

REVIEW

Some key physiological and molecular processes of cold acclimationR. JOHN¹, N.A. ANJUM², S.K. SOPORY³, N.A. AKRAM^{4*}, and M. ASHRAF⁵*Department of Botany, University of Kashmir, Hazratbal, Srinagar, 190006, Kashmir, India¹**CESAM and Department of Chemistry, University of Aveiro, 3810-193, Aveiro, Portugal²**Jawaharlal Nehru University, New Delhi, 110001, India³**Department of Botany, GC University, Faisalabad, 38000, Pakistan⁴**Department of Botany & Microbiology, King Saud University, Riyadh, 11451, Saudi Arabia⁵***Abstract**

Agricultural production worldwide has been severely impacted by cold and freezing stresses. Plant capacity to acclimate to environmental conditions in their immediate vicinity largely control their survival, growth, and productivity. Molecular as well as biochemical mechanisms underpinning plant cold acclimation are very complex and interwoven. The cold-impacted plants try to modulate expression of variety genes controlling cell membrane lipid composition, mitogen-activated protein kinase cascade, total soluble proteins, polyamines, glycinebetaine, proline, reactive oxygen species (ROS) scavengers, cryoprotectants, and a large number of cold responsive factors. To this end, this paper dissects the array of transcriptional factors/genes down- or up-regulated, their identification in different plant species, recognition of cold tolerant/resistant transgenic plants, complexity of the mitogen-activated protein kinase cascade, as well as their cross talk under different stresses and molecular mechanisms. Furthermore, it also comprehensively elucidates physio-biochemical interferences in cold acclimation with a particular emphasis on endogenous content as well as exogenously supplied different types of polyamines, ROS, and osmoprotectants. Overall, low temperature stress tolerance or cold acclimation varies greatly among species depending on the stress intensity and duration and type of plant species.

Additional key words: cryoprotectants, gene expression, glycinebetaine, MAPK, membrane lipids, polyamines, proline, ROS, transcription factors, transgenic plants.

Introduction

Low temperature is one the major environmental factors limiting growth of many plant species (Sanghera *et al.* 2011, Cruz *et al.* 2013, Jeon and Kim 2013, Chen *et al.* 2014). However, the responses of plants to low temperature stress vary considerably depending on the type of plant species as well as intensity and duration of the stress (Thomashow 1999, Fowler 2008, Chen *et al.* 2014). For example, differential tolerance in different cereal crops (barley, wheat, and rye) was reported by Fowler (2008). He was of the view that cereals monitor

temperature with more precision compared with other crops. Hardy wheat cultivars have a 5.7 °C warmer activation temperature than tender genotypes when the vernalization gene is neutralized in near-isogenic lines, and a 12 °C difference in induction temperature of hardy rye relative to tender barley cultivars emphasizes the high cold adaptation potential of rye (Fowler 2008). This early response to low temperatures indicates that hardy rye has a longer time to prepare for the extremes of winter and is in a better position to cope with unexpected frosts during

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Abbreviations: α -Gal - alpha-galactosidase; ABA - abscisic acid; ADC - arginine decarboxylase; APX - ascorbate peroxidase; BADH - betaine aldehyde dehydrogenase; bHLH - basic helix-loop-helix; CBF - C-repeat/DRE-binding factor; COR - cold-regulated; DRE - dehydration-responsive element; ERD - early-responsive to dehydration; ERF - ethylene-response factor; F_v/F_m - variable to maximum chlorophyll fluorescence ratio; GA - gibberellin; GB - glycinebetaine; GPX - glutathione peroxidase; ICE1 - inducer of CBF expression1; MAPK - mitogen-activated protein kinase; MAPKK - MAPK kinase; MAPKKK - MAPK kinase kinase; MYB - myeloblastosis; MYC - myelocytomatosis; P5CS - pyrroline-5-carboxylate synthetase; PAs - polyamines; RCI - rare cold-inducible gene; ROS - reactive oxygen species; Spd - spermidine; Spm - spermine; TF - transcription factor.

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the growing season as compared to wheat and barley. Recently, Chen *et al.* (2014) added in a review that cold is a typical environmental constraint which depends on the geographical distribution of plants. They reported that it is not completely known yet how content of unsaturated fatty acids, protective enzymes, functional genes, and regulation genes are involved in cold resistance. The plant ability to tolerate low temperature is known as cold tolerance, cold acclimation/adaptation, and cold avoidance. Chilling tolerance is the ability of a plant to tolerate low but above zero temperatures without damage or injury (Somerville 1995). However, freezing tolerance is the ability of plants to survive ice formation in extracellular spaces without a significant damage to membranes or other cell components (Blum 1988). Many plants exhibit freezing tolerance after exposure to a low non-freezing temperature (below 10 °C), a phenomenon known as cold acclimation (Thomashow 1999). At one extreme, there are plants from tropical regions that have virtually no capacity to survive even the slightest freeze. In contrast, herbaceous plants from temperate regions generally survive freezing temperatures ranging from -5.0 to -30 °C, depending on the species, whereas forest trees survive temperature below -30 °C. During freezing avoidance strategy, plant cells maintain internal fluids in a supercooled state, more stable under extreme climates, and protect the plant by avoiding ice formation (Thomashow 1998, Cruz *et al.* 2013).

Like many other abiotic stresses, cold/freezing stress also causes a variety of disorders in plant metabolism. For example, cold stress causes cell death (primarily due to cytoplasmic dehydration) and ice formation in cell wall (Lissarre *et al.* 2010), disturbance in water relations (Beck *et al.* 2007), poor seed germination, suppression of leaf expansion, stunted plant growth, loss of rigidity, chlorosis, and necrosis have been reported. Similarly, delayed energy dissipation, acceleration of oxidative stress (Beck *et al.* 2004), crop plant sterility (Jiang *et al.* 2002), and restricted seed development have been reported (Smith and Stitt 2007, Kumar *et al.* 2011).

Plant tolerance to freezing (< 0 °C) and/or chilling (0 - 15 °C) varies considerably (Levitt 1980). However, in contrast, economically essential crop plants, *e.g.*, rice, maize, tomato, soybean, cotton, and banana, are reported to be highly susceptible to low temperature stress (10 to 15 °C); those plants rapidly show symptoms of damages under such temperatures (Shinozaki and Yamaguchi-Shinozaki 1996, Thomashow 1998, Gilmour *et al.* 2000, Imin *et al.* 2004, Gammulla *et al.* 2010, Miura and Furumoto 2013). In fact, plants from subtropical or tropical areas usually have low or no cold tolerance (Carvalho *et al.* 2011) and virtually no capacity to survive even under slight freezing (Thomashow 1998, Miura and Furumoto 2013). After cold acclimation, temperate plants are able to survive over winter at freezing temperatures (Weiser 1970, Dinari *et al.* 2013). Cold acclimation in plants can be induced for a specific time period at or below 10 °C (Thomashow 1999, Hannah *et al.* 2006). It is a complex strategy which involves several biochemical

and physiological changes such as variation in lipid composition, hormonal balance, accumulation of cryoprotective molecules (sugars and proline), total soluble proteins, and activation of a large number of cold related genes such as myeloblastosis (MYB) 15, C-repeat/dehydration-responsive (DRE)-binding factor (CBF), and CBF3 (Guy 1990, Hannah *et al.* 2005, Chinnusamy *et al.* 2007, Zhu *et al.* 2007, Lissarre *et al.* 2010). In fact, the differences in cold acclimatization ability in different plants are genetically determined (Kurepin *et al.* 2013).

Cold de-acclimation (de-hardening) and re-acclimation (re-hardening) are important agronomic traits for plant survival during winter. In winter cereals, winter-hardiness/tolerance can be enhanced by exposure to low non-freezing temperatures (Guy 1990, Rapacz 2002, Kalberer *et al.* 2006, Schulze *et al.* 2012). The term de-acclimation can be used to describe losses in hardiness due to warm temperatures, phenological changes, and reactivation of growth (Kalberer *et al.* 2006). Although the volume of research on various aspects of cold acclimation is reasonably high, the processes of de-acclimation and re-acclimation are less understood particularly in late winter and spring crops (Rapacz 2002, Kalberer *et al.* 2006). Some overwintering plants can return to previous cold tolerance after de-acclimation, which has been referred to as re-acclimation (Rapacz 2002). The capability of overwintering in winter cereals results in their ability to withstand cold hardening during autumn or de-hardening during temporary warm spells and then to withstand re-hardening when cold waves return (Janmohammadi *et al.* 2012). The same authors reported that cold acclimation induces elevation in the activities of antioxidative enzymes in the leaves of winter wheat, whereas in spring wheat a considerable increase was recorded only after the de-acclimation period. Gorsuch *et al.* (2010) assessed leaf phenotypes for initially warm (25/20 °C day/night) grown plants that are exposed to cold (5 °C) before being transferred back to the warm temperature at the reproductive stage. The leaves that experience cold for extended periods during early development show less plasticity in either photosynthesis or respiration, and they do not revert to a warm-associated saccharide profile. Moreover, cold exposure of immature/developing leaves for as little as 5 d results in irreversible changes in morphology of leaves that subsequently mature in a warm, with 15-d cold being sufficient for a permanent alteration of leaf anatomy (Gorsuch *et al.* 2010).

Thus, an extensive attention has been paid to improve low temperature stress tolerance in crops during the past few decades (Thakur *et al.* 2010). Nevertheless, a number of factors including complexity of the mechanism of cold tolerance, genetic variation under cold stress, and inefficient selection for cold tolerance considerably limit progress in terms of improved cold tolerance through conventional breeding approaches such as intra-specific selection as well as inter-specific/generic hybridization. Moreover, recent literature search has displayed a number of critical gaps in our current understanding of tolerance

to low temperature stress in different plants. Hence, the present review describes major physio-biochemical processes, roles of key enzymes, cold stress proteins and

genes, as well as molecular mechanisms involved in plant cold acclimation.

Metabolic responses

Cold stress affects plant growth at all levels of organization. Whole plant level symptoms under cold stress are wilting, suppression of leaf expansion, yellowing leaves, necrosis (tissue death), and disturbance in water relations and in reproductive development (Jiang *et al.* 2002, Thakur *et al.* 2010). Cold stress-induced effects at cellular levels include: impairment in functioning electron transport, cellular dehydration, turgor loss, radical formation, and oxidative stress (Beck *et al.* 2007). Cold stress can also reduce membrane fluidity by changing content and conformation of metabolites, proteins, and nucleic acids (Chinnusamy *et al.* 2007). It also causes modifications in composition, ultrastructure, and function of plasma membranes and cell rupturing due to intercellular ice adhesion between the cell wall and membranes (Olien and Smith 1977), protein denaturation (Guy 1990), and changes in cell cycle and cell division (Chen *et al.* 2011). Also, plants introduce antifreeze proteins, *e.g.*, late embryogenesis abundant proteins (Sanghera *et al.* 2011) and undergo metabolic changes in a number of ways as accumulation of osmoprotectants [glycine-betaine (GB), proline, raffinose] that are capable of stabilizing structures of proteins and adjusting osmolarity inside cells (Zhang *et al.* 2014). Additionally, changes in antioxidant metabolism occur to efficiently remove excess reactive oxygen species (ROS) accumulation and maintain redox balance (Janska *et al.* 2010, Sanghera *et al.* 2011). Overall, the aforesaid mechanisms facilitate plants to maintain osmotic balance at low temperature (Janska *et al.* 2010, Wang *et al.* 2013).

Saccharides: Studies have shown that there is a direct correlation between cold-induced modulation of sucrose metabolism and low temperature tolerance (Tabaei-Aghdai *et al.* 2003, Mollo *et al.* 2011, Sanchez-Bel *et al.* 2012, Folgado *et al.* 2013). The high content of soluble sugars including sucrose, stachyose, sorbitol, glucose, raffinose, fructose, and mannitol have been observed in overwintering or cold acclimated plants (Mollo *et al.* 2011, Krasensky and Jonak 2012).

Accumulation of soluble sugars resist against freezing temperatures in addition to helping plants in osmoregulation under stress conditions (Qin *et al.* 2011). An elevated soluble sugar pool has been reported during cold acclimation in *A. thaliana* (Strand *et al.* 2003, Takagi *et al.* 2003, Klotke *et al.* 2004, Rohde *et al.* 2004), winter wheat (Sagisaka *et al.* 1991, Hurry *et al.* 1995, Kamata and Uemura 2004), *Alcantarea imperialis* (Mollo *et al.* 2011), barley (Tabaei-Aghdai *et al.* 2003), potato (Folgado *et al.* 2013), rice (Huang *et al.* 2012), cabbage (Sasaki *et al.* 1996), and *Brassica napus* (Hurry *et al.*

1995, Gusta *et al.* 2005). Upon cold shock, different sugars including maltotriose and maltose followed by glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate, and galactose-6-phosphate accumulate in *Arabidopsis* (Kaplan *et al.* 2007). Trehalose reversibly absorbs water and is thus suitable for protecting biological membranes from desiccation-induced damage (Fernandez *et al.* 2010). Alterations in distribution of different sugars may also protect specific compartments by regulating cell expansion and dehydration (Gerhardt and Heldt 1984). It is documented that sugars accumulate in the chloroplasts of cabbage under cold stress (Fowler *et al.* 2001).

The plant metabolome is not passively involved during cold stress, but it is vital in signaling and over-expression of cold regulated genes (Zhu *et al.* 2007). Over-accumulation of several isoforms of trehalose-6-phosphate synthase has been reported to confer enhanced resistance to abiotic stresses including cold in rice (Li *et al.* 2011). Plants with altered sugar metabolism have provided some insights into the role of sugars in cold stress tolerance. For example, the introgression of fructosyl-transferases to fructan-deficient rice and tobacco plants enhances not only production of fructan but also tolerance to cold stress (Kawakami *et al.* 2008).

Alpha-galactosidase (α -Gal) regulates different plant processes such as hydrolysis of the α -1,6-linkage of raffinose at the time of deacclimation. Pennycooke *et al.* (2003) found an increase in raffinose content whereas a decrease in α -Gal induces chilling tolerance in petunia in both cold-acclimated plants and non-acclimated plants.

Polyamines (PAs) including spermine (Spm), putrescine (Put), and spermidine (Spd) are universal polycationic (positively charged) organic compounds having two or more primary amino groups. Polyamines interact with major macromolecules, such as phospholipids, RNA, DNA, and proteins, resulting in alteration of physico-chemical characteristics of nucleic acids, membranes, and enzymes (Alcázar *et al.* 2006, Kocsy *et al.* 2011). The high content of PAs has been found to be positively related to stress tolerance (Kovacs *et al.* 2010, Ashraf *et al.* 2011). In plants, ornithine and arginine are the precursors of PAs. In *Arabidopsis*, PAs biosynthesis relies on the arginine pathway, depending on activity of arginine decarboxylase, which synthesizes Put (Cook *et al.* 2004). Putrescine acts as precursor of Spd and Spm in the presence of Spd and Spm synthases (Alcázar *et al.* 2010, Table 1 Suppl.).

The content of free PAs is strictly regulated, and fluctuates in response to developmental stages and/or to abiotic constraints (Bitrian *et al.* 2012). Despite PAs have

been evidenced to protect membranes by minimizing oxidative stress, however, their definite role in stress tolerance is not clear (Alcázar *et al.* 2011, Krasensky and Jonak 2012). Over-expression of arginine decarboxylase lead to increased Put content as well as enhanced chilling and water stress tolerance in *A. thaliana* (Alet *et al.* 2011). A Spm synthase gene *OsSPDS2* is expressed in response to long-term chilling in *Oryza sativa* roots (Imai *et al.* 2004). In cold stressed *Thlaspi arvense* (Sharma *et al.* 2007) and *O. sativa* (Yan *et al.* 2006), S-adenosyl-l-methionine transcripts are up-regulated. In low temperature exposed *Arabidopsis* (14 d at 4 °C), Cook *et al.* (2004) reported an increase in Put, ornithine, and citrulline content, the precursors of further PAs. In *O. sativa* exposed to 5 °C, transcription of *OsSAMDC* increases more for up to 72 h in cold-resistant *O. sativa* cv. Yukihiikari than in susceptible cv. TKM9 (Pillai and Akiyama 2004). Generally, the high content of polyamines is observed in plants under cold stress/cold acclimation (Racz *et al.* 1996, Hummel *et al.* 2004, Yoshikawa *et al.* 2007, Kovacs *et al.* 2010). For example at -2 °C in apple trees, the content of jasmonic acid, Put, and Spd is generally higher than at 20 °C (Yoshikawa *et al.* 2007). In another study, cold tolerance in *Pringlea antiscorbutica* is connected with arginine decarboxylase gene (*ADC1* and *ADC2*) expressions (Hummel *et al.* 2004). In wheat seedlings, incubation at 5 °C for 6 or 9 h induced a significant increase in lipid peroxidation, membrane leakage, as well as hydrogen peroxide content, however, pre-sowing seed treatment with 0.5, 1.0, and 2.0 mM Spd considerably decreases these attributes (Abdel Kader *et al.* 2011). Activities of catalase, peroxidase, and ascorbate peroxidase also decline after exposure to chilling, whereas glutathione and ascorbate content increases in wheat seedlings, however, Spd treatment alleviate injury caused by chilling stress by improving ROS scavenging (Abdel Kader *et al.* 2011). Recently, Abavisani *et al.* (2013) has observed an increase in the antioxidative properties of anthocyanin, flavonoids, phenolics, and peroxidase activity due to application of putrescine to a root medium for *Dracocephalum* plants. In another study with *Arabidopsis* under cold stress, a significant increase in accumulation of PAs, particularly putrescine, occurred due to transcriptional upregulation of *ADC1* and *ADC2*. Mutants deficient in *adc1* and *adc2* display a lower freezing tolerance than wild-type plants (Cuevas *et al.* 2009).

Glycine betaine is an important osmolyte which accumulates in most plants under different environmental cues including cold stress (Sakamoto and Murata 2000, Xing and Rajashekar 2001, Park *et al.* 2006, Shirasawa *et al.* 2006, Zhang *et al.* 2011). The accumulation of GB correlates with cold acclimation in a number of plants including tomato (Park *et al.* 2006), rye (Koster and Lynch 1992), wheat (Kamata and Uemura 2004), rice (Shirasawa *et al.* 2006), *Arabidopsis* (Xing and Rajashekar 2001), and sugar cane (Rasheed *et al.* 2010). A rapid increase in GB content is observed in strawberry

plants during cold acclimation. In addition, exogenous GB is effective in inducing cold tolerance and regrowth in strawberry plants (Rajashekar *et al.* 1999). The introgression of GB biosynthetic pathway genes into GB non-accumulators, such as *Arabidopsis*, tomato, potato, and rice (Wyn Jones and Storey 1981), can improve their ability to tolerate abiotic stresses including cold stress (Park *et al.* 2004, Waditee *et al.* 2005, Abass *et al.* 2010, Ashraf *et al.* 2011, Bansal *et al.* 2011).

The introgression of a chloroplastic betaine aldehyde dehydrogenase (BADH) gene (*SoBADH*) obtained from spinach into sweet potato improves tolerance to low temperature stress by enhancing BADH activity, GB accumulation, photosynthetic activity, and antioxidants content and by reducing ROS accumulation and maintaining cell membrane integrity (Fan *et al.* 2012). Similarly, over-expression of an *E. coli betB* gene enhances GB accumulation and protects the photosynthetic apparatus of genetically modified *N. tabaccum* plants from photoinhibition under freezing stress (Holmstorm *et al.* 2000). *A. thaliana* and rice transgenic plants incorporating a *codA* gene perform better than wild-type plants under low temperature stress at different developmental stages. Furthermore, activity of photosystem II (in terms of the variable to maximum chlorophyll fluorescence ratio, F_v/F_m) of transgenic plants is efficiently protected due to a high GB accumulation (Sakamoto *et al.* 2000). In contrast, some transgenic plants produce GB in a very low concentration which might not be sufficient for osmotic adjustment (Giri 2011). For example, rice plants over-expressing a gene for choline monooxygenase isolated from *Spinacia oleracea* are not effective in crop productivity as well as in GB accumulation under cold stress (Shirasawa *et al.* 2006). Huang *et al.* (2000) introduced choline, a ubiquitous substance for production of betaine, in three species, tobacco, *Arabidopsis*, and *B. napus*, by over-expressing a bacterial choline oxidase gene. An increasing choline content considerably enhances betaine synthesis, and electrolyte leakage is higher in non-transgenic *Arabidopsis* at -3 °C as compared to the transgenic lines. The transgenic *Arabidopsis* plants are relatively tolerant to freezing stress as compared to wild-type plants. However, none of the transgenic lines of tobacco or *Brassica* shows better freezing tolerance than their non-transgenic controls.

Proline: The accumulation of proline has been widely reported in different plants under environmental stresses. For example, proline protects plants against osmotic stress, oxidative stress, and protein oxidation (Ashraf *et al.* 2011, Hayat *et al.* 2012, Kavi Kishor and Sreenivasulu 2014). Besides acting as an excellent osmolyte, it acts as antioxidant, metal chelator, and signaling molecule (Hayat *et al.* 2012). In addition, stress-induced over-accumulation of proline maintains cell pressure potential and osmotic balance, stabilizes membranes, and reduces electrolyte leakage and oxidative stress (Hayat *et al.* 2012, Kavi Kishor and

Sreenivasulu 2014).

The induction/expression of proline-responsive element (ACTCAT) has been ascribed to elevated proline accumulation (Sato *et al.* 2002). Different studies have evidenced the key role of proline in acquisition of cold acclimation in different plants including wheat (Kamata and Uemura 2004), *Arabidopsis* (Kaplan *et al.* 2007), creeping bentgrass (Sarkar *et al.* 2009), sugarcane (Rasheed *et al.* 2010), *Poa pratensis* (Sarkar *et al.* 2009), perennial ryegrass (Sarkar *et al.* 2009), and maize (Duncan and Widholm 1987). Application of proline is also an imperative strategy to overcome stress by increasing endogenous proline in different plants (Gleeson *et al.* 2004, Kamran *et al.* 2009, Ashraf *et al.* 2011, Hayat *et al.* 2012, Jonytiene *et al.* 2012). In another study, a low temperature (4 °C) completely inhibits growth of *Quercus robur* in a culture medium, but application of 1.0, 10, and 100 mM proline alleviates to some extent this growth inhibition (Gleeson *et al.* 2004). Some reports show that positive effects of exogenous proline can be achieved if it is supplied in low concentrations, but high proline concentrations show toxic effects (Hayat *et al.* 2012).

Very few investigations have so far been conducted for genetical engineering genes involved in proline accumulation in plants under cold stress (Yamaguchi-Shinozaki *et al.* 2000). For example, over-expression of a pyrroline-5-carboxylate synthetase (*P5CS*) gene leads to a higher accumulation of proline and a better tolerance to cold at 4 °C for 2 and 24 h in tomato plants (Patade *et al.* 2013). Ma *et al.* (2009) examined that transgenic rice plants over-expressing *OsCycB1;1* show resistance to cold stress by increasing cellular free proline content as compared to wild-type plants.

Reactive oxygen species including hydroxyl radical ($\cdot\text{OH}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1\text{O}_2$) and superoxide anion ($\text{O}_2^{\cdot-}$) are generated in plants as part of their metabolism and are also induced in response to environmental stresses (Ashraf 2009, Gill and Tuteja 2010, Akram and Ashraf 2013, Chowdhury *et al.* 2013). Reactive oxygen species play a dual role; at low concentrations, they trigger signal transduction, and at high concentrations, they cause oxidative stress finally leading to programmed cell death (Shinozaki and Yamaguchi-Shinozaki 1997, Zhou *et al.* 2012). Low

temperature stress causes oxidative stress due to over-production of ROS resulting in imbalance between energy absorbed during photosynthetic reactions in photosystems I and II, and its consumption during carbon dioxide reduction through the Calvin cycle (McKersie and Bowley 1997, Ensminger *et al.* 2006).

Reactive oxygen species produced under cold stress could be helpful as stress indicators. Genes including stress responsive genes *RD29A*, *KIN1*, *KIN2*, cold regulated (*COR*) *15A*, *COR47*, dehydration-responsive (*DRE*) *B1A*, *DREB2A*, early-responsive to dehydration (*ERD*) *10*, and *FRO1* regulate ROS involved in temperature stress tolerance or acclimation in plants by interacting with different processes such as ROS sensing and defense (Suzuki and Mittler 2006). In wheat lines differing in freezing tolerance, the production of ROS in mitochondria was examined under cold stress (Mizuno *et al.* 2011). The authors found that a freezing-tolerant cultivar produces a lower amount of ROS than a freezing-sensitive cultivar suggesting that ROS production increases severity of freezing stress in wheat.

In a comprehensive review on cold stress tolerance, Einset *et al.* (2007) suggested that chilling/freezing enhances ROS production which significantly reduces root growth, although after removal of stress ROS accumulation seized. Thus, normal root growth could be attained after transferring plants back to normal temperature. Under low temperature, an elevated content of H_2O_2 is a key response in four different accessions of *Arabidopsis* (Distelbarth *et al.* 2013). An increase in glutathione reductase activity with no or a reduced change in activities of ascorbate peroxidase (APX), catalase, and superoxide dismutase is observed in all accessions. However, the activities of the antioxidant enzymes and freezing tolerance in the four *Arabidopsis* accessions are not positively correlated (Distelbarth *et al.* 2013). It was proposed that H_2O_2 is involved in plant signal transduction under stress conditions. It diffuses from a source to subcellular micro domains very rapidly, but translocation depends on its concentration. Then it induces gene expression by transmitting intracellular signals by oxidizing different transcription factors of the signaling pathway (Gadjev *et al.* 2006). In this context, in rice, a ROS-mediated regulatory pathway is considered as an early response to chilling stress (Cheng *et al.* 2007).

Molecular basis of cold sensing and acclimation

Cold-regulated genes: Plant response to cold stress depends on cold signal transduction (Chinnusamy *et al.* 2005, 2007, Miura and Furumoto 2013, Seo *et al.* 2013). Cold tolerance and acclimation are regulated by the cascade of cold-responsive genes that either transcribes into transcription factors (TFs) or effector proteins (Guy 1990, Jaglo *et al.* 2001, Lee *et al.* 2005, Chinnusamy *et al.* 2007). A number of cold-regulated (*COR*) genes have been identified including *MYB*, (*C/TAACNA/G*),

C-repeat elements/dehydration-responsive elements (*CBFs*, *A/GCCGAC*), *MYC* recognition sequences (*CANNTG*), as well as abscisic acid (ABA)-responsive element (*ABRE*, *PyACGTGGC*) (Yamaguchi-Shinozaki *et al.* 2000, Xiong *et al.* 2002). Molecular dissection of plant cold stress adaptation has revealed a number of cold-induced genes. These genes are related to transcription factors (*CBFs* and *DREB1A*), membrane lipid modification, *e.g.*, fatty acid desaturase (*fad7*),

glycerol-3-phosphate acyl-transferase (*GPAT*), nodulin-related protein, rare cold-inducible genes (*RCI2A* and *RCI2B*), biosynthesis of amino acid- and sugar-related osmoprotectants, antioxidative response, plasma membrane protein (*MpRCI*), and ROS-induced mitogen-activated protein kinase (MAPK) cascade (*AtMEKK1-AtMKK2-AtMPK4/6*). These processes regulate the cold-induced gene system such as apple basic helix-loop-helix (*bHLH*) gene *MdCibHLH1* (Feng *et al.* 2009), protein kinase ATMEKK1 (structurally related to MAPK kinase, MAPKKK), ATPK19 (structurally related to ribosomal S6 kinase), and ATMPK3 (structurally related to MAPK) (Mizoguchi *et al.* 1996). The *Arabidopsis thaliana* cold-regulated genes encode, *e.g.*, calmodulin-related proteins and xyloglucan endotransglycosylase (Polisensky and Braam 1996) and *RCI2A* and *RCI2B* (Medina and Salinas 2001). Absciscic acid-dependent and -independent pathways have also been found to induce *COR* gene expression (Heidarvand and Amiri 2010) and the inducer of CBF expression1 (*ICE1*) (Chinnusamy *et al.* 2007). The introgression of these genes could be used to improve cold stress tolerance in cold sensitive species (Feng *et al.* 2009). Table 2 Suppl. lists some plants transformed with certain genes for their improved cold stress tolerance.

The role of cell membrane in cold sensing: Cell membranes are involved in exchange of compounds such as metabolites and nutrients (Kawamura and Uemura 2003, Cruz *et al.* 2013, Takahashi *et al.* 2013). Signals from environmental stimuli also transmit through the plasma membrane so it has a primary role in regulating cellular transport and signal transduction (Takahashi *et al.* 2013). The plasma membrane is very sensitive to freezing injury. However, the lipid or protein ratio alters during cold tolerance and helps to protect the plasma membrane from cold-induced dehydration stress (Uemura and Yoshida 1984, Yamazaki *et al.* 2009). A significant relationship between cold acclimation and plasma membrane proteins has been found in *Arabidopsis*, and a set of 38 proteins during 3 d of cold acclimation is observed (Kawamura and Uemura 2003). Of these, cold-responsive plasma membrane proteins include ERD10 and ERD14 proteins, the members of dehydrins and a novel cold acclimation plant synaptotagmin 1, which protect membranes and proteins under cold-induced dehydration (Uemura *et al.* 2006, Kosova *et al.* 2007).

Chilling stress induces membrane destruction/leakage (Mahajan and Tuteja 2005, Yadav *et al.* 2010, Thakur and Nayyar 2013). There is a direct relationship between the content of unsaturated fatty acids in the cell membrane and the chilling susceptibility of plants (Thomashow 1999). Cold-tolerant plants also contain high proportions of unsaturated fatty acids. Generally, the melting point of unsaturated fatty acids is low that is why cold tolerant plant membranes have the phase transition temperature lower than that of chilling stress (Hara *et al.* 2003). Furthermore, an increase in fatty acid unsaturation and phospholipid content has been associated with

tolerance to freezing stress (Steponkus *et al.* 1993, Mahajan and Tuteja 2005, Moellering *et al.* 2010). Therefore, by decreasing the ratio of saturated fatty acids and increasing that of unsaturated fatty acids, chilling tolerance can be induced in cold-sensitive plants.

Phospholipids and sterols are common in the plasma membrane and tonoplast. However, the content of phospholipids is higher in the tonoplast, but that of total sterols is mostly similar in both the membranes (Uemura and Steponkus 1997, Schulze *et al.* 2012). A considerable increase in the content of solutes in the plant cell has been observed. In *Arabidopsis*, accumulation of proline, fructose, sucrose, and glucose has also been shown to increase during cold acclimation (Wanner and Junttila 1999, Schulze *et al.* 2012). The H^+ -ATPase is a major enzyme of the plant plasma membrane, and its activity increases during cold acclimation. Kinetic parameters (Michaelis constant, K_m , and maximum velocity, V_m) determined from assays performed at different temperatures (10 and 30 °C) indicate that V_m increases during cold acclimation, whereas K_m declines (Mattheis and Ketchie 1990). The vacuole is a major solute storage organelle, but until now, the role of tonoplast proteins in cold acclimation has not been much investigated. In a comparative tonoplast proteome analysis, several membrane proteins, such as tonoplast pyrophosphatase, subunits of vacuolar V-ATPase, and V-ATPase activity along with vacuolar concentration of dicarbonic acids and soluble sugars have been reported to increase significantly during cold acclimation (Schulze *et al.* 2012).

Mitogen-activated protein kinase cascades: Protein kinases and phosphatases are the main components of the signaling pathway that transmit stress signals both biotic and abiotic to various cellular compartments in the plant cell (Mishra *et al.* 2006, Pitzschke *et al.* 2009, Sinha *et al.* 2011, Atkinson and Urwin 2012). Mitogen-activated protein kinases are a major group of threonine/serine protein kinases. They remain conserved throughout eukaryotes and are important for transduction of different intra- and extra-cellular signals (Pitzschke *et al.* 2009, Xu *et al.* 2010, Sinha *et al.* 2011, Atkinson and Urwin 2012). Of all sequenced eukaryotes, the highest quantity of recognized MAPK cascade genes are expressed in plant genomes; *e.g.*, in *Arabidopsis* more than 100, whereas in yeast 14, and in humans 34 genes (Hamel *et al.* 2006). The MAPK cascade consists of MAPK, MAPK kinase (MAPKK), and MAPKKK (Robinson and Cobb 1997, Mishra *et al.* 2006, Pathak *et al.* 2013). Introgression of MAPKKK (*ANP1*)/*ANP1* orthologue (*NPK*) into tobacco plants enhances tolerance to different stresses including cold stress, which could be an effective strategy for increasing crop production (Kovtun *et al.* 2000). Furthermore, in another study, *Arabidopsis* protoplast MAPK 2 (*MKK2*) is over-expressed by salt and cold stress-induced MAPKKK. Plants over-expressing *MKK2* exhibit 152 stress-induced marker genes and improved salt and cold tolerance as compared to *MKK2* deficient

plants (Teige *et al.* 2004).

Plants possess different MAPKs, which are over-expressed by osmotic stress. For example in alfalfa, salt-stress-inducible MAPKs, whereas in tobacco salicylic-acid-induced protein kinase was activated on exposure to

osmotic stress (Mikołajczyk *et al.* 2000). It suggests the presence of some protein kinase pathways in plants under the control of osmotic stress (Jonak *et al.* 1996, Zhu *et al.* 2000, Sinha *et al.* 2011).

Regulation of transcription

In order to acquire cold tolerance, regulation of transcriptome has been considered necessary (Lissarrie *et al.* 2010). More than 1000 genes are induced in response to cold, out of which about 170 are transcription factors, all of which coordinate to determine transcriptomic response of a plant to cold stress (Thomashow 2010). Transmission of a cold signal in plants requires expression of both CBF-independent and CBF-dependent transcriptional pathways (Chinnusamy *et al.* 2010). Transcriptome re-programing has been observed in chilling-tolerant plants at low temperature. Four to 20 % of the *Arabidopsis* genome was reported to be constituted by cold-regulated genes (Lee *et al.* 2005). The important roles of TFs *ICE1* and CBF/DREB1 in response to cold are discussed in detail.

CBF/DREB1 responsive pathway: Up-regulation of *CBF/DREB1* and *COR* expressions has been widely reported in low temperature stressed plants (Yadav *et al.* 2010, Feng *et al.* 2012; Kurepin *et al.* 2013). Plants may vary in their response when they are exposed to cold stress for short or long periods. For example, cold resistance in wheat plants at the onset of cold stress has been attributed to different stress proteins (*Wcor15*, *Wrab17*, *Wrab19*, and *Wcs120*) and to expression of a *WRKY* transcription factor. However, prolonged adaptation of wheat plants to cold is related to expression of *Wcor15* and *Wrab17* genes (Talanova *et al.* 2011).

The CBFs/DREB1s are upstream transcription factors encoded by three homologous genes that lie in the sequence of *DREB1B/CBF1*, *DREB1A/CBF3*, and *DREB1C/CBF2* in *Arabidopsis* (Qin *et al.* 2011). Proteins CBF/DREB resemble the *Apetala2*/ethylene response factor (ERF) that is unique to plants (Qin *et al.* 2011). However, CBF1,2,3, and three CBF/DREB1 proteins (CBF4/DREB1D, DREB1E, and DREB1F) have a unique conserved amino acid sequence known as 'signature sequence' immediately upstream and downstream of the AP2/ERF domain. The CBFs bind to a CCGAC core sequence in the promoter region, termed as DRE or C-repeat, of cold responsive genes, which activates their expressions (Lee *et al.* 2001). In *Arabidopsis*, the CBFs up-regulate expression of about 12 % of cold responsive genes (Fowler and Thomashow 2002). Cold responsive genes regulate different proteins and enzymes participating in metabolism of phenylpropanoids, lipids, sugars, antioxidants, molecular chaperons, and antifreeze proteins, which presumably have a role in low temperature tolerance (Guy 1990, Thomashow 1999, Yadav *et al.* 2010, Sanghera *et al.* 2011).

In addition, DNA-binding activity of some CBFs is determined by temperature. Xue (2003) observed that *Hordeum vulgare* CBF (HvCBF2) binds to the dehydration responsive element (DRE) element at 0 °C 10 times more than at 25 °C. Much understanding of the regulatory role of CBF/DREB, known also as 'CBF regulon', has been from transgenic plants over-expressing these transcription factors (Table 3 Suppl). Plants accumulate *CBF* transcripts within 15 min of exposure to cold stress. Since the response of CBF is quite quick, Gilmour *et al.* (1998) proposed that induction of cold responsive genes is a two-step process wherein the unknown activator designated as ICE comprises the first step of CBF response. The ICE is present even at higher temperatures in plants but is inactive. However, when plants experience cold stress, a signal transduction pathway is activated and results in the modification of ICE, which in turn enables to induce *CBF* expression (Gilmour *et al.* 1998). Later, this hypothesis was confirmed by Chinnusamy *et al.* (2007) who identified *ICE1*, a MYC-like bHLH protein, as upstream transcription factor for the CBF cascade. They observed that *ICE1* mutation in *Arabidopsis* lead to the complete elimination of *CBF3* transcription and also of many genes downstream of *CBF*, thus reducing the cold tolerance of the mutants. Similarly, *high expression of osmotically responsive gene 1 (HOS1)* encodes an important negative regulator of cold responsive genes (a finger motif). Using an *HOS1*-green fluorescent protein complex in *Arabidopsis* reveals that at normal growth temperatures, *HOS1* protein is usually in the cytoplasm, but on exposure to cold stress, it starts accumulate inside the nucleus (Lee *et al.* 2001). Likewise, in *Arabidopsis* *HOS2* mutants, transcriptions of *CBF2* and *CBF3* are considerably up-regulated suggesting that *HOS2* cause up-regulation of *CBFs* (Xiong *et al.* 2004). Warren *et al.* (1996) isolated *Arabidopsis* mutants sensitive to low temperature stress (known as the sensitive to freezing, *sfr*). In one of the mutants, *SFR6*, *CBF* expression is not altered, and it was hypothesized that the *SFR6* product is cofactor of the CBF pathway or needed for maturation/posttranslational modification (Warren *et al.* 1996, Knight *et al.* 1999). Feng *et al.* (2012) have found that an apple gene *MdCibHLH1* (cold-induced bHLH1) activates an ICE-like protein in response to cold stress. Over-expressions of *MdCibHLH1*, *MdCBF2*, and *MdCibHLH1* enhance cold tolerance in transgenic *Arabidopsis*, apple, and tobacco (Feng *et al.* 2012). Similarly, Lee *et al.* (2005) examined over-expression of a total of 939 cold-regulated genes in *A. thaliana* using *Affymetrix Gene*

Chips that contain ~24 000 genes during cold stress. Out of total expressed genes, 655 are up-regulated and 284 down-regulated under cold stress. In addition to encoding transcription factors for early cold-responsive genes, late-responsive genes are also activated showing that a variety of transcriptional factors are involved in regulation of *CBF* expression 1 (*ice1*) for signaling phytohormones including ABA, gibberellins, and auxins under low temperature.

Moreover, *CBF* cold response pathways are not only activated in cold-tolerant plants, *e.g.*, canola (Jaglo *et al.* 2001), *Populus balsamifera* subsp. *trichocarpa* (Benedict *et al.* 2006), and barley (Xue 2002), but also in cold-sensitive plants, *e.g.*, tomato (Zhang *et al.* 2004) and rice (Dubouzet *et al.* 2003). In all the above-mentioned plant species, at least one *CBF* gene over-expresses at low temperature stress. It is important to point out here that *CBF* over-expression enhances chilling tolerance in *A. thaliana* (Liu *et al.* 1998), potato (Pino *et al.* 2007),

P. balsamifera subsp. *trichocarpa* (Benedict *et al.* 2006), and canola (Jaglo *et al.* 2001) but does not influence chilling tolerance in tomato as well as in rice (Dubouzet *et al.* 2003, Zhang *et al.* 2004). This contradictory difference may involve changes in the concentration of *CBF* regulons. Furthermore, *CBF* regulons in *P. balsamifera* and *Arabidopsis* consist of 63 and 85 cold-induced genes, respectively (Vogel *et al.* 2005, Benedict *et al.* 2006) whereas in rice (Dubouzet *et al.* 2003) and tomato (Zhang *et al.* 2004), there are only 10 cold-induced genes. So far, several transcription factors and genes are up/down-regulated during stress, however, there is still a huge gap in knowledge of gene regulation under cold stress. For example, specific receptors involved in cold stress tolerance are not yet known, and signals transduced up/down-stream in different plant species at different freezing temperatures vary considerably as well as their activation differs to a great extent.

Strategies for achieving cold-acclimated plants

Osmoprotectant genetic engineering: Over-expression of trehalose-6-phosphate synthase isoforms has been reported to confer enhanced resistance to abiotic stresses including cold (Li *et al.* 2011). Expression of fructosyl transferases in fructan non-accumulating plants has been evidenced to lead to fructan production, which in turn results in an increased low-temperature stress tolerance (Li *et al.* 2007, Kawakami *et al.* 2008). Down-regulation of α -Gal has also been shown to increase plant freezing tolerance in both cold acclimated plants and non-acclimated plants (Pennycooke *et al.* 2003). Generation of a precursor of putrescine synthesis - arginine - and increased activity of ADC enhance putrescine content and improve plant freezing tolerance (Alet *et al.* 2011). Up-regulation of a spermidine synthase gene - *OsSPDS2* - has been reported to protect *O. sativa* roots against long-term chilling stress (Imai *et al.* 2004). Transcriptional up-regulation of *ADC1* and *ADC2* helps *A. thaliana* against cold stress, where loss of function mutants *adc1* and *adc2* result in a lower freezing tolerance as compared to wild-type plants (Cuevas *et al.* 2009). In *A. thaliana* and *O. sativa*, cold tolerance is achieved by transforming these plants with a *codA* gene encoding choline oxidase (Sakamoto *et al.* 2000). Responsive element ACTCAT containing promoters have been ascribed to enhanced cold tolerance as result of an elevated proline accumulation (Satoh *et al.* 2002). In transgenic and wild-type tomato plants, over-expression of *Osmotin* and

related genes (*CBF1*, *P5CS*, and *APX*) was reported to increase cold tolerance due to a high accumulation of proline and ascorbate content (Patade *et al.* 2013).

Genetic engineering of MAPK and CBF/DREB1 transcription factors: A specific *Arabidopsis* MAPKKK protein ANP1 that activates a phosphorylation cascade has been confirmed in transgenic tobacco plants over-expressing *ANP1* orthologue *NPK1* where induced an increased tolerance to cold, drought, as well as ABA stresses. Thus, introgression of *ANP1/NPK1* into sensitive plants provides a strategy for engineering tolerance to multiple stresses (Kovtun *et al.* 2000). Plants over-expressing *MKK2* exhibit constitutive MPK4 and MPK6 activities, up-regulated expression of stress-induced marker genes, and increased freezing tolerance (vs. *MKK2* deficit plants, Teige *et al.* 2004). The *CBF* technology has a great potential for improving cold and freezing tolerance of plants. Over-expression of *MdCibHLH1*, *MdCBF2*, and *MdCibHLH1* genes in *Arabidopsis*, apple, and tobacco plants, respectively, enhance chilling tolerance (Feng *et al.* 2012). A *CBF* over-expression-mediated improvement of freezing tolerance has been evidenced in *P. balsamifera* subsp. *trichocarpa* (Benedict *et al.* 2006), *Arabidopsis* (Liu *et al.* 1998), *Solanum tuberosum* (Pino *et al.* 2007), and canola (Jaglo *et al.* 2001).

Cross-talks between signalling pathways

Cold, drought, and salinity share a common feature - they impose an osmotic stress and affect water relations inside the cell ultimately leading to the loss of pressure potential (Krasensky and Jonak 2012). Besides, many genes are

expressed by cold, water, and salt stresses suggesting the existence of crosstalk between their signal transduction pathways, *e.g.*, in *Arabidopsis* and *Nicotiana*, introgression of the transcription factor *DREB1* under the

stress inducible promoter *RD29* results in an improved resistance to multiple abiotic stresses (Kasuga *et al.* 1999). Similarly, *DREB1* from *Arabidopsis* under both *cam35S* and *rd29* promoters results in a greater tolerance not only to cold stress but also to salt and drought stresses (Kasuga *et al.* 1999). In addition, *DREB1/CBF* and *DREB2*, which are expressed under freezing, drought, as well as non-stress conditions, control induction of various genes involved in plant stress tolerance (Shinozaki and Yamaguchi-Shinozaki 2000). Furthermore, the transcriptional regulator SoERF3 from *Saccharum officinarum* is induced not only by cold stress, but it responds to ethylene, wounding, ABA, and salt stresses (Trujillo *et al.* 2008). Some signaling pathways may cross-talk at different stages during stress conditions. For example, Chinnusamy *et al.* (2004) identified that *ICE1* signaling

is important for controlling a cold-responsive transcriptome, however, the signaling pathways that trigger an *ICE1* transcription factor under low temperature are not clear. Furthermore, it is now well evident whether MAPK cascades may act as converging points of numerous stress signaling processes. Late embryogenesis abundant proteins are synthesized at the time of embryo maturation and also in the vegetative tissues of different plant species in response to low temperature, salinity, and drought (Ingram and Bartels 1996). Introgression of a *Hordeum vulgare* late embryogenesis protein (*HVA1*) gene in rice plants has a substantial use as molecular tool for crop improvement through genetic manipulation under different stresses (Xu *et al.* 1996).

Conclusions and future prospects

Temperature stress has overwhelming effects on plant growth, development, and metabolism, which result in reduction of agricultural production as all these processes have optimum temperature limits in different plant species. Generally, plants from temperate climatic regions are considered to be freezing tolerant to some degree. However, plants of tropical and subtropical origins are generally sensitive to chilling/freezing stresses and lack a mechanism of cold acclimation. Thus, the low temperature-induced yield loss of main crops is considerably high. Furthermore, harsh winter climatic conditions are a major constraint, particularly for perennial crops.

Cold acclimation is a complicated process including different physiological and biochemical mechanisms such as changes in ultra-structure of cellular membranes and cytoskeleton, induction of antioxidant defense system components, alteration in sugar content, synthesis of secondary metabolites and compatible solute, *etc.* The content of PAs, proline, GB, proteins, sugars, amino acids, and antioxidants generally correlate with cold

acclimation in most plants. However, there is a need to find-out the mobility and localization of all these compounds, source/sink relationship, and role of intermediary compounds in specific pathways involved in the mechanism of cold acclimation/tolerance. A plethora of reports on the activities of antioxidant enzymes and gene regulation in plants subjected to stress conditions can be deciphered from the literature, however, the genes of antioxidant enzymes need to be engineered to generate plants with enhanced freezing tolerance. A number of transgenic cold tolerant plants have been generated under controlled environmental conditions, but rarely these transgenic plants have been tested under field conditions. It is highly likely that the degree of tolerance observed under controlled conditions may not be manifested under field conditions. For resolving all these issues, the development of cultivars/species adapted to cold conditions through modern molecular biology tools as well as potential management practices may mitigate the adverse effects of freezing/chilling stress on plants.

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