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

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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 Nalbantoglum, 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 Nalbantoglum, 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 homozygous 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

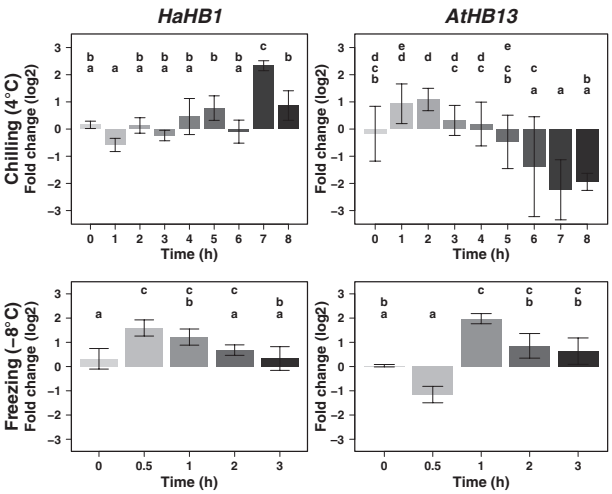

Figure 1. Sunflower *HaHB1* and Arabidopsis *AtHB13* are up-regulated by cold temperatures.
Low temperatures kinetics of expression of *HaHB1* in sunflower R1 leaves and *AtHB13* in 21-day-old seedlings. Upper panel at 4°C and lower panel at –8°C. All the quantitative polymerase chain reaction (qPCR) 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 $P < 0.05$.

significantly higher than for WT plants ($P < 0.01$; Table 1a,b). Figure 2(b) shows an illustrative image of the plants after the treatment. Furthermore, *HaHB1* and *AtHB13* plants exhibited better membrane stabilization than their controls during the freezing treatment, indicating that the phenomenon of cell membrane stabilization was responsible, at least in part, for the observed increased tolerance (Figure 2c).

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

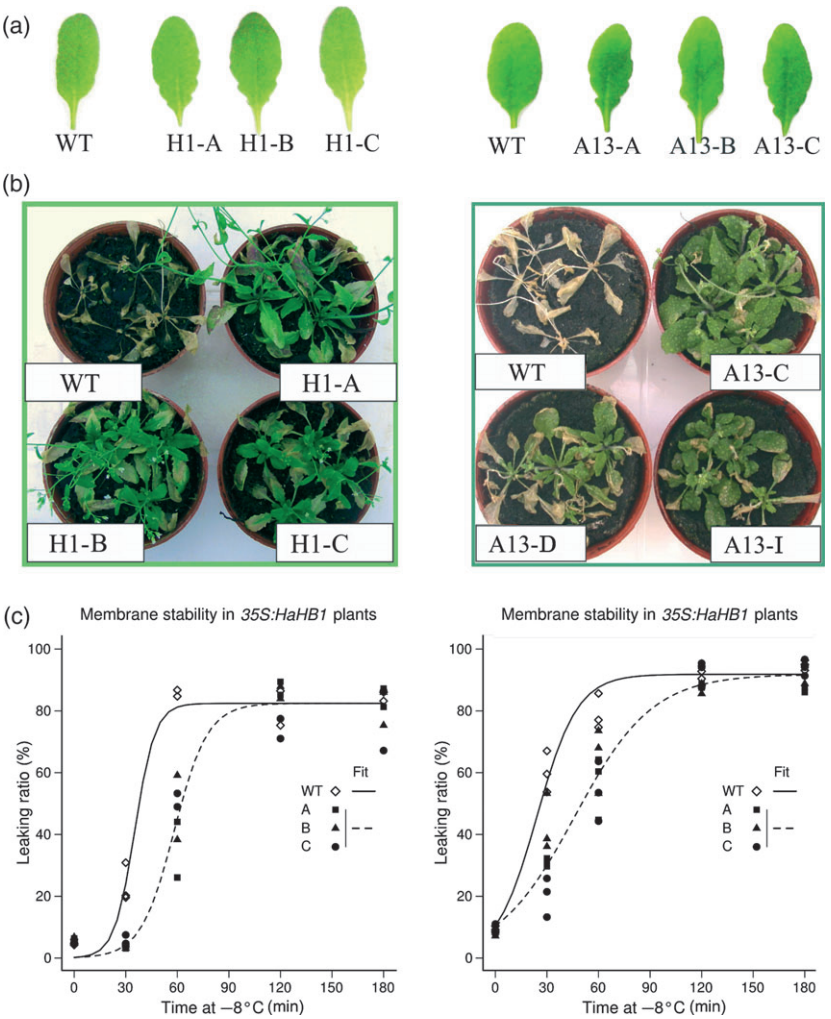

Figure 2. Transgenic plants ectopically and constitutively expressing *HaHB1* or overexpressing *AtHB13* are tolerant to freezing conditions.
(a) Leaves from transgenic 30-day-old plants expressing *HaHB1* (H1-A, H1-B and H1-C) or *AtHB13* (A13-C, A13-D and A-13 I) and wild-type (WT) plants cultured in normal growth conditions
(b) Illustrative photograph of 3-week-old *35S:HaHB1* (H1-A, H1-B and H1-C) or *35S:AtHB13* (A13-A, A13-B, A1-C) and WT plants cultured in normal growth conditions and then placed at −8°C during 6 h. The experiment was carried out with 12 or 16 plants of each genotype and repeated four times for *HaHB1* and twice for *AtHB13* plants. The photograph was taken 6 days after recovery at normal growth conditions.
(c) Membrane stability assay performed with transgenic (*35S:HaHB1* and *35S:AtHB13*) and WT plants subjected to −8°C as described in Experimental Procedures. Results were fitted with a nonlinear model, as Methods, and differences in membrane stability were found between transgenic and WT plants in both cases ($P < 0.01$).

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 ($P < 0.01$, 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

Table 1 Tolerance to freezing temperatures of plants transformed with *35S:HaHB1*, *PrH1:H1*, *35S:AtHB13*, *35S:PR2*, *35S:PR4* and *35S:GLU*

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

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.

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

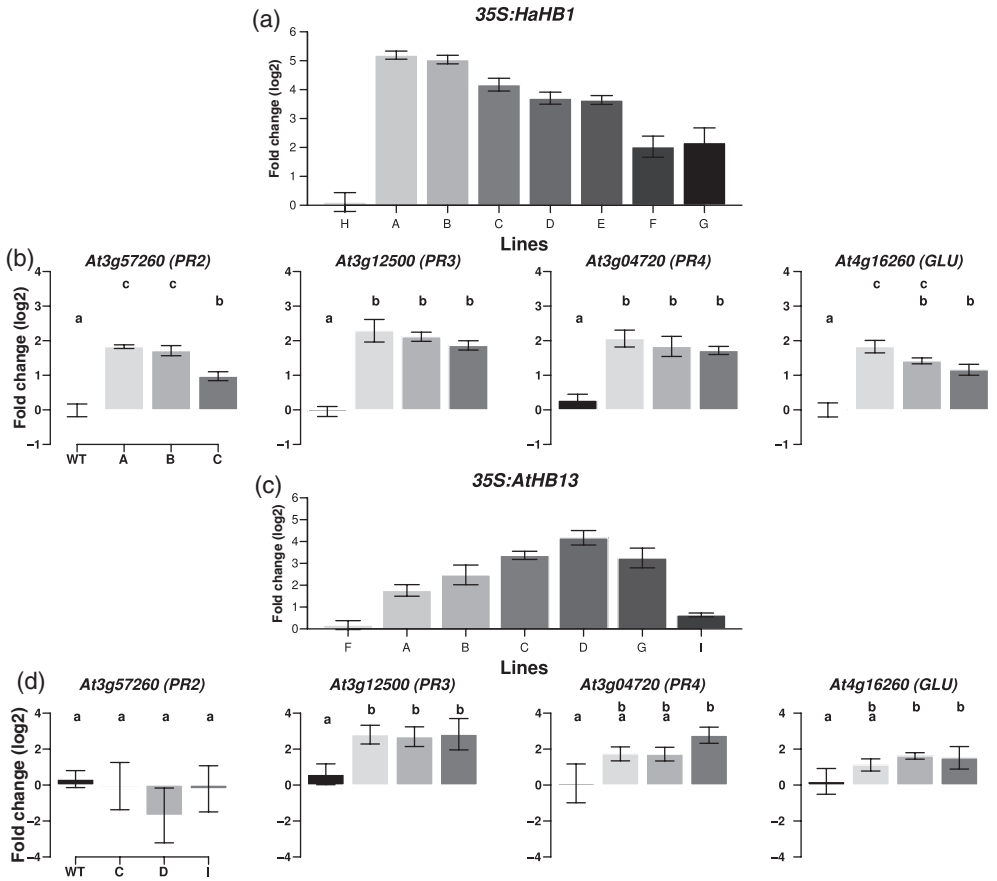

Figure 3. Expression of HaHB1 and AtHB13 regulated genes in *35S:HaHB1* and *35S:AtHB13* transgenic plants.
(a) Transcript levels of *HaHB1* in eight independent *35S:HaHB1* transgenic lines, named A to H. All the values were normalized with respect to the value measured in line H, arbitrarily assigned 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 $P < 0.05$.

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, paralogue 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

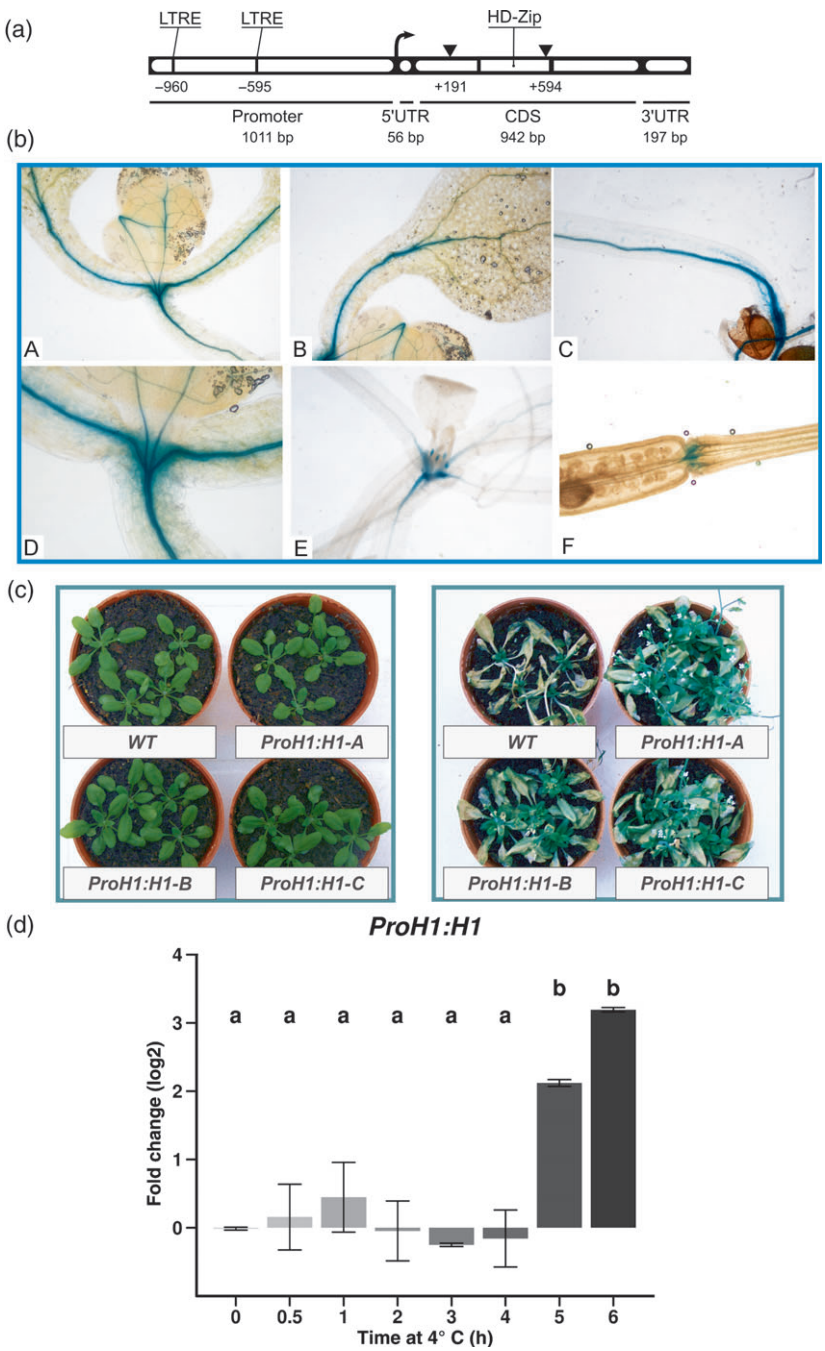

Figure 4. The *HaHB1* promoter directs the expression of the *GUS* reporter gene to meristems and siliques and together with the cDNA is able to confer freezing tolerance to transgenic plants.
(a) Schematic representation of the *HaHB1* gene. LTRE-like boxes as well as the 5′ and 3′ UTRs, transcription initiation site, introns (vertical arrows) and exons distribution are signalled. The distances expressed in bp are related to the +1.
(b) Histochemical detection of GUS enzymatic activity in *ProH1:GUS* plants. (b-A–b-D): 14-day-old seedlings; (b-E) 30 day-old plants; (b-F) 45 day-old plant. View of meristems (b-A and b-D); cotyledons (b-B), hypocotyls (b-C), apical meristem (b-E) and siliques (b-F).
(c) Morphological and developmental characteristics of 3-week-old *ProH1:H1* plants (ProH1-A, -B and -C) cultured in normal conditions (left panel). Photograph taken 6 days after placing plants (previously subjected during 10 days at 4°C) 6 h at –8°C and then 2 days at 4°C, to recover in normal growth conditions (right panel).
(d) Kinetics of induction of *HaHB1* in 3-week-old *ProH1:H1* placed at 4°C for the indicated time periods; transcript levels were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and standard deviations calculated from three independent samples. All the values were normalized with respect to the value measured at time 0, arbitrarily assigned a value of one.

HaHB1. 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

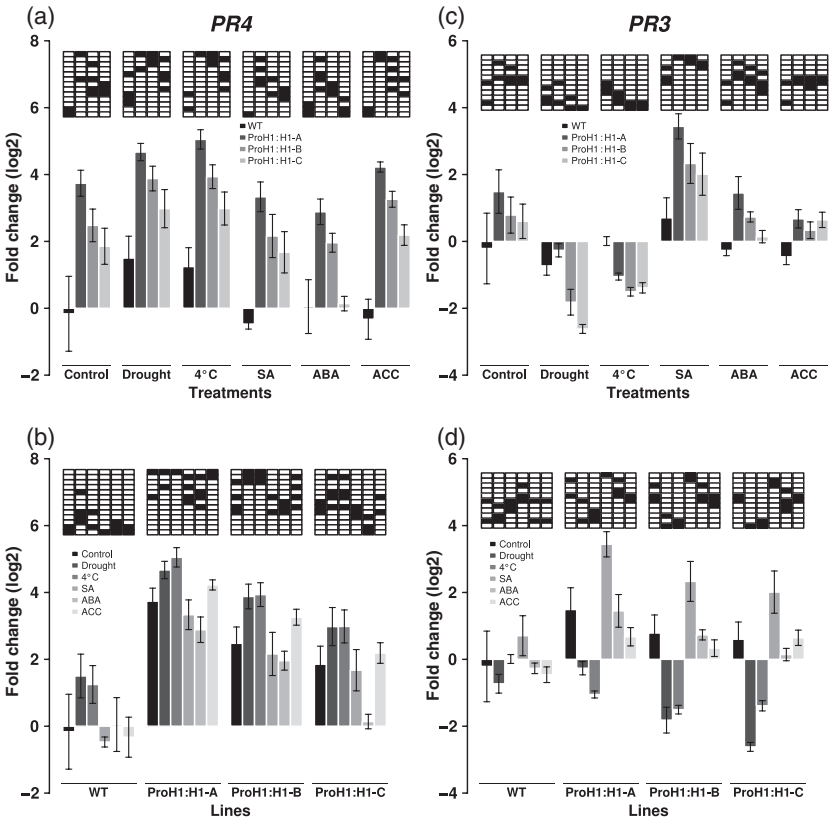

Figure 5. Expression levels of *AtPR4* and *AtPR3* in transgenic *ProH1:H1* and wild type plants subjected to different stress treatments.
(a, b) *PR4* and (c, d) *PR3* expression levels in *ProH1:H1* plants or wild-type (WT) subjected to drought, 4°C or treated with ACC, SA or ABA for 1 h. Error bars 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.

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 *PR2*, *PR3*, *PR4* and *GLU* in *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

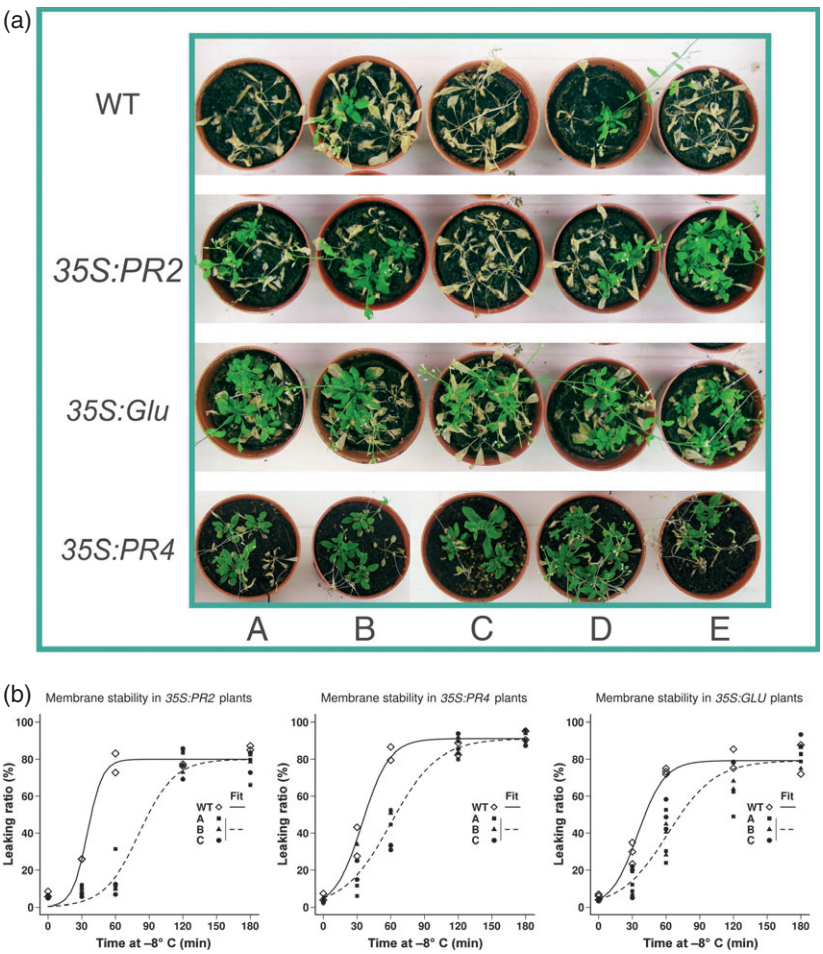

Figure 6. Transgenic plants bearing the construct *35S:GLU*, *35S:PR2* and *35S:PR4* are more tolerant to freezing conditions.
(a) Five independent homozygous lines of *35S:GLU*, *35S:PR2* and *35S:PR4* compared with wild-type (WT) plants subjected to freezing conditions (7 h at –8°C) and then placed for 6 days in normal conditions before taking the photograph.
(b) Membrane stability of the same plants subjected to freezing conditions for the times indicated in the figure. Differences in membrane stability were found between transgenic and WT plants in the three cases ($P < 0.01$).

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

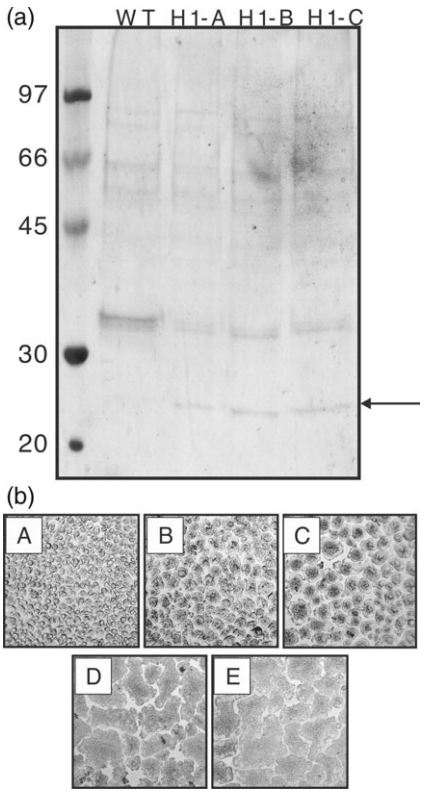

Figure 7. Differential apoplastic proteins in transgenic (*35S:HaHB1*) and wild type plants prevents the formation of large ice crystals.
(a) SDS-PAGE showing the proteins present in the cellular apoplast, isolated from *35S:HaHB1-A*, *-B* and *-C*. Twenty-five-day-old transgenic plants and wild-type (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 *35S: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.

sequenced. This fragment contained a 1011-bp fragment corresponding to the promoter region which was deposited in the Genebank (accession no. HQ287802) and was cloned in the pBI 101.3 binary vector. In this way the promoter of *HaHB1* directs the expression of the *GUS* reporter gene (construct *ProH1:GUS*).

PLACE database (http://www.dna.affrc.go.jp/PLACE/) was used to search for *cis*-elements in the promoter region.

ProH1:H: this clone was obtained by replacing the *GUS* gene in the *ProH1:GUS* construct.

35S:AtHB13: the *AtHB13* cDNA was cloned in pDONR221 using the BP Clonase™ II (Invitrogen, http://www.invitrogen.com); and subsequently transferred to the vector pEarlyGate103 (Earley *et al.*, 2006) by recombination with the LR Clonase™ II (Invitrogen).

35S:PR2, *35S:PR4* and *35S:GLU*. These Arabidopsis genes were amplified by PCR using genomic Arabidopsis DNA as probe and the oligonucleotides described in Table S3. The amplification products were cloned in the pBI121 plasmid, replacing the *GUS* reporter gene.

E. coli DH5 cells were transformed with each construct and, once a positive clone was identified and sequenced, it was used to transform *Agrobacterium tumefaciens* cells (LBA4404) (Höfgen and Willmitzer, 1988).

Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) were grown directly on soil in a growth chamber at 22–24°C under long-day photoperiods at an intensity of approximately 150 μE m^{-2} sec^{-1} in 8 cm diameter ×7 cm height pots during the periods of time indicated in the figures. Plants used for the different treatments were grown in Petri dishes containing Murashige and Skoog medium, 0.8% agar. The dishes were kept at 4°C for 2 days and then transferred to growth chamber conditions and kept for variable periods of time.

Helianthus annuus L. (sunflower CF33, from Advanta seeds) seeds were grown on Petri dishes or in soil pots in a culture room at 28°C for variable times depending on the purpose of the experiment as detailed in the figure legends.

Plant transformation

Transformed *Agrobacterium tumefaciens* strain LBA4404 was used to obtain transgenic *Arabidopsis* plants by the floral dip procedure (Clough and Bent, 1998). Fifteen positive independent lines for each construct were used to select homozygous T3 and T4 plants in order to analyze phenotypes and the expression levels of *HaHB1*. Plants transformed with pBI101.3 were used as negative controls (called WT).

Transient transformation of sunflower and soybean leaf discs was carried out as described for sunflower tissue (Manavella and Chan, 2009).

Histochemical GUS staining

In situ assays of GUS activity were performed as described by Jefferson *et al.* (1987). Whole plants were immersed in a 1 mM 5-bromo-4-chloro-3-indolyl-b-glucuronic acid solution in 100 mM sodium phosphate pH 7.0 and 0.1% Triton X-100 and, after applying vacuum for 5 min; they were incubated at 37°C overnight. Chlorophyll was cleared from green plant tissues by immersing them in 70% ethanol.

Plants *PrH1:GUS* were placed at 4°C during 24 h for cold induction when indicated (Figure S4).

RNA isolation and expression analyses by real-time RT-PCR

RNA for real-time RT-PCR was prepared with Trizol® reagent (Invitrogen™) according to the manufacturer's instructions. RNA (2 μg) was used for the RT reactions using Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega, http://www.promega.com). Quantitative PCRs were essentially carried out as described by Giacomelli *et al.* (2010). Specific oligonucleotides were designed using publicly available sequences (http://arabidopsis.org, http://www.ncbi.nlm.nih.gov web pages, Table S3). In all cases, actin transcripts (*ACTIN2* plus *ACTIN8*) were used as reference genes.

Plant treatments and freezing assays

Two different types of assays were performed as indicated in the figure legends. Non-acclimated 3-week-old plants grown in normal conditions or 2-week-old plants acclimated during 7 or 14 days at 4°C, were then incubated during 6 h at −8°C. After the freezing treatment, non-acclimated plants were placed directly in normal conditions and acclimated plants were previously placed at 4°C during 24–48 h. All the parameters measured after 6 days of recovery.

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 $2.7e^{-5}$ and these groups were merged generating a cluster of 1024 genes (Table S1).

The comparison of this cluster with the genes with altered expression found in other works (Fowler and Thomashow, 2002; Lee *et al.*, 2005; Matsui *et al.*, 2008) was performed with the datasets presented by the authors (Table S2). Sets of common altered-expression genes were obtained by analysis with the R statistical language (R Development Core Team, 2011).

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 *C*2/*C*1 (*L* = *C*1/*C*2) and used as index of injury. *C*1 is the conductance after the treatment and *C*2 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.

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, e_1 and e_2, the second affected by a vector *f* of 'dummy variables' that was zero for WT plants, and one for transgenic lines. The model formulae where:

$$\text{Leaking ratio} \sim \frac{c}{1 + e^{a - \text{time} \times e}} \quad (1)$$

$$\text{Leaking ratio} \sim \frac{c}{1 + e^{a - \text{time} \times (e_1 + f \times e_2)}} \quad (2)$$

The parameters *a*, *c*, *e*, e_1 and e_2 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.

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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.
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REFERENCES

Amme, S., Matros, A., Schlesier, B. and Mock, H.P. (2006) Proteome analysis of cold stress response in Arabidopsis thaliana using DIGE-technology. *J. Exp. Bot.* **57**, 1537–1546.

Arce, A.L., Raineri, J., Capella, M., Cabello, J.V. and Chan, R.L. (2011) Uncharacterized conserved motifs outside the HD-Zip domain in HD-Zip subfamily I transcription factors; a potential source of functional diversity. *BMC Plant Biol.* **11**, 42.

Ariel, F.D., Manavella, P.A., Dezar, C.A. and Chan, R.L. (2007) The true story of the HD-Zip family. *Trends Plant Sci.* **12**, 419–426.

Atici, O. and Nalbantoglum, B. (2003) Antifreeze proteins in higher plants. *Phytochemistry*, **64**, 1187–1196.

Baker, S.S., Wilhelm, K.S. and Thomashow, M.F. (1994) The 5′-region of *Arabidopsis thaliana* cor15a has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Mol. Biol.* **24**, 701–713.

Bravo, L.A. and Griffith, M. (2005) Characterization of antifreeze activity in Antarctic plants. *J. Exp. Bot.* **56**, 1189–1196.

Bray, E.A., Bailey-Serres, J. and Weretilnyk, E. (2000) Responses to abiotic stresses. In *American Society of Plant Biologists Biochemistry and Molecular Biology of Plants* (Gruissen, W., Buchannan, B. and Jones, R., eds). Rockville MD: American Society of Plant Biologists, pp. 1158–1249.

Chan, R.L. and Gonzalez, D.H. (1993) A cDNA encoding an HD-Zip protein from sunflower. *Plant Physiol.* **106**, 1687–1688.

Chan, R.L., Gago, G.M., Palena, C.M. and Gonzalez, D.H. (1998) Homeoboxes in plant development. *Biochim. Biophys. Acta*, **1442**, 1–19.

Chinnusamy, V., Zhu, J. and Zhu, J.K. (2007) Cold stress regulation of gene expression in plants. *Trends Plant Sci.* **12**, 444–451.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Cook, D., Fowler, S., Fiehn, O. and Thomashow, M.F. (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **101**, 15243–15248.

DeVries, A.L. (1971) Glycoproteins as biological antifreeze agents in antarctic fishes. *Science*, **172**, 1152–1155.

Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K. and Pikaard, C.S. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **45**, 616–629.

Fowler, S. and Thomashow, M.F. (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell*, **14**, 1675–1690.

Giacomelli, J.I., Ribichich, K.F., Dezar, C.A. and Chan, R.L. (2010) Expression analyses indicate the involvement of sunflower WRKY transcription factors in stress responses, and phylogenetic reconstructions reveal the existence of a novel clade in the *Asteraceae*. *Plant Sci.* **178**, 398–410.

Gibson, S., Arondel, V., Iba, K. and Somerville, C. (1994) Cloning of a temperature-regulated gene encoding a chloroplast omega-3 desaturase from *Arabidopsis thaliana*. *Plant Physiol.* **106**, 1615–1621.

Gonzalez, D.H., Valle, E.M., Gago, G.M. and Chan, R.L. (1997) Interaction between proteins containing homeodomains associated to leucine zippers from sunflower. *Biochim. Biophys. Acta*, **1351**, 137–149.

Griffith, M. and Yaish, M.W.F. (2004) Antifreeze proteins in overwintering plants: a tale of two activities. *Trends Plant Sci.* **9**, 399–405.

Griffith, M., Ala, P., Yang, D.S., Hon, W.C. and Moffatt, B.A. (1992) Antifreeze protein produced endogenously in winter rye leaves. *Plant Physiol.* **100**, 593–596.

Griffith, M., Lumb, C., Wiseman, S.B., Wisniewski, M., Johnson, R.W. and Marangoni, A.G. (2005) Antifreeze proteins modify the freezing process in planta. *Plant Physiol.* **138**, 330–340.

Guy, C.L., Huber, J.L.A. and Huber, S.C. (1992) Sucrose phosphate synthase and sucrose accumulation at low temperature. *Plant Physiol.* **100**, 502–508.

Hannah, M.A., Heyer, A.G. and Hincha, D.K. (2005) A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*. *PLos Genetics*, **1**, 179–196.

Hanson, J., Johannesson, H. and Engstrom, P. (2001) Sugar-dependent alterations in cotyledon and leaf development in transgenic plants expressing the HD Zip gene ATHB13. *Plant Mol. Biol.* **45**, 247–262.

Hanson, J., Regan, S. and Engström, P. (2002) The expression pattern of the homeobox gene ATHB13 reveals a conservation of transcriptional regulatory mechanisms between *Arabidopsis* and hybrid aspen. *Plant Cell Rep.* **21**, 81–89.

Henriksson, E., Olsson, A.S.B., Johannesson, H., Johansson, H., Hanson, J., Engstrom, P. and Soderman, E. (2005) Homeodomain leucine zipper class I genes in *Arabidopsis*. Expression patterns and phylogenetic relationships. *Plant Physiol.* **139**, 509–518.

Hiilovaara-Teijo, M., Hannukkala, A., Griffith, M., Yu, X.M. and Pihakaski-Maunsbach, K. (1999) Snow-mold-induced apoplastic proteins in winter rye leaves lack antifreeze activity. *Plant Physiol.* **121**, 665–674.

Hincha, D.K., Meins, F. Jr and Schmi, J.M. (1997) β-1,3-Glucanase is cryoprotective in vitro and is accumulated in leaves during cold acclimation. *Plant Physiol.* **114**, 1077–1083.

Höfgen, R. and Willmitzer, L. (1988) Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Res.* **16**, 9977.

Hon, W.C., Griffith, M., Mlynarz, A., Kwok, Y.C. and Yang, D.S. (1995) Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. *Plant Physiol.* **109**, 879–889.

Hothorn, T., Bretz, F. and Westfall, P. (2008) Simultaneous inference in general parametric models. *Biom J.* **50**, 346–363.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.

Johannesson, H., Wang, Y. and Engström, P. (2001) DNA-binding and dimerization preferences of Arabidopsis homeodomain leucine zipper transcription factors in vitro. *Plant Mol. Biol.* **45**, 63–73.

Khodakovskaya, M., McAvoy, R., Peters, J., Wu, H. and Li, Y. (2006) Enhanced cold tolerance in transgenic tobacco expressing a chloroplast omega-3 fatty acid desaturase gene under the control of a cold-inducible promoter. *Planta*, **223**, 1090–1100.

Kim, Y.K., Son, O., Nam, K.H., Kim, G.T., Lee, M.S., Choi, S.Y. and Cheon, C.I. (2007) ATHB23, an *Arabidopsis* class I homeodomain leucine zipper gene, is expressed in the adaxial region of young leaves. *Plant Cell Rep.* **26**, 1179–1185.

Lee, B.H., Henderson, D.A. and Zhu, J.K. (2005) The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. *Plant Cell*, **17**, 3155–3175.

Levitt, J. (1980). *Responses of plants to environmental stresses*, Vol. I and II. New York: Academic Press.

Li, N., Andorfer, C.A. and Duman, J.G. (1998) Enhancement of insect antifreeze protein activity by solutes of low molecular mass. *J. Exp. Biol.* **201**, 2243–2251.

Manavella, P.A. and Chan, R.L. (2009) Transient transformation of sunflower leaf discs via an Agrobacterium-mediated method: applications for gene expression and silencing studies. *Nat. Protoc.* **4**, 1699–1707.

Maruyama, K., Sakuma, Y., Kasuga, M., Ito, Y., Seki, M., Goda, H., Shimada, Y., Yoshida, S., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2004) Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems. *Plant J.* **38**, 982–993.

Maruyama, K., Takeda, M., Kidokoro, S. *et al.* (2009) Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. *Plant Physiol.* **150**, 1972–1980.

Matsui, A., Ishida, J., Morosawa, T. *et al.* (2008) *Arabidopsis* transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. *Plant Cell Physiol.* **49**, 1135–1149.

Mauch, F. and Staehelin, L.A. (1989) Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. *Plant Cell* **1**, 447–457.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Kakubari, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. *FEBS Lett.* **461**, 205–210.

Palena, C.M., Gonzalez, D.H. and Chan, R.L. (1999) A monomer-dimer equilibrium modulates the interaction of the sunflower homeodomain leucine-zipper protein Hahb-4 with DNA. *Biochem. J.* **341**, 81–87.

Puhakainen, T., Hess, M.W., Mäkelä, P., Svensson, J., Heino, P. and Palva, E.T. (2004) Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in Arabidopsis. *Plant Mol. Biol.* **54**, 743–753.

R Development Core Team (2011) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, Available at: http://www.R-project.org/.

Schena, M. and Davis, R.W. (1994) Structure of homeobox-leucine zipper genes suggests a model for the evolution of gene families. *Proc. Natl Acad. Sci. USA*, **91**, 8393–8397.

Seo, P.J., Lee, A.K., Xiang, F. and Park, C.M. (2008) Molecular and functional profiling of Arabidopsis pathogenesis-related genes: insights into their roles in salt response of seed germination. *Plant Cell Physiol.* **49**, 334–344.

Seo, P.J., Kim, M.J., Park, J.Y., Kim, S.Y., Jeon, J., Lee, Y.H., Kim, J. and Park, C.M. (2010) Cold activation of a plasma membrane-tethered NAC transcription factor induces a pathogen resistance response in Arabidopsis. *Plant J.* **61**, 661–671.

Strimmer, K. (2008) A unified approach to false discovery rate estimation. *BMC Bioinformatics*, **9**, 303.

Sukumaran, N.P. and Weiser, C.J. (1972) Freezing injury in potato leaves. *Plant Physiol.* **50**, 564–567.

Taiz, L. and Zeiger, E. (2002) *Plant Physiology*, 3rd edn. Sunderland, MA: Sinauer Associates, Inc., Publishers.

Takenaka, Y., Nakano, S., Tamoi, M., Sakuda, S. and Fukamizo, T. (2009) Chitinase gene expression in response to environmental stresses in *Arabidopsis thaliana:* chitinase inhibitor Allosamidin enhances stress tolerance. *Biosci. Biotech. Biochem.* **73**, 1066–1071.

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

Thomashow, M.F. (2010) Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiol.* **154**, 571–577.

Tomczak, M.M., Hincha, D.K., Estrada, S.D., Wolkers, W.F., Crowe, L.M., Feeney, R.E., Tablin, F. and Crowe, J.H. (2002) A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. *Biophys. J.* **82**, 874–881.

Yu, X.M., Griffith, M. and Wiseman, S.B. (2001) Ethylene induces antifreeze activity in winter rye leaves. *Plant Physiol.* **126**, 1232–1240.

Zhang, X., Fowler, S.G., Cheng, H., Lou, Y., Rhee, S.Y., Stockinger, E.J. and Thomashow, M.F. (2004) Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBFregulon that differs from that of freezing-tolerant Arabidopsis. *Plant J.* **39**, 905–919.

