HOS10 encodes an R2R3-type MYB transcription factor essential for cold acclimation in plants

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Communicated by André T. Jagendorf, Cornell University, Ithaca, NY, May 19, 2005 (received for review April 9, 2005)

We report the identification and characterization of an *Arabidopsis* mutant, *hos10-1* (for high expression of osmotically responsive genes), in which the expression of *RD29A* and other stress-responsive genes is activated to higher levels or more rapidly activated than in wild-type by low temperature, exogenous abscisic acid (ABA), or salt stress (NaCl). The *hos10-1* plants are extremely sensitive to freezing temperatures, completely unable to acclimate to the cold, and are hypersensitive to NaCl. Induction of *NCED3* (the gene that encodes the rate-limiting enzyme in ABA biosynthesis) by polyethylene glycol-mediated dehydration and ABA accumulation are reduced by this mutation. Detached shoots from the mutant plants display an increased transpiration rate compared with wild-type plants. The *hos10-1* plants exhibit several developmental alterations, such as reduced size, early flowering, and reduced fertility. The *HOS10* gene encodes a putative R2R3-type MYB transcription factor that is localized to the nucleus. Together, these results indicate that HOS10 is an important coordinating factor for responses to abiotic stress and for growth and development.

Arabidopsis | freezing | abscisic acid | *NCED3*

Exposure to low temperature, soil drying, or high levels of salt results in altered expression of a diverse range of plant genes (1). The products of these genes may either directly protect plants against stresses or further control the expression of other target genes. Although the signal transduction pathways responsible for the activation of these genes are still mostly unknown, transcriptional control of some stress-responsive genes is understood to involve transcription factor-binding and activation of cis elements in target genes, such as the *RD29A* (responsive to dehydration 29A) gene promoter (2), which contains the abscisic acid (ABA) responsive element (ABRE) and the dehydration-responsive element (DRE)/C-repeat (CRT) sequences (2).

Transcription factors in the ethylene response element binding protein/apetala 2 family that bind to the DRE/CRT element are termed C-repeat binding factor (CBF)/DRE-binding proteins (DREBs) (3, 4). *CBF/DREB1* genes are rapidly and transiently induced by cold stress and subsequently activate the expression of target genes. *DREB2* genes are also induced by osmotic stress and may confer osmotic stress induction of target stress-responsive genes (4). Ectopic expression of *CBF/DREB1* genes in plants was reported to improve tolerance to cold, drought, and salt stresses (5–7).

Early signaling components upstream of CBF/DREB1 may be subject to specific ubiquitination-mediated degradation, as suggested by the characterization of the *HOS1* gene in *Arabidopsis* (8). *HOS1* encodes a protein with a RING finger motif similar to that present in a group of inhibitor of apoptosis proteins in animals that act as E3 ubiquitin ligases to target certain regulatory proteins for degradation. Recently, *ICE1* (inducer of CBF expression 1) was identified with the *CBF3/DREB1A* promoter::*LUC* screening system (9). ICE1 is a MYC-like basic helix–loop–helix transcriptional activator, and it binds specifically to the MYC recognition sequences in the *CBF3/DREB1A* promoter. *ICE1* is constitutively expressed, and its overexpression enhances the expression of the *CBF* regulon in the cold, resulting in increased freezing tolerance. These results suggest that ICE1 acts as an upstream regulator that positively controls the transcription of *CBF* genes in the cold (9) and may be a target for degradation by HOS1 (8).

ABRE is another major cis element in ABA-responsive gene expression. Two ABRE motifs are important in the ABA-responsive expression of the *Arabidopsis* gene *RD29B* (10). The ABRE-binding proteins (AREBs)/ABRE-binding factors (ABFs) can bind to ABRE and activate ABA-dependent gene expression (10). The AREB/ABF proteins have reduced activity in ABA-deficient *aba2* mutants and the ABA-insensitive *abi1* mutant and enhanced activity in the ABA-hypersensitive *era1* mutant. Hence, activation of the AREB/ABF proteins has been shown to require an ABA-mediated signal (10), which probably involves ABA-dependent phosphorylation. Overexpression of *ABF3* or *AREB2/ABF4* caused ABA hypersensitivity, a reduced transpiration rate, and enhanced drought tolerance (11).

A number of lines of evidence suggest the existence of crosstalk among the drought, salinity, cold, and ABA signal transduction pathways (12, 13). Many transcription-factor genes, including MYBs, were found to be stress-inducible, suggesting that transcription regulation is a part of drought, cold, or salt stress signaling (13, 14). Although acclimation to cold has been associated with the CBF/DREB1 family of transcription factors (5), evidence has revealed a possible CBF-independent pathway that is also necessary for cold acclimation (15).

Here we report that the *Arabidopsis* mutant *hos10-1* exhibits altered expression of low temperature, salt stress, and ABA-responsive genes, some of which belong to the CBF regulon. However, mutation of the *HOS10* locus does not alter expression of the *CBF* family of genes. The *hos10-1* mutant plants show dramatically reduced capacity for cold acclimation and are hypersensitive to dehydration and NaCl. The ability to increase ABA levels in response to dehydration stress also is impaired in *hos10-1* mutant plants. *HOS10* encodes an R2R3-type MYB transcription factor that is localized to the nucleus. It appears that HOS10 is essential for cold acclimation and may affect dehydration stress tolerance in plants by controlling stress-induced ABA biosynthesis.

Materials and Methods

Isolation of *hos10-1* Mutant. *Arabidopsis thaliana* plants (ecotype C24) expressing the RD29A::*LUC* transgene (referred to as wild type) were mutagenized with an *Agrobacterium tumefaciens*-mediated (strain GV3101) T-DNA (portion of the tumor-inducing plasmid that is transferred to plant cells) transformation with the activation tagging vector pSKI015 (12, 16). Seeds

Abbreviations: ABA, abscisic acid; ABRE, ABA responsive element; CRT, C-repeat; CBF, CRT binding factor; DRE, dehydration-responsive element; DREB, DRE-binding protein; PEG, polyethylene glycol.

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www.pnas.org/cgi/doi/10.1073/pnas.0503960102

from T_2 plants were used for screening mutants with altered *RD29A*::*LUC* expression in response to low temperature, ABA, and/or osmotic stress by luminescence imaging with a charge-coupled device camera as described in ref. 12.

ABA Measurement and Real-Time PCR Analysis of *NCED3* Expression. ABA was quantified by using a radioimmuno assay essentially as described in ref. 17. For measurement of low water potential-induced ABA accumulation, 5-d-old seedlings grown on half-strength Murashige and Skoog medium (PhytoTechnology Laboratories, Lenexa, KS) (without addition of sugar) containing 2.5 mM Mes buffer were transferred using nylon mesh overlaid on the agar surface to −1.2-MPa, polyethylene glycol (PEG)-infused plates (18). Control seedlings were transferred to half-strength Murashige and Skoog medium plates. Samples for ABA measurement were collected at 8 h (peak ABA accumulation) and 96 h (steady-state ABA level) after the transfer. For cold treatment, 3-week-old, soil-grown *hos10-1* and wild-type plants were transferred to a growth chamber at 4°C under a long-day photoperiod (16 h of light/8 h of dark). ABA content of untreated and cold-treated plants was assayed at the designated time points (see Fig. 5*C*).

For real-time PCR analysis, total RNA was extracted from untreated or treated (−1.2-MPa, PEG-infused plates) seedlings with the RNeasy plant mini kit (Qiagen, Valencia, CA). First-strand cDNA synthesized with 1 μg of total RNA was used for the real-time PCR. Real-time PCR analysis of *NCED3* expression was performed as described in ref. 19 by using a Sequence Detection System 7700 instrument (Applied Biosystems). Quantification of *NCED3* copy number was conducted by amplifying a 600-bp portion of the *NCED3* cDNA containing the *Taq*man probe-binding site. The primers used were 5′-TCCAGCTCT-TCATTTCCCTAA-3′ (forward) and 5′-CGGCCATT-GAAATAGACCAA-3′ (reverse). The amplified DNA fragment was purified, quantified spectrophotometrically, and diluted appropriately to generate a standard curve for calculation of the copy number of the *NCED3* transcript.

RNA Gel Analysis and Microarray Analyses. Wild-type and *hos10-1* seedlings were grown on separate halves of the same Murashige and Skoog medium agar plates for 14 d and then left untreated or treated with low temperature, ABA, or NaCl. Total RNA was extracted from whole seedlings, and RNA analysis was conducted as described in ref. 16.

Total RNA (20 μl) extracted with the RNeasy plant mini kit (Qiagen) from 21-d-old wild-type and *hos10-1* seedlings after cold treatment (24 h at 0°C) was used to make biotin-labeled cRNA targets. Microarray analysis (Affymetrix GeneChip array) was performed as described in ref. 9.

Cloning of the *HOS10* Gene. The genomic DNA fragment flanking the left border of the inserted T-DNA in *hos10-1* plants was isolated by thermal asymmetric interlaced PCR and subcloned into the cloning vector pBluscript SK(+) (Stratagene) as described in ref. 16. The entire isolated fragment was sequenced.

The coding region of *HOS10* was amplified by RT-PCR by using primer pair 5′-ACTGGAGCTCATGGGAAGATCAC-CATGTTGTG-3′ (forward) and 5′-ACGTTCTAGACACAC-GAGCTAGTAACAAGATC-3′ (reverse). The RT-PCR product was then subcloned into pGEM-T Easy Vector (Promega) and sequenced. The *HOS10* was released from the resulting clone 10-133 and cloned into binary vector 99-1 between SacI and XbaI sites, resulting in an expression cassette of *HOS10* under the control of the cauliflower mosaic virus ^{35}S promoter. The construct was introduced into *hos10-1* mutant plants through an *A. tumefaciens*-mediated (strain GV3101) T-DNA transformation. Primary transformants, which were resistant to 50 mg/l hygromycin (Invitrogen), were transferred to soil to grow to maturity. Progenies of these transformants were examined for *RD29A*::*LUC* expression and for freezing tolerance.

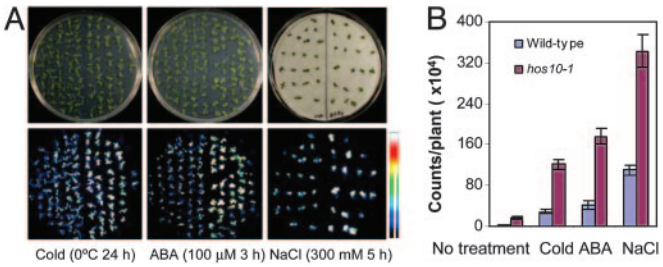

Fig. 1. Elevated *RD29A*::*LUC* expression in the *hos10-1* mutant. (*A*) *RD29A*::*LUC* expression was quantitatively measured as luminescence intensity (counts per plant). Wild-type (right side of plate) and *hos10-1* (left side of plate) seedlings and their luminescence images were taken after stress treatments. The color scale on the right shows the luminescence intensity from dark blue (lowest) to white (highest). (*B*) Quantification of the luminescence intensities of wild-type and *hos10-1* plants shown in *A*. Also shown is no treatment. Error bars represent standard deviation ($n = 20$).

GFP–HOS10 Fusion Protein Construct. The coding region of *HOS10* was amplified from the cDNA clone 10-133 by PCR and cloned in-frame into pEGAD vector between the EcoRI and BamHI sites, and the entire insert and the conjunction regions were sequenced. This construct was then introduced into *Arabidopsis* wild-type plants by floral dip transformation with *Agrobaterium* strain GV3101.

Results

Identification of the *HOS10* Locus. Through a luciferase imaging-based screen (12), we isolated one mutant, *hos10-1*, that has a 3- to 4-fold higher level of *RD29A*::*LUC* expression after low temperature, ABA, and/or NaCl stress treatments (Fig. 1). All 41 F_1 progeny of *hos10-1* backcrossed to wild-type plants exhibited a wild-type phenotype. Approximately three-quarters of the F_2 progeny of the selfed F_1 plants displayed a wild-type phenotype, indicating that *hos10-1* is a recessive mutation in a single nuclear gene.

Regulation of Stress-Responsive Gene Expression in *hos10-1* Plants. Northern hybridizations revealed that, without stress, there was no detectable *RD29A* transcript in *hos10-1* or wild-type plants (Fig. 2*A*). Induction of the endogenous *RD29A*, of the introduced luciferase gene, and of *COR15A*, *ADH*, and *KIN1* genes by cold was more rapid in *hos10-1* than in wild type (Fig. 2*A*). After ABA or NaCl treatment, the expression of the above genes was slightly higher in *hos10-1* than wild type (Fig. 2*A*). The steady-state transcript levels of *P5CS1* (Fig. 2*B*) and of the cold-specific transcription factor genes *CBF1*, *CBF2*, and *CBF3* (Fig. 2 *B* and *C*) were virtually not altered in *hos10-1*.

Analysis of Affymetrix near-full genome GeneChip arrays showed that, compared with wild-type plants, the expression levels of only six genes were higher by at least 2-fold in the *hos10-1* plants after 24 h of cold treatment (Table 1, which is published as supporting information on the PNAS web site). There were also only six genes with lower expression in *hos10-1* by at least 2-fold compared with wild-type plants (Table 2, which is published as supporting information on the PNAS web site). The altered expression of three randomly selected genes (At1g75830, At3g49620, and At5g01600) from the microarray tables was confirmed by RT-PCR analysis (Fig. 2*D*). This number of genes is unusually low compared with estimated targets of other transcription factors but could reflect the limited types of conditions used in our experiments.

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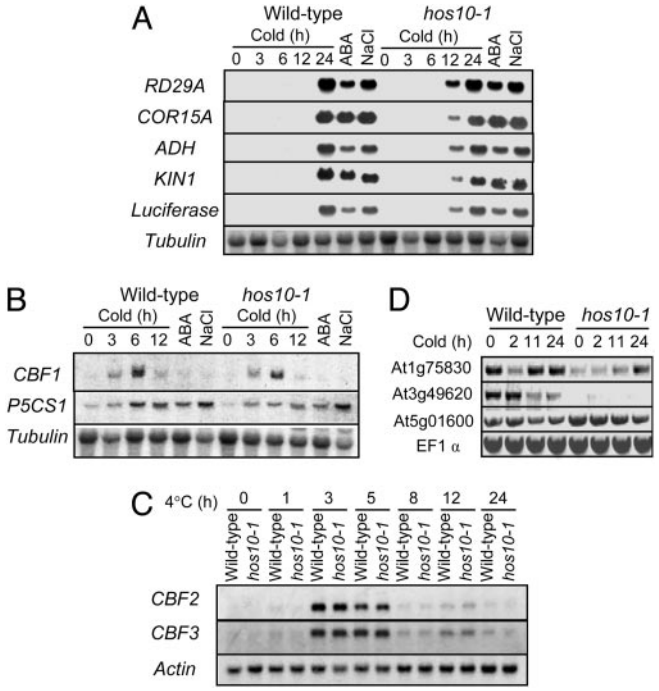

Fig. 2. Gene regulation in *hos10-1* and wild-type plants. (*A–C*) Steady-state transcript abundance of several stress-regulated genes in wild-type and *hos10-1* plants by Northern blot analysis. The blots were also hybridized with probes from t*ubulin* and *actin* genes to ensure equal loading. ''Cold'' means 0°C for indicated time points; ABA, 100 μM for 3 h; NaCl, 300 mM for 4 h. (*D*) RT-PCR analysis of three genes identified in the microarray analysis. The two genes with lower expression in the *hos10-1* than wild type in the microarray encode a plant defensin protein (At1g75830) and an oxidoreductase (At3g49620). The *hos10-1* target gene with higher expression than wild-type in the microarray encodes a ferritin 1 precursor (At5g01600). The elongation factor 1α gene (At1g07920) was used as loading control.

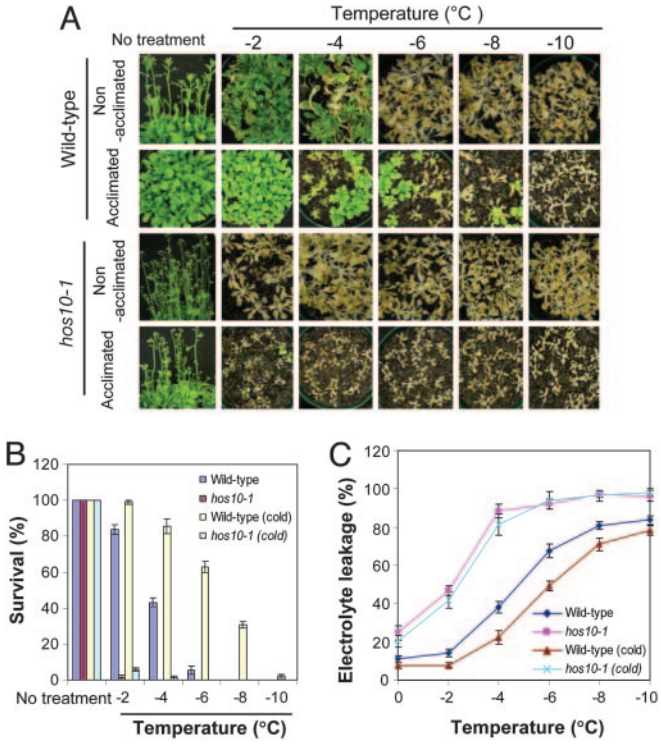

Fig. 3. *hos10-1* mutant plants are defective in cold acclimation. (*A*) Four-week-old plants grown at room temperature or at 4°C for 8 d were used for freezing treatments as described in ref. 15. The photographs were taken 7 d after the freezing treatments. (*B*) Quantitative analysis of the plant survival 7 d after the freezing treatment as shown in *A*. The error bars indicate standard deviation ($n = 60$). (*C*) Electrolyte leakage assay. Shown are wild-type (cold), *hos10-1* (cold), cold-acclimated (4°C for 8 d) wild-type, and *hos10-1* plants. Error bars represent standard deviation ($n = 8$).

***hos10-1* Plants Are Defective in Cold Acclimation.** Non-cold-acclimated young (≈3-week-old) plants of wild type could tolerate lower temperatures than *hos10-1* plants (Fig. 3 *A* and *B*). When acclimated at 4°C for 8 d, the majority of the wild-type plants tolerated freezing temperatures as low as −8°C. However, <2% of the *hos10-1* mutant plants survived freezing at −2°C, and none (nonacclimated or acclimated) survived below −2°C (Fig. 3 *A* and *B*). With or without cold acclimation, detached leaves of *hos10-1* plants also showed more injury than wild type and were unable to increase their freezing tolerance significantly when measured by electrolyte leakage (15) (Fig. 3*C*). Thus, plants carrying the *hos10-1* mutation are extremely sensitive to freezing temperatures and are unable to acclimate to the cold.

***hos10-1* Mutant Plants Are Hypersensitive to NaCl.** The *hos10-1* seedlings form shorter roots than wild-type when grown vertically in germination medium (Fig. 4*A*), and their growth was also substantially inhibited by NaCl stress (Fig. 4*B*). The *hos10-1* mutant plants accumulated essentially the same amounts of Na^+ or K^+ compared with wild type with or without NaCl treatment (Fig. 4 *C* and *D*) (16), indicating that increased sensitivity of *hos10-1* plants to these stresses was not due to impaired Na^+ homeostasis or impaired K^+ acquisition.

***hos10-1* Mutant Plants Are Impaired in ABA Biosynthesis Under Dehydration Stress.** The *hos10-1* mutant plants lost water much faster than wild-type plants during slow dehydration (Fig. 5*A*). This result suggests that, during dehydration, the *hos10-1* plants either may not make enough ABA, or their stomata fail to respond to water-deficit-induced ABA. Under normal, nonstress conditions, *hos10-1* and wild type accumulate similar amounts of ABA. However, there is a much smaller rise in ABA accumulation in *hos10-1* than in wild-type after PEG-induced dehydration stress (Fig. 5*B*). In contrast, we did not find any substantial difference in ABA accumulation between wild-type and *hos10-1* plants during cold acclima-

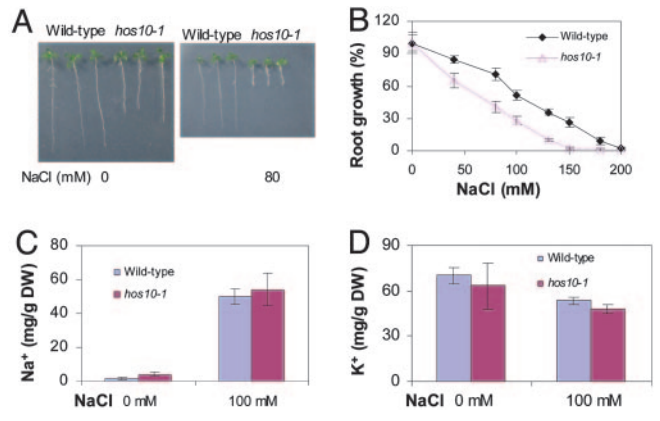

Fig. 4. *hos10-1* mutant plants are hypersensitive to NaCl. (*A*) *hos10-1* plants are hypersensitive to NaCl. (*B*) Dose–response of *hos10-1* and wild-type seedlings to NaCl. Data shown are relative root growth (growth on 0 mM NaCl was considered 100%). Error bars indicate standard deviation ($n = 16$). (*C*) Na^+ accumulation in *hos10-1* and wild-type seedlings. (*D*) K^+ accumulation in *hos10-1* and wild-type seedlings. Error bars in *C* and *D* indicate standard error ($n = 8$).

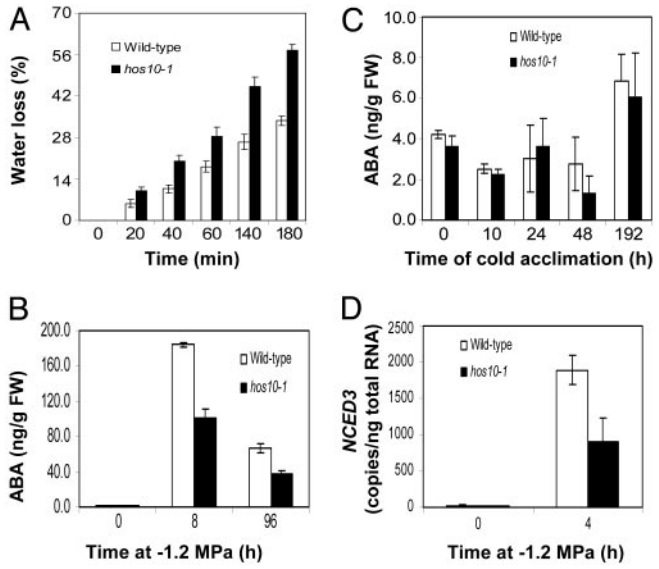

Fig. 5. Water loss and ABA biosynthesis defects in *hos10-1* plants. (*A*) Transpirational water loss as indicated by loss of the initial fresh weight is greater in the *hos10-1* mutant detached shoots. Error bars represent standard deviation ($n = 10$). (*B*) ABA accumulation in wild-type and *hos10-1* plants under PEG-induced dehydration stress. FW, fresh weight. Error bars represent standard deviation ($n = 4–6$). (*C*) Accumulation of ABA in wild-type and *hos10-1* plants during cold acclimation. Error bars represent standard error ($n = 3$). (*D*) Expression of *NCED3* in wild-type and *hos10-1* plants by real-time PCR analysis. Error bars represent standard error ($n = 4$).

tion (Fig. 5*C*) at 4°C. The transcript level of *NCED3* that encodes a key enzyme in ABA biosynthesis is much less in *hos10-1* than in wild-type after PEG-induced dehydration stress (Fig. 5*D*), suggesting that HOS10 controls expression of genes involved in ABA biosynthesis under dehydration stress.

***hos10-1* Mutant Plants Flower Early.** *hos10-1* plants are somewhat smaller than wild type (Fig. 6 *A–C*). Under long-day (16 h of light/8 h of dark) and short-day (8 h of light/16 h of dark) photoperiods, *hos10-1* plants flower earlier than wild type (Fig. 6 *B* and *D*). The vernalization responses of *hos10-1* and wild-type plants were the same (Fig. 6*H*). The expression of *FLC*, a gene encoding a critical flowering time regulator, also is not altered in *hos10-1*, consistent with its unchanged vernalization response (Fig. 6*E*). *hos10-1* plants also have reduced fertility (Fig. 6 *C* and *F*), and their seeds are more round than wild-type (Fig. 6*G*).

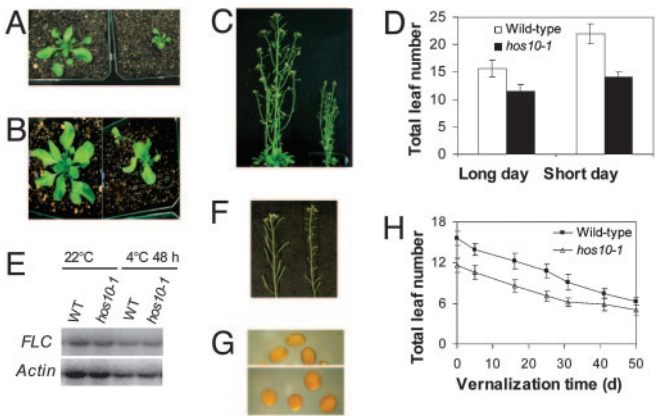

Fig. 6. *hos10-1* mutant plants flower early. (*A*) Three-week-old wild-type (left plant) and *hos10-1* (right plant) plants grown in soil. (*B*) Five-week-old, soil-grown, wild-type (left plant) and *hos10-1* (right plant) plants. (*C*) Mature plants of wild-type (left plant) and *hos10-1* (right plant). (*D*) Quantification of flowering time of *hos10-1* under different photoperiods. (*E*) Expression of *FLC* in *hos10-1* mutant plants. (*F*) Inflorescence of wild-type (left plant) and *hos10-1* (right plant). (*G*) Seeds of wild-type (*Upper*) and *hos10-1* (*Lower*). (*H*) Vernalization response of *hos10-1* mutant plants. Error bars in *D* and *H* indicate standard deviation ($n = 30$).

Identification of the *HOS10* Gene. A DNA fragment flanking the T-DNA insert in *hos10-1* mutant plants was obtained by thermal asymmetric interlaced PCR. This sequence was found to match that of the predicted *Arabidopsis* gene At1g35515. The *HOS10* cDNA was cloned by RT-PCR using RNA prepared from wild-type plants. We conducted a complementation test by constitutive expression of *HOS10* cDNA under the control of the cauliflower mosaic virus ^{35}S promoter. Twenty of 29 *hos10-1* plants transformed with the wild-type *HOS10* cDNA cassette exhibited wild-type phenotypes in the T_2 generation (Fig. 7*B*). Genetic analyses indicated that additional lines carrying T-DNA inserts in the At1g35515 gene (seed stock nos. SALK_122356, SALK_031231, and SALK_088230) are allelic to *hos10-1* (Fig. 7*A*). The expression of *HOS10* is disrupted in *hos10-1* and the other three alleles of *HOS10* (Fig. 7*C*). Excess electrolyte leakage induced by freezing treatments in the other *hos10-1* alleles, before and after cold acclimation, showed that they were all defective in cold acclimation (Fig. 7*D*). In addition, plants carrying allelic mutations of *hos10-1* flower earlier than do their wild-type background strain (*Col-0*) (Fig. 7*E*).

Comparison of the predicted HOS10 amino acid sequence with those of other gene products revealed that HOS10 shares greatest sequence similarities with R2R3-type MYB transcription factors from *Arabidopsis*, cotton, rice, and tomato within the R2 or R3 domain (Fig. 7*F*). MYP transcription factors comprise a large gene family in *Arabidopsis* that are involved in numerous functions, including response to stresses (18, 19). The *HOS10* gene is constitutively expressed (Fig. 7*G*). *HOS10* was fused in-frame to the C terminus of *GFP* and expressed under the control of the cauliflower mosaic virus ^{35}S promoter. We found that the GFP–HOS10 fusion protein accumulates in the nucleus with or without low-temperature treatment, consistent with its predicted function as a transcription factor (Fig. 7*H*) (20).

Discussion

Tolerance and acclimation to freezing temperatures is a long-observed characteristic of plants indigenous to temperate regions (21). Cold acclimation is thought to involve many changes in cellular processes. Acclimation must include changes in metabolism that ensure maintenance of essential cellular structures/functions that are directly affected by low temperature. Often-cited examples include membrane fluidity changes caused by the behavior of lipids at low versus high temperatures and temperature-dependent protein conformation changes (22).

Much research has focused on the particular details of cellular function that fails under freezing conditions. This approach has not been very instructive in the actual mechanisms of tolerance. Guy (21) has pointed out that the large volume of reports on the when and where of freezing injury in plants has not led to any particular method or approach by which increased freezing tolerance can be achieved in plants. Essentially, this earlier work has established only a catalog of the biochemical and physiological changes that occur during cold acclimation. To go beyond this catalog requires a more analytical approach. The broad variation in the ability of plant species to acquire cold hardiness bolstered the hope that the understanding of the mechanism of acclimation and, hence, the ability to control it could be achieved by uncovering genetic differences between cold-tolerant and -sensitive species (21). However, only recently, with the introduction of a plant model system (*A. thaliana*) with appropriate molecular genetic attributes that could be applied to cold

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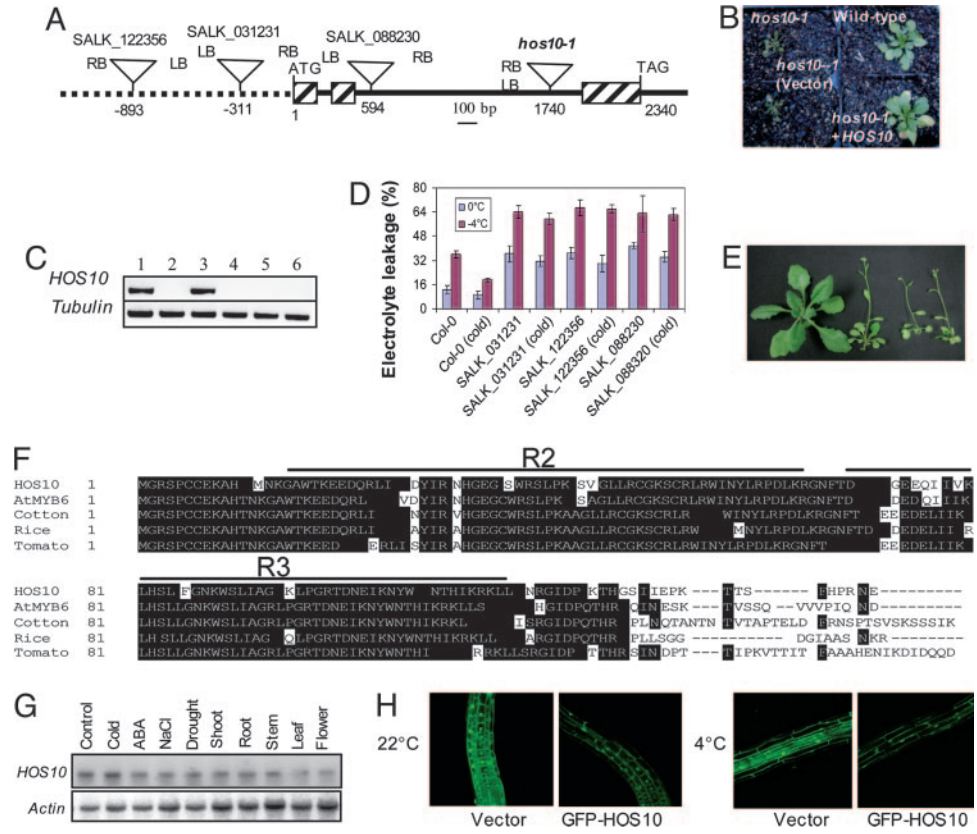

Fig. 7. *HOS10* encodes an R2R3-type MYB transcription factor. (*A*) Structure of the *HOS10* gene and positions of T-DNA inserts in the *HOS10* gene. LB and RB, T-DNA left border and right border, respectively. (*B*) Complementation of *hos10-1* mutant plant. Shown is freezing treatment (−8°C for 1 h) of wild-type, *hos10-1*, *hos10-1*(vector) (*hos10-1* plant transformed with the empty vector), and *hos10-1*+*HOS10* (one representative line of *hos10-1* plant transformed with the ^{35}S::*HOS10* cassette). The photograph was taken 1 week later. (*C*) RT-PCR analysis of *HOS10* expression. Lanes: 1, wild type; 2, *hos10-1*; 3, Col-0; 4, SALK_031231; 5, SALK_122356; and 6, SALK_088230. (*D*) Electrolyte leakage assay of *hos10-1* additional alleles. ''Cold'' indicates when the plants had been given the acclimatization treatment (4°C for 8 d). Error bars represent standard deviation ($n = 4$). (*E*) Plants of *hos10-1* additional alleles flower early in the greenhouse. (*F*) Alignment of HOS10 predicted amino acid sequence with other gene products. Compared proteins are MYB-related proteins: HOS10 (accession no. AAF20989), AtMYB6 (accession no. AAA98761), cotton (accession no. AAN28270), rice (accession no. BAA23337), and tomato (accession no. S69189). (*G*) Expression profile of the *HOS10* gene. Control, no treatment; cold, 0°C for 24 h; ABA, 100 μM for 3 h; NaCl, 300 mM for 4 h; drought, 40% humidity for 3 h. (*H*) GFP–HOS10 protein is localized in the nucleus. Shown are confocal images of transgenic plants expressing GFP–HOS10. Plants were kept at room temperature under light or at low temperature (4°C) in the dark for 12 h before imaging. The *tubulin* (*C*) and *actin* (*G*) gene were used as loading control.

acclimation studies, has this genetic and biochemical dissection of the mechanism(s) of cold acclimation begun to be realized.

Thus, decades after Weiser (23) proposed an important role for altered gene expression in cold acclimation, it has now become well established that plants respond to cold treatment by changing the transcript levels of specific genes and that these changes can be linked to phenotypic adjustment. Prominent among these changes is the induction of the CBF family of transcription factors that, when overexpressed in transgenic plants, results in increased cold tolerance (5).

Notwithstanding the clear importance of the CBFs to cold acclimation, several lines of evidence have suggested that there are other signal pathways that control gene transcription in response to cold treatment and consequently contribute to freezing tolerance. First, many cold-induced genes do not contain in their promoter regions the DRE/CRT elements that are controlled by CBFs, and the apparent CBF regulon that has been determined by microarray analysis does not include all cold-induced genes (14). Importantly, some genes that are constitutively expressed, such as *ESK1* and *HOS9*, have major effects on cold tolerance and acclimation but apparently do not require the induction of *CBF* genes for their activities (15, 24).

Although the *hos10-1* mutation affects the expression of genes in the CBF regulon, the expression of the *CBF* genes themselves is not altered by the *hos10-1* mutation (Fig. 2 *B* and *C*). The induction of the native *RD29A* gene by stress in *hos10-1* was not as dramatic as that of the *RD29A*::*LUC* transgene. Similar differences between the *RD29A*::*LUC* transgene and the *RD29A* endogenous gene have been observed previously and are probably due to the presence of additional regulatory elements that are only present in the native *RD29A* gene (25). This finding may indicate the presence of negative regulators that do not recognize the *RD29A*::*LUC* transgene promoter or cannot access the chromatin structure at the *RD29A*::*LUC* site. More rapid induction of *RD29A* and other stress-responsive genes (Fig. 2) may be the result of the increased cold sensitivity of the *hos10-1* mutation as suggested for other cold-sensitive mutants (25).

Microarray analysis revealed that HOS10 controls the expression of at least 12 genes under the conditions used (24 h of cold treatment) (Fig. 2*D* and Tables 1 and 2). Only two of these genes belong to the CBF regulon (26): At2g39030 encoding GCN5-related *N*-acetyl transferase and At1g19670 encoding coronatine-induced protein 1. *RD29A*, *COR15A*, *ADH*, and *KIN1* are positively regulated by CBF2 (26) but these are negatively regulated by HOS10 (Fig. 2). CBF2 and HOS10 positively control the transcript level of the At2g39030 gene. In the case of At1g19670, CBF2 negatively regulates its transcript level, whereas HOS10 acts in the opposite way (Table 2) (26). These results indicate that, besides the

CBFs, other transcription factors, including HOS10, also participate in the complex network controlling stress-responsive genes.

A striking feature of *hos10-1* mutant plants is their complete inability to acclimate to freezing (Fig. 3). Besides the dramatic sensitivity of *hos10-1* plants to freezing temperatures as high as −2°C, both visual and ion leakage assays revealed no acclimation at all after 4°C treatment for 8 d (Fig. 3). *hos10-1* mutant plants are also sensitive to NaCl stress, and this sensitivity does not involve altered ion accumulation (Fig. 4). This finding suggests that *hos10-1* plants have impaired ability to adjust to osmotic stress and/or injury responses (27). In fact, the water balance of *hos10-1* plants is changed, and mutant shoots lose water more rapidly during dehydration (Fig. 5*A*). Measurement of the ABA content of *hos10-1* and wild-type plants revealed that *hos10-1* plants are impaired in their ability to increase ABA level in response to dehydration stress (Fig. 5*B*).

The impaired ABA biosynthesis response of *hos10-1* plants raises the issue of whether ABA plays an important role in cold tolerance and the ability of plants to acclimate to freezing. Freezing of plant tissues may be viewed biologically in two distinct phases. Almost universally, for many reasons that have been reviewed by Guy (21), ice crystals form first in the extracellular space or apoplast of plant cells. Formation of ice crystals in the cytosol or growth of crystals through the plasma membrane into the cytosol is essentially a lethal process. Maintenance of an intact plasma membrane and the slow cooling rate of many natural environments will restrict ice crystals to the apoplast. The formation of ice crystals greatly depresses the water potential of the apoplast by the intense concentration of solutes and the large decline of the vapor pressure of water as it enters the frozen state. The reduced water potential imposes a large dehydration force on the intracellular solution (28) and explains why freezing and dehydration stresses share a physical–chemical property and involve similar biological responses.

Thus, the freezing sensitivity of *hos10-1* plants coupled with their salt sensitivity and water imbalance argues that the primary physiological lesion of this mutation is the inability to adjust to dehydration stress. Such impairment could understandably be manifested through the inability of *hos10-1* plants to normally accumulate ABA in response to dehydration as seen in Fig. 5*B*. Increased ABA accumulation during cold treatment has been correlated with acclimation ability between species (29, 30). In addition, mutants impaired in ABA biosynthesis have moderately altered freezing tolerance (31, 32), although Thomashow (22) has argued that this could be the result of pleiotropic effects. Measurement of bulk ABA in plants did not reveal any changes in ABA content in *hos10-1* plants different from wild-type plants during cold acclimation (Fig. 5*C*). However, the low level of ABA during cold acclimation makes accurate measurement difficult. Furthermore, the bulk measurements would obscure differences in ABA content in specific tissues or cells that may be critical for freezing tolerance.

Reduction in dehydration-induced ABA accumulation coupled with the inability of *hos10-1* plants to fully induce *NCED3* expression strongly implies that *HOS10* may encode a transcription factor that controls the increased expression of ABA biosynthesis genes during stress (33). As such, *HOS10* could be a crucial gene controlling an important aspect (ABA amplification) of stress responses. It is well established that partial dehydration can act like a low-temperature treatment and induce cold acclimation in plants that may link ABA to cold acclimation (21). In fact, treatment with exogenous ABA is also able to lead to freezing tolerance, sometimes very rapidly, and to levels near those induced by cold treatment (21). In view of these observations, the *hos10-1* mutation could prove very useful in further studies of the connection between freezing tolerance and ABA. Because *hos10-1* plants appear to be impaired only in the ability to increase ABA in response to dehydration stress and may actually be deficient only in an ABA-mediated amplification cycle (34), important insights into the complex role of ABA in several stress responses may be gained with additional experiments with *hos10-1*. A particular allele of *NCED3* (35) also displays reduced ability to accumulate ABA after osmotic stress, but its ability to tolerate or acclimate to cold is unknown. These special mutant alleles of genes specifically controlling ABA accumulation after stress clearly show that mutants with altered ABA accumulation are not phenotypically the same and many more such mutants are needed to fully understand the roles of ABA in growth and development during stress responses.

We thank the researchers who have generated and donated the SALK T-DNA insertional lines used in this work (36). This work was supported by National Science Foundation Grants DBI9813360, IBN-0212346, and MCB-0241450 and by United States Department of Agriculture National Research Initiative Grant 2003-00751. This is Purdue University Agricultural Program Paper 2005-17644.

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