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To dissect genetically the complex network of osmotic and cold stress signaling, we constructed lines of Arabidopsis plants displaying bioluminescence in response to low temperature, drought, salinity, and the phytohormone abscisic acid (ABA). This was achieved by introducing into Arabidopsis plants a chimeric gene construct consisting of the firefly luciferase coding sequence (LUC) under the control of the stress-responsive RD29A promoter. LUC activity in the transgenic plants, as assessed by using in vivo luminescence imaging, faithfully reports the expression of the endogenous RD29A gene. A large number of cos (for constitutive expression of osmotically responsive genes), los (for low expression of osmotically responsive genes), and hos (for high expression of osmotically responsive genes) mutants were identified by using a high-throughput luminescence imaging system. The los and hos mutants were grouped into 14 classes according to defects in their responses to one or a combination of stress and ABA signals. Based on the classes of mutants recovered, we propose a model for stress signaling in higher plants. Contrary to the current belief that ABA-dependent and ABA-independent stress signaling pathways act in a parallel manner, our data reveal that these pathways cross-talk and converge to activate stress gene expression.

INTRODUCTION

Drought, salinity, and low temperature are common adverse environmental factors encountered by land plants (Boyer, 1982; Thomashow, 1994; Bohnert et al., 1995). Water deficit caused by drought and high salinity has been a major selective force in plant evolution and an important factor limiting crop productivity. On the other hand, low temperature is perhaps the most important environmental constraint for plant distribution on land. To cope with these environmental stresses, plants execute a number of physiological and metabolic responses (Bartels and Nelson, 1994; Thomashow, 1994; Bohnert et al., 1995). Knowledge of the mechanisms by which plants perceive and transduce the stress signals is the key to understanding these responses and to genetic improvement of stress tolerance through biotechnology.

In response to osmotic stress elicited by water deficit or conditions of high salt, the expression of numerous genes is altered in plants (Skriver and Mundy, 1990; Bray, 1993; Bartels and Nelson, 1994; Zhu et al., 1997). Some of the osmotic stress–responsive (OR) genes can also be induced by low-temperature stress (Nordin et al., 1991; Thomashow, 1994; Giraudat, 1995). Both osmotic and cold stresses increase the level of the phytohormone abscisic acid (ABA; Zeewaart and Creelman, 1988; Skriver and Mundy, 1990; Chandler and Robertson, 1994). The expression of many OR genes can be induced by the application of ABA (Skriver and Mundy, 1990; Bray, 1993; Zhu et al., 1997). Accordingly, ABA is known to mediate OR gene expression in response to osmotic and cold stresses (Skriver and Mundy, 1990; Chandler and Robertson, 1994; Zhu et al., 1997). However, analysis of OR gene expression in ABA-deficient and ABA-insensitive mutants has indicated that the expression of some OR genes can act independently of ABA (Gilmour and Thomashow, 1991; Nordin et al., 1991; Gosti et al., 1995). In addition, a cis-acting DNA regulatory element, termed the dehydration-responsive element (DRE)/C-repeat, which responds to cold or osmotic stress but not to ABA, has been found in some OR promoters (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). In contrast, the ABA-responsive element/complex (ABRE) is known to mediate gene expression in response to ABA (Guiltnnan et al., 1990; Yamaguchi-Shinozaki and Shinozaki, 1994; Shen and Ho, 1995; Vasil et al., 1995). Therefore, ABA-dependent and ABA-independent signal transduction pathways have been proposed to function in a parallel manner to mediate gene expression in response to cold and osmotic stresses (Yamaguchi-Shinozaki and Shinozaki, 1994; Gosti et al., 1995; Shen and Ho, 1995; Stockinger et al., 1997). However, the results described in this study reveal that ABA-dependent and ABA-independent stress signaling
pathways do not act in a parallel manner; rather, they interact and converge to activate stress genes.

Despite the rapid progress in dissecting osmotic signaling pathways in the unicellular model eukaryote Saccharomyces cerevisiae (Brewster et al., 1993; Maeda et al., 1994; Posas and Saito, 1997) and the wealth of information on the identification and expression of plant OR genes (Zhu et al., 1997), understanding of the osmosensing mechanism in plants still remains a major challenge. Phospholipase C (Hirayama et al., 1995), many putative transcription factors (Urao et al., 1993; Stockinger et al., 1997), and protein kinases (Urao et al., 1994; Nishihama et al., 1995) have been described—some of which are encoded by OR genes—but their role in osmotic signaling is unclear. Mutants defective in osmotic responses would be valuable for dissecting osmotic signaling pathways. Traditional approaches to isolating mutants with altered osmotic responses are problematic because of difficulties in identifying a reliable phenotype for mutant screening. Hence, except for several ABA signaling mutants (e.g., ABA deficient [aba], ABA insensitive [abi], and enhanced response to ABA [era]), which were identified by their aberrant seed germination response to ABA (Koornneef et al., 1982, 1984; Finkelstein, 1994; Cutter et al., 1996; Leon-Kloosterziel et al., 1996), it has not been possible to isolate other plant mutants defective in osmotic signal transduction.

To begin a comprehensive genetic analysis of the osmotic and cold signal transduction pathways in plants, we identified Arabidopsis mutants that show altered regulation of OR gene expression. Arabidopsis plants were transformed with a chimeric gene construct (RD29A–LUC) containing a firefly luciferase reporter (Millar et al., 1992, 1995) driven by the DRE/C-repeat– and ABRE-containing RD29A promoter (Yamaguchi-Shinozaki and Shinozaki, 1994). The resulting plants emit bioluminescence in response to cold, osmotic stress, or exogenous application of ABA. Seeds from transgenic plants homozygous for the transgene were mutagenized by ethyl methanesulfonate (EMS), and the M2 seedlings were screened for mutants with altered bioluminescence. Hundreds of such mutants were obtained, and their responses to osmotic stress, cold, and ABA were characterized. Surprisingly, the phenotypes of many of the mutants cannot be explained by current models of osmotic signal transduction. Based on the analysis of these mutants, we propose an alternative scheme of osmotic and cold signal transduction in which ABA-dependent and ABA-independent pathways interact and converge to activate stress genes.

RESULTS

Regulation of Bioluminescence in RD29A–LUC
Transgenic Arabidopsis Plants by Osmotic Stress, Low Temperature, and ABA

The choice of an appropriate promoter and reporter gene is critical for utilizing a promoter–reporter approach to screen for gene regulation/signaling mutants. For the selection of osmotic signaling mutants, we chose the RD29A promoter (Yamaguchi-Shinozaki and Shinozaki, 1994) and the firefly LUC reporter gene (Millar et al., 1992). Although the function of the RD29A gene (also known as cor78 or lti78; Horvath et al., 1993; Nordin et al., 1993) product is not known, the RD29A promoter is one of the well-characterized promoters that can be activated by osmotic and cold stresses (Yamaguchi-Shinozaki and Shinozaki, 1994). In addition to an ABRE/ABA response complex mediating ABA regulation, the RD29A promoter also contains the DRE element, which can be activated by osmotic and cold stresses but not by ABA (Yamaguchi-Shinozaki and Shinozaki, 1994). Thus, this promoter makes it possible to identify mutants in both ABA-dependent and ABA-independent pathways. The LUC reporter gene was chosen because its expression in plants can be measured noninvasively by using low-light video imaging, making it practical to screen a large population of plants. Because of the presumed complex nature of osmotic and cold signaling in plants, a high-throughput video-imaging method of screening is necessary to isolate a large number of mutants to recover mutations in many signaling components.

The RD29A–LUC construct used to transform Arabidopsis is illustrated in Figure 1A. The construct was introduced into Arabidopsis (ecotype C24) via Agrobacterium-mediated root transformation (Valvekens et al., 1985). From nine independent transformants, one line was chosen for subsequent experiments because it displayed the highest LUC activity under osmotic and cold stresses (data not shown). Kanamycin-resistant and kanamycin-sensitive plants segregated 3:1 in the T1 population, indicating that there is a single functional RD29A–LUC transgene in this line. Plants homozygous for the transgene were identified from the progeny of this line. Seeds from these plants were collected and used for analysis of LUC expression and mutagenesis.

To test the suitability of the RD29A–LUC transgenic plants for isolation of mutants, we first examined the effect of low-temperature treatment on LUC expression. One-week-old seedlings grown in agar plates were exposed to cold (0°C) for 48 hr, and their luminescence was measured with a low-light CCD imaging system. As shown in Figure 1B, luminescence was detected in the RD29A–LUC plants only after imposing cold stress. In contrast, transgenic plants containing the PC–LUC transgene (LUC being under the control of the light-responsive plastocyanin promoter; Dijkwel et al., 1996) emitted luminescence without cold stress; after the cold treatment, luminescence in the PC–LUC plants decreased (Figure 1B). As expected, control plants without the RD29A–LUC or PC–LUC transgene did not emit luminescence before or after the cold stress (Figure 1B). Further imaging experiments showed that cold, ABA, or high-salt stress strongly induced bioluminescence in roots, stems, leaves, and cotyledons (data not shown).

The response of RD29A–LUC to cold, drought, or ABA is shown in Figure 2A. Cold stress, osmotic stresses (NaCl or
polyethylene glycol (PEG), or ABA strongly induced bioluminescence in the RD29A-LUC plants. Under NaCl treatments, the luminescence level became higher as the NaCl concentration increased. The luminescence reached its peak level at 300 mM NaCl (Figure 2B). Higher concentrations of NaCl resulted in decreased levels of luminescence (Figure 2B). Figures 2C and 2D show the time course of RD29A-LUC expression in response to 300 mM NaCl or 100 μM ABA treatment, respectively. The response to both treatments was rapid; significant luminescence was detected 1 hr after treatment, and the expression reached near peak levels by 3 hr after the treatments (Figures 2C and 2D). The patterns of these bioluminescence responses to ABA and various stresses are similar to that of the endogenous RD29A gene, as determined by RNA gel blot analysis (Horvath et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1993; M. Ishitani and J.-K. Zhu, unpublished data), and to responses reported in studies using a promoter-β-glucuronidase gene reporter (Horvath et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1994). We conclude that RD29A-LUC faithfully reflects stress and ABA regulation and appears to be reliable for genetic analysis of stress and ABA responses.

Isolation of Mutants with Altered Bioluminescence with Respect to Osmotic Stress, Low Temperature, and ABA Regulation

To conduct a large-scale screening of stress and ABA signaling mutants, it was necessary to have a large population of mutagenized plants. One gram of the RD29A-LUC transgenic seeds was mutagenized by EMS, divided into 28 pools, and germinated, and the resulting plants were allowed to self-pollinate. M2 seeds from each pool (~9 g of seeds representing ~1200 M1 plants) were collected independently. We were interested in three types of mutants designated as cos (for constitutive expression of osmotically responsive genes), los (for low expression of osmotically responsive genes), and hos (for high expression of osmotically responsive genes), respectively.

Figure 3 outlines the screening strategy that was used to select for the cos, los, and hos mutants. Agar plates, each containing 500 to 1000 1-week-old seedlings (Figure 4A), were first imaged for luminescence before stress treatment (Figure 4C). Individuals emitting significant luminescence were marked as putative cos mutants. The plates were then
Figure 2. Characterization of the Regulation of Luminescence from RD29A-LUC Plants by Cold, Osmotic Stress, or ABA.

(A) Induction of luminescence by NaCl (300 mM for 5 hr), PEG (30% for 5 hr), ABA (100 μM for 3 hr), or cold stress (0°C for 2 days).

(B) Concentration curve of NaCl response (5-hr treatments).

(C) Time course of NaCl (300 mM) response.

(D) Time course of ABA (100 μM) response.

Each point represents the average luminescence from 48 seedlings. Error bars represent standard deviation.

placed at 0°C in the dark for 48 hr followed by a second luminescence imaging. Seedlings with abnormally low or high luminescence levels were noted as putative los and hos mutants, respectively (Figure 4D). The plates were then placed at room temperature (20 to 22°C) for 2 days to allow the luminescence to drop to pre-cold treatment levels. The plants were then sprayed with 100 μM ABA, and a third luminescence image was taken 3 hr later. Individuals with abnormally low or high luminescence levels were again marked as putative los and hos mutants, respectively. All putative mutants were then transferred from the plates to grow in soil. Some of the remaining seedlings were transferred onto filter papers soaked with 300 mM NaCl to identify additional putative los and hos mutants.

During the screening of cos mutants, it was difficult to match accurately the luminescence image of a putative cos mutant directly with the individual seedling in a plate containing several hundred plants. To solve this problem, we took advantage of the natural fluorescence emitted by the seedlings. Green plants exposed to light emit fluorescence during the first 1 or 2 min after being transferred to the dark. Before luminescence imaging for cos mutants, a fluorescence image of all seedlings can be collected with a 30-sec exposure (Figure 4B). To identify precisely a putative cos mutant out of hundreds of seedlings in an agar plate, we simply looked for a seedling in the fluorescence image with the same coordinates as the one in the luminescence image. An example of the use of fluorescence and luminescence imaging before and after cold treatment is shown in Figure 4. Normally, to avoid the interference of fluorescence during
luminescence imaging, we spray seedlings with luciferin first and then store the plants in the dark for 5 min before collecting a luminescence image.

Approximately 300,000 M<sub>2</sub> seedlings from the 28 pools were screened, and 3000 were selected as putative mutants for growth in soil. Of these, 1768 lines survived and set seeds, and their progeny were rescreened to eliminate false positives (Figure 3). Approximately 20 seedlings from each putative mutant were examined for luminescence expression before and after cold and ABA treatments. Five to 10 seedlings from each putative mutant were also transferred from the agar plates onto a filter paper soaked with 300 mM NaCl and incubated for 5 hr. Luminescence images were then taken from the NaCl-treated seedlings. Of these, 833 were confirmed as true cos, los, or hos mutants during the rescreening process; 103 of them exhibited the strongest luminescence phenotypes and were further characterized.

### Characterization of Stress and ABA Responses in the Mutants

To categorize the mutants, we quantitated their responses to cold, NaCl, and ABA. The 103 mutants with the strongest phenotypes fall into 13 categories: cos (Figure 5A); hyperresponsive to osmotic stress, cold, and ABA (hos<sub>AB</sub>; Figure 5B); hyperresponsive to cold only (hos<sub>cold</sub>; Figure 5C); hyperresponsive to osmotic stress only (hos<sub>os</sub>; Figure 5D); hyperresponsive to ABA only (hos<sub>ABA</sub>; Figure 5E); hyperresponsive to osmotic stress and cold (hos<sub>osNaCl</sub>; Figure 5F); hyperresponsive to cold and ABA (hos<sub>cold</sub>/ABA; Figure 5G); hyperresponsive to osmotic stress and ABA (hos<sub>NaCl</sub>/ABA; Figure 5H); low response to osmotic stress, cold, and ABA (los<sub>AB</sub>; Figure 6A); low response to cold only (los<sub>cold</sub>; Figure 6B); low response to osmotic stress only (los<sub>NaCl</sub>; Figure 6C); low response to osmotic stress and cold (los<sub>coldNaCl</sub>; Figure 6D).

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**Figure 3.** The Protocol Used for the Screening for Mutants.
Figure 4. An Example of Screening cos, los, and hos Mutants for Cold Stress.

(A) Plate containing 6-day-old M2 seedlings.
(B) Fluorescence image of the seedlings.
(C) Luminescence image taken before stress treatments.
(D) Luminescence image taken after cold stress treatment.

Putative cos, los, and hos mutants are marked. The fluorescence image was taken to assist in the accurate identification of the putative cos plant. For relative luminescence intensities, refer to the color scale in Figure 1.

6D); and low response to osmotic stress and ABA (losNaciABA; Figure 6E). Table 1 lists the number of lines in all mutant classes obtained. Figure 7 shows the luminescence images of some of the cos, los, and hos mutants under various treatments.

The cos mutant shown in Figures 5A and 7 emits luminescence constitutively. However, the responses to cold, ABA, and NaCl are not changed in this mutant. This is not true in all cos mutants. One cos mutant (line 396) showed reduced responses to cold, NaCl, and ABA, whereas others had enhanced responses to these treatments (data not shown). We also recovered cos mutants with enhanced responses to
just one or two of the treatments (M. Ishitani and J.-K. Zhu, unpublished data). The majority of the cos mutants that we obtained exhibited slow growth and appeared to be "constitutively stressed."

The two classes that include the largest numbers of mutant lines are losall and hosall (Table 1). In several of the losall mutants, the responses to cold, NaCl, or ABA were nearly eliminated. Most other losall mutants still responded to the

Figure 5. Luminescence of Representative cos and hos Mutants in Response to Cold, High-Salt, and ABA Treatments.

M4 plants were grown for 10 days and then subjected to control (untreated), cold (0°C for 2 days), ABA (100 μM for 3 hr), or NaCl (300 mM for 5 hr) treatments. At the end of the treatments, luminescence was determined, and the mean level was calculated from data for 10 to 20 single seedlings. White bar, wild type; black bar, mutant. Error bars represent standard deviation.

(A) A cos mutant with constitutive expression of luminescence.
(B) A hos mutant with enhanced responses to cold, ABA, and high salt.
(C) A hos mutant with a high response to only low-temperature stress.
(D) A hos mutant with a high response to only osmotic stress.
(E) A hos mutant with an enhanced response to only ABA.
(F) A hos mutant with enhanced responses to cold and osmotic stresses.
(G) A hos mutant with enhanced responses to cold and ABA.
(H) A hos mutant with enhanced responses to ABA and osmotic stress.
stress and ABA treatments, but their responses were reduced. Nearly all hosall mutants also exhibited substantial luminescence in the absence of a stress treatment (Figures 5B and 6; M. Ishitani and J.-K. Zhu, unpublished data). However, the constitutive expression in hosall mutants generally was not as high as those in the cos class of mutants.

Expression of the endogenous RD29A gene was examined with RNA gel blot analysis in the 103 mutants with the strongest luminescence phenotypes. It is easy to envision that a mutation in the LUC coding region or even in the RD29A promoter could result in a los phenotype. These cis mutants would not be of interest. Figure 8 shows the expression of the endogenous RD29A gene in los10, los1, and los5 mutants, which represent the los1, loscold, and loscoldNaCl classes, respectively. Complementation tests showed that these mutations are not allelic to each other (data not

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**Figure 6.** Luminescence of Representative los Mutants in Response to Cold, High-Salt, and ABA Treatments. Treatment and assay conditions were the same as described for Figure 5. White bar, wild type; black bar, mutant. Error bars represent standard deviation.

(A) A los mutant with reduced responses to cold, ABA, and osmotic stress.
(B) A los mutant with a reduced response to only cold stress.
(C) A los mutant with a reduced response to only osmotic stress.
(D) A los mutant with reduced responses to cold and osmotic stresses.
(E) A los mutant with reduced responses to ABA and osmotic stress.
DISCUSSION

In this study, we present results on the isolation of hundreds of Arabidopsis mutants defective in the regulation of RD29A gene expression by cold, osmotic stress, or ABA. In addition to many expected mutants, several unexpected classes of mutants were recovered. These mutants provide a foundation for comprehensive analysis of stress and ABA signal transduction. Categorization of the mutants provides a global view of cold, osmotic stress, and ABA signaling.

A High-Throughput Method for the Selection of Stress and ABA Signal Transduction Mutants

Because of the complexity of the responses of plants to environmental stresses, it has not been possible to identify osmotic and cold stress signal transduction mutants by their morphological or physiological phenotypes. Osmotic signal transduction mutants of yeast were identified by NaCl inhibition of growth (Brewster et al., 1993; Maeda et al., 1994). Our group has isolated many sos (salt generally sensitive) mutants of Arabidopsis (Wu et al., 1996; J.-K. Zhu, unpublished data). However, these mutants are all specifically hypersensitive to Na+ concentrations and not to general osmotic stress. Arabidopsis is a glycophyte that is very sensitive to NaCl (Wu et al., 1996), with concentrations >150 mM resulting in near complete inhibition of growth. In contrast, S. cerevisiae can tolerate almost 1 M NaCl (Haro et al., 1993). Therefore, the very salt-sensitive nature of Arabidopsis makes it impossible to isolate osmotic response mutants by screening for NaCl-hypersensitive growth.

We have developed a very efficient method for the isolation of osmotic as well as cold and ABA signaling mutants by using transgenic plants with stress-inducible bioluminescence. This method takes advantage of low-light video imaging and thus makes it easy to screen large populations of plants. Gene expression in hundreds of plants can be quantitatively detected by luminescence imaging in a matter of several minutes. A Petri dish containing several hundred to 1000 seedlings can be subjected to multiple stress and hormonal treatments and imaged repeatedly. Our screening procedure (Figure 4) is comparable to replica plating in prokaryotic genetics.

Using this procedure, we were able to screen efficiently >300,000 M2 plants. The limitation in screening more plants is in time spent growing Arabidopsis in agar plates. More than 100 mutants exhibiting strong phenotypes were readily recovered. These mutants include not only constitutive expressers but also low and high expressers in response to cold, high salt, or ABA. Some of the mutants may turn out to be caused by mutations in the transgene and are not of interest. However, many have been found to be trans mutants and should be useful for studies on stress and ABA signal transduction. The frequency of these mutations may appear very high. However, considering that there are >10 categories of these mutants, the frequency for any particular mutant class is not too high. A high frequency of these mutations may also reflect the complexity of osmotic and cold responses in higher plants.

Interactions and Convergence of ABA-Dependent and ABA-Independent Stress Signaling Pathways

Recovery of those mutants defective specifically in their responses to cold, osmotic stress, or ABA is expected. Because

<table>
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<th>Mutant Classes</th>
<th>1&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> Mutants with strong luminescence phenotypes (total of 103).

<sup>b</sup> Mutants with intermediate phenotypes (total of 271).

<sup>c</sup> Mutants with weak phenotypes (total of 459).

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**Table 1. Numbers of Mutant Lines in Various Mutant Classes**
nearly all of the mutations were recessive (data not shown), the los\textsubscript{cold} and hos\textsubscript{cold} mutants most likely define positive and negative regulators, respectively, in cold signal transduction. Similarly, the los\textsubscript{NaCl} and hos\textsubscript{NaCl} mutants most likely define positive and negative regulators, respectively, in osmotic signaling.

However, the phenotypes of several other categories of mutants are not expected and cannot be explained by current knowledge of signal transduction in response to abiotic stresses. Existing schemes of osmotic and cold signaling in-
Theoretically also result in enhanced response to osmotic stress and cold, data shown in Figure 5E indicate otherwise. Mechanistically, it is not obvious how ABA-dependent and ABA-independent pathways converge, because they target independent cis-regulatory elements in the RD29A promoter. One possibility is that the convergence points represent transcriptional enhancers and repressors that interact directly or indirectly with both the ABRE and DRE/C-repeat DNA elements. In yeast, for example, a large number of different stimuli controlling mating, haploid invasive growth, or pseudohyphal development were found to converge at the transcriptional factor Ste12 (Madhani and Fink, 1997). This common factor is able to direct selective responses to a stimulus through interactions with more specific transcriptional factors (Madhani and Fink, 1997).

Our results also reveal specific interactions between cold and ABA pathways as well as between osmotic and ABA pathways. Mutations causing enhanced response to osmotic stress and ABA (Figure 5H) define common steps in osmotic and ABA signaling pathways. Interactions between osmotic and ABA pathways are also revealed by mutations that cause low response to osmotic stress and ABA but not to cold (Figure 6E). Mutations causing a hyperresponse to cold and ABA (Figure 5G) reveal a step(s) at which cold and ABA signaling pathways interact. Although it is conceivable that different mutations in one gene could result in phenotypes found in more than one category of the above mutants, allelism tests thus far have shown that mutants in different categories are complements (data not shown).

A Genetic Model of Cold and Osmotic Signal Transduction in Plants

Mutations that fail to synthesize ABA or cause ABA insensitivity or hypersensitivity have been previously isolated by using seed germination assays (Koornneef et al., 1982, 1984; Meyer et al., 1994; Cutler et al., 1996; Leon-Kloosterziel et al., 1996; Leung et al., 1997). These mutations proved instrumental in our understanding of ABA function and signaling. Our work adds many novel types of mutations to the study of ABA and stress signaling. This new collection of mutants provides the foundation for comprehensive analysis of osmotic, low-temperature, and ABA signal transduction in plants. This comprehensive genetic analysis should complement and facilitate integration of current molecular and biochemical studies of stress signal transduction (Nishihama et al., 1995; Jonak et al., 1996; Sheen, 1996). A scheme that places the various mutations in putative positions in stress signaling pathways is presented (Figure 9). In line with existing experimental evidence (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Gosti et al., 1995), the scheme retains the notion of ABA-dependent and ABA-independent cold and osmotic signal transduction. The fact that mutations affecting osmotic stress and cold but not ABA signaling were recovered in the screening (Figure 6D) strongly supports this relationship.

A distinct feature of the proposed scheme is that ABA-dependent and ABA-independent pathways for osmotic stress and low temperature interact and converge (Figure 9). The interactions and convergence may provide added levels of coordination between the stress signals and ABA in the regulation of OR gene expression. It is currently not known whether the points of interactions (i.e., HOScold/ABA, LOSNaCl/ABA, and HOSNaCl/ABA) are downstream or upstream of the ABI1 and ABI2 gene products (Leung et al., 1994, 1997; Meyer et al., 1994). However, because the abi7 mutation affects seed germination, stomata opening, and OR gene expression, ABI1 most likely functions early in the ABA pathway (Koornneef et al., 1984; Leung et al., 1994) (Figure 9). Although the cold- or osmotic-specific components are placed upstream of the respective interaction points, their relationships are also unclear (Figure 9).

We hypothesize that cos mutations may occur either at early steps of stress signal perception or at the end points of the signal transmission (e.g., transcriptional repressors). A mutation in a very early component of osmotic signaling is expected to result in pleiotropic phenotypes. It is possible that some of the cos mutants may accumulate ABA constitutively. We have observed that many of the cos mutants are seriously retarded in growth and that their leaves appear vitreous in culture.

<table>
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<td>Actin</td>
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Figure 8. RNA Gel Blot Analysis of RD29A Gene Expression in Three los Mutants.

Twenty micrograms of total RNA from the wild type (WT) or individual mutants was loaded per lane. C, untreated; cold, ABA, NaCl, and PEG treatments are as described in the legend to Figure 2.
Osmotic stress is the single most limiting environmental factor for crop yield worldwide (Boyer, 1982; Bartels and Nelson, 1994). Unlike that of the unicellular yeast (Brewster et al., 1993; Haro et al., 1993), plant responses to osmotic stress have largely been unamenable to genetic analysis. Our results establish a genetic framework for the elucidation of osmotic and cold stress sensing and intracellular signaling mechanisms and should facilitate the improvement of osmotic stress tolerance of crop plants.

**METHODS**

**Plant Materials and Growth Conditions**

In all of the experiments described here, the wild type refers to the unmutagenized RD29A-luciferase (LUC) line in the Arabidopsis thaliana ecotype C24 background. Growth conditions were as described by Wu et al. (1996). During mutant screening and subsequent experiments with the mutants, we routinely planted seeds in nutrient agar media that were supplemented with 30 μg/mL kanamycin.

**Construction of RD29A-LUC Transgenic Plants**

The RD29A promoter (Yamaguchi-Shinozaki and Shinozaki, 1994) was obtained by polymerase chain reaction by using Arabidopsis genomic DNA and two primers: 5'-TCGGGATCCGGTGAATTAAGAGAGAGGAGG-3' and 5'-GACAAGCTTTGAGTAAAACAGAGGGTGTCAC-3'. The promoter fragment was inserted into a plant transformation vector containing the firefly LUC coding sequence (Millar et al., 1992). Arabidopsis plants were transformed with the chimeric RD29A-LUC construct via Agrobacterium tumefaciens infection of the roots (Valvekens et al., 1988). Plants homozygous for the RD29A-LUC chimeric gene were selected from T1 seeds and used for subsequent experiments.

**Mutagenesis and Mutant Isolation**

RD29A-LUC seeds were mutagenized with ethyl methanesulfonate (EMS) (Redei and Koncz, 1992). M2 seeds were sterilized and planted...
individually in 150 × 15 mm plates (500 to 1000 seeds per plate) containing Murashige and Skoog salts (Murashige and Skoog, 1962), 3% sucrose, and 0.8% agar, pH 5.7. Five- to 7-day-old seedlings grown under light were sprayed with luciferin and placed immediately under a CCD camera (see next section on LUC imaging) for fluorescence imaging. Fluorescence images were collected with 30-sec exposures. Four minutes after the fluorescence imaging, a LUC image was acquired with a 5-min exposure to identify cos mutants. The plates of seedlings were then incubated at 0°C for 2 days. After the cold treatment, the plates were sprayed immediately with luciferin and then incubated at room temperature for 5 min in the dark. A luminescence image was then collected to identify los and hos mutants. The plates were returned to grow under light for ~2 days before being sprayed with 100 μM abscisic acid (ABA).

Three hours after the ABA treatment, the seedlings were sprayed with luciferin, incubated in the dark for 5 min, and placed under the CCD camera (model CCD-512SB; Princeton Instruments, Inc., Trenton, NJ) for luminescence collection. Putative cos mutants, loscos mutants, and losaba and hosaba mutants were transferred to grow in soil. For a small portion (15 plates) of the M2 population screened, the remaining plants in the plates were treated with NaCl by transferring individual seedlings onto filter papers soaked with 300 mM NaCl in a Murashige and Skoog solution. To collect luminescence, the NaCl solution was drained before spraying the seedlings. For LUC imaging, the seedlings were kept for 5 min in the dark after the luciferin application. The imaging system consists of a high-performance CCD camera mounted in a dark chamber, a camera controller, and a computer. Image acquisition and processing were performed with the WinView software provided by the camera manufacturer. Exposure time was 5 min, unless stated otherwise.

**Stress and ABA Treatments**

For cold treatment, seedlings in agar plates were incubated at 0 ± 2°C in the dark or with dim light. Osmotic stress was imposed by NaCl or polyethylene glycol (PEG) (average molecular mass 6000) treatment. Seedlings were pulled out of agar media and laid down on filter papers soaked with appropriate concentrations of NaCl or PEG solutions for specified time periods. For ABA treatment, 100 μM ABA in water was sprayed on the seedlings until solution runoff. Osmotic stress and ABA treatments were conducted under light.

**LUC Imaging**

Imaging with the firefly LUC reporter requires application of the exogenous substrate luciferin. Luciferin (Promega, Madison, WI) was dissolved in sterile water and stored frozen in small aliquots as 100 mM stock solution. One millimolar concentration of working solution of luciferin in 0.01% Triton X-100 was applied uniformly onto seedlings by spraying five times. For LUC imaging, the seedlings were kept for 5 min in the dark after the luciferin application. The imaging system consists of a high-performance CCD camera mounted in a dark chamber, a camera controller, and a computer. Image acquisition and processing were performed with the WinView software provided by the camera manufacturer. Exposure time was 5 min, unless stated otherwise.

**RNA Gel Blot Analysis**

Total RNA was extracted from seedlings and analyzed as described previously (Liu and Zhu, 1997). The RD29A gene–specific probe was from the 3' noncoding region (Liu and Zhu, 1997).

**ACKNOWLEDGMENTS**

We thank Shaw-Jye Wu and Lei Ding for technical assistance and Drs. Paul P. Dijkwel and Sjef C.M. Smeekens of Utrecht University for the PC-LUC transgenic seeds. We also thank Dr. Robert Leonard for critical reading of the manuscript. This study was supported by grants from the Southwest Consortium on Plant Genetics and Water Resources and the United States Department of Agriculture National Research Initiative Competitive Grants Program (Plant Responses to the Environment) to J.-K.Z.

Received July 10, 1997; accepted September 2, 1997.

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*PLANT CELL* 1997;9;1935-1949
DOI: 10.1105/tpc.9.11.1935

This information is current as of January 13, 2009