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Molecular genetic analysis of cold-regulated gene transcription

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Chilling and freezing temperatures adversely affect the productivity and quality of crops. Hence improving the cold hardiness of crop plants is an important goal in agriculture, which demands a clear understanding of cold stress signal perception and transduction. Pharmacological and biochemical evidence shows that membrane rigidification followed by cytoskeleton rearrangement, Ca^{2+} influx and Ca^{2+}-dependent phosphorylation are involved in cold stress signal transduction. Cold-responsive genes are regulated through C-repeat/dehydration-responsive elements (CRT/DRE) and abscisic acid (ABA)-responsive element *cis*-elements by transacting factors C-repeat binding factors/dehydration-responsive element binding proteins (CBFs/DREBs) and basic leucine zippers (bZIPs) (SGBF1), respectively. We have carried out a forward genetic analysis using chemically mutagenized *Arabidopsis* plants expressing cold-responsive RD29A promoter-driven luciferase to dissect cold signal transduction. We have isolated the *fiery1* (*fry1*) mutant and cloned the *FRY1* gene, which encodes an inositol polyphosphate 1-phosphatase. The *fry1* plants showed enhanced induction of stress genes in response to cold, ABA, salt and dehydration due to higher accumulation of the second messenger, inositol (1,4,5)- triphosphate (IP_3). Thus our study provides genetic evidence suggesting that cold signal is transduced through changes in IP_3 levels. We have also identified the *hos1* mutation, which showed super induction of cold-responsive genes and their transcriptional activators. Molecular cloning and characterization revealed that *HOS1* encodes a ring finger protein, which has been implicated as an E3 ubiquitin conjugating enzyme. HOS1 is present in the cytoplasm at normal growth temperatures but accumulates in the nucleus upon cold stress. HOS1 appears to regulate temperature sensing by the cell as cold-responsive gene expression occurs in the *hos1* mutant at relatively warm temperatures. Thus HOS1 is a negative regulator, which may be functionally linked to cellular thermosensors to modulate cold-responsive gene transcription.

Keywords: low temperature; signalling; CRT/DRE; abscisic acid-responsive element; FRY1; HOS1

1. INTRODUCTION

Adjustment is a way of life for sessile and poikilothermic land plants that endure environmental stresses such as low or high temperatures, water deficit and salinity. These abiotic stresses not only limit the temporal and spatial distribution of plants but also adversely affect the productivity and quality of agriculturally important crops. Plant body temperature changes with ambient temperature, although a few plants can control their temperature by several degrees above (through alternate oxidase respiration) or below (through transpirational cooling) the ambient temperature. Most temperate plants can acquire tolerance to freezing temperatures by prior exposure to low non-freezing temperatures, a process called cold acclimatization. This is achieved by the expression of many genes, change in the membrane lipid composition, accumulation of compatible osmolytes (proline, betaine, polyols and soluble sugars), transient rise in ABA and reduction or cessation of plant growth (Levitt 1980). Tropical and sub-tropical plants are incapable of cold acclimatization. Frost tolerance is essential for temperate crops like winter wheat, while in tropical crops like rice, maize, soybean, cotton and tomato, productivity and quality are affected by even non-freezing low temperatures. Hence engineering cold-tolerant crop plants is one of the cherished goals in agriculture. To achieve this, a thorough understanding of cold stress signal perception and transduction in plant cells, which lead to cold acclimatization, is required. Thanks to the advent of molecular biology, which has propelled the research in this area over the past two decades, today some of the events of cold signal perception, transduction and cold acclimation are defined at the molecular level (for recent reviews, see Shinozaki & Yamaguchi-Shinozaki 2000; Browse & Xin 2001; Thomashow 2001; Zhu 2001).

In nature, low temperature stress is often accompanied by dehydration (or osmotic stress), as low temperature may limit water uptake by the roots, while freezing-induced ice formation in the apoplast (due to low solute concentration) causes reduction in water potential, which leads to movement of water into the apoplast from the symplast. This process causes severe dehydration. In addition, a minor change in osmotic potential occurs due to its temperature dependency (osmotic potential is $CiRT$, where C is the concentration of solutes, i is the ionization constant, R is the gas constant and T is the absolute

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temperature). Decrease in turgor pressure is known to induce biosynthesis of the plant stress hormone ABA. Hence, depending upon the level of cold stress, in addition to cold stress, dehydration and ABA-mediated signalling operate to regulate freezing tolerance. In plants, gene expression is regulated by both developmental and environmental cues. For example, some of the dehydration-responsive genes are also expressed developmentally during late embryogenesis. Cold stress can vary in severity (how low the temperature is), rate of stress development (change in temperature per unit time), duration and fluctuations (diurnal and seasonal). So it is logical to expect plants to have various mechanisms for sensing and transduction, which means that there are multiple cold stress perception and transduction modules and interactions at different nodes in these signal transduction modules. This review focuses on recent developments in cold stress signalling and gene regulation in higher plants.

2. MEMBRANE RIGIDIFICATION: A MECHANISM OF COLD SENSING

Membrane fluidity and protein structural stability and flexibility are determined by the composition of building block molecules and their interacting environment. Temperature is one of the important environmental cues that influence the membrane fluidity and protein structural stability and flexibility. Hence these can be the primary candidates as biological thermometers. It is logical to expect that plants have thermosensors in the expressed state, irrespective of developmental and environmental cues, so that stress can be sensed at once. The folding kinetics of *Escherichia coli* cold shock protein A is temperature dependent (Leeson *et al.* 2000). The CBF1, a transcriptional activator involved in cold-regulated gene expression in *Arabidopsis thaliana*, undergoes cold-induced denaturation (when the temperature changes from 25 to 4 °C) in both N-terminal and acidic regions *in vitro* (Kanaya *et al.* 1999). Changes in the structure of protein can alter its ability for protein–protein interaction, i.e., its ability to form a multimer of its own or with other proteins, which plays an important role in gene regulation. Although so far no such low temperature sensor has been identified, these studies form a *prima facie* case for considering the possibility of protein denaturation-dependent low temperature sensors in plants.

Change in membrane fluidity is one of the immediate effects of cold stress and hence the plasma membrane is proposed as a primary sensor of low temperature (Levitt 1980). The first evidence for this hypothesis is in cyanobacterium *Synechocystis* PCC6803, where Palladium (Pd)-catalyzed plasma membrane rigidification activated the expression of the cold-inducible fatty acid desaturase A (*desA*) gene (Vigh *et al.* 1993). Ca^{2+} influx into the cytosol is an early event in cold acclimatization (Knight *et al.* 1991; Monroy *et al.* 1993; Plieth *et al.* 1999); blocking the Ca^{2+} influx by Ca^{2+} channel blockers inhibited cold acclimatization at 4 °C, and Ca^{2+} channel agonist (Bay K8644) or Ca^{2+} ionophore could induce cold acclimatization even at 25 °C in Alfalfa (*Medicago sativa*) (Monroy *et al.* 1993; Monroy & Dhindsa 1995) and *Arabidopsis* (Tähtiharju *et al.* 1997). Actin microfilament re-organization has been implicated in Ca^{2+} influx in hepatocytes (Yamamoto 1989) and tobacco protoplasts (Mazars *et al.* 1997). However, the temporal relationship between membrane rigidification, actin microfilament reorganization and Ca^{2+} influx was not known. Örvar *et al.* (2000) have demonstrated that membrane fluidity may indeed act as a thermosensor in Alfalfa cell suspension cultures using pharmacological agents and *CAS30* gene expression and cold acclimatization as end markers. At 25 °C, Ca^{2+} influx, *CAS30* expression and cold acclimatization could be achieved in alfalfa cells by treatment with membrane rigidifier (DMSO) and actin microfilament destabilizer (cytochalasin D). Conversely, treatment with membrane fluidizer (Benzyl alcohol) and actin microfilament stabilizer (Jasplakinolide) inhibited Ca^{2+} influx, *CAS30* expression and cold acclimatization even at 4 °C. Reorganization of actin microfilaments in cold signalling is downstream of membrane rigidification and above Ca^{2+} influx, as cold or DMSO induced *CAS30* expression and cold acclimatization is inhibited by the treatment of cells with Jasplakinolide (Örvar *et al.* 2000). Further strength to this proposal was provided by the study of Sangwan *et al.* (2001) in intact seedlings of *Brassica napus* transgenic plants carrying a *BN115* promoter-driven *GUS* reporter gene. The transgene was induced at 25 °C by treatment of the leaves with membrane rigidifier (DMSO), microfilament destabilizer (Latrunculin B) and microtubule destabilizer (oryzalin/colchicine), while the transgene was not expressed even at 0 °C in plants treated with membrane fluidizer (Benzyl alcohol), microfilament stabilizer (Jasplakinolide) and microtubule stabilizer (taxol). Gadolinium (Gd^{3+}), a mechanosensitive calcium channel blocker, could inhibit cold-, DMSO-, Latrunculin B-, oryzalin- and colchicines-induced reporter gene expression (Sangwan *et al.* 2001). Cold-induced membrane rigidification is thought to occur in distinct microdomains of the plasma membrane (Murata & Los 1997). Hence in higher plants, cold-induced rigidification at microdomains on the plasma membrane may lead to cytoskeleton rearrangement, induction of stretch-sensitive Ca^{2+} channels and increase in cytosolic Ca^{2+} that triggers cold-induced gene expression and cold acclimatization (Örvar *et al.* 2000; Sangwan *et al.* 2001). Isolation and characterization of a cold-inducible *TaADF* further supports the involvement of cytoskeleton rearrangement during cold signalling. *TaADF* expression is strongly induced by cold but not by ABA, dehydration, heat or NaCl and the level of expression correlates with increase in cold acclimatization and the genotypic differences in cold acclimatization. TaADF is phosphorylated by a 52 kDa protein kinase in a temperature-dependent manner. Because this TaADF is not detected at the normal growth temperature (24 °C) of wheat and is expressed at a significant level only after 2 days of cold stress, this specific TaADF may not be actively involved in the initial process of cold perception but may be involved in cold acclimatization (Ouellet *et al.* 2001).

3. REGULATION OF CA^{2+} INFLUX

The involvement of Ca^{2+} in cold signal transduction is demonstrated in many studies (Knight *et al.* 1991; Monroy *et al.* 1993; Monroy & Dhindsa 1995; Tähtiharju *et al.* 1997; Plieth *et al.* 1999; Örvar *et al.* 2000; Sangwan *et*

al. 2001). Hence it is essential to analyse how this Ca^{2+} influx occurs and what are the downstream components that translate specific Ca^{2+} signatures during cold stress. The regulators of Ca^{2+} channels in cells are IP3, cADPR and nicotinic acid adenine dinucleatide ribose. Stretch/mechanosensitive Ca^{2+} channels are also involved in Ca^{2+} influx. Mechanosensitive Ca^{2+} channels are activated by cytoskeleton rearrangement during cold stress to induce Ca^{2+} influx in alfalfa cell suspension culture (Örvar *et al.* 2000) and in seedlings of *B. napus* (Sangwan *et al.* 2001). Single-cell microinjection in tomatoes showed that cADPR could activate cold-regulated genes *RD29A* (responsive to dehydration) and *KIN2* (cold inducible). Microinjection of ABA (50 μM) or cADPR (1 μM) could induce the *RD29A*/*KIN1* promoter-driven *GUS* gene, while ADP-ribosyl cyclase (an enzyme that synthesizes cADPR) or Ca^{2+} can replace the ABA and cADPR in reporter gene induction. Conversely, induction of ABA-responsive reporter genes was inhibited by 8-amino-cADPR (a structural analogue of cADPR) or EGTA (a Ca^{2+} chelator) (Wu *et al.* 1997). In *B. napus* seedlings, cADPR treatment at 25 °C could induce the *BN115* promoter-driven *GUS* reporter gene, *BN115* transcripts and increase freezing tolerance (Sangwan *et al.* 2001). Thus pharmacological and biochemical evidence indicates that cADPR acts as a signal molecule in activating calcium channels to cause Ca^{2+} influx during cold stress.

Microinjection of heparin, a specific inhibitor of IP_3 receptors, could block the IP_3-induced *RD29A*/*KIN1* promoter-driven *GUS* reporter gene in tomatoes but not ABA-mediated expression of the reporter gene. Hence the effect of IP_3 was thought to be a secondary response in the regulation of *RD29A* and *KIN1* (Wu *et al.* 1997). However, the involvement of IP_3 in cold, ABA, salt and dehydration signal transduction was demonstrated by Zhu and his colleagues by using a molecular genetic approach. Xiong *et al.* (2001) used chemically mutagenized *Arabidopsis* transgenic plants with the *RD29A* promoter-driven luciferase (*Luc*) to dissect cold signal transduction and isolated a mutant *fry1* that showed enhanced induction of cold-regulated genes (*RD29A*, *KIN1*, *COR15A*, *COR47A* and *ADH*) in response to cold, ABA, salt and dehydration stresses. The *fry1* mutant constitutively expressed the cold-responsive genes at low abundance and the cold-responsive genes were super-induced under abiotic stresses. The *fry1* mutant is hypersensitive to these abiotic stresses, in other words, the level of stress required to induce cold-responsive genes is lower than that required for WT plants. *RD29A* promoter-driven *Luc* reporter gene expression at 15 °C was similar to that of the WT at 0 °C. Similarly, the ABA and NaCl requirements were 0.1 μM and 10 mM, respectively, in *fry1* as compared with 100 μM ABA and 250 mM NaCl required by the WT for the same level of *RD29A-Luc* expression. The *fry1* mutation was mapped to chromosome five and isolated by map-based cloning. Sequence comparison of *FRY1* revealed that it encodes a protein homologous to a bifunctional enzyme with 3′(2′), 5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities. The FRY1 protein was found to be mainly acting as an inositol polyphosphate 1-phosphatase, which functions in the catabolism of IP_3. Northern analysis revealed that *FRY1* is expressed in every tissue constitutively, but the expression is significantly higher in leaves. Analysis of the IP_3 content revealed that the *fry1* mutant accumulated significantly higher levels of IP_3 even in unstressed conditions when compared with WT plants. ABA induced a significant increase in IP_3 within 1 min in WT, which returned to the basal level within 10 min. However, the *fry1* mutant maintained its basal level of IP_3 at 1 min after ABA treatment, but accumulated a significantly higher level over 30 min. This shows that ABA induces a transient increase in cellular IP_3 in intact seedlings of *Arabidopsis* and that FRY1 is involved in the regulation of IP_3 levels during signal transduction. Although cold-responsive genes are super-induced in the *fry1* mutant, the *fry1* plant is defective in cold acclimatization and germination is highly sensitive to ABA and NaCl. This shows that the cold-responsive gene regulation and cold acclimatization processes can be unlinked. Thus the *fry1* study provides, to our knowledge, the first genetic evidence of the involvement of IP_3 in ABA and abiotic signal transduction in plants. Cold-responsive genes are regulated through CBFs and bZIP transacting factors. The expression of *CBF2* (which is a transcriptional activator of cold-responsive genes) is similar in the WT and the *fry1* mutant at 1.5 and 3 h of cold stress. In the WT, *CBF2* expression decreased drastically after 3 h to a minimum level. However, in the *fry1* mutant the *CBF2* transcript was 1.8 times higher than the WT level after 6 h of cold treatment. Hence *FRY1* is a negative regulator of cold-responsive gene expression through modulating IP_3 levels, which may also regulate the cold-induced transient changes in the transcript level of *CBF2* (Xiong *et al.* 2001).

4. SENSORS OF CA^{2+} SIGNATURES

Intracellular Ca^{2+} signatures are sensed by the calcium sensor family of proteins like calmodulin and CDPKs (Zielinski 1998). An antagonist (W7) of the CDPKs could inhibit cold-responsive gene expression and cold acclimatization in alfalfa (Monroy *et al.* 1993) and *Arabidopsis* (Tähtiharju *et al.* 1997). In rice (*Oryza sativa* L. cv. Don Juan), a constitutively expressed membrane-bound CDPK has been characterized. Cold stress (12–18 h) significantly increases the auto-phosphorylation and kinase activity of this rice CDPK, thus showing a post-translational regulation of CDPK by cold stress (Martín & Busconi 2001). Recently, a new family of calcium sensors called CBL proteins was identified in *Arabidopsis*, which are similar to the regulatory B subunit of calcineurin and the neuronal calcium sensors in animals (Liu & Zhu 1998; Kudla *et al.* 1999). AtCBLs are small Ca^{2+} binding proteins that themselves do not have any enzyme activity but act through protein kinases. Involvement of CBL proteins in salt stress signal transduction has been demonstrated by genetic analysis and molecular cloning of SOS3, which activates the protein kinase SOS2. SOS2 in turn activates SOS1, a plasma membrane Na^+/H^+ antiporter (Halfter *et al.* 2000; Ishitani *et al.* 2000; Liu *et al.* 2000; Shi *et al.* 2000; Guo *et al.* 2001). Another member of the *Arabidopsis* CBL family, *AtCBL1*, is highly inducible by cold, drought and wounding (Kudla *et al.* 1999). Using yeast two-hybrid screening, a target protein for AtCBL1 was identified from *Arabidopsis*, named as CIPK1. CIPK1 encodes a 49 kDa protein and is expressed constitutively

in all tissues. CIPK1 interacts with AtCBL1 in a Ca^{2+}-dependent manner and EGTA (a Ca^{2+} chelator) could inhibit this interaction (Shi *et al.* 1999).

The requirement of reversible phosphorylation of pre-existing proteins for cold acclimatization has been demonstrated in alfalfa and *Arabidopsis* (Monroy *et al.* 1993, 1997, 1998; Tähtiharju *et al.* 1997). Hence the role of protein kinases and phosphatases has been explored in cold signal transduction. Is there a specific set of protein kinases and phosphatases, which perceive cold-specific Ca^{2+} signatures? If these protein kinases and phosphatases are involved in cold signal transduction, they should show a cold-regulated activation/inhibition. Tomato seedlings microinjected with protein kinase inhibitor (K252a) could inhibit ABA/cADPR/Ca^{2+}-induced *RD29A* and *KIN2* expression, while microinjection with protein phosphatase inhibitor (okadaic acid) stimulated *RD29A* and *KIN2* expression even in the absence of ABA treatment (Wu *et al.* 1997). Similar responses to the inhibitors of protein kinases and phosphatases were observed in alfalfa *CAS15* expression (Monroy *et al.* 1998). Treatment with inhibitors of tyrosine kinases (genistein), protein kinase C (H7) and phosphoinositide kinases (wortmannin) on *B. napus* seedlings carrying the *BN115* promoter-driven *GUS* gene prevented reporter gene expression and freezing tolerance even after cold treatment, while treatment with inhibitors of protein phosphatases 1 (okadaic acid) and 2A (calyculin A) could induce the reporter gene at 25 °C and conferred freezing tolerance (Sangwan *et al.* 2001). Low temperature causes Ca^{2+}-dependent, rapid and dramatic decrease in protein phosphatase 2A in alfalfa (Monroy *et al.* 1998). Thus it appears that the Ca^{2+} signal is transduced by protein kinases/phosphatases to regulate cold-responsive genes during cold acclimatization. Cold stress (4 °C)-induced increase in the expression of *AtPP2CA* reached a maximum by 12 h and remained high afterwards. *Arabidopsis* transgenic plants expressing *AtPP2CA* in antisense showed that regulation of cold-responsive genes (*RAB18*, *RCI2A*/*LTI6*, *RD29A*/*LTI78*) was cold stress-dependent similar to the WT, but they are super-induced during cold stress in *AtPP2CA* antisense plants and conferred better freezing tolerance. Also the cold-responsive gene expression and cold acclimatization were accelerated in *AtPP2CA* antisense plants, i.e. less time of cold stress was required when compared with that of the WT. As the expression pattern of *CBF1*, *CBF2*, *CBF3* and *DREB2* was unaltered in *AtPP2CA* antisense plants, the enhanced expression of cold-responsive genes is not mediated through the CRT/DRE element (Tähtiharju & Palva 2001). The expression of *RAB18* and *RCI2CA* (rarely cold inducible) is regulated by an ABA-dependent pathway through ABREs (Lång & Palva 1992). Hence AtPP2CA is a negative regulator of cold stress through ABA-dependent pathways (Tähtiharju & Palva 2001).

MAPKs are serine/threonine protein kinases that play key roles in integrating multiple intracellular signals transmitted by various second messengers. A MAPK cascade consists of three protein kinases. Inactive MAPKKKs are activated by a stress signal messenger; upon activation, they activate MAPKKs by phosphorylation at conserved serine and serine/threonine (SXXXS/T motif). Activated MAPKKs activate MAPKs by phosphorylating MAPK at both tyrosine and threonine residues in the TXY motif. MAPKs enter the nucleus to regulate appropriate transacting factors. Thus activated, MAPKs can regulate specific gene expression. In plants, many MAPK family members have been cloned and proposed to be involved in environmental stress responses (Mizoguchi *et al.* 1997). A MAPK cascade regulated by cold and dehydration independently of ABA has been characterized in alfalfa. Expression of the alfalfa MAPK gene, MMK4, was strongly induced by cold and drought stress within 45 min, while salt, heat and ABA did not alter the transcript's level. Although the steady state level of protein was unaltered, kinase activity of MMK4 was enhanced by cold and dehydration (Jonak *et al.* 1996). *Arabidopsis ATMPK3* (MAPK) gene expression is highly induced by cold, NaCl and touch. *AtMPK3* expression reached a very high level within 10 min of cold stress, while the induction by NaCl and touch was less sensitive (Mizoguchi *et al.* 1996). In *Arabidopsis*, H_2O_2 can activate a specific MAPKKK, ANP1, which initiates the phosphorylation cascade involving cold stress-regulated AtMPK3. Transgenic tobacco constitutively expressing *NPK1*, an orthologue of *ANP1* showed enhanced tolerance to cold, drought and ABA (Kovtun *et al.* 2000). Hence an ANP1 cascade involving AtMPK3 might be involved in cold signal transduction. In alfalfa, a gene encoding a negative regulator of MAPKKK, a mitogen protein phosphatase type 2C, has been cloned and proposed to act as a negative regulator of cold-, drought-, touch- and wound-induced MAPK cascade (Meskiene *et al.* 1998). In *Arabidopsis*, AtMPK4 (MAPK), AtMPK6 (MAPK) and a 44 kDa MAPK are activated by phosphorylation within 5 min by cold, dehydration, wound and touch but not by ABA and heat (Ichimura *et al.* 2000). Using yeast two-hybrid analysis, a possible MAPK cascade comprising ATMEKK1 (MAPKKK), MEK1 (MAPKK)/ATMKK2 (MAPKK) and ATMPK4 (MAPK) has been proposed (Mizoguchi *et al.* 1998). How cold-induced calcium signatures activate MAPKKKs and what are the target proteins of cold stress-activated MAPK await further studies.

5. RECEPTOR PROTEIN KINASES

Receptor protein kinases (which include two-component histidine kinases, receptor-like protein kinases and G-protein associated kinases) play active roles in environmental stress signal transduction. In *Synechocystis* PCC6803, a two-component histidine kinase *HIK33* has been identified. Autophosphorylation of HIK33 occurs upon sensing the cold-induced membrane rigidity and subsequently transfers a phosphate group to HIK19, then to RER1. RER1 induces the expression of fatty acid desaturase gene (*desB*) (Suzuki *et al.* 2000, 2001). Although AtHK1, a two-component regulator, has been proposed to function as osmosensor in *Arabidopsis* (Urao *et al.* 2000), so far no two-component system involved in cold stress signalling has been identified in higher plants. Involvement of a G-protein was demonstrated in ABA signal transduction in stomata (Wang *et al.* 2001), but involvement of G-proteins and associated receptors in cold stress signal transduction are not known. In *Arabidopsis*, the receptor-like protein kinase, RPK1, contains a putative amino terminal signal sequence domain, an extracellular domain with leucine-rich repeat sequences, a

membrane-spanning domain and a cytoplasmic protein kinase domain. *RPK1* gene expression is rapidly induced by cold, salt and dehydration stress and the expression is ABA-independent as the dehydration-induced expression is not impaired in the ABA biosynthesis mutant (*aba1*) and ABA-insensitive mutants (*abi1-1*, *abi2-1* and *abi3-1*) (Hong *et al.* 1997). Whether this RPK1 participates in cold signal perception or transduction is not known.

6. REGULATION OF COLD-RESPONSIVE GENES

Cold acclimatization is accomplished by the expression of many cold-regulated genes (reviewed by Thomashow 1999; Shinozaki & Yamaguchi-Shinozaki 2000; Browse & Xin 2001; Zhu 2001). In *Arabidopsis*, these genes are called *rd* (responsive to dehydration), *erd* (early responsive to dehydration), *lti* (low-temperature induced), *kin* (cold-induced) and *cor* (cold-regulated). These genes are also induced by dehydration (due to water deficit or high salt) and ABA, and can be collectively called cold-responsive genes. Cold-responsive gene expression studies in ABA deficient (*aba*) and ABA-insensitive (*abi*) mutants of *Arabidopsis* demonstrated that expression of some cold-responsive genes is mediated by both ABA-independent and ABA-dependent pathways (Kurkela & Franck 1990; Nordin *et al.* 1991; Horvath *et al.* 1993; Yamaguchi-Shinozaki & Shinozaki 1993; Ingram & Bartels 1996). To understand the mechanism of regulation, the promoter region of *RD29A* (= *COR78*/*LTI78*) gene of *Arabidopsis* was analysed by Yamaguchi-Shinozaki & Shinozaki (1994) and they identified DRE or CRT, a *cis*-element with CCGAC as its core sequence. CRT/DRE-related motifs have also been identified in the promoters of genes regulated by osmotic, low temperature and salt stress, including *COR15a*, *KIN1*, *COR6.6*/*KIN2*, *RAB18* and *RD17*/*COR47* in *Arabidopsis* (Kurkela & Franck 1990, 1992; Lång & Palva 1992; Baker *et al.* 1994). These DRE elements are not involved in ABA-dependent gene expression (Wang *et al.* 1995; Shinwari *et al.* 1998) because these genes are expressed in *aba* and *abi* mutants of *Arabidopsis*. Hence it is thought that cold-regulated gene expression occurs through ABA-independent pathways. However, a cold-induced transient increase in intracellular ABA was observed in many plant species. Analysis of promoter regions of *RAB18*, *LTI65*, *RD29A* and *RD29B* revealed the presence of ABREs, PyACGTGGC (Nordin *et al.* 1993; Welin *et al.* 1994; Yamaguchi-Shinozaki & Shinozaki 1994). Cold-responsive accumulation of *RAB18* and *LTI65* transcripts is severely impaired in *aba1* or *abi1* mutants. Hence cold-responsive regulation of these genes may occur through an ABA-dependent pathway (Lång & Palva 1992; Nordin *et al.* 1993). By chemical mutagenesis of transgenic *Arabidopsis* plants carrying the *RD29A* promoter-driven luciferase reporter gene, Ishitani *et al.* (1997) have isolated mutants with hyper-expression or diminished expression of *RD29A* in response to both cold and ABA, and thus demonstrated that cold- and ABA-dependent regulatory pathways cross-talk at some nodes of signal transduction.

7. CRT/DRE-DEPENDENT REGULATION OF COLD-RESPONSIVE GENES

An important step towards understanding of the cold-responsive gene regulation was isolation of a gene encoding a CRT/DRE-binding protein, called CBF1, from *Arabidopsis* by Stockinger *et al.* (1997). Later, five independent genes encoding DREBs were isolated from *Arabidopsis* using a yeast one-hybrid screening. Similar to CBF1, these DREBs also contain an APETELA2/ethylene-responsive element binding protein DNA binding domain. These DREBs are classified into two classes: DREB1 (DREB1A, DREB1B & DREB1C) and DREB2 (DREB2A & DREB2B) (Liu *et al.* 1998). CBF1 homologues, namely CBF2 and CBF3, have also been cloned from *Arabidopsis* (Gilmour *et al.* 1998). Both DREB1 and DREB2 can specifically bind to the CRT/DRE elements and transactivate cold-responsive genes in yeast and *Arabidopsis* protoplasts. Expression of *DREB1A* (= *CBF3*) and its homologues, *DREB1B* (= *CBF1*) and *DREB1C* (= *CBF2*), is induced by low temperature stress, while expression of *DREB2A* and *DREB2B* is induced by dehydration and salt stresses (Liu *et al.* 1998). Thus two independent families of DREB proteins, DREB1 and DREB2, function as transcriptional factors in low temperature and dehydration signal transduction pathways, respectively, to activate CRT/DRE *cis*-elements. Constitutive over-expression of CBFs under the control of the *CaMV35S* promoter induced cold-responsive gene expression strongly and also imparted acquired freezing tolerance to the transgenic *Arabidopsis* without prior cold treatment (Jaglo-Ottosen *et al.* 1998; Liu *et al.* 1998; Kasuga *et al.* 1999). Over-expression of CBF3 driven by the *RD29A* promoter resulted in a constitutive low-level expression of cold-regulated genes and enhanced expression under cold, dehydration and salt stresses in transgenic *Arabidopsis* (Kasuga *et al.* 1999). These studies provided functional evidence for the involvement of CBFs in cold signal transduction; they act as nodes of cross-talk between cold, dehydration and salt stress signalling pathways and also offer a promising approach to engineer multi-stress-tolerant transgenic plants of agronomic value. Towards this step, Thomashow and his colleagues have over-expressed the *Arabidopsis CBF* genes in canola (*B. napus*) and found that the expression of CRT/DRE-regulated genes increased freezing tolerance in both acclimatized and non-acclimatized canola plants (Jaglo *et al.* 2001). Recently, Stockinger *et al.* (2001) have shown that transcriptional activation of CRT/DRE *cis*-elements by CBFs involves a chromatin structure modifying transcriptional adaptor complex consisting of Ada2, Ada3 and GCN5 (histone acetyltransferase). These proteins are constitutively expressed in *Arabidopsis* in all tissues with the highest expression in leaves, and cold stress did not alter the expression of the genes.

8. ABRE-MEDIATED REGULATION OF COLD-RESPONSIVE GENES

The transient increase in ABA during cold stress and enhancement of freezing tolerance by exogenous application of ABA indicate that ABA must be playing a critical role in cold acclimatization. Cold- and cold stress-

mediated dehydration lead to an increase in endogenous ABA, which might regulate cold-responsive genes through the ABRE *cis*-elements (PyACGTGGC), as these elements have been identified in the promoters of *COR15a* (Baker *et al.* 1994), *RD29A* (Yamaguchi-Shinozaki & Shinozaki 1994) and *COR6.6* (Wang *et al.* 1995). Gene expression through ABREs is regulated by bZIP-transacting proteins in plants. Cold-regulated bZIP proteins have been identified in *Arabidopsis* (Lu *et al.* 1996; Choi *et al.* 2000), rice (Aguan *et al.* 1993) and maize (Kusano *et al.* 1995). In *Arabidopsis*, three cold-induced C2H2 zinc finger proteins, *AZF1*, *AZF3* and *STZ*, have been cloned (Sakamoto *et al.* 2000). Four ABRE binding factors (ABF1, 2, 3 & 4) have been cloned from *Arabidopsis* using a yeast one-hybrid system. All four ABFs are induced by ABA, while the induction of *ABF1* is specific to cold and ABA. ABFs could transactivate ABRE-driven reporter gene expression in yeast (Choi *et al.* 2000). However, no transgenic plants have been developed to show the role of these bZIP and C2H2 zinc fingers in cold-responsive gene regulation and cold acclimatization. Recently, Kim *et al.* (2001) have cloned a novel cold-inducible zinc finger protein from soybean, *SCOF1*, using an mRNA differential display technique. The SCOF1 contains two C2H2-type zinc fingers and a putative nuclear localization signal, KRKRSKR. The *SCOF1* expression pattern differs from *DREB1* in the following ways: (i) *SCOF1* is weakly constitutive but DREB1 is cold-inducible; (ii) *DREB1* is induced by cold (4 °C) within 40 min, reaches maximum expression by 2 h and then slowly decreases to a minimum level by 24 h at 4 °C (Liu *et al.* 1998; Shinwari *et al.* 1998), while *SCOF1* induction occurs at 3 h at 4 °C and then the level of transcript tends to increase even up to 72 h (Kim *et al.* 2001); (iii) *SCOF1* could be weakly induced by exogenous application of ABA, while *DREB1* expression is not induced by ABA. These temporal sequences in the expression pattern of *DREB1* and *SCOF1* indicate that the initial induction of cold-responsive gene expression by *DREB1* is synergistically increased by *SCOF1* during cold stress (after 3 h when DREB1 decreases). The SCOF1:GUS fusion protein revealed that SCOF1 is a nuclear protein. Over-expression of *SCOF1* under the control of the constitutive CaMV35S promoter in *Arabidopsis* resulted in constitutive expression of cold-responsive genes (*COR15a*, *COR47* and *RD29B*) and constitutive freezing tolerance. However, SCOF1 did not directly bind to ABRE or DRE/CRT motifs. Transactivation experiments in *Arabidopsis* protoplasts revealed that SCOF1 enhanced the DNA binding activity of SGBF1, a bZIP transcription factor (Kim *et al.* 2001). SGBF1 is cold- and ABA-inducible (Hong *et al.* 1995). Thus SCOF1 interacts with SGBF1 to regulate the cold-responsive gene expression through activation of ABRE in ABA-dependent pathways of cold stress signal transduction (Kim *et al.* 2001).

9. REGULATION OF CBFs/DREBs AND bZIP TRANSACTING FACTORS

The *DREB1* and *DREB2* genes are expressed only under stress. *DREB1A* was induced with in 1 h at 4 °C and the expression peaked at 2 h at 4 °C. *DREB2* was induced by 250 mM NaCl within 10 min, but reached its maximum at 5 h of stress in *Arabidopsis* (Liu *et al.* 1998). Hence the question arises, how are these CBF genes regulated by cold and dehydration stresses/ABA? Analysis of promoter regions of *DREB1*-family genes of *Arabidopsis* revealed that the 5′ upstream regions contain motifs similar to G-box and ABRE sequences (T/CACGTGG/TC), and to MYB (C/TAACNA/G) and MYC (CANNTG) recognition motifs. Because *DREB1* genes were not induced by ABA, the G-box motifs do not function as ABREs (Shinwari *et al.* 1998). *Arabidopsis* cold-induced C2H2 zinc finger proteins, *AZF1*, *AZF3* and *STZ*, also have MYB and MYC *cis*-acting motifs. The transcript level of these proteins reached a maximum within 30 min of cold stress (Sakamoto *et al.* 2000). One of the largest families of transcription factors in *Arabidopsis* is the MYB-R2R3 family, which contain two imperfect repeats of the MYB motif (Riechmann *et al.* 2000). The MYB motif consists of a helix-turn-helix structure with three regularly spaced tryptophan residues. An *Arabidopsis* cDNA encoding a MYB homologue, *AtMYB2*, was cloned from a cDNA library of dehydrated rosette plants. *AtMYB2* was induced by ABA, salt and dehydration stresses, and disappeared upon rehydration. An *AtMYB2* promoter-driven GUS reporter could be activated by dehydration and salt stresses in transgenic *Arabidopsis* (Urao *et al.* 1993, 1996). AtMYB2 proteins have been shown to transactivate the *RD22B* promoter-driven GUS reporter in *Arabidopsis* leaf protoplast (Abe *et al.* 1997). However, to our knowledge, there is no evidence so far that MYB transacting factors are involved in the regulation of CBFs/bZIPs expression through MYB-related *cis*-elements present in their 5′ upstream regions.

10. GENETIC DISSECTION OF COLD SIGNAL TRANSDUCTION

A classical genetic approach on freezing tolerance led to the identification of *sfr* (sensitive to freezing) mutants in *Arabidopsis* (Warren *et al.* 1996). In the *sfr6* mutant, the cold-induced expression of *KIN1*, *COR15a* and *RD29A* was abolished, and also osmotic stress and ABA-induced expression of *KIN1* was inhibited. However, the expression of *CBF1*, *CBF2*, *CBF3* and *ATP5CS1* was not influenced by the *sfr6* mutation. Hence SFR6 specifically affects the transactivation of DRE/CRT by CBFs (Knight *et al.* 1999). It appears that *sfr6* is also involved in ABRE-regulated gene expression, as ABA and salt stress could not induce *KIN1*. Cloning and characterization of *sfr6* may shed further light on the regulation of cold-responsive genes. Identification of the *esk1* (constitutively freezing-tolerant) mutant of *Arabidopsis* revealed proline accumulation as a possible mechanism of acquired freezing tolerance (Xin & Browse 1998). The *esk1* mutant did not differ in its cold-responsive gene expression from the WT, but maintained a 30-fold higher proline level due to higher expression of the *P5CS* gene when compared with the WT plants in normal growing conditions. In *esk1*, cold acclimatization leads to differential expression of cold-responsive genes, i.e. *RD29A*, *COR47* and *COR15a* expression were similar to that of the WT and *RAB18* expression was enhanced by three to fourfold, while *COR6.6* expression was significantly reduced. Further understanding of how

these cold-responsive genes are differentially regulated needs the molecular cloning of *ESK1*.

Genetic analysis of chemically mutagenized *Arabidopsis* transgenic with *RD29A* promoter (which contains both CRT/DRE and ABRE)-driven luciferase revealed that cold, drought, salt and ABA stress signalling pathways interact at different nodes of signal transduction (Ishitani *et al.* 1997). The *hos1* (high expression of osmotically responsive genes) mutation resulted in super-induction of *RD29A*, *COR47*, *COR15a*, *KIN1* and their transacting factors (*CBF2* and *CBF3*) at 4 °C. In WT plants, these genes are also induced by ABA, high salt, or polyethylene glycol in addition to cold, but the *hos1* mutation only enhances their expression under cold stress (Ishitani *et al.* 1998). The expression of *CBF*s is transient in the WT, while in the *hos1* mutant *CBF*s mRNA abundance was maintained at a much higher level even up to 24 h during cold stress. Hence HOS1 negatively regulates the cold-responsive genes by modulating the expression level of the CRT/DRE binding factors. Molecular cloning and characterization revealed that *HOS1* encodes a ring finger protein, which has been implicated as an E3 ubiquitin conjugating enzyme. *HOS1* is constitutively expressed, shows a drastic decrease within 10 min of cold stress and recovers back to the basal level after 1 h of cold stress. HOS1 protein is present in the cytoplasm at normal growth temperatures and accumulates in the nucleus upon cold stress. The *hos1* mutation also affected the thermosensing mechanism, as is evident from the fact that *RD29A* expression occurs at relatively warmer temperatures (Lee *et al.* 2001). The cold-induced Ca^{2+} signature outputs depend on the rate of stress development (change in temperature per unit time). The *hos1* mutant reached a maximum level of *RD29A* expression within 10 h at 0 °C while WT plants reached maximal level of expression only at 24 h at 0 °C, which indicates that the rate of output signal from the cellular thermosensor is much higher in the *hos1* mutant. These results show that, being a constitutively expressed protein, HOS1 may be closely interacting with cellular thermosensors to modulate thermosensing and the rate of signal output from the cellular thermosensor (Lee *et al.* 2001). Cold-responsive genes are regulated by both ABA-independent and ABA-dependent pathways during cold stress. The expression of *RD29A:luc* showed a threefold increase if the *Arabidopsis* plants were treated with ABA after 44 h at 0 °C. Hence, at low temperatures, ABA acts synergistically with the cold signal (Xiong *et al.* 1999). However, in *hos1* and *hos2* mutants of *Arabidopsis*, cold-responsive genes are super-induced by cold stress but their expression pattern is unaltered by ABA or salt stress, indicating that cold-dependent signal transduction is specifically altered by these mutations (Ishitani *et al.* 1998; Lee *et al.* 1999). Although the signal flow through cold signal transduction modules has increased, it did not influence the signal through the ABA-dependent transduction module. Over-expression of cold-responsive genes in transgenic plants achieved through over-expression of *CBF*s or the antisense *AtPP2CA* gene conferred better freezing tolerance. By contrast, the super-induction of cold-responsive genes was not sufficient to provide cold acclimatization in *hos2* mutants. Because the expression kinetics of the *P5CS* gene in *hos2* mutants was similar to the WT, proline concentration in the cell is not responsible for decreased capacity of the *hos2* mutant to cold acclimatization. Hence it appears that *HOS2* is a negative regulator of cold signal transduction required for developing cold acclimatization.

11. CONCLUSIONS AND FUTURE PERSPECTIVES

Combined use of genetics and molecular approaches has begun to shed light on cold signal transduction modules and their components. Pharmacological and biochemical evidence shows that membrane rigidification followed by cytoskeleton rearrangement, Ca^{2+} influx and Ca^{2+}-dependent phosphorylation are involved in cold stress signal transduction. Genetic evidence provided by *Arabidopsis fry1* mutants indicates that change in IP_3 level is an important component of cold signalling. Protein dephosphorylation negatively regulates the cold-responsive genes, as evident from *AtPP2CA* antisense transgenic plants. Cold-responsive genes are regulated through CRT/DRE and ABRE *cis*-elements by transacting factors CBFs/BREBs and bZIPs (SGBF1), respectively. Constitutive over-expression or stress promoter-driven expression of these transacting factors induced cold-responsive genes and freezing tolerance in transgenic plants. Genetic evidence showed that *HOS1* is a negative regulator of CBFs/DREB1-dependent cold-regulated genes and is a modulator of the cellular thermosensor's sensitivity to temperature. Still, the components of Ca^{2+}-mediated signal transduction into the nucleus and their spatial and temporal positions in cold signalling need to be defined genetically. We have started genetic screens using *DREB1* promoter-driven luciferase, which may help to identify further the cold signalling components that regulate *DREB1* transacting factors. At the same time, it is important to continue to explore in agronomically important crops, such as rice, wheat, maize, soybean, tomato etc., which suffer from low/freezing temperatures, whether similar cold signalling modules are employed. If different mechanisms are found, then future work will identify the novel components.

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GLOSSARY

ABA: abscisic acid
ABRE: ABA-responsive element
AtCBL: *A. thaliana* calcineurin B-like
bZIP: basic leucine zipper
cADPR: cyclic adenosine 5′diphosphate ribose
CAS30: cold acclimation-specific protein
CBF: C-repeat binding factor
CBL: calcineurin B-like
CDPK: calcium-dependent protein kinase
CIPK1: CBL-interacting protein kinase 1
CRT: C-repeat
DMSO: dimethyl sulphoxide
DRE: dehydration-responsive element
DREB: dehydration-responsive element binding protein
esk1: *eskimo1*
fry1: *fiery1*
IP_3: inositol (1,4,5)-triphosphate
MAPK: mitogen activated protein kinase
MAPKK: MAPK kinase
MAPKKK: MAPK kinase kinase
RER1: response regulator 1
SCOF1: soyabean cold-inducible factor 1
SGBF1: soyabean G-box binding factor 1
TaADF: *Triticum aestivum* actin depolymerizing factor
WT: wild-type