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REVIEW

Gene regulation during cold acclimation in plants

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Cold acclimation involves precise signaling and regulation of the transcriptome. The plasma membrane may be the primary cold-stress sensor, and FRY1/HOS2 inositol polyphosphate 1-phosphatase regulates cytosolic inositol-1,4,5-triphosphate levels, which in turn control cytosolic Ca^{2+} signatures and cold acclimation. Cold-induced reactive oxygen species may activate a mitogen-activated protein kinase cascade (AtMEKK1-AtMKK2-AtMPK4/6) that regulates tolerance to freezing and other abiotic stresses. Cold acclimation induces the expression of the C-repeat binding transcription factors (CBF), which in turn activate many downstream genes that confer chilling and freezing tolerance to plants. The constitutively expressed myelocytomatosis-type basic helix-loop-helix transcription factor inducer of *CBF* expression 1 (ICE1) regulates the transcription of CBFs and other cold-induced regulons and freezing tolerance. ICE1 is probably negatively regulated by ubiquitination, which may be mediated by the HOS1 RING finger protein. The ICE1-CBF pathway positively regulates the expression of cysteine-2 and histidine-2 zinc finger transcriptional repressors, which are under the negative control of LOS2, a bi-functional enolase. In a CBF-independent pathway, the transcription factors HOS9 (a homeodomain type) and HOS10 (a R2R3 myeloblastosis type) play pivotal roles in the regulation of cold-responsive genes and freezing tolerance. The signaling process from sensors to transcription factors and to cellular responses needs further understanding. Also, cold-stress signaling in reproductive tissues is still largely unknown.

Abbreviations – ABA, abscisic acid; *ABRE*, ABA-responsive element; ada2b, transcriptional adaptor; ANP1, *Arabidopsis Nicotiana* protein kinase 1 (NPK1)-related protein kinase 1; *At*, *Arabidopsis thaliana*; AZF2, *Arabidopsis* zinc-finger 2; bHLH, basic helix-loop-helix; bZIP, basic-leucine zipper; C2H2, cysteine-2 and histidine-2; CBFs, C-repeat binding factors; CBL1, calcineurin B-like calcium-binding protein; *COR*, cold regulated; *CRT*, C-repeat elements; *DRE*, dehydration-responsive elements; DREB, dehydration-responsive element-binding protein; FRO1, FROSTBITE1; FRY, FIERY; gcn5, general control of amino acid synthesis; HOS, high expression of osmotically responsive genes; ICE1, inducer of CBF expression 1; IP3, inositol-1,4,5-triphosphate; LOS, low expression of osmotically responsive genes; MEKK, MAPKKK, mitogen-activated protein kinase kinase kinase; MKK, MAPKK, mitogen-activated protein kinase kinase; MPK, MAPK, mitogen-activated protein kinase; MYB, myeloblastosis; MYBRS, MYB recognition sequences; MYC, myelocytomatosis; MYCRS, MYC recognition sequences; RD, responsive to dehydration; ROS, reactive oxygen species; SCOF1, soybean cold-inducible zinc finger protein; SGBF1, soybean G-Box-binding factor 1; STZ, salt-tolerance zinc finger.

Introduction

Cold stress is one of the major environmental stresses that limit crop productivity, quality, and post-harvest life. Most temperate plants acquire chilling and freezing tolerance upon prior exposure to sublethal cold stress, a process called cold acclimation, although many agronomically important crops are incapable of cold acclimation. Cold acclimation involves precise regulation of expression of transcription factors and effector genes collectively called cold-regulated (*COR*) genes (Thomashow 1999, Viswanathan and Zhu 2002, Xiong et al. 2002a). Significant progress has been made in identifying transcriptional, post-transcriptional, and post-translational regulators of cold-induced expression of *COR* genes. Promoters of many of the *COR* genes contain *cis*-elements such as dehydration-responsive elements/C-repeat elements (*DRE*/*CRT,* A/GCCGAC), abscisic acid (ABA)-responsive element (*ABRE*, PyACGTGGC), and myeloblastosis (MYB) (C/TAACNA/G) and/or myelocytomatosis (MYC) recognition sequences (CANNTG) (Yamaguchi-Shinozaki and Shinozaki 2005, Zhu 2002). Freezing tolerance-based genetic screens have led to the isolation of several interesting *Arabidopsis* mutants with increased (Xin and Browse 1998) or decreased freezing tolerance (Boyce et al. 2003). Ishitani et al. (1997) developed a bioluminescent genetic screen involving stress-inducible *RD29A* promoter-driven luciferase-reporter (P_{RD29A}*::LUC*). Use of stress-responsive bioluminescent P_{RD29A}*::LUC* and P_{CBF3}*::LUC* genetic screens (Chinnusamy et al. 2002, Ishitani et al. 1997) in *Arabidopsis* led to the identification of upstream signaling components that have provided novel insight into cold-stress signaling and gene regulation, which will be discussed here.

Cold sensing and secondary signals

Plant cells may sense cold stress-induced change in membrane fluidity and protein conformation. Cold stress-induced rigidification of the plasma membrane at microdomains may lead to actin cytoskeletal rearrangement, which may be followed by activation of Ca^{2+} channels and increased cytosolic Ca^{2+} level, triggering the expression of *COR* genes during cold acclimation (Orvar et al. 2000, Sangwan et al. 2001). ABA serves as a secondary signal to transduce, at least in part, cold signals, as evidenced by the *los5* (low expression of osmotically responsive genes) mutant isolated through bioluminescent P_{RD29A}*::LUC* genetic screening. The *los5* mutant is impaired in molybdenum co-factor (MoCo) sulphurase, which synthesizes MoCo for abscisic aldehyde oxidase, and is thus defective in ABA synthesis. The *los5* mutant showed significant reduction in cold- and salt/drought-induced expression of *COR* genes (*RD29A*, *COR15*, *COR47*, *RD22*, and pyrroline-5-carboxylate synthetase) and is unable to acquire freezing tolerance. Thus, ABA plays a significant role in cold acclimation of plants (Xiong et al. 2001a). ABA may transduce cold-stress signals through second messengers such as H_2O_2 and Ca^{2+}.

Second messengers and phosphorelay

Cold-stress signals perceived by the yet to be identified sensors induce a transient increase in cytosolic Ca^{2+} level (Plieth et al. 1999, Tahtiharju et al. 1997). Results of studies involving gadolinium (Gd^{3+}, a mechanosensitive Ca^{2+} channel blocker) and cyclic ADP-ribose (cADPR) implicated the involvement of mechanosensitive and cADPR-gated Ca^{2+} channels, respectively, in *COR* gene transcription and cold acclimation in *Brassica napus* (Sangwan et al. 2001). An inhibitor of inositol-1,4,5-triphosphate (IP_3) receptor has been shown to block IP_3-induced *RD29A/KIN1* promoter-driven *GUS* expression in tomato (Wu et al. 1997). A search for mutants defective in *COR* gene expression by P_{RD29A}*::LUC* screening resulted in identification of a *FIERY1* (*FRY1*) locus involved in IP_3-mediated cold and ABA signaling. *FRY1* encodes an inositol polyphosphate 1-phosphatase, which dephosphorylates inositol phosphates such as IP_3 and thus negatively regulates IP_3 levels in cells. *fry1* mutant plants accumulated significantly higher and sustained levels of IP_3 instead of the transient increase observed in wild-type plants in response to ABA. This higher and sustained level of IP_3 led to enhanced induction of *COR* genes (*RD29A*, *KIN1*, *COR15A*, *COR47A*, and *ADH*) under ABA, cold and osmotic stress in *fry1* mutant plants compared with that in wild-type plants (Xiong et al. 2001b). A single amino acid substitution in the FRY1 protein of *Arabidopsis* caused by the *hos2* (high expression of osmotically responsive genes) mutation resulted in enhanced induction of *COR* genes and the P_{RD29A}*::LUC* reporter under cold stress but not ABA treatment. Transcript levels of C-repeat binding factors (*CBF2* and *CBF3*) and their target *COR* genes were significantly higher in *hos2* mutants than in wild-type plants under cold stress (Xiong et al. 2004). These results suggest that Ca^{2+} release from internal cellular stores mediated by IP_3 is upstream of the expression of *CBFs* and *COR* genes in the cold-signaling pathway(s). Furthermore, the calcium exchanger 1 (*cax1*) mutant of *Arabidopsis* defective in a vacuolar Ca^{2+}/H^+ antiporter exhibited enhanced expression of *CBF/DREBs* and their target *COR* genes and enhanced freezing tolerance (Catala et al. 2003). Thus, regulation of $[Ca^{2+}]_{cyt}$ levels by mechanosensitive and ligand-gated channels is upstream of the expression of *CBFs* and *COR* genes in cold-stress signaling.

Results of genetic and transgenic studies have suggested some calcium-dependent protein kinases as positive regulators (Saijo et al. 2000), with some calmodulins (Townley and Knight 2002), a protein phosphatase 2C (AtPP2CA, Tahtiharju and Palva 2001) and a salt overly sensitive 3-like or calcineurin B-like calcium-binding protein (CBL1, Cheong et al. 2003) as negative regulators of expression of *COR* genes.

Cold acclimation has been shown to induce reactive oxygen species (ROS) such as H_2O_2 (Prasad et al. 1994). ROS may alter Ca^{2+} signatures and activate mitogen-activated protein kinases (MAPKs) and redox-responsive transcription factors. Molecular analysis of the *FROSTBITE1* (*FRO1*) locus suggested that the expression of *COR* genes is regulated by ROS levels. The *fro1* mutant displayed a constitutively higher accumulation of ROS. The *FRO1* gene encodes a Fe-S subunit of complex I (NADH dehydrogenase) of the electron-transfer chain in the mitochondrion, a potential site of ROS generation under abiotic stresses. In *fro1*, constitutively higher accumulation of ROS probably triggers Ca^{2+} signaling in the absence of cold stress, which may desensitize the cells to cold-induced Ca^{2+} signaling. This could be the cause of reduced cold induction of *COR* genes and reduced cold acclimation in *fro1* mutant plants (Lee et al. 2002a).

Cold and other abiotic stresses regulate the expression and activity of various kinases of MAPK pathways, which suggests that MAPK cascades act as a converging point in abiotic stress signaling (Chinnusamy et al. 2004). Under cold stress, ROS activates the AtMEKK1/ANP1 (MAPKKK)-AtMKK2 (MAPKK)-AtMPK4/6 (MAPK) MAPK cascade that is necessary for cold acclimation in plants (Kovtun et al. 2000, Teige et al. 2004).

Regulation of cold-responsive transcriptome by CBFs

A significant step toward understanding the regulation of *COR* genes involved identification of cold-stress-inducible CBFs or DRE-binding factors in *Arabidopsis*. Cold stress induces *AtCBF1* (*DREB1B*), *AtCBF2* (*DREB1C*), and *AtCBF3* (*DREB1A*) genes. The CBF proteins activate the transcription of *DRE/CRT cis*-element containing *COR* genes (Liu et al. 1998, Stockinger et al. 1997). Constitutive or stress-inducible overexpression of *AtCBF1* or *AtCBF3* in transgenic plants resulted in constitutive or enhanced expression of *COR* genes and increased abiotic-stress tolerance, including freezing tolerance, in *Arabidopsis* (Jaglo-Ottosen et al. 1998, Kasuga et al. 1999, Liu et al. 1998). Overexpression of *AtCBF1/3* enhanced chilling, freezing, drought and/or salt-stress tolerance in *Brassica* (Jaglo et al. 2001), tomato (Hsieh et al. 2002), tobacco (Kasuga et al. 2004), wheat (Pellegrineschi et al. 2004), and rice (Oh et al. 2005). Similarly, overexpression of rice (Dubouzet et al. 2003) and maize (Qin et al. 2004) *DREB1* in transgenic *Arabidopsis* was sufficient to induce constitutive expression of CBF-target *COR* genes and conferred tolerance to freezing/drought stresses. Hence, CBF-dependent gene expression is an important, evolutionarily conserved component of cold acclimation in diverse plant species (Nakashima and Yamaguchi-Shinozaki 2006, this issue). Transcriptome analysis of *CBF*-overexpression transgenic *Arabidopsis* revealed that only about 12% of the cold-responsive genes are certain members of the CBF regulon (Fowler and Thomashow 2002). This observation suggests that other transcriptional activators/repressors also play a significant role in cold acclimation.

ICE1, a master regulator of cold acclimation

Because *CBF* genes are cold induced, an upstream transcription factor present in the cell at normal growth temperatures may be activated by cold stress, which in turn induces the expression of *CBFs*. Using the P_{CBF3}*::LUC* bioluminescent genetic screen, we identified an upstream transcription factor called inducer of CBF expression 1 (ICE1). The dominant *ice1* mutation blocks expression of *CBF3* and decreases the expression of many CBF-target genes. *ICE1* encodes a MYC-type basic helix-loop-helix (bHLH) transcription factor that binds to MYC *cis*-element in the *CBF3* promoter and may be able to activate the expression of *CBF3* upon cold stress. The *ice1* mutant showed impaired chilling tolerance and cold acclimation, while constitutive overexpression of *ICE1* enhanced the expression of *CBFs* and *COR* genes and freezing tolerance of transgenic *Arabidopsis*. *ICE1* is constitutively expressed and localized in the nucleus, but activation of *CBF* expression requires cold treatment. This observation suggests that cold-induced modification of ICE1 is necessary for activation of its target genes (Chinnusamy et al. 2003). Transcriptome analysis revealed that a large percentage of cold-induced genes are either not induced or their induction in the *ice1* mutant is less than 50% of that in wild-type plants. Thirty-two of these genes encode transcription factors, nine with highly preferable ICE1-binding *cis*-elements and five with CBF3-binding *cis*-elements in their promoters (Table 1, supplementary data from Chinnusamy et al. 2003). These results show that ICE1 is a master switch that controls many cold-

Table 1. Inducer of C-repeat binding factor (CBF) expression 1 (ICE1) and CBF3-binding sites in the promoter of some of the transcription-factor genes for which the cold-stress-induction level in *ice1* mutant is less than 50% of that in wild-type *Arabidopsis*.

Transcription factor gene	AGI	CBF3-binding *cis*-element	Position	ICE1-binding *cis*-element	Position
CBF3	At4g25480			CTGGA***CACATG***GCAGA	−193 to −188
DREB2A	At5g05410			TGAGG***CACATG***GGATT	−918 to −913
				AAGGA***CACATG***AGGCA	−764 to −759
				TGAGG***CACATG***CAAAG	−755 to −750
MYB	At1g01520			ATGTC***CACATG***GCTTG	−209 to −204
ABREB	At1g49720	TTCCG***ACCGACAT***GATAC	−815 to −808	TTTGT***CATGTGC***ATAG	−670 to −665
ATHB-12	At3g61890	ACGTA***ACCGACCT***CTAAA	−860 to −853	GCAGT***CACATG***TTAAA	−614 to −609
RGA-like protein	At5g17490			GCCGC***CACATG***TCGAC	−562 to −557
WRKY	At4g31800	ATTAT***GCCGACAT***CCATT	−139 to −132	TGAAA***CACATG***TGCAT	−662 to −657
WRKY	At1g80840			TATCC***CACATG***TCATT	−698 to −693
				AAAAG***CACATG***CTCCT	−91 to −86
Zinc finger-like protein	At3g52800			CTTTA***CACATGA***TCAA	−382 to −377
AZF2	At3g19580	ATTTG***ACCGACTT***AAAAA	−69 to −62		
STZ	At1g27730	TTATA***GCCGACCT***CTTCT	−285 to −278		
C2H2 zinc finger	At5g04340	AAGTA***GCCGACTT***AATTT	−412 to −405		
		TCTTA***GCCGACTT***CCACA	−250 to −243		
AP2-like	At2g23340	TGTCC***ACCGACCT***AATTT	−834 to −827		
Tiny-like (AP2)	At4g32800	GGGTT***GCCGACTT***GACCA	−437 to −430		

responsive CBF-dependent and independent regulons (Fig. 1). Probably, ICE1-like bHLH transcription factors may be involved in the regulation of *CBF1* and/or *CBF2* ((Van Buskirk and Thomashour 2006, this issue) Zarka et al. 2003). The *CBF2* expression appears to be under the positive control of a mitogen-activated protein kinase (MAPK)-signaling pathway, AtMEKK1-AtMKK2-AtMPK4/6, because *AtMKK2*-overexpressing *Arabidopsis* plants showed constitutive expression of *CBF2* (Teige et al. 2004).

ABA also induces the expression of *CBF1*, *CBF2*, and *CBF3* genes but to a significantly lower level than that with cold induction (Knight et al. 2004). Similarly, we have also observed that cold, ABA, and salt stress induce the expression of the P_{CBF3}*::LUC* reporter, although to a significantly lower extent than with cold induction. The *ice1* mutant showed significantly less ABA-induced expression of the P_{CBF3}*::LUC* reporter as compared with the wild-type (Fig. 2; unpublished data). In addition to cold stress, salt and ABA stress slightly enhance the expression of *ICE1* (Chinnusamy et al. 2003). Thus, ICE1 may also regulate ABA-mediated expression of *CBF* genes. Because cold-induced expression of *CBFs* is transient, ABA may activate ICE1-CBF-dependent and -independent pathways, which may be necessary to maintain the expression of *COR* genes during prolonged chilling.

Self-regulation of *CBF* expression

CBF proteins may be involved in optimization of *CBF* expression. The *los1* mutant of *Arabidopsis*, defective in the translational elongation factor 2, showed superinduction of *CBF* genes but little induction of *COR* genes in the cold. Because *los1* is defective in protein synthesis under cold temperatures, the lack of CBF proteins for feedback repression of *CBF* genes might lead to superinduction of *CBFs* (Guo et al. 2002). Characterization of the *cbf2* null mutant of *Arabidopsis* provided further evidence for CBF2-mediated downregulation of *CBF1* and *CBF3*. As compared with the wild-type, *cbf2* mutant plants showed increased expression of *CBF1* and *CBF3* and higher freezing, salt and dehydration stress tolerance. This evidence indicates that the CBF2 protein is involved in feedback regulation of *CBF1* and *CBF3* expression during cold acclimation (Novillo et al. 2004). *CBF2* expression levels may be regulated by CBF3, because impaired *CBF3* expression in the *ice1* mutant is accompanied by enhanced expression of *CBF2* (Chinnusamy et al. 2003). Analysis of null mutants of *cbf1* and *cbf3* will be required to further define the role of individual CBFs in self-regulation and cold acclimation.

Repressors of cold-induced transcriptome

Cold acclimation involves adjustment of metabolism and growth and thus involves not only induction and upregulation but also downregulation of many genes in plants (Kreps et al. 2002). Moreover, feedback repression is required to maintain the optimal protein levels of cold-induced transcriptomes. Cysteine-2 and histidine-2-type (C2H2) zinc finger proteins and a double-

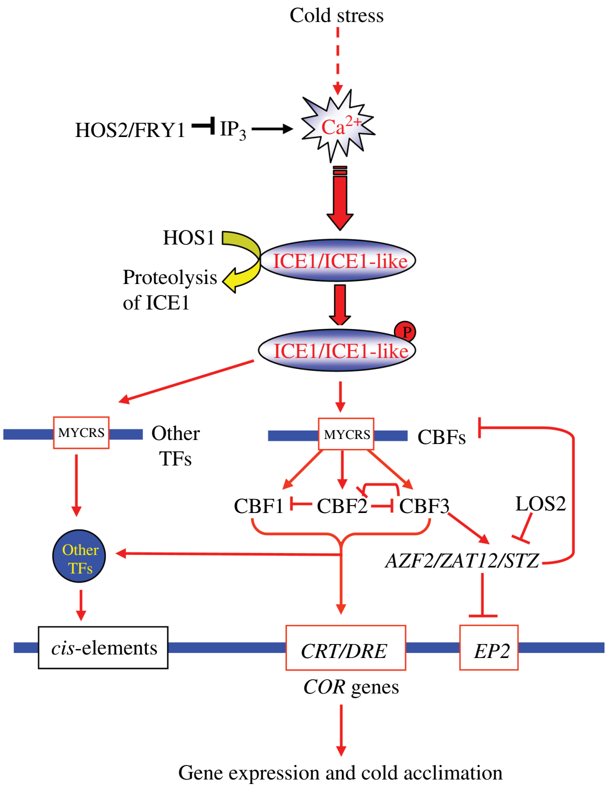

Fig. 1. Regulation of transcription factors under cold stress. Cold-stress-induced calcium signature is necessary for cold acclimation. Cold stress activates the ICE1 protein which induces transcription of CBFs and other transcription factors. CBFs also self regulate their transcription to optimize their expression levels. C2H2 zinc finger transcriptional repressors are positively regulated by CBFs and negatively regulated by the LOS2. These zinc finger transcriptional repressors downregulate the expression of CBFs and their target COR genes. (HOS2, high expression of osmotically responsive genes 2; FRY1, FIERY1, inositol polyphosphate 1-phosphatase; HOS1, high expression of osmotically responsive genes 1, a RING finger E3 ubiquitin ligase; ICE1, inducer of CBF expression 1, a myelocytomatosis (MYC)-type basic helix-loop-helix (bHLH) transcription factor; CBF, C-repeat-binding factor, AP2-type transcription factor; LOS2, low expression of osmotically responsive genes 2, a bi-functional enolase with transcriptional repression activity; AZF2, STZ, and ZAT12, cysteine-2 and histidine-2 type zinc finger transcriptional repressors; EP2, a *cis*-element originally identified in 5-enolpyruvylshikimate-3-phosphate synthase gene promoter; *CRT*, C-repeat elements; *DRE*, dehydration-responsive elements; MYCRS, MYC-type bHLH transcription factor recognition sequences; block arrow, activation; line arrow, induction of expression; line ending with bar, repression).

stranded RNA-binding protein have been identified as negative regulators of *CBF* expression.

In *Arabidopsis*, cold, ABA, drought, and salt stress induce the expression of C2H2 zinc fingers, namely, *Arabidopsis* zinc-finger 2 (*AZF2*) and salt-tolerance zinc finger ZAT10 (*STZ*) (Lee et al. 2002b, Sakamoto et al. 2004). Promoters of both *AZF2* and *STZ* contain the *DRE*, *MYB* recognition, and *MYC* recognition *cis*-elements. Hence, the expression of these genes may be under the control of transcription factors that bind to these *cis*-elements during cold stress. Transgenic plants overexpressing *CBF3* showed enhanced expression of *STZ* (Maruyama et al. 2004). Conversely, defective *CBF3* expression caused by the *ice1* mutation significantly reduced the cold induction of *STZ* and *AZF2* as compared with the wild-type (Table 1) (Chinnusamy et al. 2003). Hence, ICE1-induced *CBF3* expression may positively regulate the expression of *AZF2* and *STZ* through the *DRE cis*-element (Fig. 1).

The cold-stress induction of *STZ* is rapid and transient in wild-type plants, while its induction is stronger and more sustained in the *los2* mutant of *Arabidopsis*. Also, LOS2 bi-functional enolase binds to the *MYC* recognition sequence in the promoter of *STZ*. Thus, the expression of *STZ* is under the negative regulation of LOS2 (Lee et al. 2002b) (Fig. 1). The *Arabidopsis ada2b-1* mutant defective in a histone acetyltransferase complex also showed higher expression of *STZ* and *ZAT12* and more constitutive freezing tolerance than wild-type plants (Vlachonasios et al. 2003). Thus, the ICE1-CBF pathway positively regulates the expression of these zinc finger transcriptional repressors, while LOS2 and ADA2b negatively regulate them. STZ is also probably positively regulated by a MAPK-signaling pathway, AtMEKK1-AtMKK2-AtMPK4/6, as is evident from transcriptome profiling of *AtMKK2*-overexpression *Arabidopsis* plants (Teige et al. 2004).

These zinc finger repressors repress the expression of *COR* genes directly and/or through the repression of *CBFs*. Transient expression studies have shown that STZ is a repressor of *RD29A*. A *los2* mutation that enhanced *STZ* expression resulted in reduced cold induction of a *CRT cis*-element containing *COR* genes. STZ appears to repress *RD29A* expression by binding to the STZ recognition site at −554 to −522 (ACTAGTGTAN$_{13}$TCTAGTAAG) in the promoter of *RD29A* (Lee et al. 2002b) (Fig. 1). Gel mobility-shift assays showed that *AZF2* and *STZ* bind specifically to an A(G/C)T *cis*-element within the EP2 sequence (a *cis*-element where a negative regulator binds). Co-expression of *AZF2* and *STZ* with *DREB1A* in an *Arabidopsis* protoplast resulted in inhibition of DREB1A-induced expression of a chimeric *EP2*-fused *RD29A* promoter-driven reporter gene (Sakamoto et al. 2004). Similarly, *ada2* and *gcn5* mutants with a higher induction of *STZ* and *ZAT12* showed reduced expression of *COR47* and *COR6.6* genes (Vlachonasios et al. 2003). Transgenic plants constitutively overexpressing *CBFs* showed higher induction of *STZ*, which may repress genes involved in photosynthesis and carbohydrate

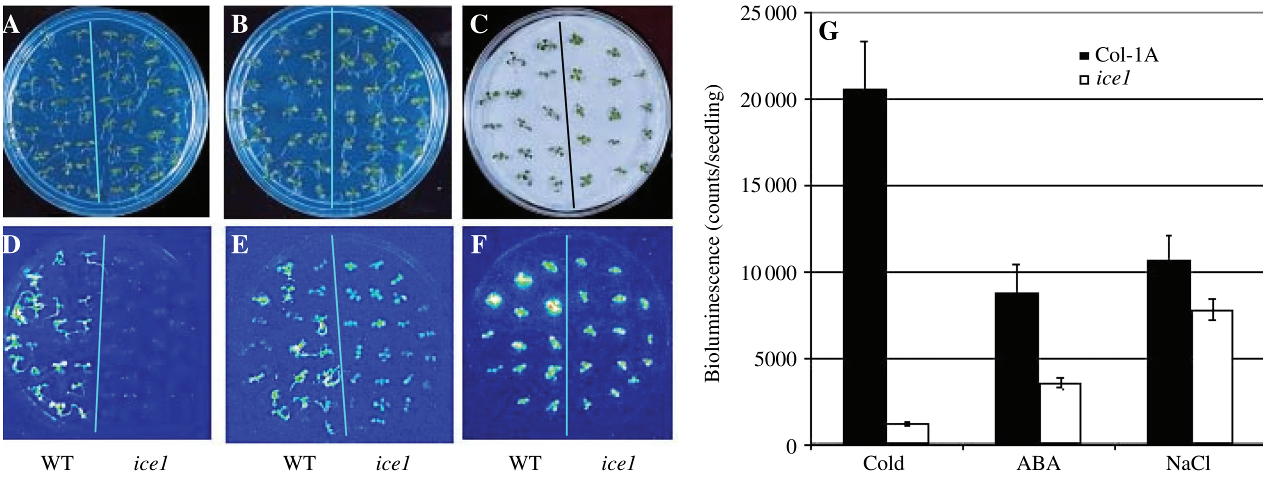

Fig. 2. Induction of PCBF3::LUC expression by cold, abscisic acid (ABA), and salt stresses. (A and B) Wild-type (WT) (on the left) and *ice1* (on the right) seedlings grown on an agar plate for 1 week. (C) WT (on the left) and *ice1* (on the right) seedlings grown on an agar plate for 1 week were transferred on to filter paper saturated with 300 m*M* NaCl. (D) Luminescence of (A) after low-temperature treatment at 0° C for 12 h. (E) Luminescence of (B) after treatment with 100 μ*M* ABA for 3 h. (F) Luminescence of (C) after treatment with 300 m*M* NaCl for 3 h. (G) Quantification of the luminescence intensity in D (cold), E (ABA), and F (NaCl).

metabolism and thus reduce the growth of these transgenic plants (Maruyama et al. 2004). CBF-induced zinc fingers also appear to be involved in feedback repression of *CBFs*, because *ZAT12*-overexpression transgenic *Arabidopsis* showed decreased expression levels of *CBFs* (Vogel et al. 2005).

Genetic analyses of *fiery2* (*fry2*) mutant of *Arabidopsis* revealed that the FRY2 RNA polymerase II C-terminal domain phosphatase, which controls transcription and mRNA processing by de-phosphorylation of RNA polymerase II, is a regulator of *COR* genes (Koiwa et al. 2002, Xiong et al. 2002b). *fry2* mutants exhibited hypersensitivity to freezing damage as compared with wild-type plants. *fry2* mutation enhanced the expression of *CBFs* and *COR* genes under cold and ABA stress. This observation suggests that FRY2 is a negative regulator of *CBFs* and their target *COR* genes (Xiong et al. 2002b). The increased freezing sensitivity in *fry2* mutant plants implies that FRY2 may positively regulate the expression of certain genes critical for freezing tolerance.

CBF-independent pathways of cold responses

Both ABA-independent and -dependent pathways regulate cold-responsive genes, and ABA acts synergistically with the cold signal (Xiong et al. 1999). ABA-dependent gene expression is regulated by transcription factors that belong to the bZIP (ABRE-binding factors or AREBs), MYC, and MYB families. A cold- and ABA-inducible *bZIP* gene, *ABRE*-binding factor 1 (*ABF1*), has been cloned from *Arabidopsis* (Choi et al. 2000), but its target genes are not known. However, a C2H2-type zinc finger protein that activates a bZIP transcription factor has been found to regulate *COR* gene expression through *ABRE* elements. Over-expression in *Arabidopsis* of a cold-inducible zinc finger protein from soybean, *SCOF1*, resulted in constitutive expression of *COR* genes and freezing tolerance. SCOF1 enhanced the DNA-binding activity of a cold-inducible bZIP transcription factor, soybean G-Box-binding factor 1 (SGBF1), which induces the expression of *COR* genes in an ABA-dependent pathway during cold acclimation (Kim et al. 2001).

In rice, a member of MYB family transcription factors, *OsMYB4*, has been shown to be inducible by cold (10–15° C) but not by ABA. Transient expression analysis showed that OsMYB4 could transactivate the expression of *COR* genes (*RD29A*, *COR15a*, and *PAL2*). Furthermore, transgenic *Arabidopsis* plants overexpressing *OsMYB4* exhibited enhanced induction of *COR* genes, increased proline content and enhanced freezing tolerance (Vannini et al. 2004). Genetic evidence for the involvement of MYB transcription factors in cold acclimation came from the analysis of a freezing hypersensitive *hos10* mutant of *Arabidopsis*. The *hos10-1* mutant showed enhanced expression of P_{RD29A}*::LUC* and *COR* genes under cold, ABA, and salt stress. The *HOS10* gene encodes a putative R2R3-type MYB transcription factor. Interestingly, HOS10 is required for ABA accumulation, because *hos10-1* mutant plants showed reduced induction of *NCED3* (9-*cis*-epoxycarotenoid dioxygenase) and thus low ABA accumulation under osmotic stress (Zhu et al. 2005).

A P_{RD29A}::*LUC* reporter genetic screen also led to the identification of a freezing-sensitive *hos9* mutant in *Arabidopsis*. *HOS9* encodes a putative homeodomain transcription factor that is constitutively expressed and localized to the nucleus. As compared with the wild-type, the *hos9* mutant is hypersensitive to freezing with or without cold acclimation, although cold induction of *CBFs* was not altered. Furthermore, transcriptome analysis of *hos9-1* mutant plants under cold stress suggested that the HOS9 regulon is different from that of the CBFs. Thus, HOS9 plays an important role in regulating cold acclimation through a CBF-independent pathway (Zhu et al. 2004).

Post-transcriptional regulation

Post-transcriptional regulation of gene expression is mediated through pre-mRNA splicing, nucleocytoplasmic transport, RNA stability, translation, post-translational modification, and proteolysis. The use of a P_{RD29A}::*LUC* genetic screen led to the identification of one of the proteins involved in RNA export, a DEAD-box RNA helicase (LOS4), as a positive regulator of *CBF* expression during cold acclimation. Cold induction of *CBF3* is blocked, while that of *CBF1* and *CBF2* is delayed in the *los4* mutant, and thus, the mutant is impaired in the cold induction of *COR* genes. The *los4-1* mutant was sensitive to chilling stress, and ectopic expression of *CBF3* rescued the mutant from chilling sensitivity. LOS4 may unwind the cold-stabilized secondary structure in the 5′-untranslated region of RNA or may directly control the transcript stability of *CBFs* or regulators of *CBF* genes (Gong et al. 2002). The *cryophyte* mutant isolated by a P_{RD29A}::*LUC* genetic screen is allelic to *los4-1*. It showed enhanced cold induction of *CBF2* and more chilling and freezing tolerance than the wild-type *Arabidopsis*. The CRYOPHYTE/LOS4 protein is enriched in the nuclear rim. *In situ* poly(A) hybridization analysis revealed that mRNA export from the nucleus is blocked in the *cryophyte/los4-2* mutant only at warm temperatures, while the *los4-1* mutation weakens mRNA export at both cold and warm temperatures. These results suggest that the LOS4 RNA helicase is crucial in mRNA export and important in the regulation of *CBF* and *COR* gene expression (Gong et al. 2005).

In the sensitive to freezing 6 (*sfr6*) mutant of *Arabidopsis*, cold-stress induction of *CBF1*, *CBF2*, *CBF3*, and *DREB2* was not impaired, but induction of their target *COR* genes was significantly less as compared with that of the wild-type. These results suggest that the *sfr6* mutation probably controls the *trans*-activation capacity of CBFs and/or post-transcriptional regulation of *COR* genes (Boyce et al. 2003). The regulatory small RNAs such as microRNAs (miRNAs) and short interfering RNAs (siRNAs) play a vital role in the post-transcriptional gene regulation. Cold and other abiotic stress-regulated regulatory RNAs have been identified in *Arabidopsis* (Sunkar and Zhu 2004), which will help in understanding post-transcriptional gene regulation during abiotic stresses.

Although ICE1 is constitutively expressed, it activates *CBF* gene expression only upon cold treatment (Chinnusamy et al. 2003), which suggests that ICE1 requires either interaction with additional factors induced by cold or post-translational regulation under cold stress for its activity. Use of a P_{RD29A}::*LUC* genetic screen identified HOS1 (Ishitani et al. 1998) as an upstream negative regulator of *CBFs*. *HOS1* encodes a RING finger ubiquitin E3 ligase, which may target certain signaling proteins for proteolysis. Since the *hos1* mutant showed superinduction of *CBF* genes under cold stress, HOS1 is a negative regulator of *CBF* expression (Lee et al. 2001). HOS1 may target upstream positive regulator(s) of CBFs such as ICE1 for proteolysis and thus negatively regulate the expression of ICE1 target genes.

Conclusions and perspectives

Although a cold sensor has yet to be identified, current evidence suggests that cold stress may be perceived by plant cells through changes in the plasma membrane fluidity state. Cold-induced calcium signaling is decoded and transduced by calcium sensory proteins. Cold and other abiotic stresses induce ROS, which can activate a MAPK cascade, AtMEKK1-MKK2-MPK4/6. However, the molecular link between the kinases and transcription factors is still unknown. Molecular analyses have shown that CBFs play a vital role in regulation of genes encoding late-embryogensis-abundant (LEA) type COR proteins and osmoprotectant biosynthesis across plant species. ICE1, a MYC-type bHLH transcription factor, and perhaps ICE1-like proteins are upstream master regulators of *CBFs* and many cold-responsive subregulons. The HOS1 RING finger protein appears to negatively regulate the ICE1-CBF pathway possibly by targeting ICE1 for proteolysis. CBFs probably repress some genes through C2H2 zinc fingers. CBF-independent pathways of gene regulation are mediated by proteins such as HOS9, a homeodomain transcription factor, and HOS10, a MYB-type transcription factor.

The reproductive phase, particularly, pollen maturation/germination is very sensitive to cold and other abiotic stresses. Many genes necessary for cold acclimation in vegetative tissues were either not or weakly induced in pollen under cold stress (Lee and Lee 2003). This observation suggests that the mechanism of cold tolerance is different in vegetative and reproductive tissues, the understanding of which warrants serious attention. A thorough understanding of cold-stress signaling will help in transcriptome engineering of crop plants for enhanced tolerance to cold and other abiotic stresses. Stress-responsive promoter::*LUC* genetic screening will continue to be of help in dissecting stress-signaling components that activate cold-responsive transcriptome.

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