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The homologous homeodomain-leucine zipper transcription factors HaHB1 and AtHB13 confer tolerance to drought and salinity stresses via the induction of proteins that stabilize membranes

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Summary

Transgenic approaches to conferring tolerance to abiotic stresses have mostly resulted in some degree of plant yield penalty under normal or mild stress conditions. Recently, we have reported that the homeodomain-leucine zipper transcription factors (TFs) HaHB1 and AtHB13 were able to confer tolerance to freezing temperatures via the induction of glucanase (GLU and PR2) and chitinase (PR4) proteins. In the present study, we show that the expression of these TFs, as well as that of their putative targets *AtPR2*, *AtPR4* and *AtGLU,* is up-regulated by drought and salinity stresses. Transgenic plants overexpressing separately these five genes exhibited tolerance to severe drought and salinity stresses, displaying a cell membrane stabilization mechanism. Under normal or mild stress conditions, these plants achieved an improved yield associated with higher chlorophyll content. Moreover, overexpression of the sunflower *HaHB1* gene from its own, inducible, promoter conferred a high drought-stress tolerance without yield penalty under normal or mild stress conditions. We propose these TFs as potential biotechnological tools to breed crops for tolerance to multiple stresses and for increased yield.

Introduction

Plants have evolved molecular and physiological mechanisms to cope with different abiotic stresses generally involving a common feature, the re-establishment of cellular homoeostasis. This re-establishment is usually achieved by accumulating protective molecules such as compatible solutes and molecular chaperones, reducing water loss, and mitigating the effects of stress-induced toxic metabolites by means of detoxifying enzymes (Bartels and Sunkar, 2005; Bray *et al.*, 2000; Hirayama and Shinozaki, 2010; Umezawa *et al.*, 2006).

Among abiotic stresses, drought is the major limiting factor in agricultural productivity. A great effort has been invested to understand the molecular basis of stress adaptation and to generate crops with increased stress tolerance (Wang *et al.*, 2003). Drought tolerance has been used as a key parameter to select for transgenic stress tolerance in model plant species and crops. However, in most cases, the successful development of transgenics with high stress tolerance has resulted in yield decrease under normal or mild stress growth conditions (Skirycz *et al.*, 2011). Most of these drought-stress tolerance mechanisms involved stomatal closure and water efficient use with the consequent decrease in photosynthetic rate (Kasuga *et al.*, 1999). Nonetheless, a few transgenic events have been described as stress tolerant with improved yield; the mechanisms by which these genes function are largely unknown (Castiglioni *et al.*, 2008; Li *et al.*, 2008; Nelson *et al.*, 2007).

Plant transcription factors (TFs) play a key role in regulating whole stress response cascades and are considered key targets for improving stress tolerance (Century *et al.*, 2008). Several TFs belonging to different families have been identified as biotechnological tools to improve plant tolerance to stress (Arce *et al.*, 2008). In a recent study, 25 Arabidopsis genes were identified to confer drought-stress tolerance without growth penalties, either in gain- or loss-of-function background (Skirycz *et al.*, 2011). Several of the corresponding transgenic lines, which showed enhanced tolerance to lethal drought, were analysed in a mild stress assay; two of the lines displayed an increase in height, while the remaining lines were reduced in growth under normal or drought conditions. This indicates that enhanced survival under severe drought is not a good indicator for improved growth performance under mild stress conditions, considering that, limited water availability rarely causes plant death in temperate climates, but it rather restricts yield (Skirycz *et al.*, 2011). Among the 25 genes tested, several encoded TFs, but none of them belonged to the HD-Zip family.

TFs from the HD-Zip I subfamily, whose members exhibit a homeodomain (HD) associated to a leucine zipper (LZ), have been characterized as active participants in the adaptive response to several abiotic stresses, and the expression of most of them is regulated by drought, salt and abscisic acid (ABA) in different tissues and organs (Ariel *et al.*, 2007; Henriksson *et al.*, 2005). Overexpression of HD-Zip genes from sunflower and Medicago conferred abiotic stress tolerance, which was associated with certain phenotypic changes. For instance, the ectopic expression of the sunflower *HaHB4* in Arabidopsis (either constitutive or inducible) resulted in an enhanced tolerance to drought, salinity and herbivore attack, with development of shorter stems and internodes, rounded leaves and more compact inflorescences (Cabello *et al.*, 2007; Dezar *et al.*,

2005; Manavella *et al.*, 2006, 2008). Furthermore, the *Medicago truncatula* MtHB1 was identified as a salt-stress-regulated gene and its loss-of-function mutant showed shorter roots. It is argued that root elongation phenotypes and lateral root inhibition are salinity avoidance mechanisms (Ariel *et al.*, 2010).

HaHB1 and *AtHB13* are homologous members of the sunflower and Arabidopsis HD-Zip I subfamily, respectively (Ariel *et al.*, 2007; Chan *et al.*, 1998; Gonzalez *et al.*, 1997). The encoded proteins are not only highly conserved within the HD-Zip domain but also outside this domain, and they are classified into the same phylogenetic group (Arce *et al.*, 2011). It was recently reported that transgenic plants overexpressing these genes showed similar phenotypes characterized by a marked tolerance to freezing temperatures mediated by the induction of pathogenesis-related (PR) proteins and the stabilization of cell membranes (Cabello *et al.*, 2012).

Pathogenesis-related proteins are a group of heterogeneous proteins encoded by genes that are rapidly induced by pathogenic infections and by the hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). The PRs include GLUs, chitinases and thaumatin-like proteins and have been widely used as molecular markers for resistance to pathogens and for the systemic acquired response (Van Loon *et al.*, 2006). Recently, PR-encoding genes were shown to be regulated by environmental factors, including light and abiotic stresses and by developmental cues (Jiang *et al.*, 2007; Seo *et al.*, 2008). A sea-island cotton thaumatin-like protein gene (*GbTLP1*) was reported to confer a considerable tolerance to abiotic stresses including salinity and drought, when overexpressed in tobacco (Munis *et al.*, 2010).

As tolerance to different abiotic stresses share common mechanisms, we investigated the role of membrane stability provided by PR proteins that are induced by the HD-Zip TFs HaHB1 and AtHB13 in the response to stresses other than those caused by freezing temperatures. In this study, we show that the ectopic expression of *HaHB1* in Arabidopsis, either from its own promoter or from the *Cauliflower mosaic virus* 35S constitutive promoter, conferred tolerance to drought and salinity stresses, with no yield penalties under normal or mild stress growth conditions. We report similar results when the Arabidopsis homologue *AtHB13* or HaHB1/AtHB13 target genes encoding a β-GLU, the β-GLU and PR protein 2 (PR2) and the chitinase PR protein 4 (PR4) were overexpressed. Moreover, these targets are shown to be down-regulated in the *athb13* mutant background, indicating that they are, at least in part, responsible for the multiple abiotic stress tolerance achieved in the overexpressing background.

Results

HaHB1 and *AtHB13* expression is up-regulated by drought

To study the expression of *HaHB1* and *AtHB13* under abiotic stress conditions, total RNA was isolated at various time points from R1 *Helianthus annuus* leaves and 25-day-old wild-type (WT) Arabidopsis rosette leaves, both subjected to drought stress for 10 and 14 days, respectively, and transcript levels of each gene were determined by quantitative RT-PCR (qRT-PCR). Expression of *HaHB1* was found to be induced by about 20-fold after 4 days of stress and that of *AtHB13* by about fivefold after 8 days of stress, both slowly decreasing after this period (Figure 1).

Ectopic expression of *HaHB1* or *AtHB13* confers tolerance to drought and is associated with increased yield

As *HaHB1*and *AtHB13* were found to be induced by drought, the two genes were overexpressed in Arabidopsis under the control of the 35S promoter to determine their growth phenotype under this stress. Plants transformed with the pBI 101.3 plasmid were used as controls (hereafter referred to as WT). Three independent lines (16 plants per line) of each genotype were grown under normal conditions for 4 weeks, and watering was stopped for the following 16 days; this treatment gradually resulted in a severe drought stress. After that, the plants were watered and allowed to recover for a period of 2 days. A significant increase in the survival rate of the *HaHB1* and *AtHB13* lines of 4.4- and 6.5-fold on average, respectively, was observed as compared with WT (Table 1, Figure 2a). Similar results were obtained when the experiment was repeated with plants in the vegetative or reproductive stage (four repetitions for each stage).

The membrane stability status of the *HaHB1* and *AtHB13* overexpressing lines was evaluated during the above-mentioned drought treatment by measuring electrolyte leakage. The results indicated that the overexpressing lines displayed a better cell membrane stabilization mechanism than their WT counterparts, especially during the later/severe stress period (Figure 2b). For instance, WT plants displayed almost 100% electrolyte leakage after 15 days of drought treatment, while the *HaHB1*- and *AtHB13*-expressing transgenic plants showed leakage of only 20%–40% (depending on the genotype and line). The chlorophyll content of the *HaHB1* and *AtHB13* overexpressing lines was also quantified during the drought treatment as an indica-

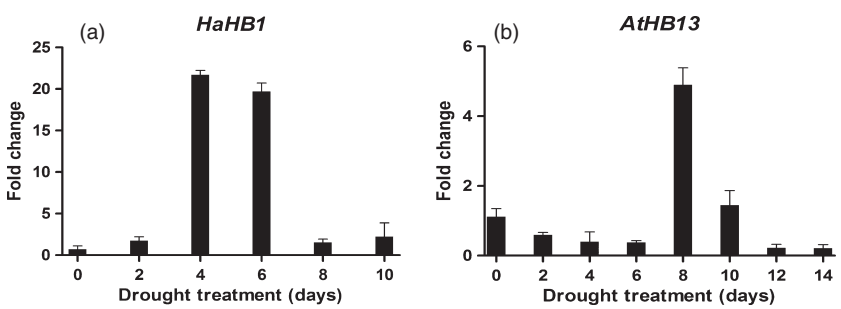

Figure 1 Sunflower *HaHB1* and Arabidopsis *AtHB13* expression is up-regulated by drought. Kinetics of expression of *HaHB1* in sunflower R1 leaves (a) and *AtHB13* in 21-day-old seedlings (b) subjected to drought stress during the indicated periods of time. Transcript levels were quantified by qRT-PCR and the values normalized with respect to that measured at time 0, arbitrarily assigned a value of one. Error bars are standard deviations calculated from three independent samples. Actin transcripts (*ACTIN2* and *ACTIN8*) were used as a reference.

Table 1 Survival rate of overexpressing lines for each of *HahB1*, *AtHB13*, *PR2*, *PR4* and *GLU*

Genotype	Average number of survivors (%)	SE	*N*
35S:HaHB1			
WT	19	7	112
H1-A	94	9	112
H1-B	81	13	112
H1-C	78	16	112
35S:AtHB13			
WT	13	11	64
A13-A	86	6	64
A13-B	85	11	64
A13-C	71	14	64
35S:PR2			
WT	31	17	64
PR2-A	72	13	64
PR2-B	50	8	64
PR2-C	60	13	64
35S:PR4			
WT	22	8	64
PR4-A	73	21	64
PR4-B	80	24	64
PR4-C	65	16	64
35S:GLU			
WT	29	7	64
GLU-A	75	21	64
GLU-B	83	20	64
GLU-C	79	10	64

WT, wild-type.

A total of three overexpressing lines for each genotype, with 16 plants per line, were assayed under severe drought. Data represent three experimental repetitions, and it is reported as the percentage of average survival rate with the standard error (SE).

tion of plant health and stress-induced senescence. These lines exhibited higher chlorophyll content (1.8- and 1.3-fold in *HaHB1* and *AtHB13* overexpressing lines, respectively) than WT during the stress treatment, a finding consistent with their more stabilized membranes status (Figure 2c).

While tolerance to a lethal stress has value as a biotechnological indicator, less extreme stress conditions are more widespread in agriculture, and plants were therefore subjected to relatively mild stress conditions in our study. Four-week-old *HaHB1* overexpressing and WT plants grown under normal conditions were given a regular watering regime or subjected to mild stress by stopping watering for 10 days. After that, the pot weight was maintained constant by checking it daily and adding water to compensate for any loss (see Experimental procedures). This mild water-stress treatment did not cause plant death; plants were able to flower and set seeds, so that seed productivity could be measured for each plant. Figure 2d shows the seed weight obtained for *HaHB1* and WT plants under normal and mild stress conditions. Under normal watering, the *HaHB1* overexpressing and WT plants produced a similar seed weight of around 160–180 mg/plant. They both showed a decrease in seed yield after a continuous mild stress; however, the decrease in seed production observed for WT plants was significantly larger (−43% average) than for the overexpressing plants (−30% average).

Arabidopsis *PR2* and *PR4* are induced by drought, and ectopic overexpression of these genes and *GLU* confers tolerance to drought

Expression of the genes encoding GLUs and chitinases was found to be up-regulated in the *HaHB1* overexpressing background, as previously revealed by transcript profiling (Cabello *et al.*, 2012). Furthermore, overexpression of the homologous *AtHB13* gene or its indirect targets *PR2*, *PR4* and *GLU* were found to confer tolerance to freezing temperatures with stabilized membranes. To verify whether the HD-Zip TF target genes were also positively regulated by drought stress, total RNA was isolated from 25-day-old Arabidopsis plants subjected to severe drought (watering was completely stopped) for a 14-day period. *PR2* transcripts were observed to increase significantly from day 12 till day 14 following drought (Figure 3a). After that, the plants were too severely damaged to extract RNA. *PR4* expression was induced after 8 days of drought before decreasing slowly, while *GLU* expression could not be detected after this stress treatment (Figure 3a).

Each of the *PR2*, *PR4* and *GLU* genes was overexpressed in Arabidopsis (under the control of the CaMV 35S promoter), and their phenotype was characterized under conditions of drought stress. Three independent lines (16 plants per line) of each genotype were grown under normal watering conditions for 25 days, and watering was stopped for the next 16 days, resulting in a potentially lethal drought condition. After this treatment, plants were re-watered, placed in the culture chamber to recover (or not), and survival rate was determined. A significant increase in the survival rate of each of the *PR2*-, *PR4*- and *GLU*-expressing transgenic lines of 2.0-, 3.3- and 2.8-fold on average was observed compared with WT, respectively (Table 1). Notably, *GLU*-expressing plants exhibited tolerance in spite of the fact that transcripts of this gene could not be detected in stressed WT leaves (Figure 3). Similar results were obtained when the experiment was performed in triplicate, using plants in the vegetative or reproductive stage.

The membrane stability status of each of the *PR2*, *PR4* and *GLU* overexpressing lines was evaluated during the drought treatment, and the results, shown in Figure 3b, indicated that overexpressing lines displayed a better cell membrane stabilization mechanism than their WT counterparts. For instance, the *PR2*-, *PR4*- and *GLU*-expressing transgenic lines had on average a 40%, 30% and 45% of electrolyte leakage, respectively, compared with 100% for WT plants after 15 days of drought treatment. The chlorophyll content of these overexpressing lines was quantified under the same conditions of drought stress. The results, shown in Figure 3c, indicated that the transgenic plants had more chlorophyll than their corresponding WTs, that is average 1.6-, 1.4- and 1.7-fold on average, respectively.

HaHB1, *AtHB13, PR2, PR4* and *GLU* genes are up-regulated by salinity stress and their overexpression confers tolerance to salinity associated with stabilized cell membranes and increased yield

As crosstalk is known to exist between abiotic stress signal transduction pathways (Nakashima *et al.*, 2009; Xiong *et al.*,

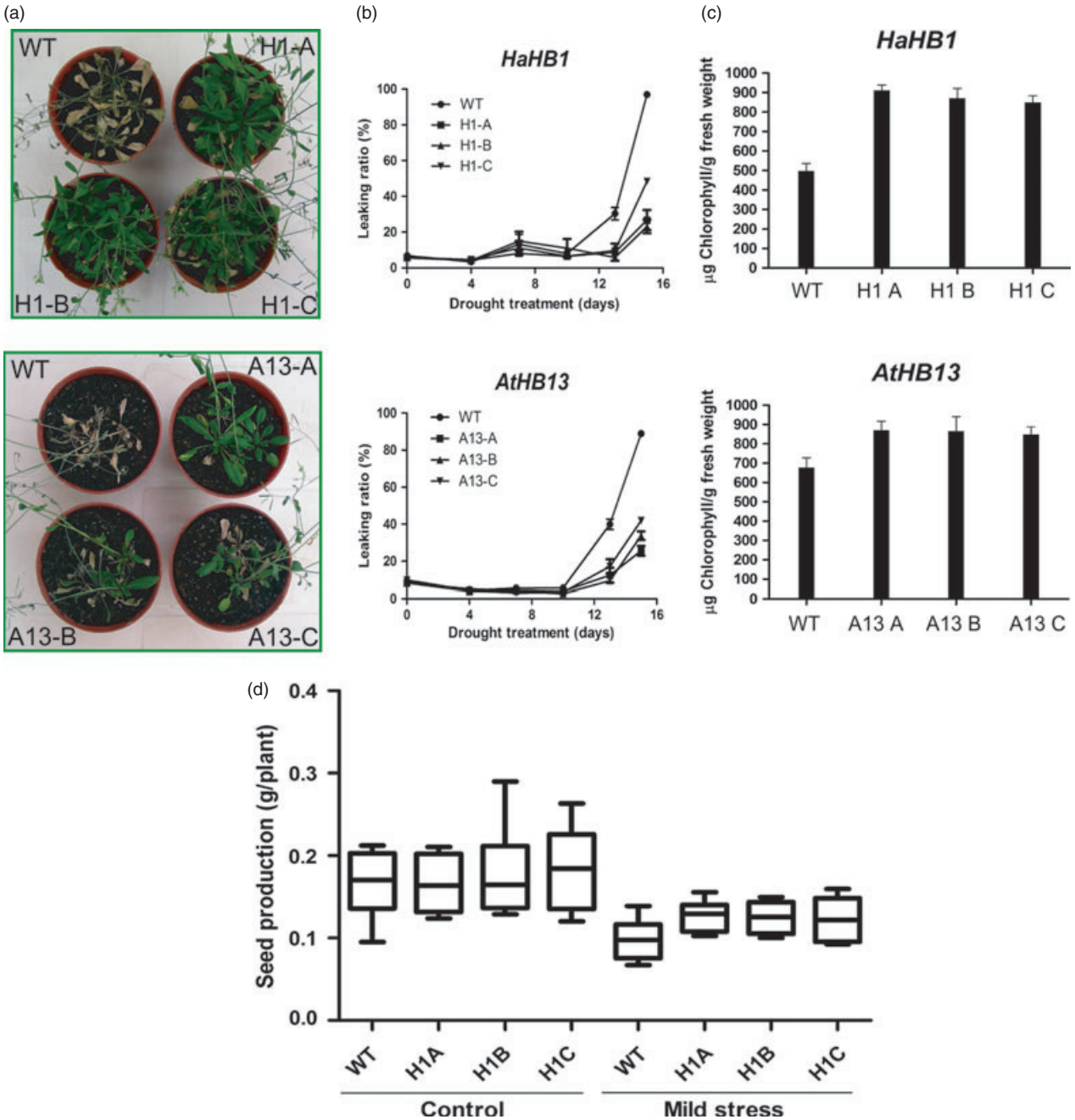

Figure 2 *HaHB1* and *AtHB13* overexpressing lines are tolerant to drought and exhibit stabilized membranes with a higher yield under mild stress. (a) Illustrative photograph of 25-day-old *HaHB1*overexpressing plants (H1-A, H1-B and H1-C), *AtHB13* overexpressing plants (A13-A, A13-B, A1-C) and wild-type (WT) plants grown under normal conditions and then subjected to drought for a period of 17 days. A total of three overexpressing lines for each genotype with 16 plants per line were assayed in triplicate. The photographs were taken 4 days after rehydration. (b) Membrane stability status of *HaHB1* overexpressing (H1-A, H1-B and H1-C) and *AtHB13* overexpressing (A13-A, A13-B, A1-C) lines subjected to drought stress during a period of 17 days. The membrane stability assay was performed on 25-day-old transgenics. Data is presented as a percentage of electrolyte leakage. (c) Chlorophyll content of *HaHB1* overexpressing lines under drought stress as related to plant fresh weight. Samples were collected from 25-day-old plants, and chlorophyll content (µg chlorophyll/g fresh weight) was quantified after 10 days of stress. (d) Seed production of *HaHB1* overexpressing lines under drought conditions. Weight of seeds (g/plant) was obtained after harvesting of 35S:*HaHB1* overexpressing and WT plants grown in under normal conditions or subjected to a mild stress throughout their life cycle, as described in the Experimental procedures. The assay was replicated three times, and standard errors were calculated from data obtained from four plants per genotype for each technical repetition. Asterisks indicate a significant difference between genotypes under the same condition with $P < 0.05$, using ANOVA test.

1999), we verified whether *HaHB1*, *AtHB13* and their target genes already participating in the cold and drought responses were also involved in the salinity-mediated pathway. *HaHB1*, *AtHB13, PR2, PR4* and *GLU* transcript levels were quantified in plants subjected to salt stress. Total RNA was isolated at different time points from R1 sunflower leaves and 25-day-old WT

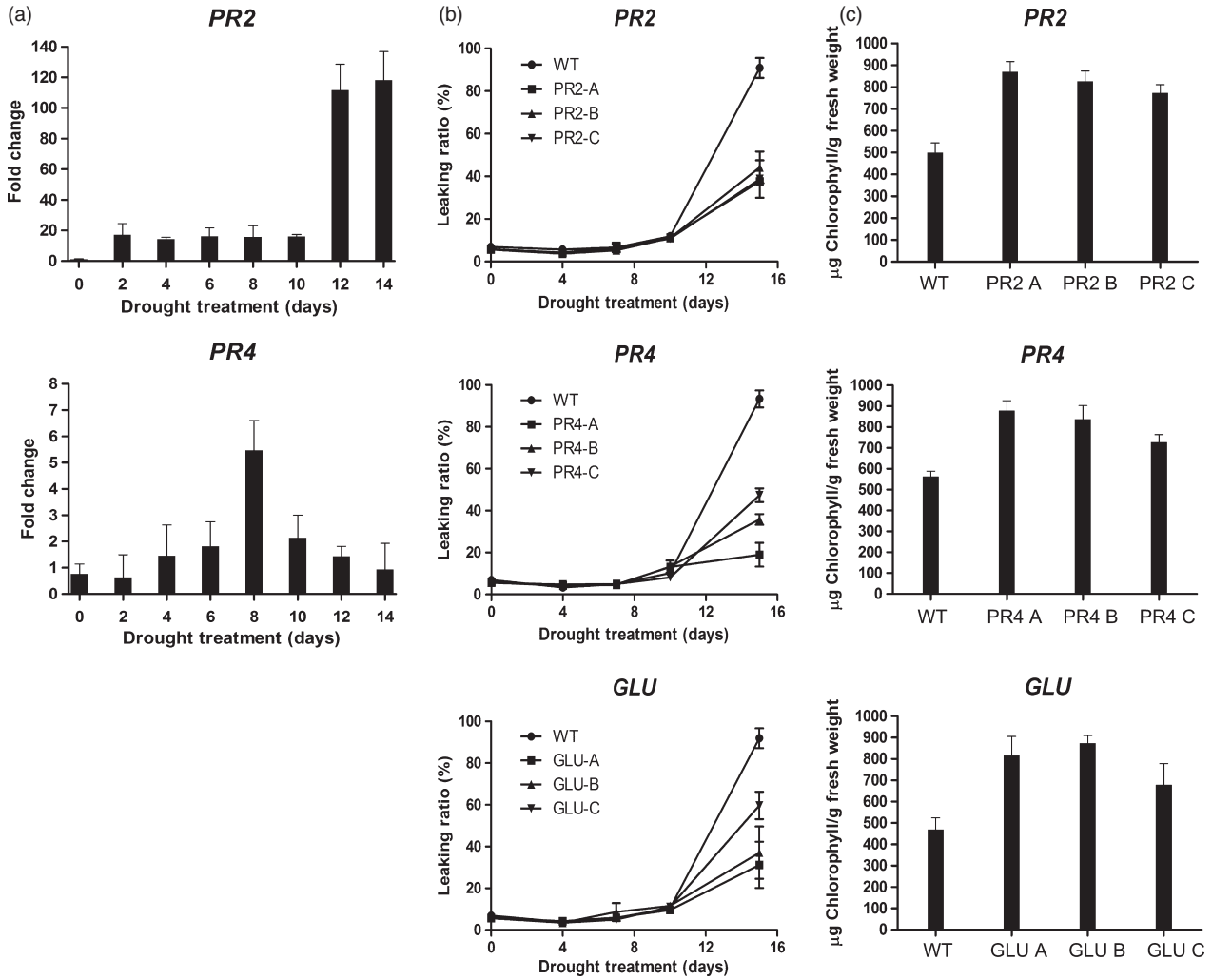

Figure 3 *PR2* and *PR4* are up-regulated by drought, and overexpression of these two genes and *GLU* confers drought tolerance associated with stabilized membranes and higher chlorophyll concentration under drought. (a) Kinetics of expression of *PR2* and *PR4* in 21-day-old seedlings subjected to drought stress during the indicated time points. Transcript levels of PR2 and PR4 were quantified by qRT-PCR and the values normalized to that measured at time 0, using actin transcripts (*ACTIN2* and *ACTIN8*) as reference. Error bars represent standard errors calculated from three independent samples. (b) Membrane stability status of *PR2*, *PR4* and *GLU* overexpressing lines subjected to drought. Membrane stability assay was performed on a total of three independent lines for each transgene, designated as PR2 A, B, C; PR4 A, B, C and GLU A, B, and on wild-type plants (25-day-old), all subjected to drought stress for a period of 16 days. Data is expressed as a percentage of electrolyte leakage. (c) Chlorophyll content of *PR2*, *PR4* and *GLU* overexpressing lines subjected to drought. Samples were collected from 25-day-old plants, and chlorophyll content (µg chlorophyll/g fresh weight) was quantified after 10 days of stress.

Arabidopsis plants as described in Experimental procedures. Figure 4 shows that *HaHB1, AtHB13, PR4* and *GLU* transcripts increased considerably 3 days after the addition of 50 mM NaCl, while *PR2* transcripts accumulated later and only in response to 200 mM NaCl. The relative increase in expression was slightly different for each gene, and it was dependent on the detectable basal expression levels (almost undetectable for *GLU* and *PR2*).

HaHB1, *AtHB13*, *PR2*, *PR4* and *GLU* lines were characterized phenotypically after salinity stress. As for the other abiotic stresses tested, all the transgenic lines displayed a better stabilized membrane status than WT under high salt concentrations, suggesting that these plants have higher tolerance to salinity. However, this enhanced cell membrane stability (CMS) was not sufficient to confer the tolerance needed to cause a difference in survival rate, at least under these particular high salt conditions.

Seed production for three selected lines overexpressing *HaHB1* was tested after a salinity stress. Two-week-old plants (30 individuals), grown under normal conditions were treated with NaCl reaching a final concentration of 300 mM (see Experimental procedures), and after that, they were watered normally. This treatment did not cause plant death, and seeds could be collected. Figure 4c shows that the *HaHB1*-expressing lines and WTs produced a similar yield of around 160–180 mg/plant under normal watering conditions, and they both showed a decrease in yield under salinity stress. However, the reduction in seed production observed for WT was significantly larger (–50% average) than for the *HaHB1* transgenics (–20% average) under this stress.

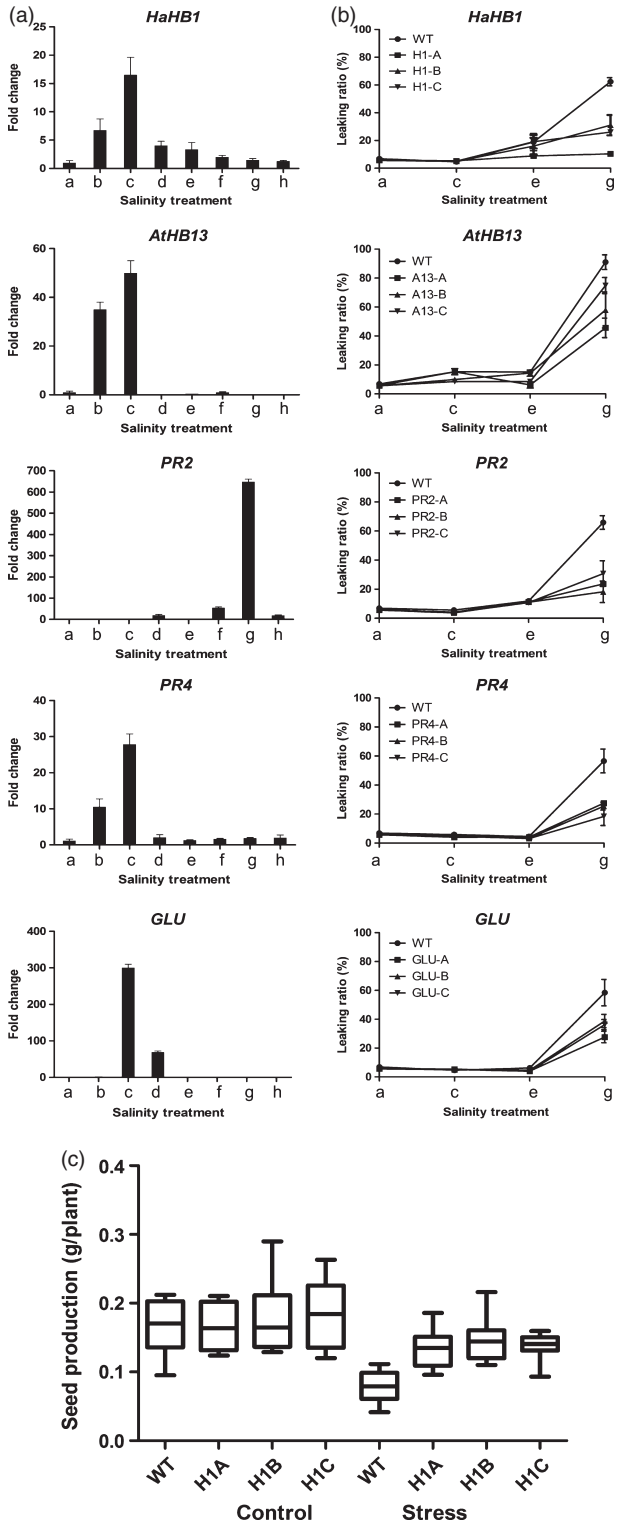

Figure 4 Sunflower *HaHB1* and Arabidopsis *AtHB13, PR2, PR4* and *GLU* are up-regulated by salinity, and overexpression of these genes confers salt tolerance associated with stabilized membranes under this stress. (a) Kinetics of expression of *HaHB1* in sunflower R1 leaves and of *AtHB13, PR2, PR3* and *PR4* in 21-day-old Arabidopsis seedlings subjected to salinity stress during the periods indicated as: a, 1 day after addition of 50 mM NaCl; b, 3 days after addition of 50 mM NaCl; c, 1 day after addition of 150 mM NaCl; d, 3 days after addition of 150 mM NaCl; e, 1 day after addition of 200 mM NaCl; f, 3 days after addition of 200 mM NaCl, and g, 7 days after addition of 200 mM NaCl. Transcript levels were quantified by qRT-PCR and the values normalized to that measured at time 0, using actin transcripts (*ACTIN2* and *ACTIN8*) as reference. Error bars are standard errors calculated from three independent samples. (b) Membrane stability assay performed with 25-day-old *35S:HaHB1, 35S:AtHB13, 35S:PR2, 35S:PR4* and *35S:GLU* (three independent lines per transgene), designated as H1-A, -B, -C; A13-A, -B, -C, PR2-A, -B, -C, PR4-A, -B,- C and GLU-A, -B, -C, respectively, and with wild-type (WT) plants, after being subjected to salinity stress during the time periods described in (a). Data is expressed as a percentage of electrolyte leakage. (c) Weight of seeds obtained from *35S:HaHB1* (H1-A; H1-B; H1-C) and WT plants grown under normal conditions or subjected to a salinity stress as described in the Experimental procedures. The assay was replicated four times, and standard deviation was calculated from data obtained from four plants per genotype for each experiment. Asterisks indicate a significant difference between transgenic and WT plants under the same conditions with $P < 0.05$, using ANOVA test.

PR2, PR4 and GLU proteins are targets of AtHB13

Homozygous lines for the *athb13* mutant (ABRC seed stock) were selected after reproduction, which is grown under normal conditions, and RNA was extracted from 25-day-old rosette leaves. Quantitative RT-PCR analysis indicated that *PR2, PR4* and *GLU* expression was down-regulated in the *athb13* mutant background compared with WT plants (Figure 5).

Overexpression of *HaHB1* from its own promoter confers drought tolerance

A 1021 bp *HaHB1* promoter fragment was previously isolated and found to contain *cis* elements such as ABRE and DRE, characterized as responsive to abiotic stresses (Cabello *et al.*, 2012). *HaHb1* was therefore overexpressed from its own 1021 bp promoter (*PrH1/HaHB1*). The phenotype displayed was more tolerant to drought and salinity than WT (Figure 6), indicating that the *HaHB1* promoter was able to sufficiently induce *HaHB1* expression, triggering tolerance to drought and salinity.

The yield of *PrH1/HaHB1* transgenics, in terms of seed production, was evaluated under normal and mild stress conditions. No significant differences between transgenics and WT were detected (Figure 6b), indicating that *PrH1/HaHB1* was less efficient in providing improved yield than the constitutive *35S:HaHB1* under stress conditions.

Discussion

Although cold, drought and salinity trigger plant responses individually, these abiotic stresses are often studied as a whole because they induce and repress common genes (Zhu, 2001). Abiotic stress signalling pathways are also known to interact with biotic stress signalling pathways in a synergistic or antagonistic manner (Fujita *et al.*, 2006). One example of crosstalk antagonism is the one that exists between the ABA-dependent signalling pathway induced by abiotic stress and the resistance mechanisms triggered by pathogen infection (Mauch-Mani and Mauch, 2005).

Genes participating in a given stress response can have a direct action like the *LEA* proteins, proline synthesis proteins and 'antifreeze' proteins or can regulate signal transduction pathways, such as those coding for kinases and TFs (Mahajan

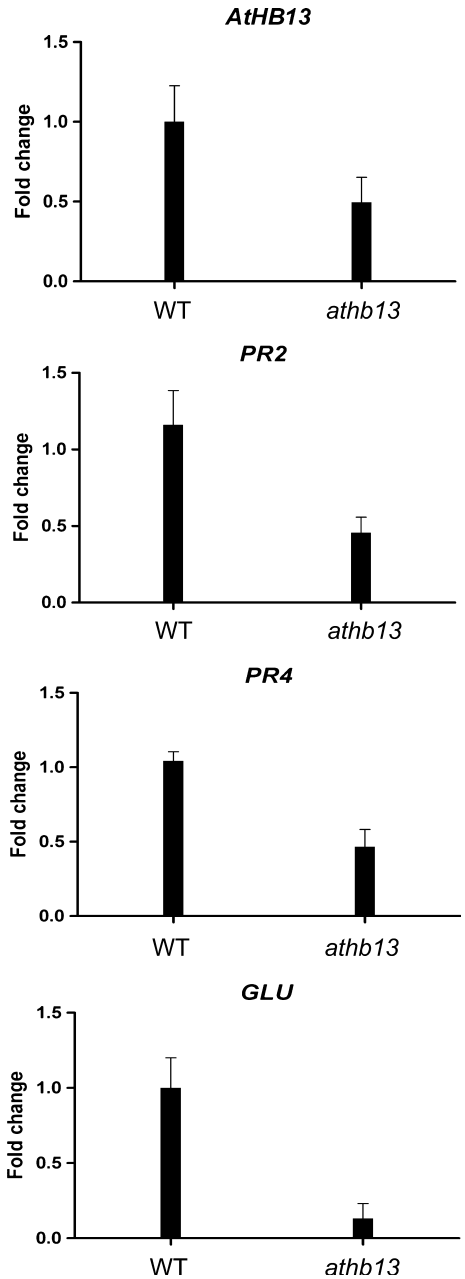

Figure 5 *PR2, PR4* and *GLU* are down-regulated in the *athb13* mutant. RNA was extracted from 25-day-old *athb13* mutant plants, and transcript levels of the putative AtHB13 targets, *PR2*, *PR4* and *GLU* were quantified by qRT-qPCR. Average and standard errors were calculated from three biological replicates.

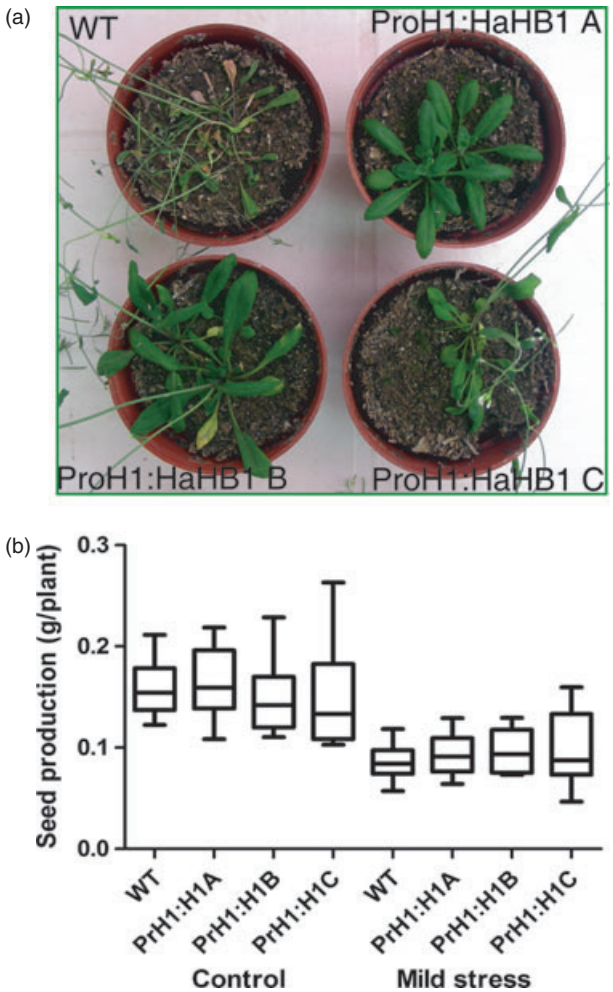

Figure 6 Overexpression of *HaHB1* from its own promoter confers tolerance to drought. (a) Illustrative photograph of 25-day-old *PrH1/HaHB1* (lines A, B and C) and wild-type (WT) plants grown under normal conditions and then subjected to drought over 17 days. The experiment was carried out with 16 plants per genotype and repeated three times. The photograph was taken after 15 days of drought treatment. (b) Weight of seeds obtained from *PrH1/HaHB1* and WT plants grown under normal conditions or subjected to a mild stress as described in the Experimental procedures. The assay was replicated three times and standard error was calculated from data obtained from four plants per genotype for each experimental repetition.

and Tuteja, 2005; Shao *et al.*, 2007). Overexpression of signal transduction pathway genes often produces complex phenotypes; a few examples are *RD26*, *OsMPK5, OsNAC5, ESKIMO1* and the sunflower *HaHB4. RD26* encodes a NAC TF that was first identified to be involved in the drought response, but it was found later to be induced by salt, hormones (ABA and JA) and pathogens (Fujita *et al.*, 2004; Zimmerman *et al.*, 2004), suggesting that it could also be participating in the biotic stress response. *OsMPK5* encodes a MAP kinase that, in rice, positively regulates tolerance to drought, salt and cold, and at the same time, it represses PR gene expression and pathogen resistance (Fujita *et al.*, 2006). *OsNAC5* confers tolerance to salinity when overexpressed, while it is induced by low temperatures and drought (Takasaki *et al.*, 2010). Mutants for the *ESKIMO1* gene exhibit tolerance to drought and cold temperatures (Bouchabke-Coussa *et al.*, 2008). Expression of the gene coding for the sunflower HD-Zip HaHB4 is induced by drought, salt and hormones (ABA, JA and ethylene) and ectopic overexpression of this gene results in a significant tolerance to drought, salt and herbivore attack (Dezar *et al.*, 2005; Gago *et al.*, 2002; Manavella *et al.*, 2006, 2008). These studies illustrate the synergistic interactions that occur between abiotic and biotic stresses, which are mediated by different regulating genes.

HaHB1 and *AtHB13,* encoding non-divergent HD-Zip TFs, were recently reported to confer tolerance to freezing tempera-

tures mediated by the induction of PR proteins, which *in vitro* exhibit antifreeze activity, and by an increase in membrane stability (Cabello *et al.*, 2012). We provide evidence in the current study for the presence of a crosstalk between freezing, drought and salinity stresses through *HaHB1* and *AtHB13*.

Expression of *HaHB1* and *AtHB13* was found to be induced by drought and salinity, concomitantly with that of several PR genes, namely *AtPR2*, *AtPR4* and *AtGLU*, indicating a correlation between expression of these TFs and that of its putative targets. *AtPR2*, *AtPR4* and *AtGLU* expression was up-regulated in the *HaHB1* and *AtHB13* overexpressing backgrounds and down-regulated in the *athb13* mutant, demonstrating the participation of both TFs in the transcriptional regulation of these three proteins. These PR proteins are therefore potential targets of *HaHB1* and *AtHB13,* even though neither of them seem to be a direct target because their promoters lack the *cis* element target site (CAATNATTG, Palena *et al.*, 1999) for HD-Zip I protein binding. *PR4* was induced under both stresses almost at the same time as *AtHB13*, suggesting a probable *AtHB13*-independent regulation. However, transcript profiling of the *HaHB1* and *AtHB13* overexpressing backgrounds strongly suggested that this gene was regulated by these HD-Zip TFs (Cabello *et al.*, 2012).

Previous groups have reported the development of transgenics with high tolerance to drought stress, but with reduced yield (Skirycz *et al.*, 2011). The most remarkable finding of the present study is that the *HaHB1*-expressing transgenic lines exhibited an enhanced tolerance to drought and salt stresses without any loss in yield under normal conditions and with a clear increase in yield under mild stress conditions. In light of this finding, it is reasonable to propose HaHB1 as a potential biotechnological tool to improve tolerance of crops to abiotic stresses, and to suggest that the molecular mechanisms triggered by this HD-Zip protein to confer drought and salt tolerance are different from those previously described.

Drought and salt stress responses in plants usually involve stomatal closure mediated by the ABA signalling pathway and osmolite accumulation (Finkelstein and Rock, 2002; Kasuga *et al.*, 1999). However, implementation of this molecular mechanism in transgenics results in stress-tolerant plants with decreased yields even when they are not stressed. This growth penalty could possibly be avoided by the use of inducible or tissue-specific promoters (Wang *et al.*, 2009). Stress-tolerant transgenic plants, which show no yield penalty when grown under normal conditions, have been developed, but the molecular mechanisms involved are largely unknown. For example, overexpression of Arabidopsis or maize genes possessing the CCAAT binding TF NF-Y conferred drought tolerance in maize with a slight increase in photosynthesis under normal conditions (Nelson *et al.*, 2007). The fresh weight of Arabidopsis plants overexpressing the gene coding for a poly (ADP ribose) polymerase increased both under normal and multiple stress growth conditions (De Block *et al.*, 2005). The heterologous expression in maize of the vacuolar H+ pyrophosphatase (TsVP) from a halophyte dicotyledonous plant resulted in drought tolerance and yield increase by a mechanism involving sugar accumulation and cell membrane stabilization (Lv *et al.*, 2009). In the present study, we have developed Arabidopsis plants overexpressing *HaHB1* or *AtHB13* that exhibit tolerance to drought and salt stresses, associated with an increased yield as compared to WT under stress conditions, and an equivalent yield to WT under non-stress conditions. Membrane stabilization seems to play an important role in this tolerance mechanism, as it was already demonstrated for cold tolerance. This is further supported by the fact that membrane stabilization is also displayed by overexpressing the putative targets of *HaHB1* and *AtHB13*, *PR2*, *PR4* and *GLU*. Furthermore, chlorophyll content was higher in all the transgenics as compared to WT under drought stress, indicating a delay in the drought-induced senescence.

Cell membrane stability has been used as a parameter for assessing tolerance to frost, heat and desiccation (Farooq and Azam, 2006), because it exhibits a positive correlation with physiological and biochemical parameters related to stress tolerance (Franca *et al.*, 2000). CMS has also been proposed as one of the mechanisms providing stress tolerance in plants overexpressing *TsVP* (Lv *et al.*, 2009). However, the molecular mechanisms triggered by this halophyte gene seem to be significantly different from those displayed by the HD-Zip I TFs. For example, no genes involved in osmolite accumulation were detected in *HaHB1* transgenics as it has been in the *TsVP* transgenics, and the resulting membrane stabilization in both transgenics was significantly different. The *TsVP* transgenics exhibited a difference of 5% in ion leakage compared with WT under drought, while *HaHB1*, *AtHB13, PR2*, *PR4* and *GLU* transgenics showed differences of 40%–60% (depending on the genotype and line) compared with WT under lethal conditions of salt and drought stresses.

It is well documented that *PR* genes are up-regulated by pathogen infection, insect attack and wounding (Van Loon *et al.*, 2006). The expression of these genes is also affected by environmental factors such as cold and osmotic stresses (Broekaert *et al.*, 2000). Moreover, in some species such as barley, wheat, grasses and Arabidopsis, a group of PR proteins is synthesized upon exposure to low temperatures (Antikainen and Griffith, 1997; Cabello *et al.*, 2012; Griffith and Yaish, 2004; Hon *et al.*, 1995), suggesting that they may also be involved in response to freezing temperatures. The regulation of PR genes has been recently explored, but to date, PR overexpressors have not been evaluated under conditions of abiotic stresses (Seo *et al.*, 2008; Takenaka *et al.*, 2009). In the present study, it is shown that three genes, *PR2*, *PR4* and *GLU*, were able to confer tolerance of drought and salinity. Taking this finding together with a previous report (Cabello *et al.*, 2012), it can be suggested that at least the three genes provide a multi-cue signalling cascade when plants are subjected to any of these stresses, triggering a CMS mechanism.

Overexpression of one of the HD-Zip TF genes, *HaHB1*, from its own inducible promoter (*PrH1/HaHB1*) produced a normal morphological phenotype under nonstress conditions as previously described (Cabello *et al.*, 2012), but a highly tolerant phenotype with no yield penalty under severe drought, indicating that the induction of *HaHB1* achieved from its own promoter under stress was enough to confer such tolerance. However, and unlike transgenics containing *HaHB1* overexpressed from the constitutive CaMV 35S promoter, *PrH1/HaHB1* transgenics did not show yield differences under mild stress conditions, as compared to WT. This result suggests that *HaHB1*-induced expression in these transgenics was enough to confer tolerance to severe stress but not to make a yield difference from WT under mild stress.

In conclusion, we confirm that *HaHB1* and *AtHB13* are up-regulated by cold, drought and salinity, displaying a

complex tolerance response to multiple abiotic stresses, which is partially achieved by CMS and mediated by the induction of PR proteins. The crosstalk between drought, salt and freezing temperatures through the HaHB1 and AtHB13 TFs is evident, as has been previously observed for other TFs regulating different pathways (Xiong *et al.*, 1999). We propose that HaHB1 and AtHB13 are involved in a conserved mechanism related to drought-, salt- and freezing-mediated enhancement with no yield penalty, functioning to improve tolerance to multiple abiotic stresses.

Experimental procedures

DNA constructs and transgenic plants

The construction of overexpressing vectors, *35S:HaHB1, 35S:AtHB13, 35S:PR2, 35S:PR4, 35S:GLU, 35S:GUS* (β-glucuronidase as control), and *ProH1/HaHB1* as well as the generation of plants transgenic for these constructs were as previously described (Cabello *et al.*, 2012).

Seeds for the *athb13* mutant were obtained from the ABRC (http://www.arabidopsis.org), and homozygous lines were selected after two complete growth cycles.

Plant growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0; Lehle Seeds, Tucson, AZ) WT and transgenic plants were grown directly in soil in a growth chamber at 22–24 °C under long-day photoperiods (16 h of illumination with a mixture of cool-white and GroLux fluorescent lamps, Sylvania, Buenos Aires, Argentina) at an intensity of approximately 150 μE/m/s in 8 × 7-cm pots during the indicated periods of time.

Helianthus annuus (sunflower CF33, Advanta) seeds were germinated on wet filter paper for 7 days, transferred to 8 × 7-cm pots, each with an equal amount of vermiculite and perlite, with one plant per pot, and water-saturated. Plants were placed in a 45-cm plastic square tray and grown normally, except that no further watering was carried out until stress was evident. Plants were harvested and frozen for RNA isolation.

RNA isolation and expression analyses

RNA used for qRT-PCR was isolated from Arabidopsis and sunflower tissues using the Trizol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First, strand cDNA was synthesized from RNA (2 μg) using M-MLV reverse transcriptase (Promega, Madison, WI). qRT-PCR was performed on a Cromos 4 apparatus (MJ Research) in a 20-μl final volume containing 1 μL of SYBR Green 10× (Invitrogen), 8 pmol of each primer, 2 mM $MgCl_2$, 10 μL of a 1 : 25 (Arabidopsis) or 1 : 50 (sunflower) dilution of cDNA and 0.25 U of Platinum Taq (Invitrogen).

Primer pairs used were as follows: H1q [5′- and GGCCGGCAGATCATCAACTTC-3′ (F) and 5′-CCAACCATGGCCAAAACCCTG-3′ (R)], A13q [5′-CTCCATGGATTTGCTTCG -3′ (F) and 5′-TCCCATTTGTGACCCATC-3′ (R)], qAtPR2 [5′-TAAGAAGGAACCAACGTATGAGAA-3′ (F) and 5′-CATAAAAAGCCCACAAGTCTCTAA-3′ (R)], qAtPR4 [5′-GTTTAAGGGTGAAGAACACAAGAAC-3′ (F) and 5′-ATTGAACATTGCTACATCCAAATC -3′ (R)], and qAtGLU [5′-ACCAAAACCTCTTTGACGCTTTAC-3′ (F) 5′-AATACGTTTCCACTCCTCTTCCAG -3′ (R).

Primers for *ACTIN2* and *ACTIN8* genes were used as a reference (Cabello *et al.*, 2012).

Water stress and salinity treatments

Early water-stress treatment in soil was carried out with 16 pots (8 × 7 cm) for each independent transgenic line. Each pot, containing a preweighed amount of a vermiculite-perlite mix, was used to germinate four seeds, water-saturated and placed in a 45-cm plastic square tray. For severe drought assays, plants were not watered again until damage was observed. During this period, samples were harvested for RNA isolation or CMS testing.

Water-stress treatment was also performed under normal conditions using 4-week-old plants, but watering was stopped for approximately additional 16 days until damage became evident. In both cases, photographs were taken 2 days after re-watering.

Mild water-stress treatments were carried out on plants grown under normal conditions for 25 days. Watering was then stopped until a mild stress level was reached, that is, 10 days later. Subsequently, the stress was maintained by watering the pots every 2 days to maintain the same weight in all the pots, a weight equal to that measured after 35 days.

For sample collection for RNA isolation and membrane stability assay, salinity stress was performed as follows. One litre of 50 mM NaCl was added to a plastic square tray with 16 pots, each containing a 25-day-old plant. After 7 days, 1 L of 150 mM NaCl was added. Fourteen days after the first NaCl treatment, 1 L of 200 mM NaCl was added. Samples were collected at one and 3 days after each NaCl treatment for RNA extraction, and 1 day after each NaCl treatment for membrane stability assay.

For measuring seed production, salinity stress was performed as follows. One litre of 100 mM NaCl was added to a plastic tray with 16 pots, each containing a 21-day-old plant. After 7 days, an additional 1 L of 100 mM NaCl was added. Fourteen days after the first NaCl treatment, another additional 1 L of 100 mM NaCl was added. After that, the plants were watered normally until harvesting.

Cell membrane stability testing

The ion leakage technique was carried out essentially as described by Sukumaran and Weiser (1972) with minor changes. Leaves were detached at the indicated periods of time following drought, and at 3 days after each NaCl addition.

Briefly, treated leaves were well washed with distilled water, placed individually in 15 mL of deionized water with continuous agitation in a water bath at 25 °C for 3 h, and conductivity of the solution (C_1) was measured. Then, the leaves were placed in a 65 °C water bath for 16 h with continuous agitation for one additional hour at 25 °C prior to measuring the conductivity of the solution (C_2). The real conductivity was calculated as C_2/C_1 and $L = C_1/C$ was used as an index of injury. L values higher than 0.5 indicate severe injury.

Chlorophyll content quantification

Chlorophyll was quantified following the method described by Chory *et al.* (1994). A total of 100 mg of rosette leaves from 25-day-old plants was pulverized with liquid nitrogen, incubated in a 1.5 mL of 80% acetone in the dark for 30 min and centrifuged for 5 min at 13.362 *g*. Absorbance of the supernatant was measured at 645 and 663 nm. Total chlorophyll content

(μg/mL) was calculated using the following formula: $20.2*A_{645} + 8.02*A_{663}$.

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