Regulation of osmotic stress-responsive gene expression by the *LOS6/ABA1* locus in Arabidopsis

Liming Xiong, Hojoung Lee, Manabu Ishitani, and Jian-Kang Zhu*

Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA

*To whom correspondence should be addressed.

For correspondence: Jian-Kang Zhu

Department of Plant Sciences

University of Arizona

Tucson, Arizona 85721

Tel. (520) 626-2229

Fax (520) 621-7186

E-mail: jkzhu@ag.arizona.edu

Running Title: Stress gene regulation by LOS6/ABA1
ABSTRACT

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.
INTRODUCTION

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) 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 3, 4, 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 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 to 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 several fold, 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.

MATERIALS AND METHODS

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 hr light and 8 hr 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 hr. 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 in MS agar plates, and incubated in the light for 3 hr 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 hour), or NaCl/PEG (transferred onto filter paper saturated with 300 mM NaCl or 30% polyethylene glycol with molecular weight of 6000 and treated for 5 hr). 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 hr before harvesting the samples. RNA blot analyzed 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 lab bench to lose 30% of the initial fresh weight (over a period of 2 hrs). Afterward, the samples were placed in a sealed plastic bag with wet paper towels for an additional 5 hrs. 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 backcrossed to the wild type RD29A-LUC plants and the F1 seedlings were tested for luminescence expression in response to stress or ABA treatment as described above. The F2 progeny of the self-pollinated F1 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 (RT-PCR) and cloned into pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA) and sequenced. The cDNA was digested with EcoRI 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. T3 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 1).

Figure 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 (Figure 1). Under cold treatment (0°C for 48 hr), los6 exhibited a higher level of luminescence than the wild type (Figure 1D). When treated with ABA (100 μM ABA, 3hr), los6 also showed an increased luminescence expression relative to the wild type plants (Figure 1E). In contrast, upon salt stress treatment (300 mM NaCl, 3hr), los6 mutant plants showed reduced luminescence expression compared to the wild-type (Figure 1F). Quantitation of
the luminescence intensities indicated that the luminescence level in los6 plants is only 17 percent of the wild type level under salt stress (Figure 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 (Figure 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 (Figure 3). Similar to what was observed with luminescence expression (Figure 1), RD29A transcript level in los6 plants was also significantly lower than that in the wild type when treated with NaCl (Figure 3). To determine whether the reduced gene expression is specific to ionic stress or to general osmotic stress, the plants were treated with polyethylene glycol (PEG) and RNA blot analysis was conducted. Figure 3 showed that the RD29A transcript level in los6 was also considerably lower than that in the wild type under PEG treatment (Figure 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 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 to the wild type under NaCl and PEG treatments (Figure 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 plants were slightly smaller in stature, and flowered about one week earlier than wild type plants under our growth conditions (Figure 4B). The leaf morphology also appeared different from that of the wild-type. Leaves of los6 plants tended to be narrower (Figure 4A) and the color was dark green when the plants were grown in soil but appeared slightly yellowish in agar plates (Figure 1). The morphology was observed in los6 mutants that had been backcrossed to the wild type plants for 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%) (Figure 3D). Measurements of transpirational water loss indicated that los6 plants lost water faster than did the wild type plants (Figure 3E). 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 hr 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 (Figure 3F), 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 2). 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 2), which resulted in the ABA content in the wild type being ten-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 F1 progenies. The result indicated that los6 is allelic to aba1 (Table 1 and data not shown).

Cloning and Expression Study of LOS6/ABA1

Previously, it was demonstrated that the tobacco ABA2 locus encodes a zeaxanthin epoxidase (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 blasted the database using tobacco ABA2 and identified that a gene (K8A10.10, GeneBank accession no. At5g67030) on chromosome V (Figure 5A) 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 the 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.

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 (Figure 5B). 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 monoxygenase domain, and a FHA motif with unknown functions (Figure 5C). The los6 mutation that occurred in the monoxygenase domain (Figure 5C) 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 monoxygenase 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 (Figure 6). At the amino acid level, LOS6/ABA1 is 62 to 71% identical to ZEP from rice, tobacco, tomato, cowpea, pepper and apricot. LOS6/ABA1 has less than 30% amino acid identities to monoxygenases in other organisms, indicating that ZEP may have evolved specifically in plants.
The *LOS6/ABA1* gene was expressed in every plant parts examined, yet the expression levels varied among them (Figure 7A). In the vegetative parts, *LOS6/ABA1* was expressed more in leaves and stems than in roots (Figures 7A and 7B). In reproductive organs, flowers had a much higher expression than siliques (Figure 7A). Additionally, although *LOS6/ABA1* was constitutively expressed, the expression levels in whole seedlings and in leaves were enhanced by drought, salt or PEG treatment (Figures 7B-D). Both in the shoot and root, *LOS6/ABA1* was up-regulated by NaCl treatment (Figure 7B), 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 *aba1-1* mutant, the *LOS6/ABA1* transcript level was very low (Figure 7D). This is probably because transcripts with a premature stop codon are known to induce 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 (MCSU) 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 (Figures 7D and 8A). In the ab1-1 mutant, although the transcript level was very low, LOS6/ABA1 gene induction could be seen under the ABA treatment (Figure 7D).

Remarkably, in 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 (Figure 8A). 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 (Figure 8). Interestingly, ABA also induced the expression of AAO3 and LOS5/ABA3 but failed to induce the expression of NCED3 (Figure 8A). In los6 mutant background, NaCl and PEG inductions of all these three genes were greatly impaired as compared to those in wild type plants (Figure 8A). 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 (Figure 8A).

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. Figure 8B showed that the expression of LOS6 and NCED3 in los5/aba3 under NaCl or PEG treatment was also
diminished compared to 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 (Figure 8C), the expression pattern for NCED3 in response to cold and ABA is different from that in the wild type C24 plants. NCED3 gene was not induced by cold or ABA in C24 wild type (Figures 8A and 8B), but was clearly induced in Landsberg wild type plants albeit at relatively low levels (Figure 8C).

The induction of these ABA biosynthesis genes by NaCl or PEG in abi1 was lower compared to that in the wild type under the same treatment conditions (Figure 8C). Moreover, the induction of LOS6 and AAO3 by ABA in abi1 is significantly lower than that in the wild type plants (Figure 8C). ABA induction of NCED3 is also abolished in abi1 (Figure 8C). In contrast, the induction of these genes in the abi2 mutant is virtually unchanged compared to that in the wild type (Figure 8C).
Previous studies suggested that part of ABA signal transduction is affected in the enhanced response to ABA mutant 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 transcripts levels were similar in the wild type and era1 mutant (Figure 8D). 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 (Figures 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 (CaMV) 35S promoter and a translational enhancer was introduced into the wild type RD29A-LUC plants by Agrobacterium-mediated flower dipping transformation. The T3 generation of these transgenic plants were 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 Figure 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 to the wild type (Figures 9A and 9B). Quantitation indicates that upon treatment with cold, there was about 20% reduction in the luminescence in the overexpression plants compared to wild type plants, a statistically significant difference (Figure 9D). When treated with ABA, the luminescence was about 10% lower in the overexpressing line than in the wild type (Figures 9B and 9D). In contrast, under NaCl treatment, there was nearly a two-fold increase in the expression level of the RD29A-LUC transgene in LOS6 overexpressing plants compared to 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
(Figures 1D 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 (Figure 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 (Figures 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 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 (Figure 3). The higher luminescence expression in los6 thus likely 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. Firstly, 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 (Figures 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 may be because ABA deficiency
in los5 is more severe.

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 (Figure 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 database (Figure 6), the role of 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 (Figures 7 and 8). More importantly, we found that LOS6/ABA1 expression was clearly enhanced by ABA (Figures 7D and 8A). Likewise, another ABA biosynthesis gene AAO3 that encodes ABA-aldehyde oxidase (19) was also induced by ABA (Figure 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 (Figure 8A), which is similar to what was recently reported (17-18), although limited induction of NCED3 by ABA was observed in the ecotypes Landsberg (Figures 8C) and Columbia (Figure 8D). The interesting observation that NCED3 was induced by ABA in the ABA-deficient mutant los5 and los6 (Figures 8A and 8B) 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 Figure 8C, ABA induction of *LOS6/ABA1*, *AAO3* as well as *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 (ROS) production was impaired in *abi1* but not in *abi2*. In contrast, H$_2$O$_2$ activates plasma membrane Ca$^{2+}$ channels and induces stomatal closure in *abi1* but not in *abi2* (25). Recently, it was suggested that ROS 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 ROS. 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 (Figure 8D), 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 *era1* mutant does not affect ABA biosynthesis/accumulation (20).
In the los6 mutant, surprisingly, enhanced expression of \textit{LOS6/ABA1} gene by osmotic stress treatment was not observed (Figure 8A). Likewise, osmotic regulation of \textit{AAO3}, \textit{LOS5/ABA3}, \textit{NCED3} as well as \textit{ABI1} was also significantly diminished in los6 (Figure 8A). This further suggests that not only exogenous ABA but also endogenous ABA elicited by osmotic stress, enhance the expression of \textit{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 \textit{LOS6/ABA1} and other ABA biosynthesis genes (Figure 8A) and in the expression of other stress-responsive genes including \textit{ABI1} in response to osmotic stress in los6 (Figures 3 and 8). Consistent with this notion, application of exogenous ABA induced a wild type level of \textit{LOS6/ABA1} expression (Figure 8A). It is also worth noting that the defects in \textit{LOS6/ABA1} expression as seen in los6 would be nearly impossible to study in \textit{aba1-1} allele, since the \textit{ZEP} transcript level was very low in that mutant (Figure 7D).

\textbf{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. 27-28). 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 their regulation by drought and salt stress, we showed in this study that the transcript levels for ABA biosynthesis genes \textit{LOS6/ABA1}, \textit{AAO3}, \textit{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 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 (Figure 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 (Figure 8C). 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 supports this notion has been circumstantial. Firstly, 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. Secondly, 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 (Figure 10). The lack or weak NCED feedback induction by ABA would in fact support that NCED might be rate limiting in regulating ABA biosynthesis, as shown in Figure 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 (Figure 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 (Figure 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) (Figure 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 (Figure 8A).

REFERENCES


**Table 1.** Genetic analysis of the *los6* mutation.

<table>
<thead>
<tr>
<th>Crosses (female x male)</th>
<th>F₁</th>
<th></th>
<th>F₂</th>
<th></th>
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<tr>
<td></td>
<td>WT</td>
<td>Mutant</td>
<td>WT</td>
<td>Mutant</td>
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<tr>
<td>Wild type x <em>los6</em></td>
<td>22</td>
<td>0</td>
<td>295</td>
<td>86</td>
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<tr>
<td><em>los5-1</em> x <em>los6</em></td>
<td>5</td>
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<td></td>
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<tr>
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<td>11</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>los6</em> x <em>aba1-1</em></td>
<td>0</td>
<td>18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values shown are number of seedlings that showed wild-type or mutant luminescence phenotypes when treated with 300 mM NaCl.
Table 2. ABA contents in wild type and los6 plants under unstressed or drought stressed treatments (µg/g fresh weight)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Wild type</th>
<th>los6</th>
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</thead>
<tbody>
<tr>
<td>Unstressed</td>
<td>0.007</td>
<td>0.008</td>
</tr>
<tr>
<td>Stressed</td>
<td>0.10</td>
<td>0.01</td>
</tr>
</tbody>
</table>

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-hr incubation at 100% of relative humidity condition.
Figures

Figure 1. *RD29A-LUC* luminescence phenotypes of *los6* plants.

(A) to (C), appearance of one-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 hr. (E) Luminescence image of seedlings in (B) after treatment with 100 µM ABA for 3 hr. (F) Luminescence image of seedlings in (C) after treatment with 300 mM NaCl for 3 hr. The color scale at right shows the luminescence intensity from dark blue (lowest) to white (highest).
Figure 2. Quantitation of luminescence intensities in \textit{los6} and wild type plants under stress or ABA treatment as shown in Figure 1. Data are means ± s.e (n = 20).
**Figure 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 hr), ABA (100 μM ABA, 3 hr), NaCl (300 mM NaCl, 5 hr), or PEG (30% PEG, 5 hr) treatment. Actin gene was used as a leading control.
Figure 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 wilted.

(D) Transpirational water loss in los6 and wild type plants at the indicated time after detachment with or without pre-treatment with ABA (100 μM ABA, 4 hr). Data are means ± s.e (n = 3).
Figure 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.
Figure 6. Homology of LOS6/ABA1 with ZEP proteins from other plant species. Amino acids in black shade indicate identity and in gray shade 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: LOS6/ABA1, At5g67030; ABA2_NICPL, Q40412; CpABA1, BAB11934; ABA2_LYCIES, P93236; ABA2_CAPAN, Q96375; OsABA2, BAB39765; ABA2_PRUAR, 081360.
Figure 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 hrs; 0 hr 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 are, control (no stress), cold (0°C, 24 hr), ABA (100 µM ABA, 3 hr), NaCl (300 mM, 5 hr); PEG (30%, 5 hr). Actin, or tublin gene, or ethidium bromide-stained ribosomal RNA are used as loading controls.
Figure 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 are, control (no stress), cold (0°C, 24 hr), ABA (100 µM ABA or at the indicated concentrations, 3 hr), NaCl (300 mM, 5 hr); PEG (30%, 5 hr). 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.
Figure 9. *RD29A-LUC* expression in seedlings overexpressing *LOS6*.

(A) Luminescence image after cold (0°C, 48 hr) treatment. (B) Luminescence image after ABA (100 µM ABA, 3 hr) treatment. (C) Luminescence image after NaCl (300 mM, 3 hr) treatment. (D) Quantitation of the luminescence intensities in (A) through (C). Data are means ± s.e (n = 19–20).
Figure 10. A model for stress induction of ABA biosynthesis. Osmotic stress first induces the expression of NCED (NCED3 in Arabidopsis), 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 dash feedback arrow), and thus the NCED step may limit further increases in ABA biosynthesis.
Regulation of osmotic stress-responsive gene expression by the LOS6/ABA1 locus in *arabidopsis*
Liming Xiong, Hojoung Lee, Manabu Ishitani and Jian-Kang Zhu

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