The Plant Journal (2004) **40**, 536–545 doi: 10.1111/j.1365-313X.2004.02225.x

A single amino acid substitution in the *Arabidopsis* FIERY1/HOS2 protein confers cold signaling specificity and lithium tolerance

Liming Xiong[1,2], Hojoung Lee[1], Rongfeng Huang[2] and Jian-Kang Zhu[1,*]
[1]*Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA, and*
[2]*Donald Danforth Plant Science Center, St Louis, MO 63132, USA*

Received 25 May 2004; revised 12 August 2004; accepted 20 August 2004.
[*]For correspondence (fax +951 827 7115; e-mail jian-kang.zhu@ucr.edu).

Summary

Low temperature induces the expression of many plant genes through undefined signaling pathways. To gain insight into cold signal transduction mechanisms, we isolated *Arabidopsis* mutants that exhibited altered regulation of low temperature-induced gene expression. One such mutant, *hos2*, was shown previously to have an enhanced induction of stress-responsive genes by cold, whereas the expression of these genes under osmotic stress or the phytohormone absciscic acid (ABA) treatments was not affected. Here we further define the targets of HOS2 by examining the regulation of upstream cold-specific *CBF* transcription factor genes. It was found that the transcript levels of *CBF2* and *CBF3* were significantly higher in *hos2* mutant plants than in the wild type under cold treatments, suggesting that HOS2 may act upstream of CBFs. The *HOS2* gene was cloned using a map-based strategy. Surprisingly, *HOS2* is identical to the *FIERY1* gene that we had described previously. FIERY1 is a general negative regulator that controls cold, osmotic stress, and ABA signal transduction and possesses inositol polyphosphate 1-phosphatase activity. The *hos2* mutation rendered the HOS2/FIERY1 recombinant protein completely inactive in the cold but did not substantially affect its activity at warm temperatures. Interestingly, the hos2 mutant protein is extremely tolerant to Li^+. This study provides a unique example of a single amino acid substitution in a critical regulator that can lead to conditional changes in protein functions and distinct plant phenotypes. The results reinforce the notion that phosphoinositols are important second messengers in cold signal transduction, and shed light on how the diversity of plant tolerance to cold and other abiotic stresses may evolve due to variations in a common molecular switch.

Keywords: abiotic stress, cold tolerance, inositiol polyphosphate 1-phosphatase, lithium tolerance, salt tolerance.

Introduction

Temperature as a key environmental factor has played important roles in the evolution and geographic distribution of life forms. Being sessile, plants have evolved sophisticated mechanisms to adapt to suboptimal temperatures (Sung *et al.*, 2003). Most plant species in the temperate region are able to increase their tolerance to freezing temperatures by a prior exposure to low non-freezing temperatures, a process referred to as cold acclimation. During cold acclimation, many plant genes that are not expressed at normal growth temperature regimes become activated (Thomashow, 1999). A group of these stress-responsive genes contain the C-repeat/dehydration responsive (CRT/DRE) *cis*-element in their promoter regions, which is the binding site for the CRT-binding factors (CBF)/DRE-binding factors (DREB). The AP2-domain transcription factors CBF1, CBF2, and CBF3 play important roles in the transactivation of cold-regulated genes whose products collectively increase plant tolerance to freezing stress (reviewed in Shinozaki *et al.*, 2003; Zhu, 2001).

Although there is now a good understanding of which genes are induced by low temperature stress and of the transcription factors that confer the induction of these genes, the upstream signaling events are still unclear. As

the expression of the CBF transcription factor genes is also induced by cold stress, there must exist upstream cold signaling circuits that culminate in the regulation of these transcription factor genes. Recently, a basic helix-loop-helix transcription factor, termed ICE1, was identified through genetic analysis. ICE1 activates *CBF* gene expression upon cold treatments, but its transcript level was not substantially regulated by low temperatures (Chinnusamy *et al.*, 2003). This suggests that its regulation occurs either post-translationally and/or through interaction with additional factors. Therefore, additional upstream regulators must exist to respond to cold signals. Genetic analysis of cold signal transduction has shown that the *HOS1* (high expression of osmotic stress-regulated gene expression 1) locus plays a negative role in regulating cold signaling (Ishitani *et al.*, 1998). *HOS1* encodes a protein that shows a limited homology to some RING finger proteins found in animals, which target certain signaling molecules for degradation (Lee *et al.*, 2001). HOS1 may have a similar function in degrading positive regulators upstream of CBFs (such as ICE1) as the expression of CBF transcription factor genes is higher in the *hos1* mutant (Lee *et al.*, 2001).

Early events in cold responses further upstream of ICE1 and HOS1 may involve Ca^{2+} signaling. Cold treatment results in a transient increase in cytosolic free Ca^{2+} concentrations and increasing cellular Ca^{2+} levels could activate low temperature-responsive gene expression at warm temperatures (Monroy and Dhindsa, 1995). Cold-induced Ca^{2+} transients may result either from external influx of Ca^{2+} or from release of internal stores (Knight *et al.*, 1996). In neurons, a TRP Ca^{2+}/cation channel was suggested to be a cold sensor (Peier *et al.*, 2002). However, similar TRP channel proteins have not been identified in plants based on sequence similarity. Furthermore, ligand-gated Ca^{2+} release from internal stores has been implicated in stress signaling in several biochemical and pharmacological studies (Sanders *et al.*, 2002). One major ligand with this property is inositol 1,4,5-trisphosphate (IP_3) (Sanders *et al.*, 2002); however, until recently genetic evidence to support the involvement of phospholipids in cold signaling has been lacking.

The *Arabidopsis* FIERY1 protein is an inositol polyphosphate 1-phosphatase that is conserved in higher eukaryotes (Xiong *et al.*, 2001). The FIERY1 homolog in humans regulates phosphoinositol turnover and is a potential target of lithium therapy for bipolar disorder (Williams and Harwood, 2000). In plants, FIERY1 may control cellular levels of IP_3 by regulating its degradation (Xiong *et al.*, 2001). IP_3, as a second messenger, is able to release Ca^{2+} from internal stores and activate the expression of certain stress-responsive genes (Burnette *et al.*, 2003; Sanchez and Chua, 2001). The *fiery1* mutant seedlings showed much enhanced induction of stress-responsive genes upon treatments with cold, drought, salt stress, or ABA. In addition, the *fiery1* mutant had higher and more sustained IP_3 levels upon treatment with ABA, which may explain its increased ABA-responsive gene expression (Xiong *et al.*, 2001).

We previously identified a genetic locus, *HOS2*, which appears to specifically control cold signal transduction in the activation of stress-responsive genes (Lee *et al.*, 1999). The *hos2* mutant plants showed an enhanced induction of stress-responsive genes specifically by cold, but the expression of these genes under osmotic stress or ABA treatment was not affected. In the present study, we further defined the target of the *HOS2* locus by examining the regulation of upstream CBF transcription factor genes. Furthermore, using a map-based strategy, we cloned the *HOS2* gene. Surprisingly, we found that *HOS2* is identical to the *FIERY1* gene. We mapped the enzymatic activities of the HOS2/FIERY1 proteins under different temperature regimes and found that the *hos2* mutation rendered the HOS2/FIERY1 protein completely inactive in the cold; however, the mutation did not substantially affect the enzymatic activity at warm temperatures. Interestingly, the hos2 mutant protein is also extremely tolerant to Li^{+} and showed reduced sensitivity to salt inhibition. Our results strongly support phosphoinositols as second messengers mediating cold signal transduction. This study provides a unique example of a single amino acid mutation that can lead to conditional changes in protein function and distinct phenotypes in an organism. In addition, the *hos2* mutant, with its conditional phenotypes, should serve as an excellent tool for further dissecting stress signal transduction mechanisms in higher plants.

Results

Enhanced cold-induction of the CBF/DREB1 transcription factor genes in hos2

The *hos2* mutant was isolated based on enhanced cold-induction of the *RD29A* promoter-driven luciferase reporter gene (*RD29A-LUC*) (Ishitani *et al.*, 1997; Lee *et al.*, 1999). *hos2* mutant plants showed enhanced expression (luminescence) of the *RD29A-LUC* transgene specifically at low temperatures and the expression was similar to that of the wild type under salt, drought, or ABA treatment (Lee *et al.*, 1999). Consistent with the luminescence phenotypes, the transcript levels of *DRE/CRT* stress-responsive genes were at higher levels in *hos2* than in wild type plants upon cold treatments; however, levels in the mutant and wild type were similar under salt or ABA treatment (Lee *et al.*, 1999). The data suggested that the HOS2 protein negatively regulates cold signal transduction.

As the *DRE/CRT* class of stress-responsive genes is regulated by the CBF family of transcription factors, we analyzed whether the *CBF* genes are regulated by HOS2. RNA blot analysis indicated that the transcript of the *CBF2* gene accumulated to significantly higher levels in *hos2* than

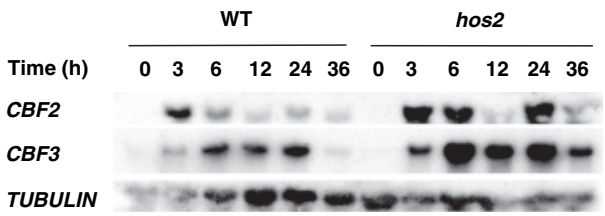


Figure 1. Regulation of the CBF transcription factor genes by HOS2 under cold treatments. Transcript levels for *CBF* genes after cold treatment (0°C) for different time periods are shown. A β tubulin gene was used as a loading control.

in the wild type upon cold treatment (Figure 1 and data not shown), although we failed to detect strong signals from *CBF1* under our experimental conditions. Time course analysis of the induction of *CBF2* transcript levels suggested that higher transcript levels of *CBF2* were seen at all the time points examined (Figure 1). The transcript levels of *CBF3* were also higher at these time points in *hos2* than in the wild type (Figure 1). These data suggest that HOS2 may function at a very early step of cold signal transduction pathways, upstream of the induction of *CBF* genes.

Chilling sensitivity of hos2 *mutant plants*

Because the *hos2* mutation affects the expression of cold-specific transcription factor genes (Figure 1) as well as their downstream target stress-responsive genes (Lee *et al.*, 1999), and because these gene products are known to control plant cold tolerance, the *hos2* mutation may affect chilling and freezing sensitivity of the plants. Measurement of freezing-induced ion leakage indeed suggested that *hos2* mutant plants were more sensitive to freezing stress and less capable of cold acclimation (Lee *et al.*, 1999). However, it is not known whether the *hos2* mutation also affects chilling sensitivity.

To test the chilling sensitivity of *hos2* mutant plants, we grew wild type and *hos2* plants in the soil under normal temperatures and in the cold to compare their growth responses. We found that under normal growth conditions (22 ± 2°C), there is no clear phenotypic difference between mutant and wild type plants (Figure 2a). However, when the plants were placed in a cold room (4°C) under white light for an extended period of time (e.g. more than 3 months), wild type plants were able to grow and finally set seeds. In contrast, *hos2* mutant plants were stunted and unable to set seeds (Figure 2b and data not shown); when moved to room temperatures, however, these mutant plants were able to complete their life cycle and set limited amounts of seeds (data not shown).

Map-based cloning of HOS2

The molecular and physiological phenotypes of *hos2* mutant plants clearly suggest an important role for HOS2 in cold signaling and in chilling and freezing tolerance. To clone the *HOS2* gene, a map-based strategy was taken. The *hos2* mutant in the C24 background was crossed to wild type *Arabidopsis* of the Columbia ecotype. The F_2 population resulting from selfed F_1 was screened by luminescence imaging for seedlings with the *hos2* mutant phenotype in response to cold treatment. Genomic DNA from these samples was used

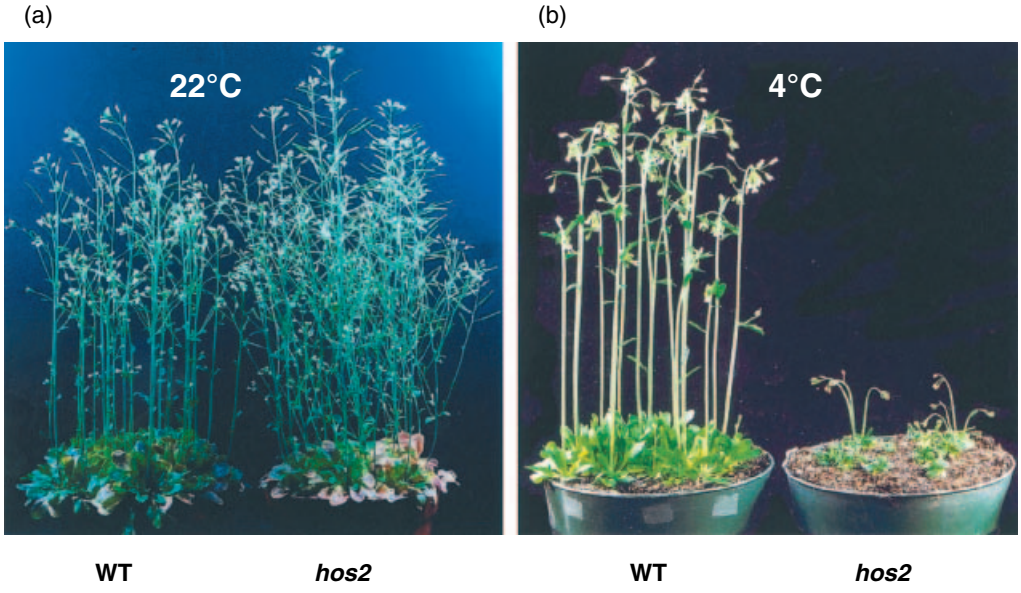


Figure 2. Chilling sensitivity of *hos2* mutant plants. Wild type and *hos2* mutant plants were grown either at normal growth condition (22 ± 2°C) for 2 months (a) or in the cold (4 ± 2°C) for 4 months (b) before taking the pictures.

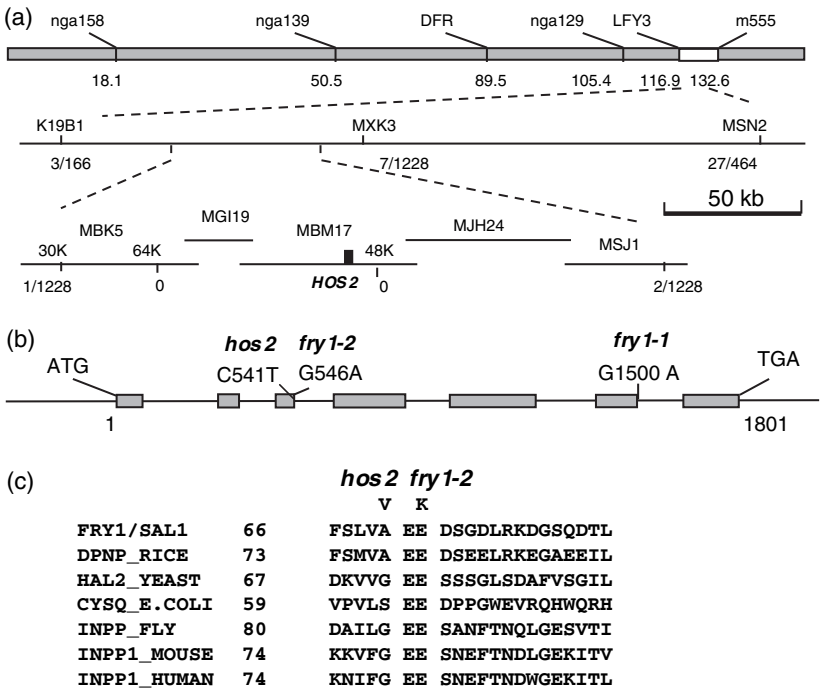


Figure 3. Positional cloning of the *HOS2* locus.
(a) The *HOS2* locus was mapped to the lower arm of chromosome V. Fine mapping delimited the locus to the interval between the markers MBK5-30K and MSJ1, the same region where *FIERY1* is localized.
(b) Candidate gene sequencing identified a mutation in the *FIERY1* gene in the *hos2* mutant. Also shown are the *fry1-1* and *fry1-2* mutations (Xiong *et al.*, 2001). Numbers indicate nucleotide position relative to the translation start site.
(c) Alignment of HOS2/FIERY1 homologs around the EE motif (York *et al.*, 1995) and the nature of the hos2 and fry1-2 mutations. Sequence accession numbers are as follows: FIERY1, At5g63980/AY034894; DPNP_rice, Q40639; MET22/HAL2_yeast, P32179; CYSQ_*E. coli*, P22255; INPP_fly, AAC24856; INPP_mouse, P49442; and INPP1_human, NP_002185.

as templates for PCR-based mapping. Initial mapping indicated that the *HOS2* locus is localized on the low arm of chromosome V. Further mapping using simple sequence length polymorphism (SSLP) markers delimited the *HOS2* locus to a region between the SSLP markers MBK5-64K and MJH24-48K. This interval was covered by three BAC clones: MGI19, MBM17, and MJH24 (Figure 3a).

Intriguingly, this is in the same chromosomal region where the *FIERY1* (*FRY1*) locus resides (Xiong *et al.*, 2001). *fiery1* mutant plants had luminescence phenotypes quite different from *hos2*: *fiery1* showed enhanced luminescence in response to cold, salt, drought, and ABA (Xiong *et al.*, 2001), whereas *hos2* showed enhanced luminescence expression specifically under cold treatment (Lee *et al.*, 1999). Despite this clear difference in mutant phenotypes, the close link between *hos2* and *fiery1* map positions prompted us to speculate that the two genes might be identical. We therefore decided to sequence the *FIERY1* gene in the *hos2* mutant plants. DNA sequence analysis indeed indicated that there is a single nucleotide change in the *FIERY1* gene in the *hos2* mutant plants. This C to T mutation occurred at the third exon and was at the position 541 from the translation initiation start site (Figure 3b). The mutation would result in the change of an alanine residue (GCT) to valine (GTT) at position 70 from the first methionine (Figure 3c). This *hos2* mutation occurred very close to the *fry1*-2 mutation (Figure 3c): the two mutations are actually only one residue apart.

To further confirm that the *HOS2* locus is allelic to *FIERY1*, the genomic DNA of *FIERY1* was cloned into a binary vector and transferred into *hos2* mutant plants via *Agrobacterium*-mediated flower-dip transformation. Analysis of the T_1 generation indicated that all the lines analyzed showed a wild type luminescence phenotype under cold treatment. T_1 seedlings were allowed to self-pollinate, and the T_2 generation was obtained. Analysis of the T_2 generation found that, using the criterion of hygromycin resistance, most of the T_2 plants had one insertion of the transgene. Furthermore, all the hygromycin-resistant seedlings exhibited a wild type luminescence phenotype after cold treatment (data not shown). Luminescence expression assays with seedlings growing on normal Murashige and Skoog (MS) agar plates without hygromycin (Figure 4a) indicated that in these T_2 lines the population segregated approximately at 3:1 wild type:mutant ratio (Figure 4b). Quantitation of the luminescence intensity of seedlings with a wild type luminescence phenotype indicated that the wild type *FIERY1* gene completely restored the gene expression phenotype of *hos2* plants to that of the wild type (Figure 4c). These complementation data proved that the *HOS2* locus is indeed identical to *FIERY1*.

hos2 mutant protein loses activity specifically in the cold

FIERY1 encodes a bifunctional enzyme with both nucleotidase activities and inositol polyphosphatase activities (Xiong *et al.*, 2001). With the *fiery1* mutant, we further showed that the mutation affects the catabolism of IP_3. In the *fiery1* mutant, IP_3 levels were higher and more sustained relative to the wild type plants upon treatment with

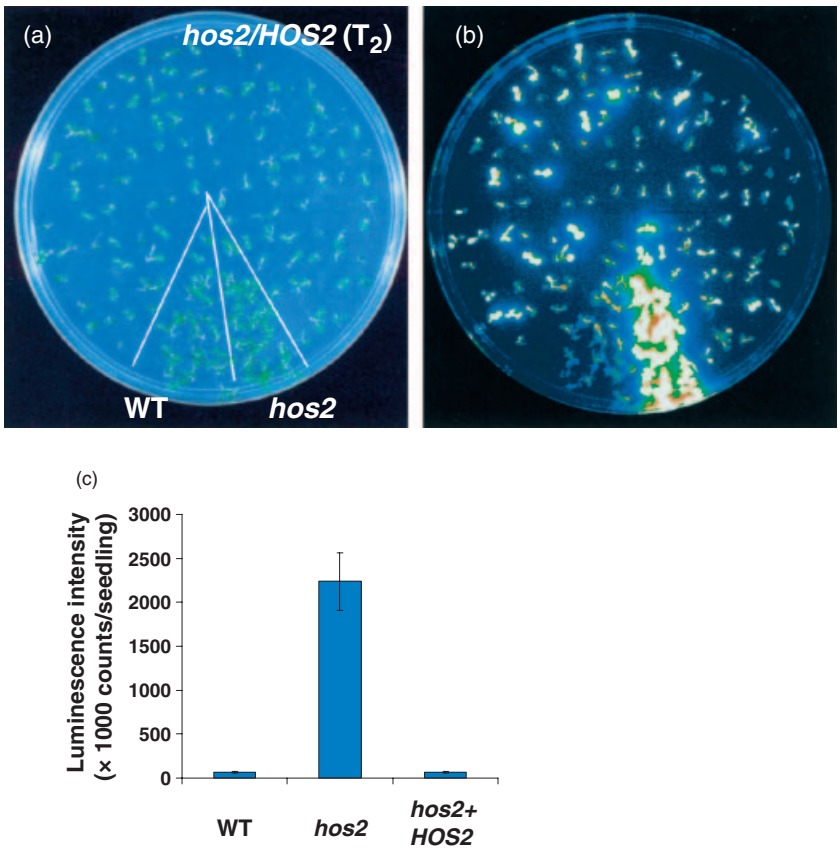


Figure 4. Complementation of the *hos2* mutant by the wild type *HOS2/FIERY1* gene. Genomic DNA of *HOS2/FIERY1* containing 1.3 kb promoter sequence was amplified from wild type Columbia plants and cloned into a binary vector and transferred into *hos2* mutant plants. Seedlings of the wild type (WT), *hos2*, and the T_2 progeny of *hos2* transformed with wild type *HOS2* gene (*hos2* + *HOS2*) were assayed for luciferase expression.
(a) The seedlings on an MS agar plate.
(b) Luminescence image of the plate in (a) after being treated at 0°C for 2 days.
(c) Quantitation of the luminescence intensity in (b). Data are mean and standard error ($n = 15$).

ABA. This sustained IP_3 level may contribute to an enhanced expression of stress-responsive genes under cold, drought, or salt stress. However, higher expression of stress-responsive genes in *hos2* was only found under cold treatment (Lee *et al.*, 1999). We thus hypothesized that the *hos2* mutation may affect the activity of the enzyme specifically in the cold.

To test whether this *hos2* mutation has an impact on the enzymatic activity of the hos2 mutant protein, we obtained the recombinant protein by expressing the HOS2-GST translation fusion construct in *Escherichia coli* and assayed its activities under a variety of conditions. Measurement of the recombinant protein activities against 3′-phosphoadenosine 5′-phosphate (PAP) suggests that both wild type and hos2 mutant proteins had similar activities on PAP at 30°C. However, although the wild type protein's activity decreased from levels at 30°C, its activity at 0°C was clear and measurable after a prolonged incubation. In contrast, the hos2 mutant protein lost its activity completely under the same assay condition at 0°C (Figure 5). These results strongly suggest that *hos2* is a temperature-sensitive mutation that causes the protein to lose its activity specifically under cold conditions.

To further reveal the impact of the hos2 mutation on HOS2/FIERY1 enzyme activity, we mapped the temperature response of the hos2 protein. The hos2 mutant protein and the wild type protein were both incubated at a series of temperatures, and their activities were assayed. At temperatures higher than 20°C, the activity of the hos2 mutant protein was not substantially different from that of the wild type protein. However, when the temperatures were below 20°C, hos2 activities started to fall noticeably. Although no critical temperature that gave rise to significant drops in the activity were detected, there were more significant decreases in the activities when temperatures fell below 10°C. When the temperature reached 6°C, the protein completely lost its activity (Figure 5a). Consistent with the loss of hos2 mutant protein activity at temperatures below 6°C, we have found that the *hos2* mutant plants stopped growing and stunted at around 7°C; those grown at 4°C behaved similarly (Figure 2b and data not shown).

Mechanisms for the cold sensitivity of the hos2 mutant protein

The loss of activity of the hos2 mutant protein at low temperatures might result from either a mild change in the structure of the protein that renders the protein vulnerable to cold denaturation, or a change in the free energy status that may affect its proper folding under low

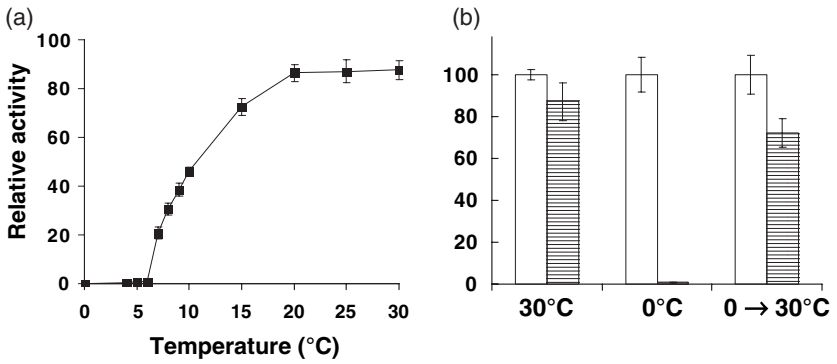


Figure 5. Loss of enzymatic activity of the hos2 mutant protein in the cold.
(a) Temperature dependence of the hos2 mutant protein activity. Wild type HOS2/FIERY1 and hos2 mutant proteins were incubated at the respective temperatures with 3′-phosphoadenosine 5′-phosphate (PAP) for the designated time, and the activities of the protein were assayed by measuring phosphate release.
(b) Wild type HOS2 and mutant hos2 recombinant proteins were incubated with PAP either at 30°C for 30 min or 0°C (on ice) overnight, or incubated at 30°C for 5 min after 0°C overnight (0 → 30°C); released phosphate was measured. White bars, wild type; filled bars, hos2. In both (a) and (b), the activity of the hos2 mutant protein was expressed relative to that of the wild type protein (which was designated as 100 at each temperature). Data are mean and standard errors ($n = 4$).

temperatures. To determine whether the loss of activity is due to cold denaturation, we first incubated the protein in the cold (0°C overnight) and then assayed the activity at 30°C immediately following cold incubation. The activity of the hos2 mutant protein was restored to near the level of non-cold-incubated sample (Figure 5b). This suggests that the hos2 mutation did not render the protein prone to cold denaturation. It is therefore likely that mutation affects the structural microenvironment around the lesion site.

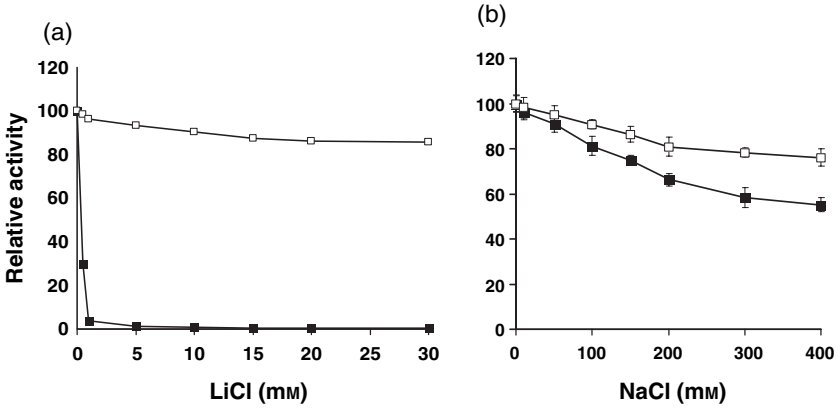
The hos2 mutant protein is highly resistant to lithium inhibition and is more tolerant to salt

FIERY1 belongs to a family of proteins that are conserved among bacteria, yeast, plants, and animals. This group of proteins is sensitive to Li^+. In humans, the FIERY1 homolog, inositol polyphosphate phosphatase 1 (INPP1), was considered a target of Li^+ therapeutic treatment for bipolar disease (Williams and Harwood, 2000). The FIERY1 protein, which is identical to the SAL1 protein that was isolated by its ability to confer salt tolerance in yeast cells, is also very sensitive to Li^+ inhibition (Gil-Mascarell *et al.*, 1999; Quintero *et al.*, 1996). To test whether the hos2 mutation has any effect on Li^+ and Na^+ tolerance, we assayed its sensitivity to both cations.

Wild type HOS2/FIERY1 protein was very sensitive to Li^+. In fact, 5 mM Li^+ completely inhibited its activity. The IC_{50} value for Li^+ (the concentration at which 50% of activity is inhibited) was 0.3 mM. This sensitivity is similar to previous reports on FIERY1 and its homologs in other organisms (Gil-Mascarell *et al.*, 1999; Lopez-Coronado *et al.*, 1999; Spiegelberg *et al.*, 1999). Under the same experimental conditions, surprisingly, the hos2 mutant protein was virtually insensitive to Li^+. At 10 mM Li^+, where the wild type protein completely lost its activity, the hos2 mutant protein retained over 90% of its activity. At 30 mM Li^+, hos2 mutant protein activity still had 86% of that of the control with 0 mM Li^+ (Figure 6a).

The sensitivity of the hos2 mutant protein to salt was also assayed. The hos2 mutant protein was more resistant to salt

Figure 6. Sensitivity of the HOS2/FIERY1 wild type and hos2 mutant protein to Na^+ and Li^+ inhibition. The wild type and hos2 mutant protein activities were assayed at 30°C in the presence of indicated concentrations of (a) Li^+ or (b) Na^+. Black symbols, wild type; white symbols, hos2. Data are mean and standard errors ($n = 4$).



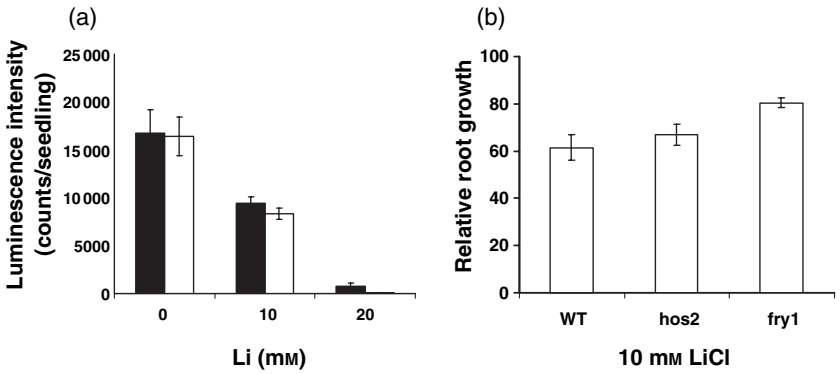


Figure 7. Luminescence expression and root growth of *hos2, fiery1-1,* and wild type seedlings in the presence of Li^+.
(a) Luminescence intensity of seedlings treated with 300 mM NaCl for 3 h after incubation with Li^+ at the indicated concentrations for 10 h. Data are mean and standard errors from 20 seedlings. Black bars, wild type; white bars, *hos2*.
(b) Relative root growth on MS medium supplemented with 10 mM Li^+ (those on MS with 0 mM Li^+ were 100). Seedlings were first grown on regular MS medium for 5 days and then transferred to the Li^+-containing media. Root length was measured 10 days after the transfer. Data are mean and standard errors ($n = 15$).

than the wild type protein, although the magnitude of the increase in resistance was not as dramatic as that for Li^+ (Figure 6b).

Is HOS2/FIERY1 the primary target of Li^+ or Na^+ toxicity in plant cells?

The dramatic increase in Li^+ and Na^+ tolerance of the hos2 mutant protein prompted us to determine whether the hos2 mutant plants have altered sensitivity to Li^+ or Na^+. To address this question, two sets of experiments were conducted. First, we studied the gene expression in *hos2* and wild type plants treated with Li^+. If HOS2/FIERY1 is the primary target of Li^+ toxicity, then treatment with Li^+ would inhibit the FIERY1 activity and would confer a phenotype in wild type plants similar to that of the *fiery1* mutant. However, the *hos2* mutant, due to its insensitivity to Li^+, would still have gene expression phenotypes similar to the wild type.

To test this possibility, we incubated wild type and *hos2* seedlings for 3 h on filter papers saturated with LiCl at concentrations of 1, 10, 20, and 30 mM. After incubation, the seedlings were sprayed with 100 μM ABA or incubated further with 300 mM NaCl for two more hours to activate *RD29A-LUC*. After the treatments, seedlings were imaged for luminescence expression from the *RD29A-LUC* gene. Li^+ treatment did not significantly enhance the expression of *RD29-LUC* (data not shown). Because it takes time for the Li^+ ions to enter the plant cells, we treated the plants with Li^+ at the above concentrations for 10 h; the seedlings were then treated with NaCl for two more hours. It appears that the Li^+ treatments did not kill the plants as, after the Li^+ solution was rinsed away, most of the plants were able to recover. Although the control treatment without Li^+ (water only) showed luminescence expression, Li^+-treated plants either did not emit luminescence or the luminescence intensities were very low (Figure 7a). This suggests that HOS2/FIERY1 is not the primary target *in planta*; rather, a positive signaling molecule(s) may be the target(s) of Li^+.

The sensitivity of the *hos2* mutant to Li^+ and Na^+ was also measured *in planta* by assaying for their root growth on agar Petri dishes supplemented with different concentrations of Li^+ or Na^+. For comparison, the *fiery1* mutant was also included. The growth of *fiery1* seedling was more sensitive to salt (Na^+) stress than that of the wild type plants (Xiong *et al.*, 2001), whereas that of the *hos2* mutants was not (data not shown). Yet, no significant difference was found in root elongation between the *fiery1* or *hos2* mutants and wild type (data not shown). *hos2* plants were not substantially different from the wild type in their sensitivity to Li^+. On the contrary, the *fiery1–1* mutant seedlings were slightly more tolerant to Li^+ at 10 mM Li^+ (Figure 7b). However, at or above 20 mM of Li^+, all seedlings were stunted and were eventually killed by the treatment (not shown).

Discussion

Plant responses to cold share some common mechanisms with salt and drought responses. Some stress-responsive genes are activated by all these stresses (Bray, 2002; Shinozaki *et al.*, 2003; Xiong *et al.*, 2002). Nevertheless, cold signaling also has its specificity. Cold signaling activates specific transcription factors for the regulation of stress-responsive genes (Shinozaki *et al.*, 2003). In addition, mutants that are affected in cold but not osmotic stress signaling have been identified. These include the *Arabidopsis hos1* (Ishitani *et al.*, 1998) and *hos2* mutants (Lee *et al.*, 1999). *HOS1* encodes a protein with a variant RING finger domain, which may participate in the ubiquitination of signaling proteins (Lee *et al.*, 2001). The target proteins of HOS1 could include positive signaling components

upstream of CBF transcription factors as the expression of *CBF* genes in *hos1* is higher than in the wild type (Lee *et al.*, 2001). Like *hos1*, the *hos2* mutation specifically affects low temperature signal transduction but does not alter drought, salt, or ABA signaling. The *hos2* mutation also reduces the capability of the mutant plants to cold-acclimate (Lee *et al.*, 1999) and results in increased sensitivity to chilling temperatures (Figure 2). It is unclear why *hos1* and *hos2* (and *fiery1*) mutants are more sensitive to cold despite their enhanced expression of stress-responsive genes under cold treatments. Perhaps, these signaling components have additional cellular targets that also contribute to stress tolerance. Alternatively, enhanced expression of stress-responsive genes may represent a consequence of increased stress damages (Xiong and Zhu, 2002), although these gene products are able to enhance stress tolerance in the wild type background where stress signaling is not compromised. Our data suggest that both HOS1 and HOS2 proteins play important roles in cold signaling and plant chilling and freezing tolerance.

In the present study, we cloned the *HOS2* gene. Unexpectedly, the *HOS2* locus was found to be allelic to *FIERY1*, which encodes a bifunctional enzyme with both 3′(2′),5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities (Quintero *et al.*, 1996; Xiong *et al.*, 2001). Loss of the inositol polyphosphate 1-phosphatase activity in *fiery1* mutants might be responsible for the mutant phenotypes. Measurement of the IP_3 levels in plants upon treatment with ABA also showed that *fiery1* mutant plants had higher and more sustained IP_3 levels than wild type plants. The data suggest that the FIERY1 protein negatively regulates stress and ABA signal transduction by mediating the catabolism of IP_3 (Xiong *et al.*, 2001). It thus appears unusual that the *hos2* mutation only affects cold signaling and not drought, salt, or ABA signaling. An assay of the recombinant protein activity showed that the *hos2* mutation causes the mutant protein to lose its activity completely in the cold, whereas it retains most of the activity at warm temperatures. This amount of activity must be sufficient for the protein to perform its function as there is no physiological or gene expression phenotypes associated with the *hos2* mutation under warm temperature conditions (Figure 2a).

It is remarkable that the *hos2* mutation, which is adjacent to the *fiery1-2* mutation (Figure 3c), should confer altered cold signaling and cold sensitivity specifically. The amino acids valine and alanine are not very dissimilar in biochemical properties, yet the change confers such dramatic conditional phenotypes. This suggests that the structure around the mutation site is critical for the catalytic activity of the protein. Structural analysis indeed suggested that although this EE consensus site in the β1 sheet (York *et al.*, 1995) does not directly bind metals, it is required for the coordination of metal ions for the catalytic activity of the enzyme (Albert *et al.*, 2000). Such a mutation might cause a loss of catalytic activity as a result of the structural alteration at low temperatures.

Although many temperature-sensitive mutant alleles have been reported in bacteria and yeast (Zeidler *et al.*, 2004), such mutants are not commonly found in higher eukaryotes such as plants. One mechanism for temperature sensitivity in these lower organisms is low temperature-induced protein denaturation (Privalov and Gill, 1988). As the hos2 mutant protein is not more prone to direct cold denaturation (Figure 5b), an alternative mechanism must be responsible for its cold sensitivity. At normal temperatures, entropy from hydrophobic residues greatly facilitates and drives protein folding. However, hydrophobic interactions become significantly weaker at lower temperature (Baldwin, 1986; Privalov and Gill, 1988). This diminished entropy at low temperatures may affect proper folding of the protein. The availability of the crystal structure of the yeast HAL2 protein (Albert *et al.*, 2000) makes it possible to infer the significance of this single residue change (A70V). By analogy to the structure of HAL2, the V69 in HOS2 is required to make hydrophobic interaction with tryptophan 287 (W287) in the WD-X_{11}-GG motif. Perhaps the A70V mutation, which is in the immediate vicinity of V69, may interfere with the formation of this hydrophobic interaction, and thus destabilize the folding of the hos2 mutant protein in the cold. Increasing temperatures can restore the proper folding of the protein and thus recover its activity (Figure 5b).

It is interesting that the hos2 mutant protein is extremely tolerant to Li^+ inhibition, compared with the wild type protein (Figure 6a). Previous studies have demonstrated that Li^+ is a non-competitive inhibitor of this group of phosphatases. By analogy to the structure of the yeast HAL2 protein, Li^+ is coordinated by Asp288 (D288) and D134 – a cavity held by W287. Although the hos2 mutation does not directly affect the metal binding site, mutation may directly affect metal coordination by modifying the interaction between V69 and W287 (see above). Interestingly, one of the two yeast *hal2* mutants that are Li^+-tolerant has a V70A mutation (the position equivalent to V69 in the HOS2 protein), which may be similar to the hos2 mutation A70V. Therefore, the mechanisms of increased Li^+ tolerance are likely the same for both mutants. This mutation may indirectly modify the metal binding sites of the Asp cavity and make it much less accessible to Li^+. Thus, the mutant protein becomes much less sensitive to Li^+.

Although the HOS2/FIERY1 protein is very sensitive to Li^+ inhibition, unlike in yeast, however, this enzyme may not be the primary target of Li^+ toxicity in plants for the following two reasons. First, the *fiery1–1* null mutant seedlings were slightly more tolerant to Li^+ at 10 mM (Figure 7b). Thus, inhibition of the FIERY1 activity does not appear to be the cause of Li^+ toxicity in plants. Secondly, the *hos2* mutant plants are not more resistant to Li^+, although the hos2 mutant protein is very tolerant to

Li^+ and Na^+ inhibition (Figure 6). Alternatively, Li^+ under our treatment conditions may not be able to gain access to the HOS2/FIERY1 protein *in planta*, or there are *in vivo* components that could increase the tolerance of the HOS2/FIERY1 protein to Li^+. This is suggested by the observation that Li^+ at concentrations that completely inhibited HOS2/FIERY1 activity *in vitro* failed to confer *hos2/fiery1* mutant luminescence phenotypes in the wild type plants (data not shown). In addition to HOS2/FIERY1, there may be other targets that are more sensitive to Li^+ inhibition. Of the six FIERY1 family members, three have been examined for their Li^+ sensitivity. Among these three proteins, FIERY1/SAL1 is the most sensitive, with an IC_{50} equal to 0.1 mM (Gil-Mascarell *et al.*, 1999), which is similar to the IC_{50} value obtained in the current study. Other likely targets of Li^+ toxicity include inositol monophosphatases and GSK3-like kinases. A GSK3-like kinase, BIN2, is involved in brassinosteriod signal transduction (He *et al.*, 2002; Li and Nam, 2002), but its sensitivity to Li^+ has not been reported. Currently, it is not clear which is the primary target of Li^+ toxicity. Likewise, the fact that *hos2* mutant plants are not more tolerant to Na^+, although the hos2 protein is, indicates that HOS2/FIERY1 may not be the primary target of salt inhibition in plants.

The identification of *hos2* as a conditional allele of *FIERY1* provides an excellent example in plants of variations in a single, key molecular switch that may result in distinct plant phenotypes and differential adaptation to environmental conditions. Because HOS2/FIERY1 is a critical component in stress and hormonal signal transduction and controls various developmental programs, the availability of the *hos2* conditional allele will greatly facilitate understanding of the role of this important group of proteins in development and responses to the environment. For example, a distinct advantage of the conditional *hos2* allele compared with our previously reported *fiery1* null alleles is that the *in planta* function of the FIERY1/HOS2 protein can be examined at all developmental stages without the complication of constitutive changes, by exposing the *hos2* mutant plants to cold temperatures.

Experimental procedures

Plant materials, mutant screen, and stress treatments

Seeds from *Arabidopsis* plants (ecotype C24) expressing the *RD29A-LUC* transgene were mutagenized with ethyl methanesulfonate, and mutants with changed luminescence expression under cold, drought, salt, or ABA treatment were isolated as described (Ishitani *et al.*, 1997). For stress treatments, plants growing in MS medium in agar Petri dish were either treated for a designated time in the cold (0°C), sprayed with ABA, or transferred to filter paper saturated with NaCl solution. After the stress treatment, seedlings were either sprayed with luciferin for luminescence imaging or used for RNA preparations (Ishitani *et al.*, 1997). Specific treatment conditions are stated in the text.

For the chilling sensitivity assay, wild type, *hos2*, and *fiery1-1* mutant plants at the rosette stage growing in potted soil were transferred to a cold room (4°C ± 2°C) and incubated under light. Pictures were taken at different times after incubation in the cold was initiated.

RNA analysis

Seedlings growing in agar Petri dish were either not treated or treated with cold, ABA, or NaCl at the indicated dosage and for the time indicated in the text. RNA extraction and blot analysis were conducted as described (Ishitani *et al.*, 1998). Probes for Northern analysis were as described (Lee *et al.*, 2001).

Map-based cloning

The *hos2* mutant was crossed with the *Arabidopsis thaliana* Columbia ecotype, and the F_1 plants were allowed to self-pollinate. The resulting F_2 progeny were treated with cold (0°C, for 24 h), and seedlings with the *hos2* mutant phenotype were isolated and transferred to the soil. Genomic DNA from these seedlings was extracted and used as the template for PCR-based mapping using SSLP markers. SSLP markers were developed by surveying the genomic sequence with potential simple sequence repeats, as described (Xiong *et al.*, 2001). These markers were evaluated for polymorphism between C24 and Columbia ecotypes, and those that generated size differences were used for mapping. Candidate genes in the mapping interval were amplified from *hos2* mutant and wild type plants and were sequenced and compared.

For the complementation assay, the *FIERY1/HOS2* genomic DNA including 1.3 kb region upstream of the initiation codon was obtained by PCR and cloned into the binary vector pCAMBIA1200. The plasmid was then introduced into *hos2* mutant plants by floral dipping as described (Xiong *et al.*, 2001). The *hos2* cDNA was obtained by reverse transcriptase-PCR and was confirmed by direct sequencing as described (Xiong *et al.*, 2001).

HOS2 *enzyme activity assay*

The *HOS2/FIERY1* cDNA was cloned into pGEX-2TK bacterial expression vector. The recombinant protein was purified using a GST column as described (Xiong *et al.*, 2001). The HOS2/FIERY1 wild type, hos2 mutant, and *fiery1-2* mutant protein activities against PAP (Cat. A-5763, Sigma, St Louis, MO, USA) were assayed using 0.2 μg protein either at 30°C (incubation time 30 min) or at 0°C (incubation overnight) as described (Murguia *et al.*, 1995; Xiong *et al.*, 2001). The activities of the hos2 mutant protein relative to the wild type protein were calculated.

Acknowledgements

We thank Dr Tom Smith (DDPSC) for his helpful suggestion on cold denaturation assay and Janet Oriatti (DDPSC) for helping prepare the manuscript. This study was funded by National Science Foundation grants IBN-0212346 and MCB-0241450 (J.-K. Z), United States Department of Agriculture National Research Initiative grant 2003–00751 (J.-K. Z), and by faculty startup funds from the Donald Danforth Plant Science Center (L. X).

References

Albert, A., Yenush, L., Gil-Mascarell, M.R., Rodriguez, P.L., Patel, S., Martinez-Ripoll, M., Blundell, T.L. and Serrano, R. (2000) X-ray structure of yeast Hal2p, a major target of lithium and sodium toxicity, and identification of framework interactions determining cation sensitivity. *J. Mol. Biol.* **295**, 927–938.

Baldwin, R.L. (1986) Temperature dependence of the hydrophobic interaction in protein folding. *Proc. Natl Acad. Sci. USA*, **83**, 8069–8072.

Bray, E.A. (2002) Abscisic acid regulation of gene expression during water-deficit stress in the era of the *Arabidopsis* genome. *Plant Cell Environ.* **25**, 153–161.

Burnette, R.N., Gunesekera, B.M. and Gillaspy, G.E. (2003) An *Arabidopsis* inositol 5-phosphatase gain-of-function alters abscisic acid signaling. *Plant Physiol.* **32**, 1011–1019.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.H., Hong, X., Agarwal, M. and Zhu, J.K. (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.* **17**, 1043–1054.

Gil-Mascarell, R., Lopez-Coronado, J.M., Belles, J.M., Serrano, R. and Rodriguez, P.L. (1999) The *Arabidopsis HAL2*-like gene family includes a novel sodium-sensitive phosphatase. *Plant J.* **7**, 373–383.

He, J.X., Gendron, J.M., Yang, Y., Li, J. and Wang, Z.Y. (2002) The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **99**, 10185–10190.

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

Ishitani, M., Xiong, L., Lee, H., Stevenson, B. and Zhu, J.-K. (1998) *HOS1*, a genetic locus involved in cold-responsive gene expression in *Arabidopsis*. *Plant Cell*, **10**, 1151–1161.

Knight, H., Trewavas, A.J. and Knight, M.R. (1996) Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. *Plant Cell*, **8**, 489–503.

Lee, H., Xiong, L., Ishitani, M. and Zhu, J.-K. (1999) Cold-regulated gene expression and freezing tolerance in an *Arabidopsis thaliana* mutant. *Plant J.* **17**, 301–308.

Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B. and Zhu, J.-K. (2001) The *Arabidopsis HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. *Genes Dev.* **15**, 912–924.

Li, J. and Nam, K.H. (2002) Regulation of brassinosteroid signaling by a GSK3/SHAGGY-like kinase. *Science*, **295**, 1299–1301.

Lopez-Coronado, J.M., Belles, J.M., Lesage, F., Serrano, R. and Rodriguez, P.L. (1999) A novel mammalian lithium-sensitive enzyme with a dual enzymatic activity, 3′-phosphoadenosine 5′-phosphate phosphatase and inositol-polyphosphate 1-phosphatase. *J. Biol. Chem.* **274**, 16034–16039.

Monroy, A.F. and Dhindsa, R.S. (1995) Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25°C. *Plant Cell*, **7**, 321–331.

Murguia, J.R., Belles, J.M. and Serrano, R. (1995) A salt-sensitive 3′(2′), 5′-bisphosphate nucleotidase involved in sulfate activation. *Science*, **267**, 232–267.

Peier, A.M., Moqrich, A., Hergarden, A.C. *et al.* (2002) A TRP channel that senses cold stimuli and menthol. *Cell*, **108**, 705–715.

Privalov, P.L. and Gill, J. (1988) Stability of protein structure and hydrophobic interaction. *Adv. Protein Chem.* **39**, 191–234.

Quintero, F.J., Garciadeblas, B. and Rodriguez-Navarro, A. (1996) The *SAL1* gene of *Arabidopsis*, encoding an enzyme with 3′(2′), 5′-bisphosphate nucleotide and inositol polyphosphate 1-phosphatase activities, increases salt tolerance in yeast. *Plant Cell*, **8**, 529–537.

Sanchez, J.P. and Chua, N.H. (2001) *Arabidopsis* PLC1 is required for secondary responses to abscisic acid signals. *Plant Cell*, **13**, 1143–1154.

Sanders, D., Pelloux, J., Brownlee, C. and Harper, J.F. (2002) Calcium at the crossroads of signaling. *Plant Cell*, **14**, S401–S417.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr. Opin. Plant Biol.* **6**, 410–417.

Spiegelberg, B.D., Xiong, J.P., Smith, J.J., Gu, R.F. and York, J.D. (1999) Cloning and characterization of a mammalian lithium-sensitive bisphosphate 3′-nucleotidase inhibited by inositol 1,4-bisphosphate. *J. Biol. Chem.* **274**, 13619–13628.

Sung, D.Y., Kaplan, F., Lee, K.J. and Guy, C.L. (2003) Acquired tolerance to temperature extremes. *Trends Plant Sci.* **8**, 179–187.

Thomashow, M.F. (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 571–599.

Williams, R.S. and Harwood, A.J. (2000) Lithium therapy and signal transduction. *Trends Pharmacol. Sci.* **21**, 61–64.

Xiong, L. and Zhu, J.-K. (2002) Molecular and genetic aspects of plant responses to osmotic stress. *Plant Cell Environ.* **25**, 131–139.

Xiong, L., Lee, B., Ishitani, M., Lee, H., Zhang, C. and Zhu, J.-K. (2001) *FIERY1* encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in *Arabidopsis*. *Genes Dev.* **15**, 1971–1984.

Xiong, L., Schumaker, K.S. and Zhu, J.-K. (2002) Cell signaling for cold, drought, and salt stresses. *Plant Cell*, **14**, S165–S183.

York, J.D., Ponder, J.W. and Majerus, P.W. (1995) Definition of a metal-dependent/Li+-inhibited phosphomonoesterase protein family based upon a conserved three-dimensional core structure. *Proc. Natl Acad. Sci. USA*, **92**, 5149–5153.

Zeidler, M.P., Tan, C., Bellaiche, Y., Cherry, S., Hader, S., Gayko, U. and Perrimon, N. (2004) Temperature-sensitive control of protein activity by conditionally splicing inteins. *Nat. Biotechnol.* **22**, 871–876.

Zhu, J.-K. (2001) Cell signaling under salt, water and cold stresses. *Curr. Opin. Plant Biol.* **4**, 401–406.