The homologous HD-Zip I transcription factors HaHB1 and AtHB13 confer cold tolerance via the induction of pathogenesis-related and glucanase proteins

Julieta V. Cabello, Agustín L. Arce and Raquel L. Chan*
Instituto de Agrobiotecnología del Litoral, Universidad Nacional del Litoral, CONICET, CC 242 Ciudad Universitaria, 3000 Santa Fe, Argentina

Received 1 August 2011; accepted 5 September 2011; published online 14 October 2011.
*For correspondence (fax +54 342 4575219; e-mail rchan@fbcb.unl.edu.ar).

SUMMARY

Plants deal with cold temperatures via different signal transduction pathways. The HD-Zip I homologous transcription factors HaHB1 from sunflower and AtHB13 from Arabidopsis were identified as playing a key role in such cold response. The expression patterns of both genes were analyzed indicating an up-regulation by low temperatures. When these genes were constitutively expressed in Arabidopsis, the transgenic plants showed similar phenotypes including cell membrane stabilization under freezing treatments and cold tolerance. An exploratory transcriptomic analysis of HaHB1 transgenic plants indicated that several transcripts encoding glucanases and chitinases were induced. Moreover, under freezing conditions some proteins accumulated in HaHB1 plants apoplasts and these extracts exerted antifreeze activity in vitro. Three genes encoding two glucanases and a chitinase were overexpressed in Arabidopsis and these plants were able to tolerate freezing temperatures. All the obtained transgenic plants exhibited cell membrane stabilization after a short freezing treatment. Finally, HaHB1 and AtHB13 were used to transiently transform sunflower and soybean leading to the up-regulation of HaHB1/AtHB13-target homologues thus indicating the conservation of cold response pathways. We propose that HaHB1 and AtHB13 are involved in plant cold tolerance via the induction of proteins able to stabilize cell membranes and inhibit ice growth.

Keywords: freezing tolerance, antifreeze proteins, HD-Zip, HaHB1, AtHB13, glucanase, chitinase, pathogenesis-related proteins, abiotic stress.

INTRODUCTION

Plants have to cope with a range of biotic and abiotic stresses during their life cycle. Among abiotic stress-causing factors, drought, soil salinity and extreme temperatures are the most harmful (Bray et al., 2000). Stresses caused by extreme temperatures produce different damages and they can be divided in those generated by high temperatures and those generated by chilling and freezing temperatures (Atici and Nalbantoglu, 2003). Freezing temperatures (below 0°C) cause the movement of water from the protoplast to the extracellular space, resulting in the growth of extracellular ice crystals and ultimately, cell dehydration (Taiz and Zeiger, 2002). Some species like winter cereals tolerated better freezing temperatures when they were previously subjected to chilling temperatures (above 0°C) during several days, thanks to the acclimation phenomenon (Levitt, 1980; Thomashow, 1999, 2010; Chinnusamy et al., 2007). The major regulatory signalling pathway that controls gene expression during acclimation is the CBF regulon (Hannah et al., 2005; Chinnusamy et al., 2007; Thomashow, 2010). Furthermore, this regulon also plays a prominent role in the reconfiguration of the Arabidopsis metabolome in response to low temperatures (Cook et al., 2004).

In Arabidopsis, tolerance to cold temperatures involves the accumulation of sugars as raffinose (Guy et al., 1992), amino acids as proline (Nanjo et al., 1999), the increase of the concentration of compatible solutes that function to stabilize enzymes (Puhakainen et al., 2004), membranes and other cellular components or the modification of the membrane lipid composition to optimize the liquid/crystalline physical structure necessary for proper membrane function via the up-regulation of desaturases (Gibson et al., 1994; Khodakovskaya et al., 2006).
Antifreeze proteins (AFPs) have been found and characterized in organisms of several kingdoms, including fishes, insects, bacteria and fungi (DeVries, 1971; Li et al., 1998). They were also identified in overwintering plants and it was proposed that these proteins could be involved in cold tolerance (Atici and Nalbantoglu, 2003; Griffith and Yaish, 2004). These proteins are secreted into the apoplast during cold acclimation and on the basis of in vitro experiments, it was proposed that they bind to ice crystals, change their shape and inhibit their growth. A second way of AFPs action as antifreeze factors is the inhibition of recrystallization (the formation of large crystals from small ones; Griffith and Yaish, 2004). Although most experimental data supporting the antifreeze action of AFPs were obtained ex planta, Griffith et al. (2005) reported that AFPs lowered the temperature at which the leaves froze in the presence of an ice nucleator after observing these leaves by infrared video thermography. AFPs are constitutively expressed as part of a freezing tolerance mechanism in Deschampsia antarctica, one of the only two vascular plants that colonized the Maritime Antarctic (Bravo and Griffith, 2005). Winter rye AFPs, the first plant AFPs identified (Griffith et al., 1992), are similar to members of three classes of pathogenesis-related (PR) proteins, namely endochitinases, endo-β-1,3-glucanases and thaumatin-like proteins (Hon et al., 1995). PR proteins are induced by pathogens or by cold temperatures. When pathogens induced them at warm temperatures, they exhibited enzymatic activity but lacked antifreeze activity, whereas when they were induced by cold temperatures, these proteins exhibited both, enzymatic and antifreeze activities, suggesting the existence of different isoforms (Hiilovaara-Teijo et al., 1999). Although the antifreeze activity of these proteins was deeply characterized, the molecular pathway by which these proteins are induced and how they function in vivo is largely unknown.

Transcription factors (TFs) play a crucial role in plant adaptation to environmental stresses. Among others, members of the HD-Zip I subfamily of TFs have been proposed as developmental regulators responsive to environmental conditions (Schena and Davis, 1994; Henriksson et al., 2005).

HD-Zip TFs are characterized by the presence of a homeodomain (HD) associated with a leucine zipper (LZ; Ariel et al., 2007; Chan et al., 1998; Schena and Davis, 1994). This family was divided in four subfamilies (I to IV) and members of subfamily I form homo- or heterodimers as a prerequisite to interact with the same pseudopalindromic DNA sequence CAAT(A/T)ATTG (Johannesson et al., 2001; Palena et al., 1999).

HaHB1 is a member of the sunflower HD-Zip I subfamily and the protein encoded by this gene exhibits a high similarity with AtHB13 and AtHB23 from Arabidopsis (Gonzalez et al., 1997). Moreover, a recent phylogenetic and functional analysis resolved these three proteins in the same group taking into account conserved motifs outside the HD-Zip domain (Arce et al., 2011). Transgenic plants overexpressing AtHB13 and AtHB23 genes were previously described and their phenotype indicated that they are involved in the regulation of cotyledon and leaf development and are components of the sucrose signalling pathway (Hanson et al., 2001; Kim et al., 2007).

In this paper we show that the ectopic expression of HaHB1 in transgenic Arabidopsis plants, either under the control of its own promoter or under the control of the constitutive promoter CaMV 35S, confers cold tolerance via the increase of proteins, which stabilizes membranes and could be inhibiting the growth of large extracellular ice crystals. Similar results were obtained when the Arabidopsis homologue AtHB13 was overexpressed. Moreover, the overexpression of the HaHB1 up-regulated genes encoding PR2, PR4 and a glucanase confers partial tolerance to temperatures below 0°C, indicating that these HD-Zip proteins provide cold tolerance, probably via the induction/export of proteins that stabilize cell membranes and inhibit ice recrystallization.

RESULTS

HaHB1 and AtHB13 were up-regulated by low temperatures

In an exploratory experiment aiming to characterize HaHB1 expression, total RNA was isolated from several tissues/organs and subjected to different abiotic stress factors and phytohormones, and qRT-PCRs (quantitative reverse-transcriptase polymerase chain reaction) carried out (data not shown). The most interesting observation was the strong up-regulation of HaHB1 by cold temperatures. Thus, kinetics of induction at chilling (4°C during 8 h) and freezing temperatures (–8°C during 3 h) were performed on the sunflower gene and its Arabidopsis homologue AtHB13. Figure 1 shows that HaHB1 was up-regulated by both chilling and freezing temperatures while AtHB13 was up-regulated only by temperatures below 0°C.

Transgenic plants ectopically expressing HaHB1 or AtHB13 were more tolerant of freezing temperatures than WT (wild type) plants

In order to determine whether the up-regulation by cold temperatures indicated a role of these genes in response to such stress condition, 35S:HaHB1 and 35S:AtHB13 transgenic plants were obtained. Three independent homzygous lines exhibiting different expression levels for each genotype were used for the analysis (Figure 3a,c). Notably, both transgenic genotypes exhibited similar phenotypes with particular characteristics such as serrated leaves (Figure 2a). These and WT plants were subjected to freezing conditions during 6 h and then placed in normal conditions during 6 days to recover themselves. After this treatment, carried out either in the vegetative and the reproductive stage, survival rates for HaHB1 and AtHB13 plants were...
Cold tolerance mediated by HD-Zip transcription factors occurred via the up-regulation of glucanase and chitinase encoding genes

To elucidate the molecular mechanisms by which HaHB1 conferred tolerance to cold temperatures, we performed an exploratory transcriptomic analysis comparing RNAs of transgenic and WT 3-week-old plants. Validation of the results was performed by qPCR (Figure S1). From the 28 500 genes analyzed, we selected a cluster of 1024 genes showing altered-expression levels in at least one of the replicas using an approach based on false discovery rate (FDR) correction (see Experimental Procedures; Table S1).

This cluster of genes was contrasted with different cold-related transcriptomic analyses (Fowler and Thomashow, 2002; Lee et al., 2005; Matsui et al., 2008). Around 10% of the genes previously detected as regulated by cold conditions were present in our selected cluster. Among them, only a few belonged to the CBF regulon (Table S2). Interestingly, in the unshared group of genes, some encoding glucanases, chitinases and PR proteins were up-regulated. This type of proteins had been previously associated with cold tolerance in overwintering plants (Griffith and Yaish, 2004).

Taking into account the microarray data obtained with HaHB1 plants, the data reported by other authors (Griffith and Yaish, 2004) and the cold tolerance phenotype observed in HaHB1 and AtHB13 plants (Figure 2b), four genes encoding PR2, PR3, PR4 and a glucanase (thereafter named GLU) were selected to be quantified in independent transgenic lines of the 35S:HaHB1 (Figure 3a) and 35S:AtHB13 (Figure 3c) genotypes. This analysis (Figure 3b,d) indicated that the four genes were up-regulated in HaHB1 plants while in AtHB13 plants, PR3 and GLU were up-regulated; only one line showed differences for PR4, and PR2 did not show changes in regulation.

In view of the microarray data and the cold tolerance conferred by HaHB1, we wondered if similar events involving chitinase and glucanase proteins occur in other plant species. Profiting from known expressed sequence tags (ESTs) and genes, we identified sequences in sunflower and soybean which were homologous to those identified in the Arabidopsis microarray as induced by HaHB1. In order to quantify these genes, transient transformations of sunflower and soybean leaf discs with 35S:HaHB1 and 35S:AtHB13 were performed. Four sunflower and three soybean genes were selected as putative homologues. Figure S3 shows that the selected genes responded similarly to the stimulus provided by HaHB1 or AtHB13.

Altogether, the results indicated that there are conserved mechanisms of action in Arabidopsis, soybean and sunflower species controlled by HaHB1 and/or its homologues.

Transgenic plants expressing HaHB1 under the control of its promoter were tolerant to cold temperatures

A 1011-bp fragment corresponding to the HaHB1 promoter region (Figure 4a) was isolated and inserted upstream of the β-glucuronidase (GUS) reporter gene to transform Arabidopsis plants. This promoter region exhibited two cis-acting elements known as Low Temperature Responsive Element (LTRE, Baker et al., 1994). In all the analyzed developmental stages, GUS expression was evident in the meristems and in the base of the siliques (during the reproductive stage) in normal growth conditions (Figure 4b). This GUS expression pattern, directed by HaHB1 promoter, remained invariant when the plants were placed 24 h at 4°C; however, the intensity of the signal increased, indicating an up-regulation of this promoter by cold in the meristems (Figure S4).

To investigate whether the cold tolerant phenotype observed in Arabidopsis plants was the result of an effect produced by the constitutive expression of HaHB1 (35S:HaHB1), the HaHB1 cDNA was cloned downstream of its native promoter and used to generate new transgenic plants (ProH1:HaHB1). No differences were observed in their morphology compared with controls, e.g. in leaf serration (Figure 4c). However, when these plants were subjected to

Figure 1. Sunflower HaHB1 and Arabidopsis AtHB13 are up-regulated by cold temperatures. Low temperatures kinetics of expression of HaHB1 in sunflower R1 leaves and AtHB13 in 21-day-old seedlings. Upper panel at 4°C and lower panel at −8°C. All the quantitative polymerase chain reaction (qPCR) values were normalized with respect to the value measured at time 0, arbitrarily assigned a value of one. Error bars correspond to standard deviation (SD) among biological replicates. Analysis of variance (ANOVA) was performed and distinct letters denote statistical differences with Tukey post-hoc test at P < 0.05.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>HaHB1 Fold change (log2)</th>
<th>AtHB13 Fold change (log2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>0.5</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>1</td>
<td>c</td>
<td>b</td>
</tr>
<tr>
<td>2</td>
<td>a</td>
<td>d</td>
</tr>
<tr>
<td>3</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>4</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td>5</td>
<td>a</td>
<td>e</td>
</tr>
<tr>
<td>6</td>
<td>b</td>
<td>f</td>
</tr>
<tr>
<td>7</td>
<td>c</td>
<td>e</td>
</tr>
<tr>
<td>8</td>
<td>a</td>
<td>f</td>
</tr>
<tr>
<td>9</td>
<td>b</td>
<td>g</td>
</tr>
<tr>
<td>10</td>
<td>c</td>
<td>f</td>
</tr>
</tbody>
</table>

© 2011 The Authors
freezing temperatures after a 10 days acclimation period at 4°C (Figure 4(c), right), a significant difference in the percentage of survivors was observed \( (P < 0.01, \text{Table 1c}) \). ProH1:HaHB1 plants showed higher survival rates than WT plants, indicating that the HaHB1 promoter was able to induce HaHB1 expression at low temperatures, consequently triggering the tolerance to freezing temperatures. Furthermore, HaHB1 was up-regulated in ProH1:HaHB1 transgenic plants incubated at 4°C (Figure 4d).

**Putative HaHB1 target genes were regulated in ProH1:-HaHB1 transgenic plants in response to different abiotic factors and hormones**

Considering the model presented by Griffith and Yaish (2004) for winter rye in which it was proposed that the activity of glucanase, chitinase and thaumatin-like proteins (antifreeze and/or enzymatic) was dependent on the external factor that induced their expression, we wondered if HaHB1 targets were regulated by cold, 1-aminocyclopropane-1 carboxylic acid (ACC) and drought or by Salicylic acid (SA) and Abscisic acid (ABA) in ProH1:HaHB1 plants. PR4 (At3g04720) expression was higher in transgenic plants than in WT in control conditions. After exposure to low temperatures these levels further increased while when the plants were treated with SA or ABA, the basal levels slightly decreased (Figure 5a,b).

PR3 (At3g12500) expression was higher in transgenic plants than in WT in normal conditions, and it was significantly induced in the presence of SA but not when ABA or ACC were applied, whereas drought and 4°C produced a decrease in the transcript levels (Figure 5c,d).

PR2 (At3g57260) was not induced in transgenic plants under normal conditions, ACC, SA, low temperatures or drought; but it was induced in the presence of ABA (Figure S5).

The expression pattern of the GLU gene (At4g16260) in front of the same treatments was similar to that of PR4 (Figure S6). Finally, AtHB13 expression was also tested in front of the same treatments. The results indicated that its transcripts increased when the plants were subjected to
drought and SA suggesting that it might be regulating these glucanase and chitinase proteins in front of these conditions (Figures 1 and S7).

These observations led us to evaluate the effects of PR4 and GLU themselves as transgenes under freezing temperatures. In spite of no significant statistical changes were observed with PR2 under cold treatments (Figure S5), this gene was also chosen for the analysis due to results derived from the proteomic analysis performed by Amme et al. (2006) in which PR2 seemed to be up-regulated in cold treated (4°C) plants.

PR2, PR4 and GLU were partially responsible for the cold tolerance conferred by HaHB1 and AthB13

Regarding the results described above and in order to corroborate our hypothesis stating that the induced GLU and PR proteins were responsible for the tolerance to cold temperatures shown by HaHB1 and AthB13 plants, we obtained transgenic plants overexpressing independently PR2, PR4 and GLU (Figure S8). Five independent homozygous lines for each construct were analyzed in normal growth conditions as well as after freezing treatments. Neither PR2 nor GLU nor PR4 plants exhibited differential morphological or developmental phenotypes in normal growth conditions while all these genes appeared to confer cold tolerance (Figure 6a). The survival rates under severe freezing conditions for PR2, PR4 and GLU plants are presented in Table 1(d–f) respectively. They indicated that it is probable that the three genes participate in the cold tolerance response conferred by HaHB1 and AthB13. Cell membrane stability (Figure 6b) was higher in PR2, PR4 and GLU plants than in WT, thus indicating that this physiological mechanism was, at least in part, responsible for the behavior at freezing temperatures.

Antifreeze proteins present in the apoplast of transgenic plants expressing HaHB1 contributed to the observed cold tolerance

Freezing temperatures induce the formation of ice crystals outside the cell and these crystals cause cell dehydration, resulting in plant death. To check the potential molecular differences between WT and 35S:HaHB1 plants, apoplast proteins were isolated. No significant differences in the protein pattern between these genotypes were observed in plants grown in normal conditions or acclimated 16 h or 10 days at 4°C, but at least four polypeptides were secreted to the cellular apoplast in all the plants subjected to 4°C (Figure S9). Of these, two were identified by MS-MALDI TOF as PR2 and PR5. On the other hand, when the plants were subjected to freezing temperatures, the apoplastic protein pattern observed was clearly different between genotypes. 35S:HaHB1 plants exhibited an additional 23 kDa polypeptide, indicating a differential molecular response in front of the adverse condition (Figure 7a).

To evaluate the antifreeze activity of apoplastic proteins in transgenic and WT plants, an in vitro recrystallization assay was performed. Figure 7(b) shows that apoplastic extracts from transgenic acclimated plants inhibited the recrystallization of ice crystals while WT extracts treated in the same way, did not.

DISCUSSION

HaHB1 and AthB13 cDNAs encode 313 and 294 amino acid proteins respectively, that are non-divergent members of the HD-Zip I subfamily. Sequence comparison (Figure S10) and phylogenetic trees indicated a high structural homology between these TFs as well as with other HD-Zip proteins from various species (Chan et al., 1998; Arce et al., 2011).

The current knowledge about HD-Zip I subfamily indicates that its members are involved in the responses to biotic and abiotic stresses (Henriksson et al., 2005; Ariel et al., 2007). Aiming to elucidate HaHB1 and its Arabidopsis homologue AthB13 functions, we first screened their expression levels under different stress factors, revealing that both genes were up-regulated by low temperatures, especially by freezing temperatures. After isolating and sequencing the HaHB1 promoter, cis-acting elements were investigated and

Table 1 Tolerance to freezing temperatures of plants transformed with 35S:HaHB1, PrH1:H1, 35S:AthB13, 35S:PR2, 35S:PR4 and 35S:GLU

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Survivors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>48</td>
<td>21</td>
</tr>
<tr>
<td>H1 A</td>
<td>48</td>
<td>83</td>
</tr>
<tr>
<td>H1 B</td>
<td>48</td>
<td>71</td>
</tr>
<tr>
<td>H1 C</td>
<td>48</td>
<td>56</td>
</tr>
<tr>
<td>1b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>A13 C</td>
<td>32</td>
<td>66</td>
</tr>
<tr>
<td>A 13 D</td>
<td>32</td>
<td>60</td>
</tr>
<tr>
<td>A 13 I</td>
<td>32</td>
<td>66</td>
</tr>
<tr>
<td>1c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>48</td>
<td>31</td>
</tr>
<tr>
<td>ProH1:H1 A</td>
<td>48</td>
<td>90</td>
</tr>
<tr>
<td>ProH1:H1 B</td>
<td>48</td>
<td>81</td>
</tr>
<tr>
<td>ProH1:H1 C</td>
<td>48</td>
<td>69</td>
</tr>
<tr>
<td>1d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>48</td>
<td>22</td>
</tr>
<tr>
<td>PR2 A</td>
<td>48</td>
<td>60</td>
</tr>
<tr>
<td>PR2 B</td>
<td>48</td>
<td>49</td>
</tr>
<tr>
<td>PR2 C</td>
<td>48</td>
<td>43</td>
</tr>
<tr>
<td>1e</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>48</td>
<td>22</td>
</tr>
<tr>
<td>PR4 A</td>
<td>48</td>
<td>71</td>
</tr>
<tr>
<td>PR4 B</td>
<td>48</td>
<td>59</td>
</tr>
<tr>
<td>PR4 C</td>
<td>48</td>
<td>36</td>
</tr>
<tr>
<td>1f</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>48</td>
<td>17</td>
</tr>
<tr>
<td>GLU A</td>
<td>48</td>
<td>68</td>
</tr>
<tr>
<td>GLU B</td>
<td>48</td>
<td>58</td>
</tr>
<tr>
<td>GLU C</td>
<td>48</td>
<td>40</td>
</tr>
</tbody>
</table>

Pearson’s chi-squared test of independence was used to assess the significance of the results observed and the differences in tolerance were highly significant for the six constructs ($P < 0.01$). WT, wild type; $N$, number of plants for each genotype.
compared with those found in the AtHB13 promoter. Both promoters share cis-elements like ARFAT (auxin response), GARE (gibberellin response), MYB, MYC and WRKY recognition sites. Two LTRE boxes, previously described as responsive to cold (Baker et al., 1994), were identified only in the HaHB1 promoter. Even though LTRE elements are absent in the promoter of AtHB13 (Hanson et al., 2002), its expression was up-regulated by freezing conditions (Figure 1). This regulation suggested the existence of different unknown cis-acting elements that might be responsive to temperatures below zero. Further work will be necessary to investigate which is/are this/these putative cold-responsive cis acting elements. On the other hand, AtHB23, another HD-Zip I encoding gene, parologue of AtHB13 and potentially orthologue of HaHB1 (Arce et al., 2011) exhibits in its promoter one LTRE-box slightly different from that of HaHB1, but presented a different expression pattern compared with the sunflower gene (Kim et al., 2007), indicating another role for this gene. Although HaHB1 and AtHB13 were differentially regulated by low temperatures they produced very similar phenotypes when they were ectopically expressed in transgenic plants, in accordance with their encoded proteins sequence homology (Figure S10). Both transgenic genotypes share certain distinctive, though not negative, morphological features as well as a marked cold tolerance partially mediated by increased membrane stability. Furthermore, the tissue/organ expression patterns directed by HaHB1 and AtHB13 promoters fused to the GUS reporter gene were very similar and different from the observed for AtHB23 promoter (Hanson et al., 2002; Kim et al., 2007; Figure 4) supporting that AtHB13, and not AtHB23, is the true orthologue of

Figure 3. Expression of HaHB1 and AtHB13 regulated genes in 35S:HaHB1 and 35S:AtHB13 transgenic plants.

(a) Transcript levels of HaHB1 in eight independent 35S:HaHB1 transgenic lines, named A to H. All the values were normalized with respect to the value measured in line H, arbitrarily assigned a value of one.

(b) Transcript levels of the Arabidopsis PR2, PR3, PR4 and GLU were quantified by qRT-PCR using RNAs isolated from wild-type (WT) or the three independent 35S:HaHB1 transgenic lines A, B, C and D. All the values are normalized with respect to the value measured in WT, arbitrarily assigned a value of one.

(c) Transcript levels of AtHB13 in seven independent 35S:AtHB13 transgenic lines, named A to I. All the values were normalized with respect to the value measured in line F, arbitrarily assigned a value of one.

(d) Transcript levels of the Arabidopsis PR2, PR3, PR4 and GLU were quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using RNAs isolated from WT or the three independent 35S:AtHB13 transgenic lines C, D and I. All the values were normalized with respect to the value measured in WT RNAs, arbitrarily assigned a value of one. Error bars are standard deviations were calculated from three biological replicas for each genotype. For (b) and (d), distinct letters denote statistical differences with Tukey post-hoc test at $P < 0.05$. 

© 2011 The Authors
The leaf serration was not observed in ProH1:HaHB1 plants while the cold tolerance remained, indicating that the cold-responsive elements present in the promoter of this gene might be functional. The induction of the GUS reporter gene controlled by the HaHB1 promoter in chilling temperatures supported this conclusion (Figure S4).

Microarray data obtained from HaHB1 plants indicated the up-regulation of genes encoding chitinases, glucanases and cell wall modifying proteins, and other genes like CBF3 (Maruyama et al., 2004) and CBF1 (Zhang et al., 2004), previously reported as participating in the response to cold temperatures. Transcriptomic analysis of plants over-expressing CBF1 and CBF3 were reported (Fowler and Thomashow, 2002; Maruyama et al., 2004, 2009). Notably, a small overlap was detected between the genes whose expression is altered in HaHB1 overexpressors and those altered in CBF overexpressors (Table S2). This finding would indicate that HaHB1 is not upstream of the CBFs. It is tempting to hypothesize that the induction of these genes in HaHB1/AtHB13 plants could be due to the induction of some
sort of stress-related signal, which was sufficient enough to alter their expression, but not to trigger an important proportion of the CBF regulon, suggesting a different response pathway triggered by HaHB1/AtHB13.

Previous reports suggested that some PR proteins could act alternatively in biotic or abiotic stresses depending on external conditions. In this sense, Yu et al. (2001) showed that non-acclimated rye plants treated with SA accumulated apoplastic proteins lacking antifreeze activity. In contrast, when these plants were exposed to ethylene (Ethephon or ACC), the concentration of apoplastic proteins increased in leaves and these proteins exhibited antifreeze activity in vitro. Additionally, when these plants were exposed to cold (5°C) or drought, endogenous ethylene production and antifreeze activity were detected. Taking into account these results, Griffith and Yaish (2004) proposed a model in which PR proteins have a dual-function (antifreeze and/or enzymatic) depending on the factor inducing them. We analyzed transcript levels of ProH1:HaHB1 and in WT plants treated with different effectors. From the results, shown in Figures 5 and S5, we suggest that PR2, PR4, and GLU could act as AFPs while PR3 probably not and, most likely it participates exclusively in the pathogen response.

It still remains unclear why PR2 is not induced in AtHB13 plants (Figure 3d) while it is in HaHB1 plants (Figure 3b) and seems to be involved in the cold response. Taking into account that both TFs show a high degree of conservation, are resolved in the same phylogenetic group of HD-Zip proteins (Arce et al., 2011) and bind in vitro the same target sequence (Johannesson et al., 2001 and this work) we are tempted to speculate that the expression levels detected in AtHB13 plants, that cannot be compared with HaHB1 transcript levels, are not enough to detect changes in some of the indirect target genes.

More recently, Seo et al. (2010) reported that PR proteins act enhancing disease resistance when plants were exposed to low temperatures. The analysis of these results, together with the reported here, and those describing the expression patterns of PR encoding genes in front of abiotic stress (Seo et al., 2008; Takenaka et al., 2009), strongly suggests a complex relationship between both stress responses and indicates the participation of PR proteins in plants cold adaptation.

Freezing temperatures induce the growth of ice crystals resulting in plant death. AFPs have been purified from the winter rye leaf cell apoplast and they either reduced ice crystals growth (Griffith et al., 1992) or inhibited their recrystallization, both in vitro. Furthermore, Hincha et al. (1997) demonstrated that a cold induced glucanase was able to stabilize membranes and later, Tomczak et al. (2002) showed that AFPs were able to directly interact with

**Figure 5.** Expression levels of AtPR4 and AtPR3 in transgenic ProH1:H1 and wild type plants subjected to different stress treatments. (a, b) PR4 and (c, d) PR3 expression levels in ProH1:H1 plants or wild-type (WT) subjected to drought, 4°C or treated with ACC, SA or ABA for 1 h. Error bars are standard deviations calculated from three independent samples. All the values were normalized with respect to the value measured in WT, arbitrarily assigned a value of one. The grid shows the results of the statistical analysis; those treatments (columns) which were different after a Tukey post-hoc test at $P < 0.05$ do not share black squares in the same row.
membranes, thereby inhibiting leakage. Accordingly, all the transgenic genotypes reported here exhibited increased membrane stability which could be explained by the action of these proteins. Supporting this, apoplastic extracts isolated from HaHB1 plants exhibited an additional 23 kDa polypeptide compared with WT and were able to inhibit recrystallization in vitro. Although it cannot be ruled out that this polypeptide was released following membrane damage instead actively transported, this mechanism is unlikely since membrane damage would generate an enrichment of the apoplastic fraction in more than one protein. Even though the antifreeze activity exerted by these apoplast extracts cannot be directly associated to GLU and/or PR action, the fact that transgenic plants expressing GLU and PRs showed cold tolerance and also increased membrane stabilization tempts us to suggest that some of these proteins are responsible for the tolerance observed in HaHB1 and AthB13 plants. However, further experimental work must be performed to elucidate this point.

The cold tolerance mechanism mediated by HaHB1/ AthB13 seems to be conserved in other plant species such as soybean. The transient transformation of leaf discs from this species with either 35S:AthB13 or 35S:HaHB1 induced the expression of two HaHB1-target homologues, indicating that an up to now unidentified homologous gene in soybean could be exerting the same type of regulation. Altogether, the results presented here indicated that HaHB1 and AthB13 participate in a cold tolerance mechanism via the induction of genes encoding proteins that might act inhibiting the recrystallization of ice and stabilizing membranes, and thus, preventing the cell death caused by freezing temperatures. This mechanism seems to be conserved between species like Arabidopsis, sunflower and soybean. Moreover, the results described in this work suggest that HaHB1/AthB13 trigger a novel pathway in response to low temperatures.

**EXPERIMENTAL PROCEDURES**

**Constructs**

35S:HaHB1: The HaHB1 cDNA (Chan and Gonzalez, 1993) was cloned in the pBI 121 plasmid under the control of the 35S CaMV promoter.

HaHB1 promoter: A 2000-bp fragment isolated from a bacterial artificial chromosome (BAC) clone (BAC Library HA_HBa, CUGI-Clemson University Genome Institute) was subcloned and
Figure 7. Differential apoplastic proteins in transgenic (35S:HaHB1) and wild type plants prevents the formation of large ice crystals.

(a) SDS-PAGE showing the proteins present in the cellular apoplast, isolated from 3SS:HaHB1-A, -B and -C. Twenty-five-day-old transgenic plants and wild-type (WT) plants placed during 3 h at -8°C. The samples loaded were obtained from 3 g leaf tissue. The arrow indicates the approximately 23 kDa band differentially expressed.

(b) Inhibition of recrystallization in apoplastic protein extracts. The samples were observed and photographed with an optical microscope. Crystals formed in the presence of apoplastic proteins: b-A, b-B and b-C, isolated from the three independent lines 3SS:HaHB1-A, -B and -C acclimated 10 days at 4°C; b-D, isolated from WT plants acclimated 10 days at 4°C; E, crystals formed in the presence of 26% sucrose without proteins.

The Plant Journal. © 2011 The Authors

**Helianthus annuus** seeds were germinated on wet filter paper for 7 days and then transferred either to a MS medium dish to perform chilling assays at 4°C or to soil to perform freezing treatments at −8°C as detailed in the figure legends. For transient transformation, 7-day-old sunflower and soybean seedlings were transferred to soil and the leaf discs were cut from leaves in R1 developmental state.

Three-week-old Arabidopsis plants grown in MS Petri dishes were transferred to a fresh MS medium dish supplemented with 200 μM ABA, 20 μM ACC or 200 μM SA or were exposed to −8 or 4°C during the time periods indicated in the figure legends or to a dry filter paper during 15 min. Seedlings were harvested and immediately frozen in liquid nitrogen to proceed with RNA isolation.

### Microarray assay and data analysis

Total RNA was extracted from the vegetative aerial part of 3-week-old plants using RNeasy® Plant Mini Kit (Qiagen, http://www.qiagen.com) following manufacturer’s instructions. The expression profiles of two independent pooled samples of RNAs from transgenic line H1-A and two from H1-B were tested versus WT RNAs in a two-color 4×44 K Arabidopsis Gene Expression Array (V3; Agilent Technologies). The whole process, from sample preparation and hybridization, to scanning and feature extraction was performed in the UHN Microarray Centre (http://www.microarrays.ca/) according to GE2v5.5 manufacturer’s protocol. Microarray data was deposited on the NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31100) as Series GSE31100.

Mainly with exploratory purposes, a FDR approach to correct for multiple testing was employed on each of the four arrays in order to obtain four groups of altered genes (Strimmer, 2008). The cutoff q-value used was 2.7e−05 in all cases, using R statistical language (R Development Core Team, 2011) combined with the ‘multcomp’ package (Hothorn et al., 2008). A compact letter display was generated from each analysis in order to ease visualization and interpretation.

The results from the membrane stability assays were analyzed using a nonlinear model fit with a logistic function. To test for differences, two models were compared: a simple logistic model fitting the measures for WT and transgenic plants (Model 1); and Model 2 in which the parameter controlling the shape of the curve between the minimum and maximum asymptotic values, e in Model 1, was the sum of two estimators, e1 and e2, the second affected by a vector f of ‘dummy variables’ that was zero for WT plants, and one for transgenic lines. The model formulae were:

\[
\text{Leaking ratio} = \frac{c}{1 + e^{-a-C1/t} + e^{-a-C2/t}}
\]

(1)

\[
\text{Leaking ratio} = \frac{c}{1 + e^{-a-C1/t} + e^{-a-C2/t} + e^{-a-Cf/f}}
\]

(2)

The parameters a, c, e, e1 and e2 were estimated for each model. Finally both models were compared with an ANOVA. Differences between model fits implied differences in the behavior of WT and transgenic plants in front of the treatments.

### Injury evaluation by the ion leakage technique

The ion leakage technique was carried out essentially as described by Sukumaran and Weiser (1972) with minor changes. The conductance was calculated as the ratio between C2/C1 (L = C1/C2) and used as index of injury. C1 is the conductance after the treatment and C2 is the maximal conductance of each sample. L values higher than 0.5 indicate a severe injury.

### Apoplastic protein extraction

Apoplastic protein extraction was carried out essentially as described by Mauch and Staehelin (1989).

The proteins were analyzed in 12% SDS (sodium dodecyl sulphate)-PAGE carried out according to Laemmli technique and visualized with Coomassie Brilliant Blue R-250 (Sigma). As molecular weight marker an Amersham Biosciences LMW calibration kit was used (Amersham Biosciences, http://www.gehealthcare.com/worldwide.html). To identify some of the polypeptides, the bands were excised and identified using MS-MALDI TOF (Pasteur Institute, Montevideo, Argentina).

### Recrystallization of ice

Recrystallization of ice was performed essentially as described by Griffith et al. (2005). Apoplastic extracts (10 μl) from transgenic and WT cold-acclimated Arabidopsis plants were mixed with 10 μl 26% (w/w) of sucrose. The mix was treated as follows: 2 min at −80°C, 10 min at −20°C, 15 min at −8°C, 30 sec at 4°C and finally placed 1 h at −8°C. The control solution contained the same amount of buffer as the samples plus 26% (w/w) of sucrose. The samples were observed and photographed with an NIKON optical microscope.

### Statistical analyses

The results of the qRT-PCR assays were studied using analysis of variance (ANOVA) followed by a Tukey post-hoc test with P-value <0.05 in all cases, using R statistical language (R Development Core Team, 2011) combined with the ‘multcomp’ package (Hothorn et al., 2008). A compact letter display was generated from each analysis in order to ease visualization and interpretation.

### ACKNOWLEDGEMENTS

We thank Dr Jan Chojecki (Plant Biologyce Limited, PBL) for critical reading of this manuscript and for his helpful suggestions; and Dr Liliana Forzani (IMAL-Conicet) for her helpful advice in statistical analyses. This work was supported by ANPCyT (PICT 2005 38103, PICT-PAE 37100 and PICT 2008 1206), Conicet (PIP 6383) and UNL. RLC is a member of CONICET; JVC and ALA are Fellows of the same Institution. We thank Eva Rueda for cloning the HaHB1 cDNA in the pBI vector.

### Supporting Information

Additional Supporting Information may be found in the online version of this article:

- **Figure S1.** Validation of the microarray data by qPCR in independent HaHB1 transgenic lines.
- **Figure S2.** Sequence specificity of DNA recognition in vitro by recombinant HAHB1.
- **Figure S3.** Sunflower and soybean leaf discs transformed with 35S:HaHB1 and 35S:AtHB13 over-express genes putatively related to the cold response.
- **Figure S4.** Histochemical detection of GUS enzymatic activity in Arabidopsis ProH1::H1 transgenic plants.
- **Figure S5.** **PR2** expression levels in ProH1::H1 transgenic and wild type plants subjected to different stress treatments.
- **Figure S6.** Expression levels of GLU in transgenic plants (ProH1::H1) and WT plants subjected to different treatments.
- **Figure S7.** Expression levels of AtHB13 in WT plants subjected to different treatments.
Figure S8. Transcript expression levels in independent transgenic lines expressing AtPR2, AtPR4 and AtGLU.

Figure S9. SDS-PAGE showing the proteins present in the cellular apoplast.

Figure S10. Protein sequence alignment between HaHB1 and AtHB13.

Table S1. Cluster of 1024 selected genes with altered expression in HaHB1 plants.

Table S2. Comparison between the cluster of HaHB1 regulated genes with the data obtained in other microarray analyses (Fowler and Thomashow, 2002; Lee et al., 2005; Matsui et al., 2008).

Table S3. Oligonucleotides used for cloning and qPCR measurements.

Appendix S1. References cited in Supporting Information and Accession Numbers.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES


