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Cold-regulated gene expression and freezing tolerance in an *Arabidopsis thaliana* mutant

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

Summary

Low temperature is an important environmental factor influencing plant growth and development. In this study, we report the characterization of a genetic locus, *HOS2*, which is defined by three *Arabidopsis thaliana* mutants. The *hos2–1*, *hos2–2* and *hos2–3* mutations result in enhanced expression of *RD29A* and other stress genes under low temperature treatment. Gene expression in response to osmotic stress or ABA is not affected in the *hos2* mutants. Genetic analysis indicates that the *hos2* mutations are recessive and in a nuclear gene. Compared with the wild-type plants, the *hos2–1* mutant plants are less capable of developing freezing tolerance when treated with low non-freezing temperatures. However, the *hos2–1* mutation does not impair the vernalization response. These results indicate that *HOS2* is a negative regulator of low temperature signal transduction important for plant cold acclimation.

Introduction

Plants are cessile organisms and cannot avoid harmful environments. Therefore, plants have developed unique mechanisms to adapt to continuously changing environments. Plant responses to environmental stresses have long occupied the attention of humans (Levitt, 1941). Low temperature is one of the most common adverse environmental factors affecting plant development and crop productivity. Freezing tolerance is a requirement for the winter survival of herbaceous annuals such as *Arabidopsis thaliana* (Fennel *et al.*, 1990; Hurry *et al.*, 1995; Strand *et al.*, 1997). Plants from temperate regions can be acclimated to freezing temperatures by pre-treatment with low non-freezing temperatures, a process known as cold acclimation (Guy, 1990). Cold acclimation in temperate plants is genetically programmed (Guy, 1990) and numerous biochemical and physiological changes occur during this process. For example, changes in leaf ultrastructure (Ristic and Ashworth, 1993), membrane composition (Lynch and Steponkus, 1987; Miquel *et al.*, 1993), protein composition (Raison, 1973), the activities of enzymes, and accumulation of sugars and polyamines (Levitt, 1980; Strand *et al.*, 1997) and activation of ion channels (Knight *et al.*, 1996) have been correlated with cold acclimation.

Recently, many studies have been conducted to understand and eventually manipulate cold stress signalling in plants. Abscisic acid (ABA) has long been believed to be involved in cold signalling and to have an important role in cold acclimation. Freezing tolerance of a wide range of plants was increased after ABA treatment at normal growth temperatures and the increase of ABA levels after exposure to low temperature was reported in a number of plant species, including *Arabidopsis* (Chen *et al.*, 1983; Guy and Haskell, 1988; Lang *et al.*, 1994; Mohapatra *et al.*, 1988). In addition, transient cytosolic calcium increases have been reported to play a role in an early step of cold stress signalling (Knight *et al.*, 1991; 1996; Monroy and Dhindsa, 1995). Recently, two stress responsive genes, *RD29A* (Yamaguchi-Shinozaki and Shinozaki, 1993) and *KIN2* (Kurkela and Borg-Franck, 1992), were reported to be activated by cyclic ADP-ribose which releases calcium from cell organelles (Wu *et al.*, 1997). Nevertheless, the mechanisms of low temperature perception and intracellular signalling largely remain to be elucidated.

The expression of many genes are up-regulated by cold stress in plants (Dhindsa and Monroy, 1994; Guy *et al.*, 1994; Hajela *et al.*, 1990; Kurkela and Franck, 1990; Nordin *et al.*, 1993; Palva *et al.*, 1994; Thomashow, 1994). These cold-responsive genes encode proteins such as lipid transfer proteins, late-embryogenesis-abundant proteins, alcohol dehydrogenase, and translation elongation factors (Nishida and Murata, 1996). We are interested in the regulation of cold responsive genes as a way to analyze low temperature signalling in plants. Our group has developed a genetic screen for *Arabidopsis* mutants with aberrant gene transcription in response to low temperature (Ishitani *et al.*, 1997). We present here the characterization of several mutants (i.e. *hos2–1*, *hos2–2* and *hos2–3*) which define an important negative regulator of cold stress signalling. Stress-responsive genes are induced to higher levels specifically by low temperature in these mutants. The *HOS2* locus is also critical for the development of freezing tolerance and the mutant plants are impaired in cold acclimation.

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*For correspondence (fax +520 621 7186; e-mail jkzhu@ag.arizona.edu).

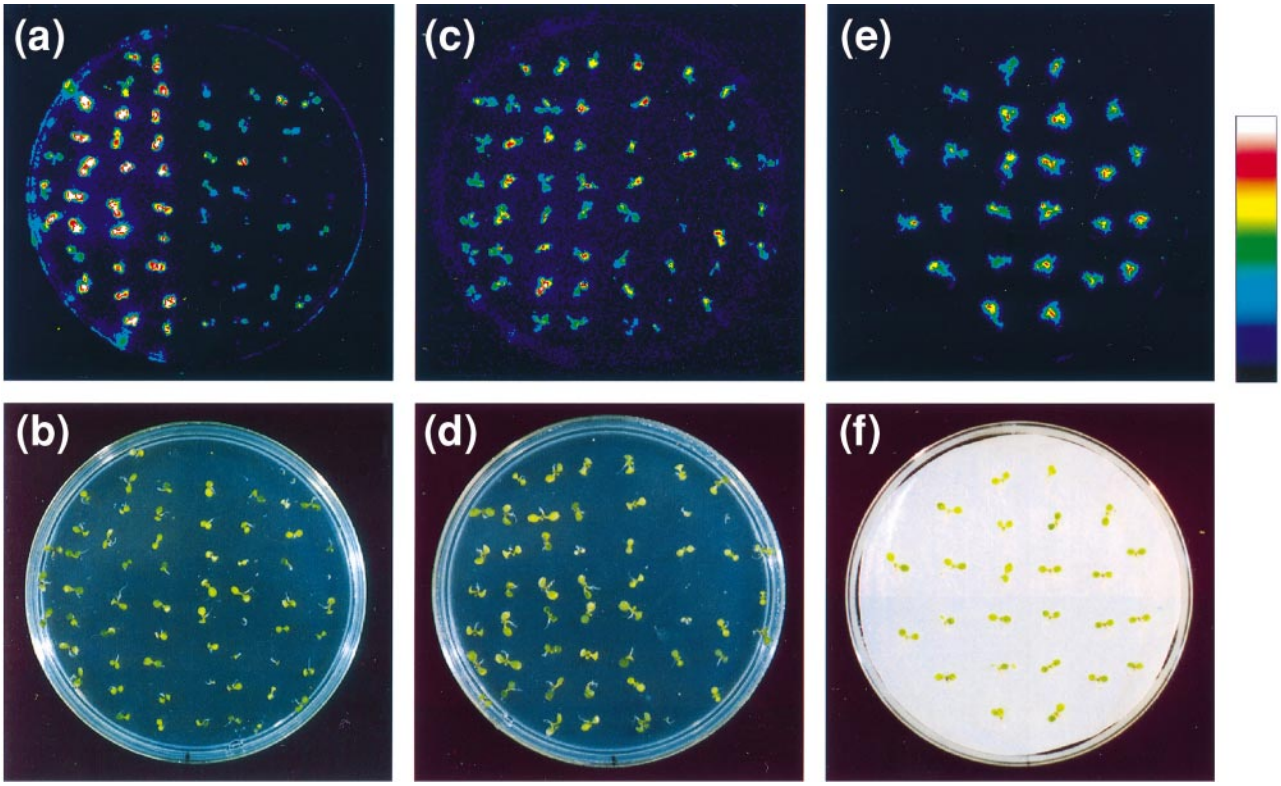

Figure 1. Luminescence images of *hos2–1* and the wild-type plants.
The colour scale on the right shows the luminescence intensity from dark blue (lowest) to white (highest).
(a) Luminescence of the *hos2–1* (left) and wild-type (right) seedlings after low temperature treatment, 0°C for 24 h.
(b) Picture of the *hos2–1* (left) and wild-type (right) seedlings that were imaged in (a).
(c) Luminescence of the *hos2–1* (left) and wild-type (right) seedlings after ABA treatment, 100 μM ABA for 3 h.
(d) Picture of the *hos2–1* (left) and wild-type (right) seedlings that were imaged in (C).
(e) Luminescence of the *hos2–1* (left) and wild-type (right) seedlings after NaCl treatment, 300 mM for 5 h.
(f) Picture of the *hos2–1* (left) and wild-type (right) seedlings on filter paper saturated with 300 mM NaCl which were imaged in (e).

Results

Identification of the HOS2 *locus*

Our genetic screen for identifying cold responsive mutants utilizes transgenic *Arabidopsis thaliana* plants expressing the *RD29A-LUC* transgene. The chimeric *RD29A-LUC* gene was constructed by fusing the cold-, osmotic stress- and ABA-responsive *RD29A* promoter with the firefly luciferase coding sequence (Ishitani *et al.*, 1997). One such mutant which we designated as *hos2–1* (for high expression of osmotically responsive genes) was chosen for detailed characterization because preliminary experiments indicated that the mutation affects not only *RD29A-LUC* transgene but also the expression of the endogenous *RD29A* gene.

Figure 1 presents the luminescence images of *hos2–1* and wild-type seedlings when treated with low temperature, ABA and NaCl. Without stress treatment, neither *hos2–1* nor wild-type plants show any expression of the *RD29A-LUC* transgene (data not shown). Substantial luminescence was induced in both *hos2–1* and wild-type plants by low temperature, ABA or NaCl treatment. However, the luminescence in response to cold stress was much brighter in *hos2–1* seedlings. Quantitation of the luminescence intensities confirmed that *RD29A-LUC* expression in *hos2–1* seedlings is specifically hypersensitive to cold stress (Figure 2a). In response to cold treatment at 0°C for 48 h, the luminescence level in *hos2–1* seedlings was approximately 10.6 times greater than that in wild-type plants (Figure 2a). The luminescence intensities in *hos2–1* plants are 0.8 and 1.4 times, respectively, of those in wild-type plants when treated with ABA and NaCl (Figure 2a).

To determine whether the *hos2–1* mutation also affects the expression of the endogenous *RD29A* gene, RNA gel blot analysis was carried out on RNA extracted from *hos2–1* and wild-type plants that were treated in the same manner as those used for luminescence imaging. Figure 2(b) shows that the pattern of endogenous *RD29A* expression was qualitatively similar to the *RD29 A-LUC* transgene, i.e. *RD29A* expression was specifically hypersensitive to cold stress. In comparison, the control actin gene did not show higher expression in *hos2–1* under any of the treatments (Figure 2b).

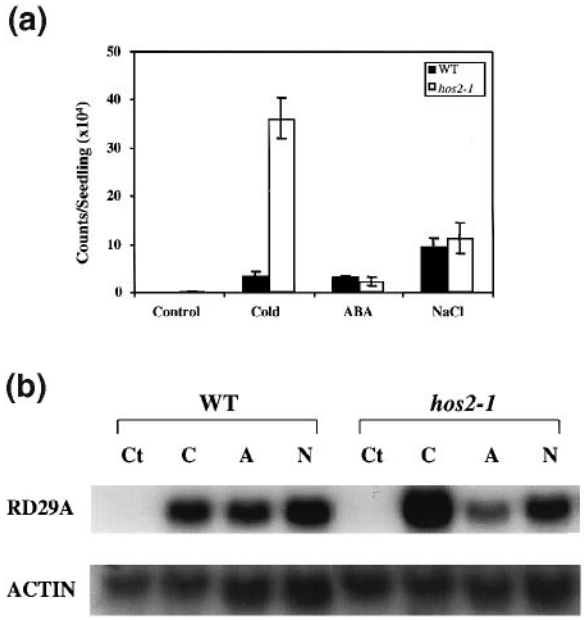

Figure 2. Comparison between the expression of *RD29A-LUC* transgene and the endogenous *RD29A* gene.
(a) *RD29A-LUC* expression (luminescence intensity) in *hos2–1* and wild-type plants. Data represent the average of 20 individual seedlings. Control: room temperature without treatment; Cold: 0°C for 48 h; ABA: 100 μM ABA for 3 h; NaCl: 300 mM NaCl for 5 h.
(b) Expression of the endogenous *RD29A* gene in *hos2–1* and the wild-type plants. Plants were treated with low temperature (0°C) for the indicated times; ABA, 100 μM ABA for 3 h; NaCl and 300 mM NaCl for 5 h. Ct: room temperature without treatment; C: 0°C for 48 h; A: 100 μM ABA for 3 h; N: 300 mM NaCl for 5 h

Table 1. Genetic analysis of Arabidopsis *hos2* mutants

Crosses	F_1		F_2		χ_2
	WT	Mutant	WT	Mutant	
WT × *hos2–1*	20	0	509	169	0.001
WT × *hos2–2*	18	0	154	48	0.16
WT × *hos2–3*	16	0	153	45	0.5
hos1–1 × *hos2–1*	11	0			
hos2–1 × *hos2–2*	0	20			
hos2–1 × hos2–3	0	20			

The *hos2–1* mutant was backcrossed with the wild-type. The resulting F_1 plants all exhibited the wild-type phenotype in *RD29A-LUC* expression (Table 1). The progeny of the selfed F_1 segregated approximately 3:1 of wild-type over mutant (Table 1). The results indicate that *hos2–1* is a recessive mutation in a nuclear gene. Complementation analysis showed that *hos2–1* is not allelic to *hos1–1*, a previously described *Arabidopsis* mutant (Ishitani *et al.*, 1998) with increased *RD29A-LUC* expression specifically in response to low temperature treatment (Table 1). The complementation tests also revealed two additional alleles of the *HOS2* locus, i.e. *hos2–2* and *hos2–3* (Table 1). Like the *hos2–1* mutation, *hos2–2* and *hos2–3* are recessive and result in increased *RD29A-LUC* expression specifically

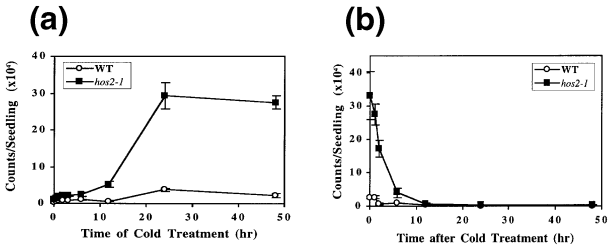

Figure 3. *RD29A-LUC* expression in *hos2–1* and the wild-type plants during cold acclimation and deacclimation.
(a) Increase in luminescence intensities with time during cold treatment. *hos2–1* and wild-type plants on the same agar plate were placed at 0°C for cold acclimation. At the specified timepoints, the plants were removed from 0°C, imaged and the luminescence intensities quantified.
(b) Decrease in luminescence intensities with time after cold treatment. After 24 h at 0°C, the plate was taken out and incubated at room temperature under light. Luminescence images were taken at the specified times and intensities were quantified. Data represent the average of 20 individual seedlings.

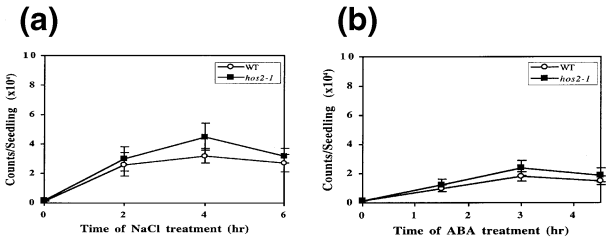

Figure 4. *RD29A-LUC* expression of *hos2–1* and the wild-type plants during NaCl (a) and ABA (b) treatment.
hos2–1 and wild-type plants on the same agar plate were treated with NaCl and ABA. ABA: 100 μM ABA; NaCl: 300 mM NaCl. Luminescence intensities were determined at the indicated times. Data represent the average of 20 individual seedlings.

under cold treatment (data not shown). Because the effect of *hos2–2* and *hos2–3* mutations on *RD29A-LUC* expression is not as strong as that of *hos2–1*, only backcrossed *hos2–1* plants were chosen for subsequent characterization.

RD29A-LUC *expression during cold acclimation and deacclimation*

To examine the kinetics of *RD29A-LUC* expression during cold acclimation and deacclimation, agar plates containing wild-type and mutant plants were treated at 0°C for 48 h and the plates were placed at room temperature. The expression of *RD29A-LUC* was examined at various timepoints during the treatment by luciferase imaging. As shown in Figure 3(a), *RD29A-LUC* expression in *hos2–1* plants was higher than in the wild-type plants throughout the cold treatment. *RD29A-LUC* expression in both wild-type and *hos2–1* plants reached peak levels after 24 h of cold treatment. The high levels of *RD29A-LUC* expression in the wild-type and *hos2–1* plants persisted for at least 7 days (data not shown). Figure 4(a,b) shows that there was no substantial difference in *RD29A-LUC* expression between wild-type and *hos2–1* plants at any timepoints

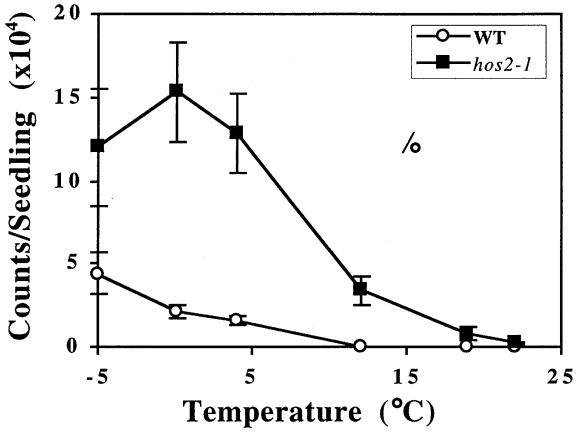

Figure 5. *RD29A-LUC* expression in *hos2–1* and wild-type plants under different temperature treatments.
hos2–1 and wild-type plants were planted on the same agar plates and allowed to grow for 1 week under constant lighting at room temperature (22 ± 2°C). The plates were then treated at indicated temperatures (± 0.1°C) for either 3 h (–5°C treatment) or 24 h (all other temperature treatments) and luminescence images were taken and the intensities quantified (see Experimental procedures for details).

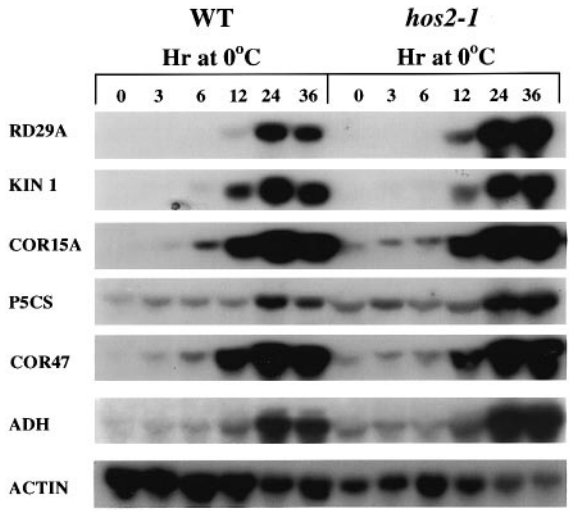

Figure 6. Time course of expression of endogenous *RD29A* gene and other stress-responsive genes in *hos2–1* and the wild-type plants in response to low temperature treatment.
Plants were treated with low temperature (0°C) for the indicated times.

when treated with either 300 mM NaCl or 100 μM ABA. The results further demonstrate that the defect in *hos2–1* is cold-specific.

During deacclimation, *RD29A-LUC* expression decreased rapidly in both wild-type and *hos2–1* plants (Figure 3b). Twenty-four h after the plants were taken from 0°C to room temperature, *RD29A-LUC* expression dropped to levels before the cold treatment (Figure 3b). These results indicate that the luciferase activity and its transcript are not more stable in *hos2–1* plants, at least at normal growth temperatures.

RD29A-LUC *expression in* hos2–1 *at different temperatures*

To investigate whether or not *hos2–1* plants express cold-responsive genes at normally non-inducing low temperatures, the mutant and wild-type seedlings on the same agar plates were transferred from room temperature (22°C) to 19°C, 10°C, 4°C, 0°C or –5°C and their *RD29A-LUC* expression determined by luminescence imaging. Wild-type plants showed the highest expression at –5°C (Figure 5). As the treatment temperature became higher, the expression decreased gradually and dropped to non-detectable levels at 19°C. Maximal *RD29A-LUC* expression in *hos2–1* plants occurred at 0°C. At –5°C, the expression was slightly lower. *RD29A-LUC* expression in *hos2–1* plants also decreased as the treatment temperature became higher than 0°C (Figure 5). *RD29A-LUC* expression dropped to non-detectable levels at 22°C. The results indicate that cold-responsive gene expression in *hos2–1* mutant plants is also dependent on a downshift in temperature, but that the expression is higher than in the wild-type plants at any low temperature treatment.

The hos2–1 *mutation affects many cold-responsive genes*

Figure 6 shows the time course of steady state mRNA levels for the endogenous *RD29A* gene in *hos2–1* mutant and wild-type plants during cold acclimation. Significant induction of *RD29A* transcript in both wild-type and the mutant was detected after 12 h or longer cold treatment. *hos2–1* mutant plants exhibited higher levels of induction throughout the cold treatment (Figure 6).

In addition to *RD29A*, the expression of several other cold-responsive genes were investigated in the *hos2–1* mutant plants. These genes include *COR47* (Gilmour *et al.*, 1992), *COR15A* (Lin and Thomashow, 1992), *KIN1* (Kurkela and Franck, 1990), *ADH* (Jarillo *et al.*, 1993) and *P5CS* (Delauney *et al.*, 1993). As a control, the expression of the actin gene was also determined. The expression levels of the cold responsive genes were quantified and normalized against the actin gene (Figure 7). The data show that all of the cold responsive genes had higher levels of expression in the *hos2–1* mutant when treated with cold. For all of the genes, greater differences between the mutant and wild-type were found with longer cold treatment. After 36 h of cold treatment, the differences ranged from 2.2- to 11-fold depending on the nature of the genes, with *COR15A* and *COR47* showing smaller differences.

The hos2–1 *mutant is more sensitive to freezing stress*

Because the *hos2–1* mutation affects low temperature regulation of genes, we investigated its effect on the

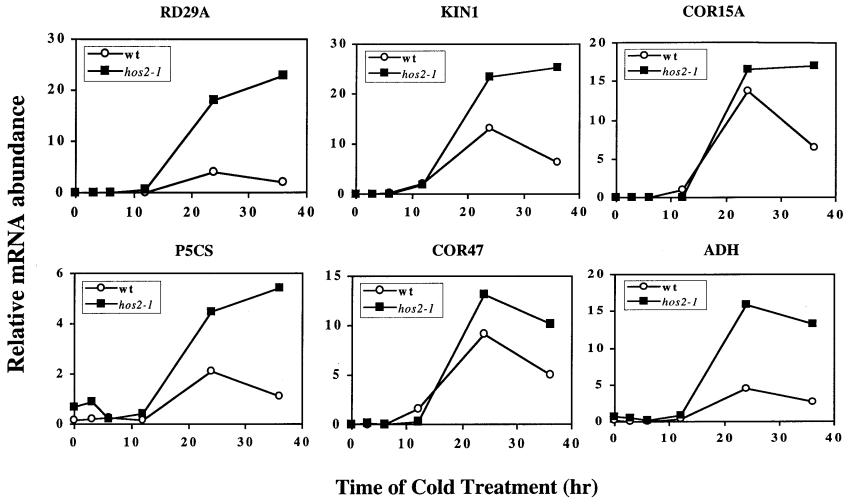

Figure 7. Quantitative representation of the data in Figure 6.
The radiograms were scanned and analyzed using ImageQuant software.

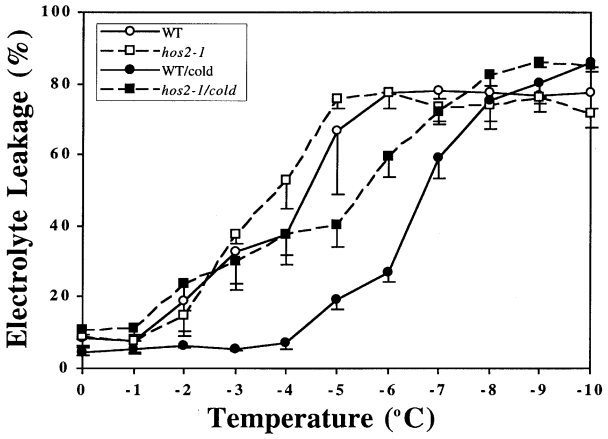

Figure 8. Leakage of electrolytes in leaves from *hos2–1* and wild-type plants when treated at temperatures below freezing.
For cold acclimation treatment, plants were incubated at 4°C for 1 week under white fluorescent light.

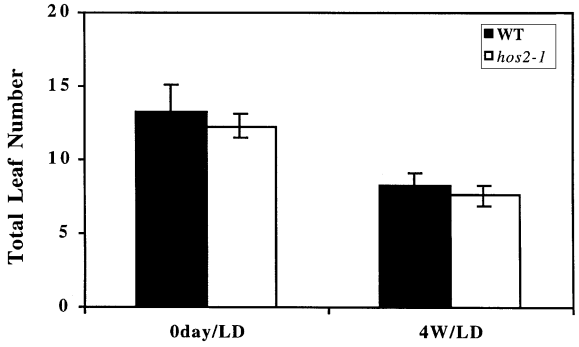

Figure 9. *hos2–1* plants are capable of vernalization.
For vernalization, seeds of *hos2–1* and the wild-type were sown in pot medium and placed in a cold room (4°C) for 4 weeks before moving to a growth chamber under either short day or long day conditions. At the appearance of the first flower bud, 10–20 plants were counted for total leaf numbers.

freezing tolerance of *Arabidopsis* plants using the electrolyte leakage test (Ristic and Ashworth, 1993; Sukumaran and Weiser, 1972). Without acclimation treatment, *hos2–1* plants were slightly more sensitive to freezing stress (Figure 8). The temperatures causing 50% of total ion leakage (LT_{50}) were approximately –3.7°C and –4.1°C, respectively, for *hos2–1* and wild-type plants. Since *Arabidopsis* is known to be fully acclimated after 7 days of cold treatment (Ristic and Ashworth, 1993), we tested the freezing tolerance of *hos2–1* and wild-type plants after 7 days of cold treatment. The cold treatment increased the freezing tolerance of both wild-type and *hos2–1* plants (Figure 8). However, the increase was much smaller for *hos2–1* mutant plants. The LT_{50} values were approximately –5.4°C and –6.8°C, respectively, for cold acclimated *hos2–1* and wild-type plants. Therefore, the difference in LT_{50} between *hos2–1* and wild-type plants increased to 1.4°C after acclimation. The results show that the *hos2–1* mutation impairs freezing tolerance and cold acclimation.

hos2–1 *mutant plants are capable of vernalizaion*

The time to flower in *Arabidopsis* can be influenced by long periods of low temperature treatment, a process known as vernalization. We examined the flowering time of wild-type and *hos2–1* mutant plants before and after 4 weeks of cold treatment in order to analyze their vernalization response. The flowering phenotype was presented as the total number of leaves (LN) at first flower anthesis (Chandler *et al.*, 1996). As shown in Figure 9, the LN value for *hos2–1* plants is nearly the same as that for the wild-

type. Four weeks of cold treatment substantially accelerated flowering for *hos2–1* as well as for the wild-type plants (Figure 9). After vernalization, the mutant plants flowered at about the same time as the wild-type did.

Discussion

In the last decade, the expression of many plant genes have been shown to be regulated by cold stress (Dhindsa and Monroy, 1994; Guy *et al.*, 1994; Hajela *et al.*, 1990; Kurkela *et al.*, 1990; Nordin *et al.*, 1993; Palva *et al.*, 1994; Thomashow, 1994). However, the signal transduction pathway leading to cold-responsive gene expression is poorly understood. In this paper, we present evidence which strongly suggests that *HOS2* is a negative regulator of cold signalling. Recessive mutations in the *HOS2* locus enhance gene induction specifically under low temperature. Our data indicate that the enhanced gene expression is due to increased transcription.

The majority of cold-inducible genes are also responsive to regulation by ABA and osmotic stress (Lang and Palva, 1992; Nordin *et al.*, 1993). This does not necessarily mean that ABA mediates the expression of these genes in the cold. First, the expression of *RD29A* and several other cold-responsive genes are not impaired in ABA-deficient mutants (Gilmour and Thomashow, 1991; Nordin *et al.*, 1993). Second, several groups have identified a sequence motif (termed DRE or C-repeat) in the promoters of cold-regulated genes and showed that the element responds to activation by cold or osmotic stress but not by ABA (Stockinger *et al.*, 1997; Yamaguchi-Shinozaki and Shinozaki, 1994). Therefore, an ABA-independent pathway shared by cold and osmotic stress is largely responsible for the cold regulation of these genes. The phenotype of the *hos2–1* mutant plants suggests that the mutations affect the cold-specific portion of this ABA-independent signalling pathway.

hos2–1 mutant plants exhibited reduced freezing tolerance. This was true for cold acclimated as well as non-acclimated *hos2–1* plants. In fact, cold-acclimation treatment augmented the difference in freezing tolerance between *hos2–1* and the wild-type plants. Ishitani *et al.* (1998) reported that *hos1–1* mutant plants also show enhanced gene expression specifically under cold treatment and that the mutation causes reduced freezing tolerance without cold acclimation. However, the *hos1–1* plants exhibit the same level of freezing tolerance as wild-type plants after cold acclimation treatment (Ishitani *et al.*, 1998). Therefore, it appears that in *hos1–1* plants, the enhanced gene induction by cold treatment correlates with increased capacity to cold acclimate. But in the *hos2–1* mutant plants, enhanced gene induction in the cold did not bring about an increase in freezing tolerance. Rather, the increase in freezing tolerance after a 7 day cold treatment was much less in *hos2–1* mutant plants than in the wild-type (Figure 8). The results suggest that the *hos2* and *hos1* mutations may cause enhanced gene induction in the cold through very different mechanisms.

Differences between the *hos2* and *hos1* mutations are also evident when their effects on flowering time are compared. The *hos1–1* mutation accelerates the onset of flowering (Ishitani *et al.*, 1998) whereas the *hos2* mutations have no substantial effect. Therefore, if cold acclimation and vernalization share some common signalling components, the *hos2* mutations may define a branch point between the two responses. The *HOS2* locus was mapped to chromosome V, approximately 20 cM from the *nga129* (our unpublished data).

Recently, Jaglo-Ottosen *et al.* (1998) and Liu *et al.* (1998) reported that transgenic overexpression of the CBF1/DREB1 family of transcription factors leads to constitutive expression of *RD29A* and other cold regulated genes in the absence of cold treatment. The transgenic plants show enhanced freezing tolerance. Therefore, increased cold-regulated gene expression appears to correlate with enhanced freezing tolerance. However, this correlation does not seem to hold true in the *hos2–1* mutant plants. The explanation for this apparent discrepancy is unclear at this point. One possibility is that the *HOS2* gene, besides being a negative regulator for cold-responsive gene expression, may play a positive role in the regulation of some other cellular changes (e.g. changes in membrane composition and sugar metabolism) that contribute to freezing tolerance.

Experimental procedures

Plant materials and growth conditions

Ecotype C24 of *Arabidopsis thaliana* was transformed with the chimeric gene *RD29A-LUC* and the seeds from the selfed progenies of the resulting transgenic plants containing homozygous transgene (referred as wild-type in this study) were mutagenized with ethylmethane sulfonate (Ishitani *et al.*, 1997). M_2 seeds were grown on 0.8% agar plates containing MS salt (Murashige and Skoog salt base, JRH Biosciences, Lenex, KS) under constant white fluorescent light at room temperature. As described by Ishitani *et al.* (1997), 1-week-old seedlings were screened for an altered luminescence image in response to low temperature, exogenous abscisic acid (ABA) or osmotic stress using a CCD camera video-imaging system. For genetic analysis, leaf number counting, or freezing tolerance assays, plants were grown in soil (Metro-Mix 350, Scott-Sierra Horticultural Products Co., Marysville, OH, USA) in growth chambers with 16 h light at 22°C, 8 h dark at 18°C and 70% relative humidity.

Cold acclimation, vernalization and other stress treatments

For mutant screening and primary characterization, low temperature treatment was conducted at 0°C for 48 h in the dark. For

detailed characterization of *RD29A-LUC* expression in response to low temperature treatment, agar plates with 1-week-old seedlings of *hos2–1* and the wild-type were placed in an incubator set at the designated temperature (± 0.1°C) for 48 h as stated in the text. At the completion of the treatment, plates were removed from the incubator and luminescence images were taken, as described in Ishitani *et al*. (1997). As longer treatment at –5°C will result in freezing of the agar plates, this treatment only lasted for 3 h and, after the treatment, the plate was placed at room temperature for 1 h to thaw before taking luminescence images. For ABA treatment, 100 μM ABA (mixed isomers) in water was sprayed on leaves of the seedlings and luminescence images taken 3 h after the treatment. NaCl treatment was conducted on 3 M filter paper saturated with MS salt solution that was supplemented with 300 mM NaCl and the luminescence images were taken 5 h after the treatment.

To determine the vernalization response, seeds of the mutant and wild-type plants were sown in pot media and the pots were kept at 4°C for 4 weeks. After vernalization, the pots were placed in a growth chamber until flowering. At the emergence of the first bud, the number of rosette and cauline leaves were counted together as total leaf number.

Freezing tolerance

For freezing tolerance assay, seeds of the mutant and wild-type plants were sown in pot medium and grown for 4 weeks. For cold acclimation treatment, plants at the rosette stage were placed in a cold room at 4°C under white fluorescent light for 1 week before sampling the leaves for freezing tolerance assay.

Fully developed rosette leaves were used to determine freezing-caused electrolyte leakage essentially as described by Sukumaran and Weiser (1972) and Ristic and Ashworth (1993). Briefly, for each treatment, one excised leaflet was placed in a test-tube containing 100 μl deionized H_2O and the tube was placed in a refrigerated circulator (freezing bath) (Model 1187, VWR Scientific) with the temperature set at 0°C. There were three replicates for each temperature treatment. The temperature of the bath was programmed to decrease to –10°C with 1°C decrement in 30 min. The tubes were removed from the bath when the designated temperature was reached and placed immediately on ice to allow gradual thawing for 18 h. The leaflet was then carefully transferred to another tube containing 10 ml deionized water and shaken overnight and the conductivity of the solution was measured. The tubes with the leaflets were then autoclaved for 10 min at 121°C and after cooling down to room temperature and shaking for 5 h, conductivities of the solutions were measured again. Percent electrolyte leakage was calculated as the percentage of the conductivity before autoclaving over that after autoclaving.

RNA analysis

Ten-day-old seedlings grown on MS agar plates were treated with either low temperature, NaCl or ABA. Respective treatment conditions were as stated in the text. Total RNA from control or stressed plants was extracted as described by Liu and Zhu (1997). The *P5CS* probe was a 1.6 kb cDNA fragment showing sequence identity to the *P5CS* gene reported in Yoshiba *et al*. (1995). Other probes and hybridization conditions are the same as described in Ishitani *et al*. (1998). To quantify the result of RNA gel-blot analysis, developed films were scanned and analyzed by ImageQuant software program. The results were represented as relative mRNA abundance by dividing each signal level with cognate actin level.

Acknowledgements

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