The Plant Journal (1999) **19**(5), 569–578

HOS5 – a negative regulator of osmotic stress-induced gene expression in *Arabidopsis thaliana*

Liming Xiong, Manabu Ishitani, Hojoung Lee and Jian-Kang Zhu*
Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA

Summary

Osmotic stress activates the expression of many plant genes through ABA-dependent as well as ABA-independent signaling pathways. We report here the characterization of a novel mutant of *Arabidopsis thaliana*, *hos5-1*, which exhibits increased expression of the osmotic stress responsive *RD29A* gene. The expression of several other stress genes are also enhanced by the *hos5-1* mutation. The enhanced expression is specific to ABA and osmotic stress because low temperature regulation of these genes is not altered in the mutant. Genetic analysis indicated that *hos5-1* is a recessive mutation in a single nuclear gene on chromosome III. Double mutant analysis of *hos5-1* and the ABA-deficient *aba1-1* as well as the ABA-insensitive *abi1-1* mutant indicated that the osmotic stress hypersensitivity of *hos5-1* is not affected by ABA deficiency or insensitivity. Furthermore, combined treatments of *hos5-1* with ABA and osmotic stress had an additive effect on *RD29A-LUC* expression. These results suggest that the osmotic stress hypersensitivity in *hos5-1* may be ABA-independent. The germination of *hos5-1* seeds was more resistant to ABA. However, the *hos5-1* mutation did not influence stomatal control and only slightly affected the regulation of growth and proline accumulation by ABA. The *hos5-1* mutation reveals a negative regulator of osmotic stress-responsive gene expression shared by ABA-dependent and ABA-independent osmotic stress signaling pathways.

Introduction

The expression of many plant genes is regulated by osmotic stress (Bray, 1993; Skriver and Mundy, 1990; Zhu *et al.*, 1997). Most of the osmotic stress responsive (OR) genes identified to date can also be induced by the application of exogenous abscisic acid (ABA). Since osmotic stress causes the accumulation of endogenous ABA, ABA is therefore often considered as a mediator of OR gene expression in response to osmotic stress treatments such as drought and high salt. However, the study of OR gene expression has also revealed ABA-independent regulation by osmotic stress. First, the expression of some OR genes cannot be regulated by the application of ABA (Guerrero *et al.*, 1990). Second, osmotic stress regulation of those OR genes that are responsive to exogenous ABA may not be dependent on endogenous ABA. This has been demonstrated by examining OR gene expression in ABA-deficient or ABA-insensitive mutants (Gilmour and Thomashow, 1991; Gosti *et al.*, 1995; Nordin *et al.*, 1991). For example, although the *RD29A* gene can be induced by exogenous ABA, its expression in response to osmotic stress is not abolished in the ABA deficient *aba1* mutant (Nordin *et al.*, 1991; Yamaguchi-Shinozaki and Shinozaki, 1993). Furthermore, Yamaguchi-Shinozaki and Shinozaki (1994) have identified a novel *cis*-element (i.e. DRE/C-repeat) in the *RD29A* promoter that responds to osmotic stress but not ABA. A transcriptional factor that binds to the DRE/C-repeat DNA element has been cloned (Stockinger *et al.*, 1997). Interestingly, overexpression of this transcriptional factor led to constitutive expression of some OR genes (Jaglo-Ottosen *et al.*, 1998). None of the other components in the ABA-independent osmotic signaling pathway(s) have yet been identified genetically or biochemically.

Many mutants affected in ABA biosynthesis or sensitivity have been described previously (Cutler *et al.*, 1996; Finkelstein, 1994; Koornneef *et al.*, 1982; Koornneef *et al.*, 1984; Leon-Kloosterziel *et al.*, 1996; Neil *et al.*, 1986), and some of the mutated genes have been isolated (Cutler *et al.*, 1996; Leung *et al.*, 1994; Leung *et al.*, 1997; Marin *et al.*, 1996; Meyer *et al.*, 1994). In contrast, little is known about mutations that affect ABA-independent osmotic stress responses. Recently, we have developed a reporter-gene based approach to identify Arabidopsis mutants affected in ABA-independent osmotic signaling (Ishitani *et al.*, 1997). This was achieved by screening mutagenized plants that express the *RD29A-LUC* (firefly luciferase driven by the *RD29A* promoter) transgene. We report here the characterization of a novel ABA and osmotic stress signal transduction mutant, *hos5-1*. In this mutant, the expression of some OR genes is hypersensitive to osmotic stress and the application of exogenous ABA. We present evidence which suggests that osmotic stress hypersensitivity in *hos5-1* is independent of ABA. The *hos5-1* mutant thus defines a point of interaction between ABA-dependent and ABA-independent osmotic signaling pathways.

Received 25 January 1999; revised 4 June 1999; accepted 20 July 1999.
*For correspondence (fax +520 621 7186; e-mail jkzhu@ag.arizona.edu).

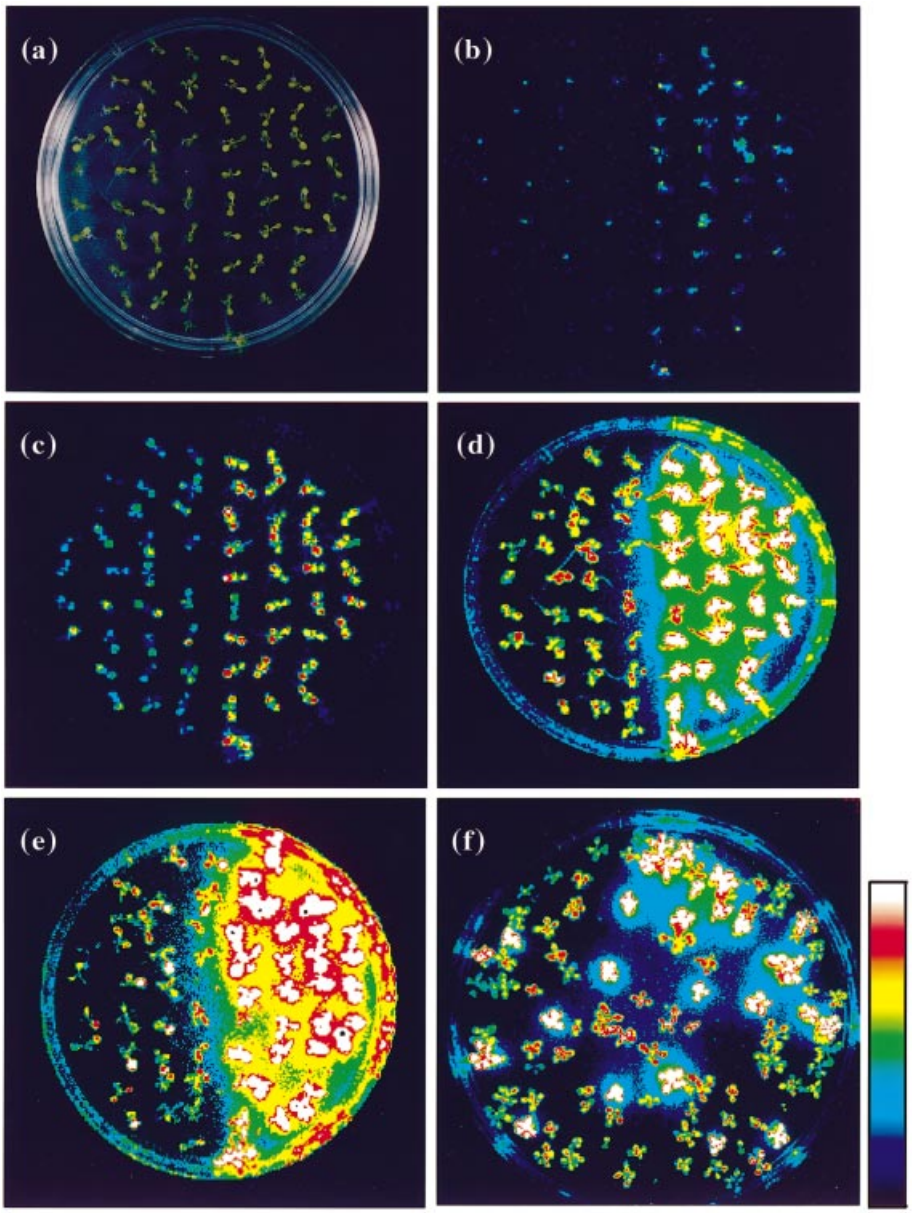

Figure 1. Luminescence images of *hos5-1* and the wild-type plants.
Seedlings of the wild type and *hos5-1* on the same MS agar plate were treated successively with low temperature, ABA and NaCl. The plants were allowed to recover for 2 days between treatments. The colour scale on the right shows the luminescence intensity from dark blue (lowest) to white (highest).
(a) Picture of the wild type (left) and *hos5-1* (right) seedlings. (b) Luminescence without treatment. (c) Luminescence after low temperature treatment at 0°C for 48 h. (d) Luminescence after treatment with 100 μM ABA for 3 h. (e) Luminescence after flooding the plate with 300 mM NaCl for 5 h. (f) Demonstration of segregation of luminescence phenotypes in F_2 populations (wild type × *hos5-1*). Plants were sprayed with 100 μM ABA and the image was taken 3 h after the treatment.

Results

Identification of the HOS5 *locus*

Transgenic *Arabidopsis thaliana* plants containing the *RD29A-LUC* transgene (referred to here as wild-type) emit luminescence in response to low temperature, osmotic stress or ABA treatment (Ishitani *et al.*, 1997). Mutants with altered luminescence responses to one or combinations of the inducers were selected from mutagenized *RD29A-LUC* plants by luciferase imaging (Ishitani *et al.*, 1997). Preliminary analysis identified a group of mutants with increased responses to both ABA and osmotic stress (high salt) but not to cold. One of these mutants, designated *hos5-1* (for high expression of osmotically responsive genes), was chosen for detailed characterization. Figure 1(a–e) shows a comparison of luminescence images

Table 1. Genetic analysis of *hos5-1* mutant (cross[a]: wild type × *hos5-1*)

Generation	Seedlings tested	Mutant	Wild type	χ^2
F_1	46	0	46	
F_2	737	187	550	0.065

[a]Female × male.

Table 2. Chromosome mapping of *hos5-1*

Markers	Chromosome	n[a]	Recombination frequency[b] (% ± SE)
nga 111	I	30	55.0 ± 6.4
nga168	II	39	44.9 ± 5.6
nga162	III	29	29.3 ± 6.0
GL1	III	41	23.2 ± 4.7
ATHGAPAb	III	43	18.6 ± 4.2
nga8	IV	40	47.5 ± 5.6
nga158	V	43	45.3 ± 5.4

[a]Number of samples analysed.
[b]Calculated by the Kosambi function (Koornneef and Stam, 1992).

of *hos5-1* and wild-type seedlings under cold, ABA or high salt treatment. Quantitation of the luminescence intensities demonstrated that *hos5-1* plants displayed an enhanced response to ABA, NaCl and polyethylene glycol (PEG) treatments, however, their response to cold was not significantly different from that of the wild type (data not shown). The increased response to both NaCl and PEG stresses indicates that the mutant phenotype is elicited by osmotic stress.

Table 1 presents data from the genetic analysis of *hos5-1*. The results suggest that *hos5-1* is a recessive mutation in a single nuclear gene. Figure 1(f) shows a sample luminescence image of a segregating F_2 population resulting from a cross between wild-type and the *hos5-1* mutant. The luciferase expression shown was in response to ABA treatment. Mutants can be clearly distinguished from plants with the wild-type phenotype because of their brighter luminescence. Allelism tests showed that *hos5-1* is a single allele and complemented all other mutants we isolated (Ishitani *et al.*, 1997) that display enhanced *RD29A-LUC* expression to ABA and osmotic stress treatments (i.e. mutant line nos 92, 213, 354, 370, 488, 602, 1071, 1415 and 1300) (data not shown).

To genetically map the *hos5* mutation, the mutant (in the C24 ecotype) was crossed with wild-type Columbia and the resulting F_1 plants were selfed to obtain a segregating F_2 population. Homozygous *hos5* mutants were selected, and genomic DNA was extracted from each of the plants. Mapping was carried out using microsatellite markers (Bell and Ecker, 1994). As shown in Table 2, *hos5* showed linkage to markers *nga162*, *GL1* and *ATHGAPAb* on chromosome III. By using the Kosambi function (Koornneef and Stam, 1992), *hos5* was estimated to be 19.5 centimorgans from *ATHGAPAb*.

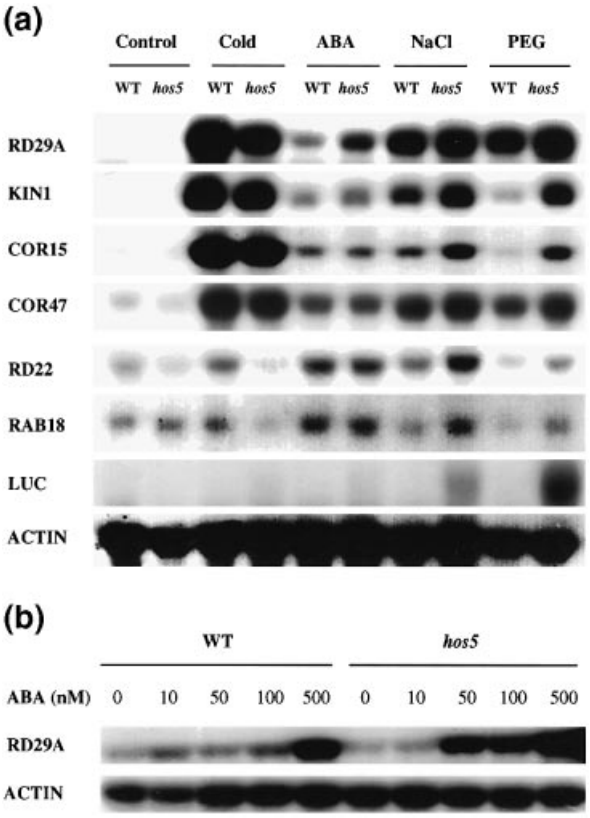

Figure 2. The expression of the endogenous *RD29A* gene and other stress-responsive genes in *hos5-1* and the wild-type plants.
(a) Control, room temperature; Cold, 0°C for 48 h; ABA, 100 μM ABA for 30 min; NaCl, 50 mM NaCl for 1 h; and PEG, 30% PEG (average molecular weight 6000) for 1 h.
(b) Treatments with lower concentrations of ABA for 1 h.

Stress gene expression in the hos5-1 *mutant*

Expression of the endogenous *RD29A* and other OR genes was analyzed in *hos5-1* seedlings. The *RD29A* message accumulated in both wild-type and the *hos5-1* mutant in response to cold (0°C for 48 h), ABA (100 μM for 30 min) and osmotic stress (50 mM NaCl or 30% PEG for 1 h) treatments (Figure 2a). However, the level of induction by ABA or osmotic stress was higher in *hos5-1* compared to the wild type. In contrast, the expression under cold stress was not higher but lower in *hos5-1*. The expression of *KIN1, COR15*, *COR47, RD22* and *RAB18* were all higher in the *hos5-1* mutant under osmotic stress. The level of *KIN1* expression was also higher in the *hos5-1* mutant with ABA treatment. However, the ABA-induced expression of *RD22*,

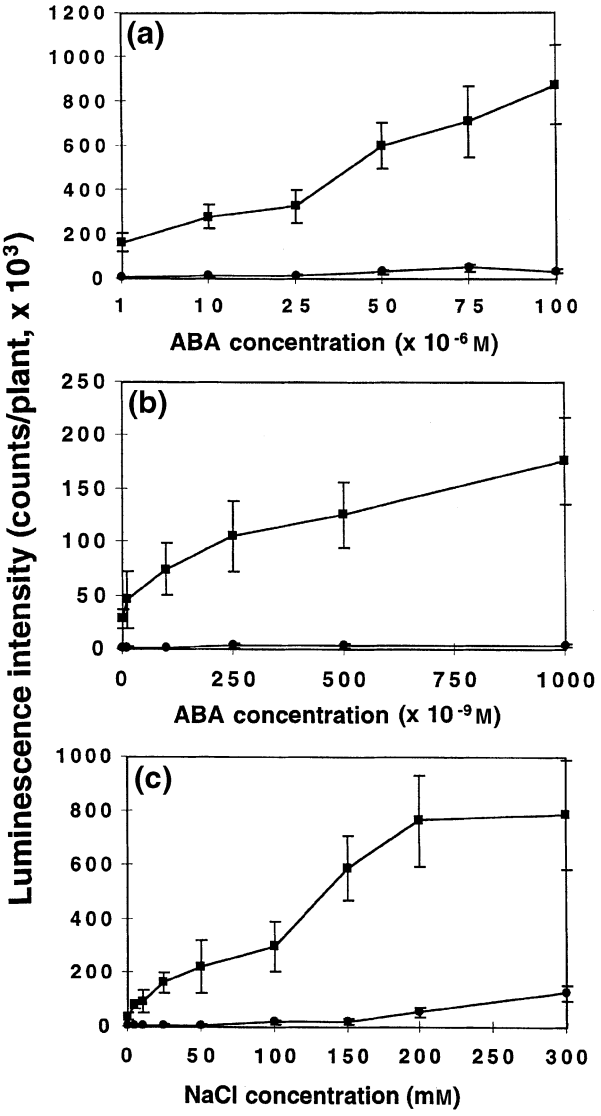

Figure 3. *hos5-1* is hypersensitive to ABA and osmotic stress.
(a) *RD29A-LUC* expression in *hos5-1* and the wild-type plants in response to μM concentrations of ABA.
(b) *RD29A-LUC* expression in *hos5-1* and the wild-type plants in response to nM concentrations of ABA.
(c) *RD29A-LUC* expression of *hos5-1* and the wild-type plants in response to NaCl. Error bars represent standard deviation ($n = 20$). ●, wild-type plants; ■, *hos5-1.*

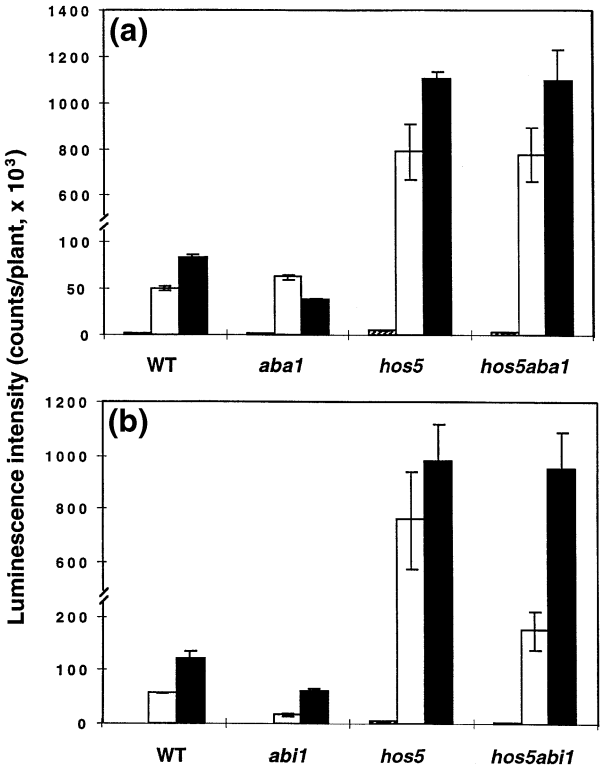

Figure 4. *RD29A-LUC* expression in *hos5-1 aba1-1* and *hos5-1 abi1-1* double mutants in response to ABA and NaCl treatments.
(a) Luminescence intensity in wild-type *RD29A-LUC* (WT), *aba1-1* (with *RD29A-LUC* transgene), *hos5-1,* and *hos5-1 aba1-1* double mutants.
(b) Luminescence intensity in wild-type *RD29A-LUC* (WT), *abi1-1* (with *RD29A-LUC* transgene), *hos5-1*, and *hos5-1 abi1-1* double mutants. Error bars represent standard deviation ($n = 20$). Striped bars, control (without treatment); open bars, 100 μM ABA for 3 h; black bars, 100 mM NaCl for 4 h. Please note the luminescence intensities of the controls (no treatment) are very low compared with ABA or NaCl treatments.

COR15, *COR47* and *RAB18* in *hos5-1* were not substantially different from those in the wild type. None of the OR genes had elevated expression in the mutant in response to cold treatment (Figure 2a). Cold induction of *KIN1*, *COR15* and *COR47* in *hos5-1* were the same as in the wild type. Furthermore, *RD29A*, *RD22* and *RAB18* also had lower steady state mRNA levels in the *hos5-1* mutant in response to cold treatment (Figure 2a).

The expression of the endogenous *RD29A* gene was also determined at lower ABA concentrations. As shown in Figure 2(b), significant *RD29A* induction was observed in *hos5-1* plants with as low as 50 nM ABA. In comparison, for wild-type plants, substantial *RD29A* induction was observed only at 500 nM ABA. As controls, the mRNA levels of actin and the luciferase transgene were also determined (Figure 2). Although the luciferase transcript was more difficult to detect than the other genes examined, its level was clearly higher in the *hos5-1* mutant when treated with high salt or PEG (Figure 2a). On the other hand, the expression of actin was not substantially different between *hos5-1* and wild-type plants under any of the treatments (Figure 2).

Sensitivity of hos5-1 *to ABA and osmotic stress*

Because the amplitudes of the responses to ABA and high salt are higher in *hos5-1* mutant plants (Figure 1), we were interested in determining whether the mutant also has increased sensitivities to these signals. Figure 3(a,b) shows the luminescence response to different concentrations of ABA in *hos5-1* seedlings. The response in wild-type plants

peaked at 75 μM ABA whereas it was not saturated even at 100 μM ABA in *hos5-1*. The response in *hos5-1* is many times higher than that in the wild type at all ABA concentrations (Figure 3a,b). The wild type did not exhibit significant response to ABA at concentrations below 250 nM (Figure 3a). In contrast, substantial response can be detected in *hos5-1* mutant at ABA concentrations as low as 10 nM (Figure 3b).

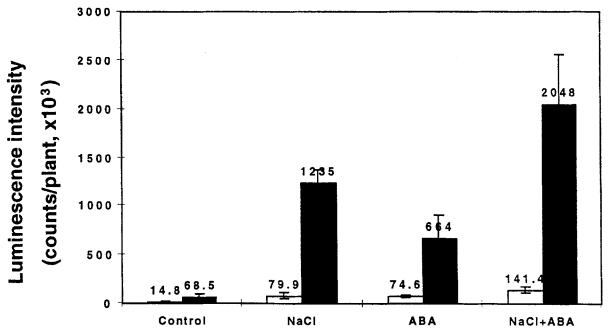

Figure 5. Osmotic stress (NaCl treatment) and ABA are additive in inducing *RD29A-LUC* expression in *hos5-1*.
One-week-old seedlings were placed on filter paper saturated with either MS salt solution (control or ABA treatments) or MS salt plus 200 mM NaCl (NaCl or NaCl plus ABA treatments). For ABA treatment, 100 μM ABA was sprayed on the leaves. Images were taken 4 h after beginning the treatments. Error bars represent standard deviation (n = 20). Open bars, wild type; black bars, *hos5-1*.

The *hos5-1* mutant also showed an increased sensitivity as well as higher amplitude towards osmotic stress (Figure 3c). There was no significant expression in wild type at 50 mM or less NaCl. In comparison, the response in *hos5-1* at 50 mM NaCl is nearly twice as much as the wild-type peak response at 300 mM NaCl. Substantial induction of luciferase expression was detected in *hos5-1* mutant plants with as low as 5 mM NaCl treatment (Figure 3c).

The time course of bioluminescence expression in *hos5-1* and the wild type in response to ABA or NaCl treatment was also measured (data not shown). The responses were rapid and transient in both wild type and the mutant. The expression in *hos5-1* is higher at all time points. The transient nature of luciferase expression in *hos5-1* also suggests that the higher luminescence levels in the mutant are not due to increased stability of the luciferase enzyme.

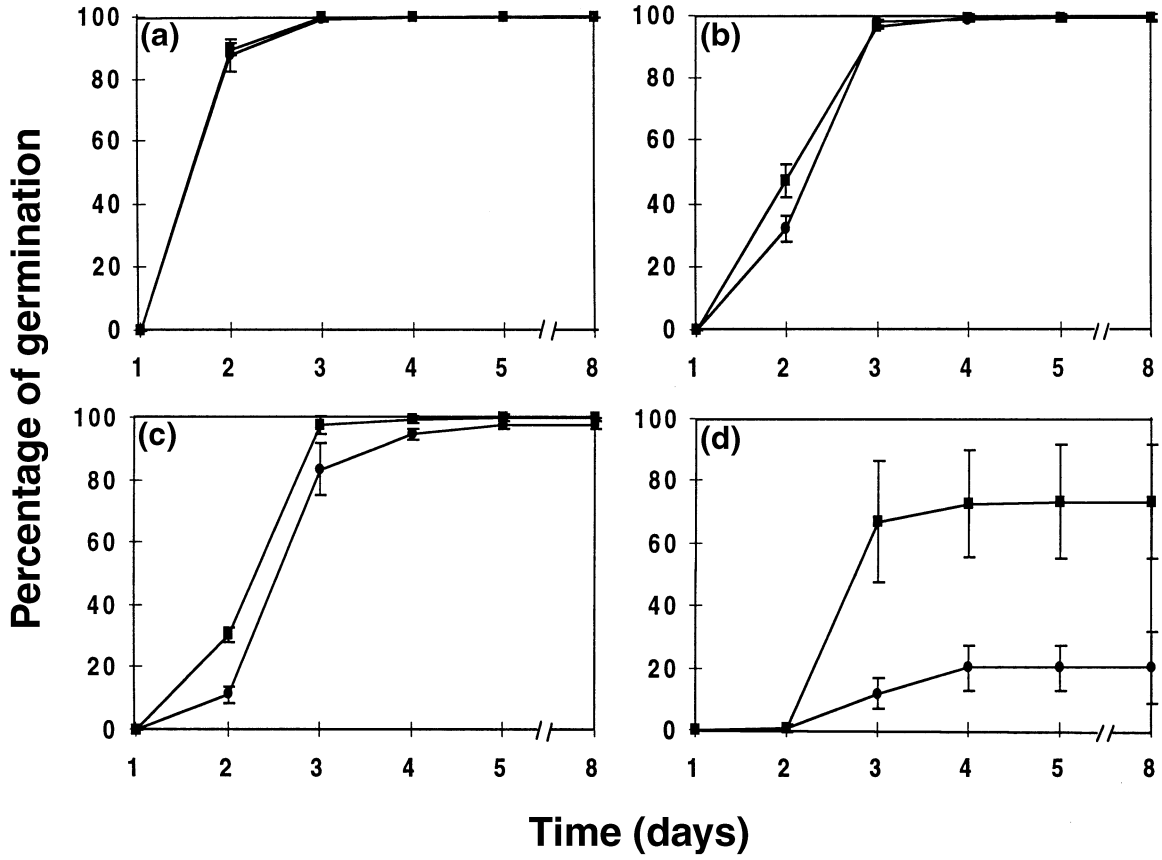

Figure 6. Germination of hos5-1 mutant seeds in the presence of ABA.
One hundred seeds from each of the *hos5-1* and the wild type were placed on the same filter paper saturated with either H_2O or different concentrations of ABA and incubated at 4°C for 48 h before being placed at room temperature for germination. Germination (with complete penetration of radicals) was scored at different times (days) after being incubated at room temperature. Error bars represent standard deviation (n = 3). ●, wild type; ■, *hos5-1*. (a) Control, with H_2O only. (b) 0.5 μM ABA in H_2O. (c) 1.0 μM ABA in H_2O. (d) 2.0 μM ABA in H_2O.

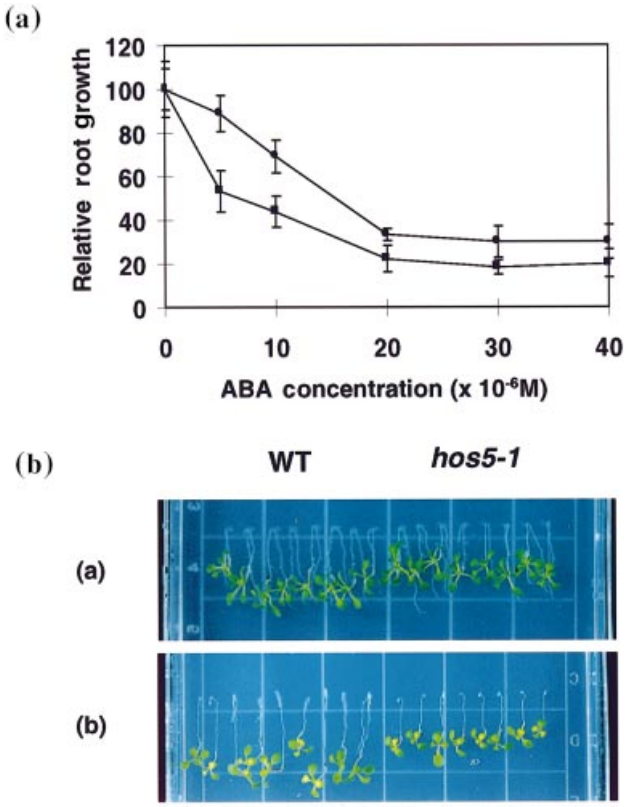

Figure 7. Root growth of *hos5-1* and the wild type on ABA media.
(a) Five-day-old seedlings were transferred from normal MS medium to MS media supplemented with different concentrations of ABA and root elongation was measured the fifth day after the transfer. Error bars represent standard deviation ($n = 15$). ●, wild type; ■, *hos5-1*.
(b) Root-bending of the *hos5-1* and the wild-type plants on ABA medium. Five-day-old seedlings were transferred from normal MS medium to either normal MS medium (a) or MS medium supplemented with 30 μM ABA (b). The pictures were taken 5 days after the transfer.

Hypersensitivity to osmotic stress in hos5-1 *is ABA-independent*

Because osmotic stress induces ABA synthesis in plants, hypersensitivity to ABA could theoretically lead to a hypersensitive response to osmotic stress. To determine whether the increased osmotic stress sensitivity of OR gene expression in *hos5-1* is due to ABA-hypersensitivity, we crossed *hos5-1* with the ABA-deficient *aba1* mutant (Koornneef *et al.*, 1982) and with the ABA-insensitive *abi1* mutant (Koornneef *et al.*, 1984). Double mutants homozygous for *hos5-1* and *aba1* or *abi1* mutations were selected from the segregating F_2 progeny resulting from the crosses and the phenotypes were confirmed in the F_3 progeny. As controls, we also obtained *aba1* and *abi1* mutants that contain the *RD29A-LUC* transgene by crossing wild-type *RD29A-LUC* plants with the respective mutants. Figure 4(a) shows that *RD29A-LUC* expression in the *hos5 aba1* double mutant in response to ABA and high salt treatments is similar to that in *hos5-1*. Therefore, the *aba1* mutation did not impair the osmotic stress hypersensitivity of *hos5-1* mutant. In the wild-type background, the *aba1* mutation reduced the response to osmotic stress but not ABA (Figure 4a). This is consistent with previous reports (Gilmour and Thomashow, 1991; Yamaguchi-Shinozaki and Shinozaki, 1993) on the effect of *aba1* mutation on *RD29A* expression using Northern analysis.

RD29A-LUC expression in the *hos5 abi1* double mutant in response to ABA is lower than in the *hos5-1* single mutant (Figure 4b). However, the *abi1* mutation had little effect on *RD29A-LUC* expression in the *hos5-1* mutant in response to osmotic stress treatment (Figure 4b). In the *HOS5* background, the *abi1* mutation reduced *RD29A-LUC* expression in response to both ABA and osmotic stress treatments. These results show that ABA deficiency or insensitivity does not impair the enhanced osmotic stress response conferred by the *hos5-1* mutation and thus suggest that the osmotic stress hypersensitivity in *hos5-1* is ABA-independent.

The ABA-independence of osmotic stress hypersensitivity in *hos5-1* is also supported by analysis of the interaction of osmotic stress and ABA application. When both ABA and salt were near the concentrations that conditioned the maximum induction of *RD29A-LUC*, combined treatment with ABA and high salt gave a response that approximates the sum of the individual treatments (Figure 5). In wild-type plants, combined treatment with ABA and high salt also had an additive effect on *RD29A-LUC* expression. This may be explained by the notion that ABA and osmotic stress activate *RD29A* expression through two separate pathways, i.e. the ABA-dependent and -independent pathways. The additive hypersensitive response observed with *hos5-1* indicates that the mutation affects ABA and osmotic stress responses through separate pathways.

Germination of hos5-1 *seeds is less sensitive to ABA inhibition*

ABA has an inhibitory effect on seed germination. Using one batch of wild-type seeds, our initial experiments showed that *hos5-1* seed germination was slightly more sensitive to inhibition by ABA. However, subsequent experiments using different batches of wild-type seeds and *hos5-1* seeds that had been backcrossed several times revealed that the *hos5-1* mutation actually reduces the sensitivity of seed germination to ABA (Figure 6). The reduced sensitivity was especially evident at 2.0 μM ABA (Figure 6d). At ABA concentrations of 3.0 μM or above, germination of both the wild-type and *hos5-1* seeds were greatly inhibited and their difference became less clear (data not shown). At 1.0 μM ABA, the *hos5-1* seeds were only slightly less sensitive (Figure 6c). With 0.5 μM ABA or without ABA, little difference was observed between the

germination rates of wild-type and *hos5-1* seeds (Figure 6a,b). Little difference was found between *hos5-1* and the wild type in their seed germination responses to NaCl (data not shown).

Other phenotypes of hos5-1

In addition to OR gene expression and seed germination, ABA and osmotic stress affect many other physiological and biochemical processes in plants. It has been documented that ABA inhibits root growth and fresh weight gain, and it also induces proline synthesis in Arabidopsis (Finkelstein and Somerville, 1990; Savoure *et al.*, 1997). Figure 7 shows that root growth inhibition by ABA was more pronounced in the *hos5-1* mutant. The difference in root elongation was most evident at relatively low concentrations of ABA (Figure 7a). When *hos5-1* and the wild-type seedlings were compared using the root bending assay (Wu *et al.*, 1996), a difference in their root growth was also observed (Figure 7b). Both *hos5-1* and the wild-type seedlings exhibited normal root bending in the absence of ABA. However, in the presence of 30 μM ABA in the agar medium, the wild-type seedlings still showed some root bending whereas the *hos5-1* plants did not. The tips of *hos5-1* roots swelled in the presence of 30 μM ABA, indicating very strong inhibition by the hormone.

Proline content is slightly higher in *hos5-1* than in the wild type when treated with either ABA or NaCl (data not shown). No significant difference in seedling growth as measured by fresh weight gain or root growth was found between the mutant and the wild type under either NaCl or PEG treatments (data not shown). The effect of *hos5-1* mutation on stomatal control was evaluated by measuring water loss from excised rosette leaves. The rate of water loss as indicated by fresh weight measurement was not different between *hos5-1* and the wild type (data not shown). Thus, the *HOS5* locus does not seem to play a role in stomatal control.

The general growth of *hos5-1* plants is similar to that of the wild type and there is no striking visible phenotype associated with this mutation.

Discussion

hos5-1 is a novel mutation that affects the regulation of OR gene expression by ABA and osmotic stress but not by cold. The *hos5* mutation dramatically increases the ABA and osmotic stress sensitivity of *RD29A-LUC* expression. The *RD29A-LUC* expression in *hos5-1* could be induced by a few nM ABA or a few mM NaCl (Figure 3). The low level of 'constitutive' *RD29A-LUC* expression observed in *hos5-1* is probably due to induction by unavoidable mild osmotic stress encountered during normal growth conditions or by low levels of ABA present in normal unstressed plants. Using an immunoassay, we found that *hos5-1* plants did not have a higher ABA content whether stressed or unstressed (0.007 $\mu g\, g^{-1}$ fresh weight for both unstressed wild type and unstressed *hos5-1,* and 0.10 and 0.11 $\mu g\, g^{-1}$ fresh weight for stressed wild type and stressed *hos5-1*, respectively). Despite this extreme sensitivity of *RD29A-LUC* expression, the mutant is only slightly altered in its sensitivity to ABA with respect to seed germination, root growth and proline accumulation. Mutant plants are not altered in stomatal control by ABA or osmotic stress. Thus, the results suggest that *HOS5* is a negative regulator specifically for ABA and osmotic stress regulation of OR gene expression, and it may indirectly have only a small impact on some other ABA-and osmotic stress-regulated processes.

One possible explanation for the enhanced osmotic stress induction of the OR genes is due to increased ABA sensitivity of *hos5-1* plants. However, our results suggest otherwise. The increased osmotic stress sensitivity of *hos5-1* mutant is not impaired by the *aba1* mutation (Figure 4a). The *aba1-1* mutation is leaky and the mutant plants still synthesize a low level of ABA (Koornneef *et al.*, 1982; Leon-Kloosterziel *et al.*, 1996). Nevertheless, if the osmotic stress hypersensitivity of *hos5-1* plants is ABA-dependent, the *aba1-1* mutation is expected to significantly reduce the osmotic stress induction of *RD29A-LUC* because the induction in *hos5-1* plants was not saturated even at 100 μM ABA (Figure 3a). The *abi1* mutation also did not affect the osmotic stress sensitivity conferred by the *hos5-1* mutation (Figure 4b). Therefore, we believe that the osmotic stress hypersensitivity of *hos5-1* plants is ABA-independent. The additive effect of ABA and osmotic stress treatments on *RD29A-LUC* expression in *hos5-1* is consistent with this notion that the *hos5-1* mutation negatively regulates osmotic stress responsive gene expression through an ABA-independent pathway (Figure 5). We propose that *HOS5* is one of the components shared by an ABA-independent osmotic signaling pathway and a pathway downstream of ABA.

The *hos5* mutation not only enhanced *RD29A* gene expression in response to ABA and osmotic stress, it also increased the induction of several other OR genes by osmotic stress (Figure 2). It should be noted that although the endogenous *RD29A* mRNA levels are six times and two times higher in *hos5-1* than in the wild type, respectively, when treated with ABA or osmotic stress, the difference is not as great as that seen with the *RD29A-LUC* reporter. The recessive nature of the *hos5-1* mutation suggests that it acts in *trans-*. To ascertain that the *hos5* phenotype is not caused by a *cis*-mutation in the introduced *RD29A* promoter, we have isolated this promoter from *hos5-1* and wild-type plants by PCR using transgene-specific primers. The sequence of the transgene *RD29A* promoter in *hos5-1* plants is identical to the one from the wild-type

RD29A-LUC plants (data not shown). This confirms that *hos5-1* is not a *cis*-mutation. The discrepancy between the *RD29A-LUC* expression and the endogenous *RD29A* mRNA level suggests that the endogenous *RD29A* mRNA level is under post-transcriptional control in addition to transcriptional regulation. A comparison of *RD29A-LUC* and the endogenous *RD29A* expression in the wild-type plants under cold and ABA or osmotic stress treatments also suggests post-transcriptional control of endogenous *RD29A* mRNA levels. As shown in Figure 1, *RD29A-LUC* expression in the wild type is much lower under cold than under ABA or high NaCl treatments. However, the endogenous *RD29A* mRNA level in the wild type is clearly higher under cold treatment than under either ABA or osmotic stress treatments. Plants may employ this post-transcriptional control (e.g. mRNA degradation) to prevent overaccumulation of OR gene messages under certain conditions such as ABA or high salt treatment. Post-transcriptional regulation of OR genes has frequently been observed whenever attempts were made to correlate mRNA levels with transcriptional activities (for review, see Zhu *et al*., 1997).

It is intriguing that the *hos5-1* mutation seemed to enhance the steady state mRNA levels of *RD29A* and *KIN1* but not *COR15*, *COR47*, *RD22* or *RAB18* in response to ABA (Figure 3). One possibility is that *hos5-1* affects the transcriptional induction of all of these genes but post-transcriptional regulation may prevent their over-accumulation. It is also possible that *RD29A* and *KIN1* may be regulated by ABA through a different pathway from the other OR genes.

Germination of *hos5-1* seeds is slightly less sensitive to ABA inhibition. Although the *hos5-1* mutation enhances the expression of several OR genes in seedlings, it is not known whether it has a similar role in seeds. The *abi1* mutation alters ABA sensitivity in seeds as well as in vegetative tissues (Finkelstein and Somerville, 1990). In this respect, the *hos5-1* mutation is clearly different. Perhaps this is because *hos5-1* affects not only ABA-dependent but also ABA-independent stress gene regulation. Future isolation of the *HOS5* gene may help explain its seemingly contradictory roles in vegetative tissues and seeds.

Experimental procedures

Plant materials

Transgenic *Arabidopsis thaliana* expressing the *RD29A-LUC* transgene were obtained by *Agrobacterium*-mediated transformation as described previously (Ishitani *et al*., 1997). Ethyl methanesulfonate mutagenized M_2 seeds were planted on 0.8% agar plates containing full strength MS salt (Murashige and Skoog salt base, JRH Biosciences, Lenexa, KS, USA). Seedlings which were 1-week-old were screened for abnormal *LUC* expression in response to low temperature, ABA or osmotic stress with a video-imaging system comprised of a CCD camera (CCD-512SB, Princeton Instruments, Inc., Trenton, NJ, USA), a controller (Princeton Instruments, Inc., Trenton, NJ, USA) and a computer with WinView image processing software. Detailed procedures on screening and image acquisition and processing were described by Ishitani *et al*. (1997).

The isolated *hos5-1* mutant was backcrossed with the wild-type *RD29A-LUC* plant (see section below on genetic analysis) and mutant seedlings were selected from segregated F_2 population by their enhanced luminescence in responsiveness to ABA treatment. All subsequent physiological tests were done with mutants that had been backcrossed at least once.

Stress treatment

One-week-old wild-type and mutant seedlings on the same MS agar plate were used to determine the stress dosage–response curve. Different concentrations of ABA ((+/–)-cis, trans-abscisic acid, Sigma Chem. Co. (St. Louis, MO, USA) dissolved in sterile H_2O was sprayed uniformly on the leaves of the seedlings. Control treatment was sprayed with sterile H_2O only. The plates were then covered and kept at room temperature under cool-white light for a designated time period before luminescence imaging. For NaCl or PEG treatment, seedlings on MS plates were transferred to filter paper saturated with different concentrations of NaCl or 30% of polyethylene glycol (molecular weight 6000) in the MS background solution and placed under light at room temperature. For time course determination, luminescence images of the same plates were taken at different time points during the treatment. For luminescence imaging, plants were sprayed uniformly with 1 mM luciferin (Promega, Madison, WI, USA) in 0.01% Triton X-100 and then kept in the dark for 5 min. All images were acquired with 5 min exposure time. The luminescence intensity of each seedling was quantified with the WinView software. Each quantitation is intended to come from similar pixels to ensure uniform background counting.

The study of the interaction between osmotic stress and ABA in inducing *RD29A-LUC* expression was conducted on filter paper saturated with either MS salt solution (control treatment or ABA treatment) or with MS salt solution plus 200 mM NaCl (NaCl treatment or NaCl plus ABA treatment). One-week-old seedlings of *hos5-1* and the wild type were placed on the filter paper. ABA (100 μM) was sprayed uniformly on leaves. All images were taken 4 h after the beginning of the treatment.

Genetic analysis

Mutant plants were backcrossed with the wild-type *RD29A-LUC* plants and the resulting F_1 seeds were tested for responses to 100 μM ABA. F_2 seeds were obtained from self-pollinated F_1 plants. The F_2 populations were scored for segregation in responses to low temperature, 100 μM ABA and 300 mM NaCl treatments.

For genetic mapping of *hos5* mutation, *hos5-1* was crossed with ecotype Columbia with the *glabrous 1* mutation. The resulting F_1 plants were allowed to self and the F_2 seeds were collected. Homozygous *hos5* mutants in the segregated F_2 population were selected for their ABA-hypersensitive luminescence. Mapping of the mutation was carried out following the procedures of Bell and Ecker (1994).

Mutant *hos5-1* was also crossed with *aba1-1* (obtained from the Arabidopsis Biological Research Center, Columbus, OH, USA) and *abi1* (provided by Dr M. Koornneef). The F_2 seedlings along with *aba1-1*, *hos5-1* and the wild type were grown on MS agar plates

for 1 week. Seedlings homozygous for *aba1-1* were selected from the F_2 population by their wilty phenotype in response to low humidity. Those containing *abi1-1* were selected by their ability to germinate and grow on MS agar plates supplemented with 10 μM ABA. Selected putative double mutant seedlings were then transferred to soil and allowed to grow and set seeds. The progeny were tested again for *hos5*, *aba1* and *abi1* phenotypes. Those that did not segregate with the *abi1* phenotypes in the F_3 progeny were considered homozygous for the *abi1-1* mutation. Selected double mutant lines were then used for NaCl or ABA treatment to assay *RD29A-LUC* expression as described above.

RNA analysis

For RNA analysis, seedlings were grown in MS agar plates for 8–9 days. The seedlings were treated as indicated in the text and total RNA was extracted and analyzed as described previously (Liu and Zhu, 1997). The *RD29A* gene-specific probe was from the 3′ non-coding region (Liu and Zhu, 1997). *COR15* and *COR47* cDNA (Gilmour *et al*., 1992; Lin and Thomashow, 1992) were provided by Dr M.F. Thomashow. DNA probes for *RD22* (Yamaguchi-Shinozaki *et al*., 1992) were cloned from genomic DNA of wild-type Columbia plants by polymerase chain reaction. Probe for *KIN1* (Kurkela and Franck, 1990) was a 0.4 kb *Eco*R1 fragment of the Arabidopsis expressed sequence tag (EST) clone YAP368T7. Probe for *RAB18* (Lång and Palva, 1992) was a 0.8 kb *Sal*I-*Not*I fragment of the Arabidopsis EST clone 246K10T7.

Germination and water loss measurements

Around 100 seeds from each of *hos5-1* and the wild type (collected and dried at the same time and stored under identical conditions) were planted in triplicates on filter paper saturated with distilled water or different concentrations of ABA or NaCl. The seeds were then placed at 4°C for 48 h before incubation at room temperature. Germination was scored daily for 10 days after being placed at room temperature. For water loss measurement, plants at rosette stage were detached from soil surface and weighed immediately in a plastic weigh boat. The boat with the plants was then placed on a laboratory bench (relative humidity 20–30%) and weighed at designated time intervals. There were four replicates for each line. Percentage loss of fresh weight was calculated based on the initial weight of the plants.

Growth and proline assays

Germinated seeds on normal MS agar plates were transferred to MS agar plates containing different concentrations of ABA, NaCl or PEG. Each plate contained half wild type and half mutant plants. There were three replicates for each treatment. The seedlings were allowed to grow for 10 days. To harvest, whole seedlings were pulled out of the agar medium and weighed immediately. The samples were then frozen in liquid nitrogen and kept at –80°C for proline assay. Proline concentration was determined as described by Bates *et al*. (1973).

Acknowledgements

We thank Becky Stevenson for excellent technical assistance, and Drs Don Nelson and Frans Tax for critical reading of the manuscript. This work was supported by grants from the National Science Foundation and the United States Department of Agriculture National Research Initiative Competitive Grants Program to J.-K. Zhu.

References

Bates, L.S., Waldren, R.P. and Teare, I.D. (1973) Rapid determination of free proline for water-stress studies. *Plant Soil*, **39**, 205–207.

Bell, C.J. and Ecker, J.R. (1994) Assignment of 30 microsatellite loci to the linkage map of Arabidopsis. *Genomics*, **19**, 137–144.

Bray, E.A. (1993) Molecular responses to water deficit. *Plant Physiol.* **103**, 1035–1040.

Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S. and McCourt, P. (1996) A protein farnesyl transferase involved in abscisic acid signal transduction in *Arabidopsis*. *Science*, **273**, 1239–1241.

Finkelstein, R.R. (1994) Mutations at two new *Arabidopsis* ABA response loci are similar to the *abi3* mutations. *Plant J.* **5**, 765–771.

Finkelstein, R.R. and Somerville, C.R. (1990) Three classes of abscisic acid (ABA) -insensitive mutations of *Arabidopsis* define genes that control overlapping subsets of ABA responses. *Plant Physiol.* **94**, 1172–1179.

Gilmour, S.J., Artus, N.N. and Thomashow, M.F. (1992) cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. *Plant Mol. Biol.* **18**, 13–21.

Gilmour, S.J. and Thomashow, M.F. (1991) Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana*. *Plant Mol. Biol.* **17**, 1233–1240.

Gosti, F., Bertauche, N., Vartanian, N. and Giraudat, J. (1995) Abscisic acid-dependent and -independent regulation of gene expression by progressive drought in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **246**, 10–18.

Guerrero, F.D., Jones, J.T. and Mullet, J.E. (1990) Turgor-responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted: sequence and expression of three inducible genes. *Plant Mol. Biol.* **15**, 11–26.

Ishitani, M., Xiong, L., Stevenson, B. and Zhu, J.-K. (1997) Genetic analysis of osmotic and cold stress signal transduction in *Arabidopsis thaliana*: interactions and convergence of abscisic acid- dependent and abscisic acid-independent pathways. *Plant Cell*, **9**, 1935–1949.

Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O. and Thomashow, M.F. (1998) *Arabidopsis CBF1* overexpression induces *cor* genes and enhances freezing tolerance. *Science*, **280**, 104–106.

Koornneef, M., Jorna, M.L., Brinkhorst-van der Swan, D.L.C. and Karssen, C.M. (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive of *Arabidopsis thaliana* (L.) Heynh. *Theor. Appl. Genet.* **61**, 385–393.

Koornneef, M., Reuling, G. and Karssen, C.M. (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiol. Plant.* **61**, 377–383.

Koornneef, M. and Stam, P. (1992) Genetic analysis. In *Methods in Arabidopsis Research* (Koncz, C., Chua, N.-H. and Schell, J., eds). Singapore: World Scientific, pp. 83–99.

Kurkela, S. and Franck, M. (1990) Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. *Plant Mol. Biol.* **15**, 134–144.

Lång, V. and Palva, E.T. (1992) The expression of a *rab*-related gene, *rab18*, is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L.) Heynh. *Plant Mol. Biol.* **20**, 957–962.

Leon-Kloosterziel, K.M., Alvarez Gil, M., Ruijs, G.J., Jacobsen,

S.E., Olszewski, N.E., Schwartz, S.H., Zeevaart, J.A.D. and Koornneef, M. (1996) Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. *Plant J.* **10**, 655–661.

Leung, J., Bouvier-Durand, M., Morris, P.-C., Guerrier, D., Chefdor, F. and Giraudat, J. (1994) *Arabidopsis* ABA-response gene *ABI1*: Features of a calcium-modulated protein phosphatase. *Science*, **264**, 1448–1452.

Leung, J., Merlot, S. and Giraudat, J. (1997) The Arabidopsis *ABSCISIC ACID-INSENSITIVE2* (*ABI2)* and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *Plant Cell*, **9**, 759–771.

Lin, C. and Thomashow, M.F. (1992) DNA sequence analysis of a complementary DNA for cold-regulated *Arabidopsis* gene *cor15* and characterization of the COR15 polypetide. *Plant Physiol.* **99**, 519–525.

Liu, J. and Zhu, J.-K. (1997) Proline accumulation and salt stress-induced gene expression in a salt-hypersensitive *Arabidopsis* mutant. *Plant Physiol.* **114**, 591–596.

Marin, E., Nussaume, L., Quesada, A., Gonneau, M., Sotta, B., Hugueney, P., Frey, A. and Marion-Poll, A. (1996) Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to *ABA* locus of *Arabidopsis thaliana*. *EMBO J.* **15**, 2331–1342.

Meyer, K., Leube, M.P. and Grill, E. (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science*, **264**, 1452–1455.

Neil, S.J., Horgan, R. and Parry, A.D. (1986) The carotenoid and abscisic acid content of viviparous kenels and seedlings of *Zea mays* L. *Planta*, **169**, 87–96.

Nordin, K., Heino, P. and Tapio Palva, E. (1991) Separate signal pathways regulate the expression of a low-temperature-induced gene in *Arabidopsis thaliana* (L.) Heynh. *Plant Mol. Biol.* **16**, 1061–1071.

Savoure, A., Hua, X.-J., Bertauche, N., Van Montagu, M. and Verbruggen, N. (1997) Abscisic acid-independent and abscisic acid-dependent regulation of proline biosynthesis following cold and osmotic stresses in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **254**, 104–109.

Skriver, K. and Mundy, J. (1990) Gene expression in response to abscisic acid and osmotic stress. *Plant Cell*, **2**, 503–512.

Stockinger, E.J., Gilmour, S.J. and Thomashow, M.F. (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proc. Natl Acad. Sci. USA*, **94**, 1035–1040.

Wu, S.-J., Ding, L. and Zhu, J.-K. (1996) *SOS1*, a genetic locus essential for salt tolerance and potassium acquisition. *Plant Cell*, **8**, 659–666.

Yamaguchi-Shinozaki, K., Koizumi, M., Urao, S. and Shinozaki, K. (1992) Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. *Plant Cell Physiol.* **33**, 217–224.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (1993) Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. *Mol. Gen. Genet.* **236**, 331–340.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell*, **6**, 251–264.

Zhu, J.-K., Hasagawa, P.M. and Bressan, R.A. (1997) Molecular aspects of osmotic stress in plants. *Crit. Rev. Plant Sci.* **16**, 253–277.