OsABA2, BAB39765; ABA2_PRUAR, 081360.

era1 (20, 21). We thus determined whether the regulation of ABA biosynthesis genes by ABA is affected in *era1* mutant. The transcript levels for *los6* and *NCED3* in Columbia wild type and *era1* mutant seedlings treated with either 10 μM or 100 μM ABA or 300 mM NaCl were examined. It was found that the transcript levels were similar in the wild type and *era1* mutant (Fig. 8*D*). Therefore, *era1* does not appear to affect stress or ABA regulation of these ABA biosynthesis genes.

Enhanced Osmotic Stress Induction of RD29A-LUC in Plants Overexpressing LOS6—The above studies show that a loss-of-function mutation in the *LOS6/ABA1* gene results in a significant reduction of osmotic stress activation of gene expression (Fig. 3), indicating an essential role of *LOS6/ABA1* in the full activation of osmotic stress-responsive genes. We hypothesized that overexpression of *LOS6/ABA1* might strengthen gene induction by osmotic stress. To test this, a construct consisting of the *LOS6/ABA1* cDNA under control of cauliflower mosaic virus 35S promoter and a translational enhancer was introduced

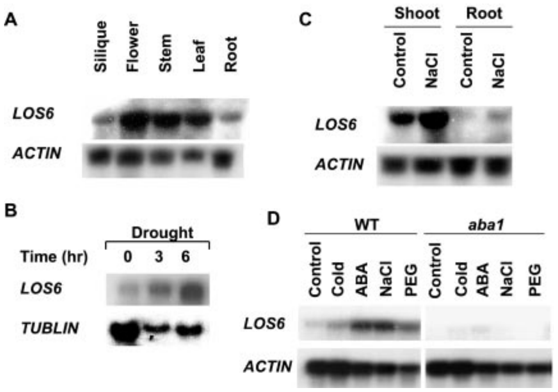

FIG. 7. **Expression and regulation of the *LOS6* gene.** *A*, expression in different plant parts. *B*, induction of *LOS6* by drought treatment (loss of 20% fresh weight and incubated for either 3 or 6 h; 0 h indicates without drought stress). *C*, expression of *LOS6* in the shoot and root with or without 200 mM NaCl treatment. *D*, expression of *LOS6* in wild type and *aba1-1* mutant. Stress treatments were as follows: control (no stress), cold (0 °C, 24 h), ABA (100 μM ABA, 3 h), NaCl (300 mM, 5 h), and PEG (30%, 5 h). Actin, tublin gene, or ethidium bromide-stained ribosomal RNA is used as loading control.

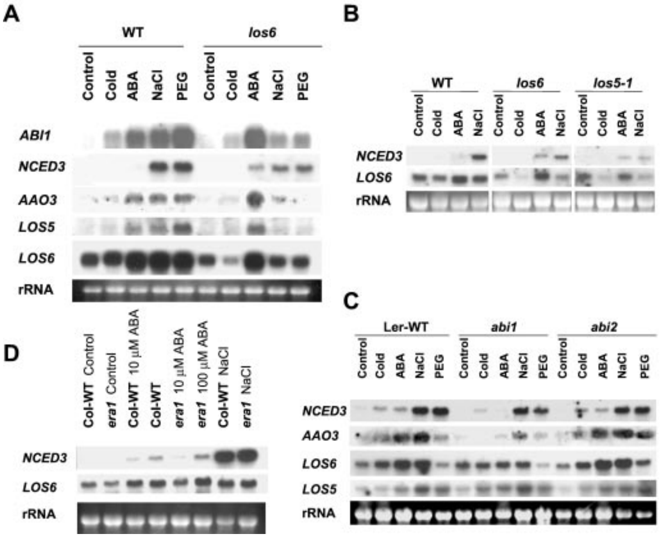

FIG. 8. **Regulation of *LOS6* and other ABA biosynthesis genes in ABA-deficient and ABA response mutants.** *A*, expression of *LOS6* and other ABA biosynthesis genes in wild type and *los6* mutant. *B*, expression of *LOS6* and *NCED3* in *los6* and *los5* mutants. *C*, expression of ABA biosynthesis genes in *abi1* and *abi2* mutants. *D*, expression of *LOS6* and *NCED3* in the *era1* mutant. Stress treatments were as follows: control (no stress), cold (0 °C, 24 h), ABA (100 μM ABA or at the indicated concentrations, 3 h), NaCl (300 mM, 5 h), and PEG (30%, 5 h). *WT* in *A* and *B* refers to *RD29A*::*LUC* (ecotype C24) wild type. *Ler-WT* in *C* refers to Landsberg wild type, and *Col-WT* in *D* refers to Columbia wild type. Ethidium bromide-stained ribosomal RNA is used as a loading control.

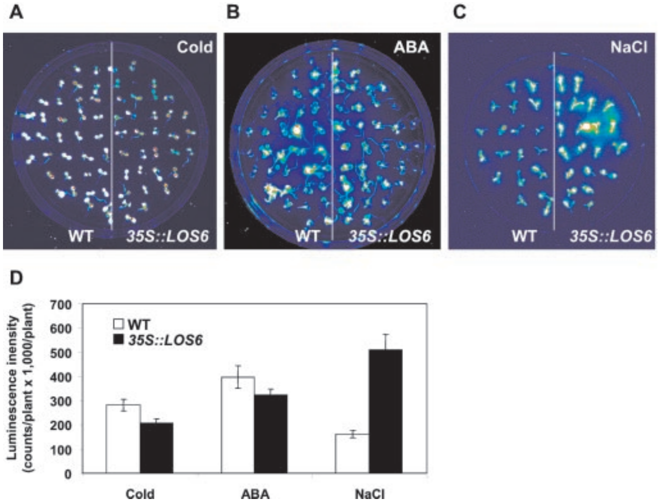

FIG. 9. ***RD29A-LUC* expression in seedlings overexpressing *LOS6*.** *A*, luminescence image after cold (0 °C, 48 h) treatment. *B*, luminescence image after ABA (100 μM ABA, 3 h) treatment. *C*, luminescence image after NaCl (300 mM, 3 h) treatment. *D*, quantitation of the luminescence intensities in *A*–*C*. Data are means ± S.E. (n = 19–20).

into the wild type *RD29A-LUC* plants by *Agrobacterium*-mediated flower dipping transformation. The T_3 generation of these transgenic plants was examined with respect to *RD29A-LUC* expression in response to low temperature, salt, and ABA treatments. Among 31 independent lines examined, 28 lines exhibited similar *RD29A-LUC* expression patterns that were different from those in the wild type. An example from one such line is presented in Fig. 9.

Like the wild type, the *LOS6/ABA1* overexpression line did not exhibit obvious luminescence without stress treatment (data not shown). When treated with cold or ABA, the overexpression plants showed slightly lower luminescence compared with the wild type (Fig. 9, *A* and *B*). Quantitation indicates that upon treatment with cold, there was about 20% reduction in the luminescence in the overexpression plants compared with wild type plants, a statistically significant difference (Fig. 9*D*). When treated with ABA, the luminescence was about 10% lower in the overexpressing line than in the wild type (Fig. 9, *B* and *D*). In contrast, under NaCl treatment, there was a nearly 2-fold increase in the expression level of the *RD29A-LUC* transgene in *LOS6*-overexpressing plants compared with that in the wild type.

DISCUSSION

LOS6 Encodes an Enzyme Functioning in ABA Biosynthesis—In the present study, we characterized and cloned an *Arabidopsis* mutation, *los6*, which was identified by virtue of its reduced osmotic stress induction of the *RD29A-LUC* reporter gene. Besides a reduced luminescence expression, the expression of the endogenous *RD29A* gene and other stress-responsive genes *RD29B*, *COR15A*, *KIN1*, *COR47*, *RD19*, *RD22*, *ADH*, and *P5CS* under osmotic stress was all reduced to various extents in *los6* mutant plants relative to the wild type. Phenotypic studies suggest that *los6* is ABA-deficient. Genetic analysis and gene cloning indicate that *LOS6* encodes a zeaxanthin epoxidase and is allelic to *ABA1* that has not been cloned previously. These results demonstrate that ABA is critical in gene regulation under osmotic stress.

In addition to the regulation of gene expression under osmotic stress, the *los6* mutation also alters ABA and cold responsiveness. Both the luminescence intensity (Figs. 1*D* and 2) and the expression level of stress-responsive genes examined are clearly higher in *los6* than in the wild type in response to ABA treatment. A similar observation was made with *los5* mutants (9), although the effect is less dramatic in *los5*. This suggests that when ABA is deficient, plant cells may become more sensitive to ABA so as to mitigate the deleterious effects of ABA deficiency.

The expression of the endogenous *RD29A* and *COR15A*, *KIN1*, *COR47*, and *RD19* was lower in *los6* mutant plants than in the wild type when exposed to cold stress (Fig. 3). This is similar to what was observed with *los5* mutants (9). In contrast to what was seen in *los5*, the luminescence level in *los6* plants is higher than in the wild type under cold treatment (Figs. 1 and 2). The discrepancy between the luminescence level and endogenous gene expression in this particular case suggests that something other than gene expression is responsible for the higher luminescence in *los6*. Since the steady state level of luciferase transcript under cold treatment was very low (data not shown), it was hard to distinguish whether there was a difference between *los6* and wild type in the *LUC* transcript level. However, it is unlikely that the *RD29A-LUC* transcript level would be higher in *los6* than in the wild type, since the endogenous *RD29A* expression is low in *los6* (Fig. 3). The higher luminescence expression in *los6* thus probably resulted from an enhanced activity of the luciferase enzyme. It is known that light emission from the oxidation of luciferin catalyzed by luciferase is an oxidative process. Defects in *LOS6/ABA1* were shown to lead to the accumulation of zeaxanthin (13, 14). Zeaxanthin as an antioxidant may affect the redox status within organelles (22, 23), which in turn may affect the luciferin oxidation reaction.

With regard to osmotic stress-regulated gene expression, the phenotypes of the *los6* mutant are similar to those of *los5-1* and *los5-2*, which are alleles of *aba3* (9). However, there are some differences between *los5* and *los6* mutants. First, the magnitude of reduction in gene expression is higher in *los5* than in *los6*. In *los5* mutant plants, the *RD29A-LUC* reporter gene was virtually not expressed under salt treatment, whereas in *los6* the luminescence could be seen, albeit the intensity is significantly lower than that in wild type plants (Figs. 1 and 2). Similarly, the expression of other stress-responsive genes under osmotic stress conditions was almost completely blocked in *los5* (9), whereas in *los6* the transcript levels of these genes are reduced but not completely. The reason for these differences is not entirely clear, but the differences may be due to the greater severity of ABA deficiency in *los5*.

Role of ABA in Osmotic Stress Signaling—The fact that both *los6* (this study) and *los5* (9) are impaired in osmotic stress-regulated gene expression and that both mutants are defective in ABA biosynthesis indicates that ABA deficiency is a common reason for the defects in gene regulation by osmotic stress. An intimate connection between ABA and osmotic stress gene regulation is further suggested by the observation that overexpressing *LOS6* led to enhanced expression of *RD29A-LUC* under osmotic stress (Fig. 9). Notwithstanding the existence of ABA-independent signaling pathways, these lines of genetic evidence together underscore the determinative role of ABA in gene regulation under osmotic stress.

Regulation of ABA Biosynthesis Genes—Although *ZEP* genes were cloned from several other plant species (initially from tobacco (12), recently from rice (24), and several sequences from other plants were deposited in the data base (Fig. 6)), the role of the *ZEP* gene in modulating stress-responsive gene expression and the regulation of *ZEP* gene expression are not well understood. Audran *et al.* (15) observed that the expression of the tobacco ZEP gene *ABA2* was regulated diurnally and that its transcript level increased in the root but not in the shoot in response to drought stress. Our study indicates that drought, salt, and PEG all increase the expression level of *LOS6/ABA1* in the shoot (Figs. 7 and 8). More importantly, we found that *LOS6/ABA1* expression was clearly enhanced by ABA (Figs. 7*D* and 8*A*). Likewise, another ABA biosynthesis gene, *AAO3*, that encodes ABA-aldehyde oxidase (19) was also induced by ABA (Fig. 8). These observations suggest that there may exist a positive feedback regulatory mechanism whereby initial increases in ABA level may stimulate further biosynthesis of ABA through up-regulation of some of the biosynthesis genes. Such a regulation has also been found for the *LOS5/ABA3* gene (9). The expression of *NCED3* was not induced by exogenous ABA in wild type *RD29A-LUC* plants (Fig. 8*A*), which is similar to what was recently reported (17, 18), although limited induction of *NCED3* by ABA was observed in the ecotypes Landsberg (Fig. 8*C*) and Columbia (Fig. 8*D*). The interesting observation that *NCED3* was induced by ABA in the ABA-deficient mutants *los5* and *los6* (Figs. 8, *A* and *B*) may reflect an enhanced sensitivity to ABA in these mutants as discussed above. Together, these data suggest that the regulation of *NCED3* by ABA may be finely tuned by plant sensitivity to ABA and that the thresholds of its ABA induction may vary among ecotypes.

To gain insight into the signal transduction mechanisms by which ABA regulates ABA biosynthesis genes, we studied the induction of these genes in the ABA response mutants *abi1*, *abi2*, and *era1*. As shown in Fig. 8*C*, ABA induction of *LOS6/ABA1*, *AAO3*, and *NCED3* was diminished or reduced in the *abi1* mutant but was not changed in *abi2*, indicating that *abi1* mutation impairs this feedback regulation. Despite their homology and overlapping functions, as suggested by their similar mutant phenotypes, ABI1 and ABI2 are often found to have different roles in ABA signaling. For example, it was shown that ABA-induced reactive oxygen species production was impaired in *abi1* but not in *abi2*. In contrast, H_2O_2 activates plasma membrane Ca^{2+} channels and induces stomatal closure in *abi1* but not in *abi2* (25). Recently, it was suggested that reactive oxygen species might also be involved in the regulation of ABA biosynthesis (26). It is likely that the observed ABA regulation of ABA biosynthesis genes may be mediated by reactive oxygen species. This possibility can be tested in future studies.

The ABA hypersensitive mutant *era1* was known to have opposite phenotypes to those of *abi1* and *abi2* in seed germination and stomatal regulation in response to ABA (20, 21), but its sensitivity to ABA at the level of gene expression is not known. We examined the expression of *LOS6* and *NCED3* in *era1* and found that *era1* mutation does not affect significantly the expression of ABA biosynthesis genes under either ABA or salt treatment (Fig. 8*D*), suggesting that the signal transduction pathway for the ABA regulation of ABA biosynthesis genes may not be mediated by ERA1. This observation is consistent with the finding that the *era1* mutant does not affect ABA biosynthesis/accumulation (20).

In the *los6* mutant, surprisingly, enhanced expression of *LOS6/ABA1* gene by osmotic stress treatment was not observed (Fig. 8*A*). Likewise, osmotic regulation of *AAO3*, *LOS5/ABA3*, *NCED3*, and *ABI1* was also significantly diminished in *los6* (Fig. 8*A*). This further suggests that not only exogenous ABA, but also endogenous ABA elicited by osmotic stress, enhances the expression of *LOS6/ABA1* and other ABA biosynthesis genes. Because osmotic stress-induced increase in ABA level does not happen in *los6* as a result of its defect in ABA biosynthesis, this ABA deficiency should be the primary reason for the defect in the induction of *LOS6/ABA1* and other ABA biosynthesis genes (Fig. 8*A*) and in the expression of other stress-responsive genes including *ABI1* in response to osmotic stress in *los6* (Figs. 3 and 8). Consistent with this notion, application of exogenous ABA induced a wild type level of *LOS6/ABA1* expression (Fig. 8*A*). It is also worth noting that the defects in *LOS6/ABA1* expression as seen in *los6* would be nearly impossible to study in the *aba1-1* allele, since the *ZEP* transcript level was very low in that mutant (Fig. 7*D*).

A Model for Stress Induction of ABA Biosynthesis—The biosynthesis of ABA may be largely regulated at the transcriptional level, since blocking transcription by using inhibitors prevents drought-induced ABA biosynthesis (*e.g.* Refs. 27 and 28). Additionally, as shown in the present study and elsewhere (9, 15, 17–19), the expression of all cloned ABA biosynthesis genes is induced by drought stress. However, the mechanisms by which these genes are induced are unknown. In addition to

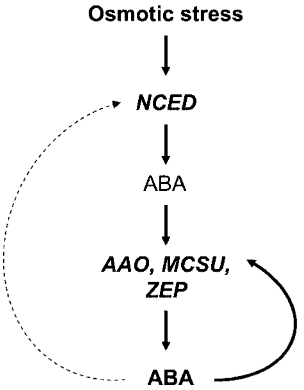

FIG. 10. **A model for stress induction of ABA biosynthesis.** Osmotic stress first induces the expression of *NCED* (*NCED3* in *Arabidopsis*, codes for 9-*cis*-epoxycarotenoid dioxygenase), which represents an early limiting step in controlling drought stress-induced ABA biosynthesis. The initial accumulation of ABA potentiates the expression of other ABA biosynthesis genes *AAO* (*AAO3* in *Arabidopsis*, codes for ABA aldehyde oxidase), *MCSU* (*LOS5/ABA3* in *Arabidopsis*, codes for molybdate cofactor sulfurase), and *ZEP* (*LOS6/ABA1* in *Arabidopsis*, codes for zeaxanthin epoxidase) (by order of decrease in inducibility). Increased expression of these genes leads to more ABA biosynthesis. This ABA then further stimulates the expression of these biosynthesis genes, leading to a sustained increase in ABA biosynthesis (indicated with a *solid feedback arrow*). ABA has a relatively limited ability in inducing the expression of *NCED* (indicated with a *dashed feedback arrow*), and thus the NCED step may limit further increases in ABA biosynthesis.

their regulation by drought and salt stress, we showed in this study that the transcript levels for ABA biosynthesis genes *LOS6/ABA1*, *AAO3*, and *LOS5/ABA3* are enhanced by exogenous ABA and greatly diminished in ABA-deficient mutants under osmotic stress. The magnitudes of the induction by either stress or ABA are different among these genes. The *LOS6/ABA1* gene, which codes for a ZEP that functions in the first committed step of ABA biosynthesis, has a relatively high basal transcript level. The other genes have a relatively low transcript abundance under unstressed conditions. Osmotic stress elicited by either drought/PEG or salt stress treatments dramatically up-regulates all of these genes. In contrast, low temperature has a limited role in inducing these genes, which is consistent with the notion that low temperature has little effect on ABA accumulation (9, 29).

Among the ABA biosynthesis genes, *NCED3* appears to be the one most drastically induced by osmotic stress (Fig. 8). In the Landsberg ecotype, PEG treatment induced *NCED3* expression to similar levels as salt treatment. In contrast, the induction of *AAO3*, *LOS6*, and *LOS5* by PEG treatment was lower than that of salt stress (Fig. 8*C*). There has been a prevailing notion that the rate-limiting step in osmotic stress-induced ABA biosynthesis is the cleavage of 9-*cis*-epoxycarotenoid catalyzed by NCED (29, 30). However, evidence that supports this notion has been circumstantial. First, it is thought that the precursor 9-*cis*-epoxycarotenoid is abundant in photosynthetic tissues (31), and thus the steps responsible for the generation of the precursor would not be rate-limiting. Second, examination of the conversion of xanthoxin to ABA in cell-free extracts found that the conversion rates between stressed and unstressed plants were similar, which suggests that the steps downstream of the xanthoxin conversion would not be rate-limiting either (32). However, the fact that *NCED3* is rapidly and strongly induced by osmotic stress implies that under stress NCED level would become rather high and thus should not limit ABA biosynthesis. An interesting observation is that *NCED* is either not induced by ABA or not as strongly induced by ABA as other ABA biosynthesis genes (Fig. 10). The lack or weak NCED feedback induction by ABA would in fact suggest that NCED might be rate-limiting in regulating ABA biosynthesis, as shown in Fig. 10.

Based on experimental evidence obtained in the present study and in other related studies, we propose that drought/osmotic stress first induces a relatively low level of NCED expression. Since other ABA biosynthesis genes have certain levels of basal transcripts under unstressed conditions, the induced NCED and basal levels of the other enzymes would allow an initial round of ABA biosynthesis. The limited amount of newly synthesized ABA would then stimulate the expression of *AAO* and *MCSU* as well as *ZEP* and, to a lesser extent, *NCED*. This coordinated increase in the transcription of all ABA biosynthesis genes would result in a more rapidly and sustained increase in ABA biosynthesis (Fig. 10). This model could also explain why overexpressing any individual ABA biosynthesis genes can result in increased ABA biosynthesis (22, 33–35) and affect ABA-regulated gene expression (Fig. 9) or physiological processes (33–35); an initial increase in ABA from overexpressing one ABA biosynthesis gene could result in an increased expression of other ABA biosynthesis genes, which then would lead to a coordinated increase in *de novo* ABA biosynthesis. Since *NCED* expression is less regulated by ABA, it may therefore limit the feedback regulation of ABA biosynthesis by ABA (*i.e.* late round of ABA biosynthesis) (Fig. 10). It should be pointed out that *NCED* does require endogenous ABA for full stress activation, because osmotic stress induction of *NCED3* was significantly compromised in *los6* and *los5* mutants (Fig. 8*A*).

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