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A critical review on morpho-physiological and molecular aspects associated with cold stress in plants

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ABSTRACT

Plants respond with changes in their pattern of gene expression and protein products when exposed to low temperatures. Thus ability to adapt has an impact on the distribution and survival of the plant, and on crop yields. Many species of tropical or subtropical origin are injured or killed by nonfreezing low temperatures, and exhibit various symptoms of chilling injury such as chlorosis, necrosis, or growth retardation. In contrast, chilling-tolerant species are able to grow at such cold temperatures. Conventional breeding methods have met with limited success in improving the cold tolerance of important crop plants involving inter-specific or inter-generic hybridization. Recent full-genome transcript profiling studies, in combination with mutational and transgenic plant analyses, have provided a snapshot of the complex transcriptional network that operates under cold stress. The changes in expression of hundreds of genes in response to cold temperatures are followed by increases in the levels of hundreds of metabolites, some of which are known to have protective effects against the damaging effects of cold stress. Various low temperature-inducible genes have been isolated from plants. Most appear to be involved in tolerance to cold stress and the expression of some of them is regulated by C-repeat/dehydration-responsive element binding (CBF/DREB1) transcription factors. Genetic analysis has revealed important roles for cellular metabolic signals, and for RNA splicing, export and secondary structure unwinding, in regulating cold-responsive gene expression and chilling and freezing tolerance. Numerous physiological and molecular changes occur during cold acclimation which reveals that the cold resistance is more complex than perceived and involves more than one pathway. The findings summarized in this review have shown potential practical applications for breeding cold tolerance in crop and horticultural plants suitable to temperate geographical locations.

Introduction

Abiotic stresses adversely affect growth, productivity and trigger a series of morphological, physiological, biochemical and molecular changes in plants. Cold stress is a major environmental factor that limits the agricultural productivity of plants in hilly areas. Low temperature has a huge impact on the survival and geographical distribution of plants. It often affects plant growth and crop productivity, which causes significant crop losses (Xin and Browse, 2000). Plants differ in their tolerance to chilling (0-15ºC) and freezing (< 0ºC) temperatures. Plants from temperate regions are chilling tolerant, although most are not very tolerant to freezing but can increase their freezing tolerance by being exposed to chilling, non freezing temperatures, a process known as cold acclimation (Levitt, 1980), which is associated with biochemical and physiological changes (Shinozaki and Yamaguchi-Shinozaki, 1996; Thomashow, 1998; Gilmour et al. 2000). By contrast, plants of tropical and subtropical origins, including many crops such as rice, maize and tomato etc. are sensitive to chilling stress and largely lack the capacity for cold acclimation. Freezing temperature greatly limits the geographical distribution of cultivated plants and often causes severe losses in agriculture production and productivity. Conventional breeding methods have met with limited success in improving the cold tolerance of important crop plants involving inter-specific or inter-generic hybridization. Besides, in vitro induced variations have also been applied to improve the abiotic stress tolerance of various crop plants but without much success. The conventional breeding approaches are limited by the complexity of stress tolerance traits, low genetic variance of yield components under stress condition and lack of efficient selection criteria. It is important, therefore, to look for alternative strategies to develop cold stress tolerant crops Biotechnology offers new strategies that can be used to develop transgenic crop plants with improved tolerance to cold stress. A number of genes have been isolated and characterized that are responsive to freezing stress. Many studies have suggested that cold regulated gene expression is critical in plants for both chilling tolerance (Hsieh et al. 2002) and cold acclimation (Knight et al. 1999, Tamminen, 2001). The molecular tool makes it possible to select directly at the gene label without waiting for the phenotype to show up.

Therefore it is important to use most appropriate tools that help in reaching the goals. The designed genotype should be better than the available ones and must reach the farmers.

An attempt has been made in this article to review the various mechanisms and genes involved in cold acclimatization

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and the possibilities where transgenic technology has been explored for breeding cold tolerance in crop plants.

Morpho-physiological Basis of Cold Tolerance

A large number of studies have evaluated different plant species, and to different stresses such as drought, salinity and cold. However, less detail is given with regard to the methods used to evaluate the stress response; these studies may bring about some misleading conclusions from an agronomic or physiology perspective (Sanghera and Wani 2008). This is particularly important, in order to closely mimic the life span of most crops under cycles of stress, rather than short exposure to very severe stresses, although we agree that short exposures to stress are certainly adequate if the purpose is to assess gene expression only. In this section, we focus on the agronomic/physiological perspective and don't mean to challenge the quality of the work done to assess gene expression. Our intention is to try to reconcile both approaches (agronomic and molecular) toward a common focus: breeding cold tolerance. Though precise details about the protocols used to evaluate the performance of plants to any given stress are very essential to assess the performance of materials.

The temperate and cool regions are those where altitudes ranged from 1600-2500 m amsl (above mean sea level) and temperature during crop growth period ranged from 5-20^{0}C (Anonymous, 1997). In temperate regions, low temperature is the primary abiotic stress which limits the crop productivity. The low temperature at seedling and reproductive stages is the major problem, results in slow establishment and low seed set which leads to poor yield of the crop (Sanghera and Wani 2008).

The low temperature limits the crop productivity when temperatures remain above freezing, i.e. > 0^{o}C, it is called as chilling stress. Chilling sensitive cultivars are typically tropical genotypes. There is wide range of cold stress in temperate areas differing in both timing and intensity of low temperature. Yield losses are more severe when cold stress occurs during reproductive stage/ anthesis in rice which lead to high spikelet sterility (Sanghera et al. 2001).

Ability of crop genotypes / lines to survive / perform better under low temperature than other genotypes is called as cold tolerance. Ordinarily, it is the consequence of cold hardening i.e. an earlier exposure to a low temperature for a specific period as a result of which chilling tolerance of the concerned plants increases. Cold tolerance involves increased chlorophyll accumulation, reduced sensitivity of photosynthesis, improved germination, pollen fertility and seed set which are desirable as:

Increased Chlorophyll Accumulation

Low temperature inhibits chlorophyll accumulations in activity growing leaves. In rice, cold tolerant lines, e.g. japonica accumulate more chlorophyll under cold stress than do cold sensitive line e.g. of indica rices (Glaszmann et al. 1990). Rasolofo (1986) evaluated 181 accessions to identify donor and out standing cold tolerant lines using leaf discolouration score (1-3) and found 19 remained green (dark) after 10 days in the 12^{o}C cold water tank. Sanghera et al. (2001) found 18 cold tolerance IRCTN rice genotypes based on dark green colour and high spikelet fertility (>90%) under temperate conditions.

Reduced Sensitivity of Photosynthesis

Chloroplast and photosynthesis is major site of cold injury. Tolerance in these aspects is expressed in native vegetation adapted to growing under cool conditions. The reduced sensitivity of photosynthesis to cold has been observed in maize inbreds adapted to low temperature which is partly related to specific enzymes of the process (Singh, 2000).

Improved Germination

Genetic variation in cold tolerance at germination and seedling stage has been documented. Saini and Tandon (1985) found that L62G, Heng Jodo, Jodo, Heugdo, IRAT 102, Khonorullo, K 78-28, Daegaldo, Mujudo and L62-2A genotypes were cold tolerant having more than 85% germination and good seedling vigour (score 3) at an average of 11^{o}C field temperature

Improved Pollen Fertility and Seed Set

Cold tolerance at reproductive stage is expressed as improved seed set and pollen fertility. It is largely a function of floral structure and function under stress. Lia et al. (1998) reported plant cold tolerance in rice is associated with anther size, number of pollen grain, diameter of fertile pollen grains at booting stage. However, Sanghera et al. (2001) reported that cold tolerance is associated with high spikelet fertility (>90%) and well panicle exsertion under temperate conditions.

Cold snaps cause a reaction in the plant that prevents sugar getting to the pollen. Without sugar there is no starch build-up which provides energy for pollen germination. And without pollen, pollination cannot occur so no grain is produced. CSIRO has found that all the ingredients for starch are present but they are not getting into the pollen grain where they are needed. A cell layer surrounding the pollen, called the 'tapetum', is responsible for feeding the pollen with sugar. The tapetum is only active for 1-2 days – so if a cold snap occurs at this time then there is no further chance for pollen growth. But the sugar can't freely move into the tapetum and pass through it to the pollen. Instead the sugar has to be broken down then transported in bits to the pollen. 'Invertase' is the catalyst that helps break down the sugar to transport it into the tapetum before it is transported to the pollen (Oliver et al. 2005). Quantities of invertase are decreased in conventional rice when it is exposed to cold temperatures, but they remain at normal levels in a cold tolerant variety when it experiences cold. By comparing a cold tolerant strain of rice with conventional rice CSIRO has found that the gene responsible for invertase looks exactly the same in the cold tolerant variety as it does in conventional rice. So the invertase gene itself does not make the rice plant cold tolerant – but instead a mechanism that regulates the invertase gene is different. Early research is indicating that the invertase gene is regulated by the hormone abscisic acid (ABA). Oliver et al., (2007) has experimented with injecting plants with ABA – the resulting rice plants are sterile, just like they would be if they experienced a cold snap. Also, ABA levels increase when conventional rice is exposed to cold, but they remain the same in the cold tolerant variety. Recent studies have indicated that the difference between cold-sensitive and tolerant rice is due to a different ability to control ABA levels. It has also been shown that this mechanism may require interactions with other plant hormones like auxins (Bhatnagar-Mathur, 2008).

Ample genetic variation for cold tolerance is available in well adapted breeding populations, germplasm collected from high altitude and low temperature areas, cold tolerant mutant, somaclonal variants and wild species (Sharma et al. 2009) that can be exploited for breeding improved cold tolerant genotypes in hilly areas.

Mechanisms for Tolerance to Cold Injury

Cold temperature affects a broad spectrum of cellular components and metabolism, and temperature extremes impose stresses of variable severity that depend on the intensity and

duration of the stress. Wide ranges of studies indicate that the membrane systems of the cell are the primary site of freezing injury in plants (Levitt, 1980; Steponkus, 1984). In addition, it is well established that freeze-induced membrane damage results primarily from the severe dehydration associated with freezing (Steponkus, 1984; Steponkus et al. 1993). As temperatures drops below 0ºC, ice formation is generally initiated in the intercellular spaces due, in part, to the extracellular fluid having a higher freezing point (lower solute concentration) than the intracellular fluid. Because the chemical potential of ice is less than that of liquid water at a given temperature, the formation of extracellular ice results in a drop in water potential outside the cell. Consequently, there is movement of unfrozen water down the chemical potential gradient from inside the cell to the intercellular spaces. At 10ºC, more than 90% of the osmotically active water typically moves out of the cells, and the osmotic potential of the remaining unfrozen intracellular and intercellular fluid is greater than 5 osmolar.

Multiple forms of membrane damage can occur as a consequence of freeze induced cellular dehydration including expansion-induced-lysis, lamellar-to-hexagonal-II phase transitions, and fracture jump lesions (Steponkus et al. 1993). Thus, a key function of cold acclimation should be to stabilize membranes against freezing injury. Indeed, cold acclimation prevents expansion-induced-lyses and the formation of hexagonal II phase lipids in rye and other plants (Steponkus et al. 1993). Multiple mechanisms appear to be involved in this stabilization. The best documented are changes in lipid composition (Steponkus et al. 1993). Membrane fluidity is largely dictated by the composition of lipid molecular species, the degree of lipid saturation and temperature environments. Temperature induced change in membrane fluidity is one of the immediate consequences in plants during temperature stresses and might represent a potential site of perception and/or injury (Horváth et al. 1998; Orvar et al. 2000). The importance of proper membrane fluidity in temperature tolerance has been delineated by mutation analysis, transgenic and physiological studies. At low temperature, greater membrane lipid unsaturation appears to be crucial for optimum membrane function. An Arabidopsis fatty acid biosynthesis FAB1 mutant with more saturated membranes showed decreased quantum efficiency of photosystem II (PSII), chlorophyll content and the amount of chloroplast glycerolipids after prolonged exposure to low temperature (Wu et al. 1997). A triple mutant fatty acid desaturation (fad3-2 fad7-2 fad8) devoid of trienoic fatty acids (18:3 or 16:3) produced a phenotype similar to FAB1 when plants were subjected to prolonged low temperature exposure (Routaboul et al. 2000). Similarly, fad5 and fad6 mutants with more saturated membranes became chlorotic and showed growth retardation during low temperature incubation (Hugly and Somerville, 1992). In addition to membrane unsaturation, it appears that lipid asymmetry in the membrane also contributes to membrane physical structure at low temperature (Gomès et al. 2000). When overexpressed in yeast, aminophospholipid ATPase1 (ALA1), a putative aminophospholipid translocase in Arabidopsis, restored phosphatidylserine internalization from the outer leaflet of the plasma membrane. The finding supports the fact that internalization of phosphatidylserine was tightly linked to the rescue of a cold sensitivity phenotype of the yeast drs2 mutant (Gomès et al. 2000).

The accumulation of sucrose and other simple sugars that typically occurs with cold acclimation also seems likely to contribute to the stabilization of membranes as these molecules can protect membranes against freeze-induced damage in vitro (Strauss and Hauser 1986; Anchordoguy et al. 1987). In addition, there is emerging evidence that certain novel hydrophilic and late embryogenesis abundant (LEA) polypeptides also participate in the stabilization of membranes against freeze-induced injury. These hydrophilic and late embryogenesis abundant polypeptides are predicted to contain regions capable of forming amphipathic α-helices which are shown to have strong effect on intrinsic curvature of monolayers and their propensity to form hexagonal II phase. They are said to defer their formation at lower temperatures (Epand et al. 1995). Whether the regions predicted to form amphipathic α-helices actually form such structures is uncertain. An additional hypothesis suggests that the extensive water binding capacity of these hydrophilic proteins might provide a protective environment in the proximity of membranes during freezing and result in membrane stabilization. Although freezing injury is thought to result primarily from membrane lesions caused by cellular dehydration, additional factors may also contribute to freezing-induced cellular damage. There is evidence that freeze-induced production of reactive oxygen species contributes to membrane damage and that intercellular ice can form adhesions with cell walls and membranes and cause cell rupture (Olien and Smith, 1977). In addition, there is evidence that protein denaturation occurs in plants at low temperature (Guy et al. 1998) which could potentially result in cellular damage. In these cases, the enhancement of antioxidative mechanisms (Aroca et al. 2003), increased levels of sugars in the apoplastic space (Livingston and Henson, 1998), and the induction of genes encoding molecular chaperones (Guy and Li, 1998), respectively, could have protective effects.

Both cold-stress-induced transcripts and constitutively expressed transcripts need to be processed, exported to the cytoplasm and kept in conformations that are competent for translation. RNA can fold into extensive secondary structures that could interfere with its function, and cold temperatures exacerbate this interference. In bacteria, nucleic-acid-binding cold shock proteins (CSPs) accumulate at cold temperatures and function as transcription antiterminators or translational enhancers by destabilizing RNA secondary structure (Jones and Inouye, 1994). Some CSP-domain-containing proteins in plants are upregulated by cold stress, and might function as RNA chaperones in the regulation of translation (Nakaminami et al. 2006; Kim et al. 2007). A different cold-responsive nucleic-acid-binding protein, a zincfinger- containing glycine-rich RNA-binding protein from Arabidopsis designated atRZ-1a, is also upregulated by cold stress, and genetic analysis supports its function in freezing tolerance (Kim et al. 2005). Another group of RNA chaperones, RNA helicases, are involved in every step of RNA metabolism. In cyanobacteria, a cold-induced DEAD-box RNA helicase was suggested to unwind cold-stabilized secondary structure in the 5'-untranslated region of RNA during cold stress (Yu and Owttrim, 2000). Compared to other organisms, plants have the largest number of DEAD-box RNA helicase genes. One of these helicases, which is encoded by the Arabidopsis low expression of osmotically responsive genes4 (LOS4) gene, is essential for plant tolerance of chilling and freezing stress (Gong et al. 2002). LOS4 is required for efficient export of RNA from the nucleus to the cytoplasm (Gong et al. 2005). The Arabidopsis nucleoporin AtNUP160 suppressor of auxin resistance1 (SAR1) also controls RNA export, and is

crucial for chilling and freezing tolerance (Dong et al. 2006). Both LOS4 and AtNUP160 proteins are enriched at the nuclear rim (Gong et al. 2002; Dong et al. 2006). Defects in the nucleocytoplasmic transport of RNA seem to affect cold tolerance preferentially, because the LOS4 and AtNUP160 mutant plants do not have severe growth or developmental phenotypes, nor are they strongly altered in the tolerance of other abiotic stresses.

Pathways Involved in Cold Responsiveness

The discovery of gene expression change during cold acclimation was the starting of exploration of antifreezing molecular mechanisms. Global transcript profiling analyses indicate that > 10% of genes in the Arabidopsis genome are regulated during cold acclimation (Fowler and Thomashow, 2002; Kreps et al. 2002; Seki et al. 2002; Vogel et al. 2005). Transcriptome analysis using microarray technology is a powerful technique, which has proven very useful for discovering many stress-inducible genes involved in stress response and tolerance (Shinozaki et al. 2003; Seki et al. 2004). Numerous genes that are induced by various abiotic stresses have been identified using various microarray systems (Kawasaki et al. 2001; Kreps et al. 2002; Rabbani et al. 2003; Bray, 2004; Maruyama et al. 2004; Vogel et al. 2005). Genes induced during stress conditions function not only in protecting cells from stress by producing important metabolic proteins, but also in regulating genes for signal transduction in the stress response. Thus, these gene products are classified into two groups (Fowler and Thomashow, 2002; Kreps et al. 2002; Seki et al. 2002). The first group includes proteins that probably function in stress tolerance, such as chaperones, LEA proteins, osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis such as proline, water channel proteins, sugar and proline transporters, detoxification enzymes, enzymes for fatty acid metabolism, proteinase inhibitors, ferritin, and lipid-transfer proteins. Some of these stress-inducible genes that encode proteins, such as key enzymes for osmolyte biosynthesis, LEA proteins, and detoxification enzymes have been overexpressed in transgenic plants and produce stress-tolerant phenotypes in the transgenic plants (Holmberg and Bulow, 1998; Cushman and Bohnert, 2000). These results indicate that the gene products of the stress-inducible genes really function in stress tolerance. The second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response. They include various transcription factors that regulate various stress-inducible genes cooperatively or separately, and may constitute gene networks. Some of these regulatory pathways are also involved in other stress responses including those of drought-, cold-, or high-salinity (Seki et al. 2003). Functional analysis of these stress-inducible transcription factors should provide more information on the complex regulatory gene networks that are involved in responses to drought, cold, and high-salinity stresses. The others are proteins kinases, protein phosphatases, enzymes involved in phospholipids metabolism, and other signaling molecules such as calmodulin-binding protein and 14-3-3 proteins. At present, the functions of most of these genes are not fully understood. Some of these stress-inducible regulatory genes that encode proteins such as transcription factors have been overexpressed in transgenic plants and generate stress-tolerant phenotypes in them (Zhang et al. 2004; Tester and Bacic, 2005; Vinocur and Altman, 2005).

The large number of genes identified in these studies raises the question of exactly which genes are most central to increasing freezing tolerance. One approach towards answering this question has been to focus on a set of genes that encode a related family of cold-regulated (COR) proteins, which are massively induced during cold acclimation (Hajela et al. 1990; Gilmour et al. 2004). Some of these COR genes have also been named low temperature-induced (LTI), cold acclimation-specific (CAS), cold-induced (KIN), and responsive to drought (RD) genes (Kurkela and Franck, 1990; Yamaguchi-Shinozaki et al. 1992; Monroy et al. 1993; Nordin et al. 1993). The COR genes were used by two research groups to identify a family of Arabidopsis transcription factors known as either C-repeat binding factors (CBF) (CBF1, CBF2 and CBF3) or dehydration responsive element-binding factors (DREB) (DREB1B, DREB1C and DREB1A) (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998). CBFs/DREBs are upstream transcription factors that bind to promotor cis element CRT/ DRE and activate the expression of these cold responsive genes (Thomashow, 1999). Ectopic transgenic overexpression of CBF1/DREB1B, CBF2/DREB1C or CBF3/DREB1A in Arabidopsis activates a suite of CBF/DREB target genes at warm temperatures (Gilmour et al. 2004) and results in increased freezing, drought and salt tolerance (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999; Gilmour et al. 2004). CBF transcripts begin accumulating within 15 min of plants being exposed to low temperature strongly suggests that the low temperature "Thermometer" and "Signal Transducer" are present at warm non-inducing temperatures (Gilmour et al. 1998). They have, therefore, proposed that there is a transcription factor already present at warm temperature that recognizes the CBF promoters. This factor would not appear to be the CBF proteins themselves as the promoters of the CBF genes lack the CRT/DRE sequence and overexpression of CBF1 does not cause accumulation of CBF3 transcripts (Gilmour et al. 1998). Further, Gilmour et al. 1998 have, therefore, proposed that COR gene induction involves a two-step cascade of transcriptional activators in which the first step, CBF induction, involves an unknown activator that they tentatively designated "ICE" (inducer of CBF expression (ICE)). ICE presumably recognizes a cold-regulatory element, the "ICE Box," present in the promoters of each CBF gene. At warm temperature, ICE is suggested to be in an "inactive" state, either because it is sequestered in the cytoplasm by a negative regulatory protein or is in a form that does not bind to DNA or does not activate transcription effectively. Upon exposing a plant to low temperature, however, a signal transduction pathway is suggested to be activated those results in modification of either ICE or an associated protein, which, in turn, allows ICE to induce CBF gene expression. Indeed, in Arabidopsis ICE is phosphorylated in response to cold treatment (Teige et al. 2004). As noted by Gilmour et al.1998, it is possible that ICE may not only regulate the expression of the CBF genes, but might induce expression of other genes ("X") that may also have roles in cold acclimation. A dominant mutation in ICE1 leads to the irregular induction of CBF3 transcription factor, an alteration of CBF-regulated genes, and a loss of freezing tolerance (Chinnusamy et al. 2003; Lee et al. 2005). HOS1, a negative regulator of the CBF regulon, was identified from a genetic screen for mutants with deregulated expression of CBF target genes (Ishitani et al. 1998; Lee et al. 2001). It is ubiquitously expressed in all plant tissues and HOS1 protein residues in the cytoplasm at normal

growth temperatures. However, in response to low temperature treatments, it accumulates in the nucleus (Lee et al. 2001). The cold induction of CBF genes and their downstream COR genes is enhanced in loss-of-function HOS1 mutant plants (Ishitani et al. 1998). HOS1 encodes a 915-amino acid protein that contains a short motif near the amino terminus that is similar to the really interesting new gene (RING)-finger domain found in the inhibitor of apoptosis (IAP) group of animal proteins (Lee et al. 2001). In vitro ubiquitination assays demonstrated that Arabidopsis HOS1 is a functional RING-finger protein that has ubiquitin E3 ligase activity. HOS1 physically interacts with ICE1, suggesting that HOS1 might ubiquitinate ICE1 and target it for proteosomal degradation. Indeed, both in vitro and in vivo ubiquitination assays showed that HOS1 mediates the polyubiquitination of ICE1 (Dong et al. 2006). Cold-induced degradation of the ICE1 protein was observed in Arabidopsis plants and this degradation is blocked by the HOS1 mutation, indicating that HOS1 is required for the degradation of ICE1, which functions to attenuate cold responses in Arabidopsis (Dong et al. 2006). ICE1 and perhaps related transcription factors that control the expression of CBF genes are present in the absence of cold stress, but probably undergo certain posttranslational modification(s) (e.g. phosphorylation) in response to cold stress, thereby becoming active in switching on the expression of CBF genes (Chinnusamy et al. 2003). The active, modified form of ICE1 might be more efficiently recognized by HOS1 and then degraded through the ubiquitination/ proteasome pathway.

Feedback repression of transcription factors that regulate cold-responsive gene expression appears to be a key to maintaining an optimal cold-induced transcriptome. Molecular analysis of a CBF2 null mutant of Arabidopsis suggested that CBF2 is a negative regulator of CBF1 and CBF3 expression during cold acclimation (Novillo et al. 2004). Conversely, CBF3 might negatively regulate CBF2 expression, because reduced expression of CBF3 in the ice1 mutant is accompanied by an enhanced expression of CBF2 (Chinnusamy et al. 2003). These results suggest that cross-regulation and, perhaps, also self-regulation have an important role in the expression levels of CBFs during cold acclimation. Furthermore, CBFs are negatively regulated by an upstream transcription factor, MYB15 (an R2R3-MYB family protein) in Arabidopsis. MYB15 is expressed even in the absence of cold stress, and MYB15 can bind to MYB recognition elements (MYBRS) in the promoters of CBFs. MYB15 TDNA knockout mutant plants show enhanced expression of CBFs during cold acclimation and enhanced freezing tolerance, whereas transgenic Arabidopsis overexpressing MYB15 show a decreased expression of CBFs and a reduction in freezing tolerance. Thus, MYB15 is an upstream transcription factor that negatively regulates the expression of CBFs (Agarwal et al. 2006). Interestingly, ICE1 can negatively regulate MYB15 as indicated from the increased MYB15 transcript level in ICE1 mutant compared with wild-type plants under cold stress (Chinnusamy et al. 2003; Agarwal et al. 2006). Yeast two-hybrid and in vitro pull-down assays showed that MYB15 can interact with ICE1, but the functional significance of ICE1–MYB15 interaction in cold acclimation is unknown (Agarwal et al. 2006). In Arabidopsis, a cold-induced C2H2 zinc finger transcription factor gene, ZAT12, also appears to function as a negative regulator of CBFs. Transgenic overexpression of ZAT12 decreases the expression of CBFs under cold stress. Transcriptome analysis of ZAT12-overexpressing Arabidopsis revealed that the ZAT12 regulon consists of at least 24 cold standard set (COS) genes, of which nine are cold-induced and 15 are cold-repressed genes (Vogel et al. 2005). Molecular analysis of the LOS2 mutant of Arabidopsis revealed that another C2H2 zinc finger protein, ZAT10/STZ, might act as a negative regulator of CBF-target genes. LOS2, a bifunctional enolase, binds to the MYC recognition elements in the ZAT10 promoter in vitro and LOS2 mutant plants showed an enhanced and more sustained induction of ZAT10 during cold stress (Lee et al. 2002). Thus LOS2 appears to be a negative regulator of ZAT10 expression during cold acclimation. Transient expression assays showed that ZAT10 could bind specifically to A(G/C)T promotor cis element within the EP2 sequence (a cis element where a negative regulator binds) of RD29A, a target gene of CBFs, and repress its expression (Lee et al. 2002). CBFs might have a role in mediating or modulating cold-stress induction of ZAT10 because transgenic plants overexpressing CBF3 showed an enhanced expression of ZAT10 (Maruyama et al. 2004). Furthermore, impairment of CBF3 expression caused by the ICE1 mutation also led to a significant decrease in the cold induction of ZAT10, as is evident from microarray data (Chinnusamy et al. 2003; Chinnusamy et al. 2006). Thus, ZAT10 could be a subregulon of CBFs and might regulate a subset of genes involved in cold acclimation. ZAT10 and ZAT12 might serve as converging nodes in abiotic stress-regulated transcriptional networks, because these transcription factors are induced by cold and other abiotic stresses, and transgenic plants overexpressing these genes exhibit enhanced osmotic and oxidative stress tolerance (Davletova et al. 2005; Mittler et al. 2006).

The homologous components of the Arabidopsis CBF cold response pathway have been found in many plants, including rape (Brassica napus), soybean (Glycine max), broccoli (Brassica oleracea), tomato (Lycopersicon esculentum), alfalfa (Medicago sativa), tobacco (Nicotiana tabacum), cherry (Prunus avium), wheat (Triticum aestivumfrom), rye (Secale cereale), corn (Zea mays), rice (Oryza sativa), strawberry (Fragaria ananassa) and barley (Hordeum vulgare) (Jaglo et al. 2001; Choi et al. 2002; Gao et al. 2002; Owens et al. 2002; Dubouzet et al. 2003; Vágújfalvi et al. 2003; Xue, 2003; Francia et al. 2004). Many of the putative orthologs have been structure analyzed and functionally tested. The expression patterns of the CBFs and CORs in response to low temperature are similar in a variety of plants species, involving rapid cold-induced expression of the CBFs followed by expression of CBF-targeted genes that increase freezing tolerance. Moreover, constitutive overexpression of the Arabidopsis CBF genes in other plants resulted in increased freezing tolerance. Similarly, constitutive overexpression of CBF homologs from other plants in transgenic Arabidopsis also results in salt, cold, and drought tolerance. More and more data suggest that components of the Arabidopsis CBF cold-responsive pathway are conserved in higher plants.

Understanding the molecular mechanisms that plants have evolved to tolerate environmental stresses has the potential to provide new tools and strategies to improve the environmental stress tolerance of crops. Since freezing tolerance is a multigenic trait (Thomashow, 2001), transformation of a single functional gene like cor15a appears to have a limited effect on crop freezing tolerance (Artus et al. 1996). Because many aspects of cold adaptation process are under transcriptional control, many transcription regulatory factors were chosen as one of the best targets for engineering crops to achieve enhanced cold tolerance.

Over expression of Arabidopsis CBF/DREB1 genes, which locate the nodes of regulatory network in cold response, or homologs from other plants can activate a group of downstream functional genes, such as KIN1, COR6.6, COR15a, RD17, RD29a and ERD10 in Arabidopsis. For example, constitutive expression of CBF3 genes in Arabidopsis not only elevated levels of COR proteins, but also elevated levels of proline and total sugars, resulting in an increase in both freezing and drought tolerance (Gilmour et al. 2000). Thus, the CBFs appear to be ‘‘master switches’’ that integrate activation of multiple components of the cold acclimation response (Thomashow, 2001). However, constitutive over expression of the CBF genes using the cauliflower mosaic virus 35S promoter can result in undesirable agronomic traits. In Arabidopsis, CBF over expression can cause a ‘‘stunted’’ growth phenotype, a decrease in seed yield and a delay in flowering (Liu et al. 1998, Gilmour et al. 2000). Using stress-inducible (Kasuga et al. 1999) or artificial cold-inducible promoters may be an ideal approach to improve cold tolerance without causing negative agronomic effects. Stress inducible promoters that have low background expression under normal growth condition have been used in conjunction with the DREB1/CBF genes to achieve increased stress tolerance without growth retardation (Kasuga et al. 1999). Constitutive over expression of Arabidopsis DREB1A improved drought and low-temperature stress tolerance in tobacco, and regulation of transgene expression via the stress-inducible RD29A promoter minimized the negative effects on plant growth (Kasuga et al. 2004). Similarly, the Arabidopsis DREB1A gene was placed under control of the RD29A promoter and transferred via biolistic transformation into bread wheat (Pellegrineschi et al. 2004). In comparison with controls, plants expressing the DREB1A gene exhibited a 10-day delay in wilting when water was withheld. This substantial increased resistance to water stress indicates that a combination of the RD29A promoter and DREB1A is useful for improvement of various kinds of transgenic plants that are tolerant to environmental stress. Though many transcription regulatory factors were cloned and identified, only CBF genes have been successfully used to engineer cold stress tolerance in several species. Transgenic attempts with many structural genes have also been made with fair degree of success. The over expression of genes encoding LEA proteins can improve the stress tolerance of transgenic plants. Expression of the citrus gene encoding a LEA protein, CuCOR19 increased the cold tolerance of transgenic tobacco (Hara et al. 2003). Likewise, the freezing tolerance of Arabidopsis was increased by the ectopic expression of the wheat gene WCS19 (Dong et al. 2002), the Arabidopsis gene COR15A (Artus et al. 1996), and the co-expression of the genes RAB18 and COR47 and XERO2 and ERD10 (Puhakainen et al. 2004). The freezing tolerance of strawberry leaves was enhanced by expression of the wheat dehydrin gene WCOR410 (Houde et al. 2004). On the other hand, the expression of two cold-induced LEA proteins from spinach (Kaye et al. 1998) and three desiccation-induced LEA proteins from C. plantagineum (Iturriaga et al. 1992) in tobacco did not induce any significant changes in the freezing or drought tolerance of the respective transgenic plants. This may indicate either that not all LEA proteins make a significant contribution to plant stress tolerance, or that they need a particular background to function in, as suggested for transgenic strawberry plants (Houde et al. 2004).

Previous studies have established that the CBF cold responsive pathway is an integral component of the cold acclimation response (Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow et al. 2001). However, the transcriptome data showed that additional cold-regulatory pathways also exist (Fowler and Thomashow, 2002; Kreps et al. 2002). Transcriptome comparisons indicated that only 12% of the cold-responsive genes are certain members of the CBF regulon. Moreover, at least 28% of the cold-responsive genes were not regulated by the CBF transcription factors, including 15 encoding known or putative transcription factors, indicating that these cold-responsive genes are members of different low-temperature regulons. Information about the complexity of cold acclimation also comes from genetic studies using a luciferase gene driven by the COR78/RD29A promoter (Ishitani et al. 1998). A large number of mutants were isolated that are defective in the induction of this fusion gene in response to cold, drought, salinity and ABA treatment. These mutants fall into three major classes based upon the response of osmotically regulated genes: HOS have high expression, LOS display low expression and COS show constitutive activity of these genes (Ishitani et al. 1997). Forward genetic analysis in Arabidopsis identified two transcription factors, high expression of osmotically responsive genes 9 (HOS9) and HOS10, which are required for basal freezing tolerance (Zhu et al. 2004; Zhu et al. 2005). The HOS9 and HOS10 genes encode homeodomain and MYB (AtMYB8) transcription factors, respectively, and their transcript levels are not cold responsive. Loss-of-function mutations in these genes cause significant decreases in basal and acquired freezing tolerance. Interestingly, the mutants show stronger or earlier cold-induction of several CBF-target genes, such as RD29A and COR15A, but no effects on the expression of CBFs. These results suggest a crucial role in freezing tolerance for regulons that are not cold responsive, and these presumably constitutive regulons have a negative effect on the cold responsive CBF regulon.

Micro array analysis led to the identification of the cold-stress-inducible AP2 family transcription factor gene related to ABI3/VP1 (RAV1) (Fowler and Thomashow, 2002; Vogel et al. 2005) that might regulate plant growth under cold stress. RAV1 is down regulated by epibrassinolide, and transgenic Arabidopsis over expressing RAV1 exhibits a retardation of lateral root and rosette-leaf development, whereas antisense RAV1 plants show an early-flowering phenotype (Hu et al. 2004).

The importance of CBF-independent pathways is also supported by analysis of mutants that have increased freezing tolerance. Mutations in eskimo1 (ESK1), a protein of unknown function, result in constitutive freezing tolerance, but the genes that are affected by the ESK1 mutation are distinct from those of the CBF regulon (Xin et al. 2007). Similarly, mutations in the transcriptional adaptor protein ADA2 also cause constitutive freezing tolerance but not constitutive expression of COR genes (Vlachonasios et al. 2003).

Although, a great majority of the highly cold-inducible genes are regulated by CBF transcription factors and other transcription factors ZAT12 and RAV1 identified from transcript profiling studies, the basis for the regulation of the remaining 70% of the cold-induced COS genes and 95% cold-repressed COS genes is unknown (Vogel et al. 2005). Elucidation of the mechanism regulating these cold-regulated genes is an important goal in achieving a full understanding of

cold acclimation. Taken together, these results demonstrate the complex and interactive relationships among different pathways regulated by cold acclimation.

Cold Tolerant Genes and Hill Agriculture

Cold is an environmental factor that limits the geographical distribution and growing season of many plant species, and it often adversely affects crop quality and productivity (Thomashow, 1999). Most temperate plants can acquire tolerance to freezing temperature by a prior exposure to low nonfreezing temperature, a process known as cold acclimatization (Guy 1990, Hughes and Dunn, 1996, Browse and Xin, 2001). Plants of tropical and subtropical origins are sensitive to chilling temperature (0oC-10oC) and are incapable of cold acclimation. Many studies have suggested that cold regulated gene expression is critical in plants for both chilling tolerance a (Gong et al. 2002 and Hsieh et al. 2002) and cold acclimation (Knight et al. 1999: Thomashow 1999, Tamminen, 2001). Cold responsive genes encode a diverse array of proteins such as enzymes involved in respiration and metabolism of carbohydrates, lipids, phenylpropanoids and antioxidants: molecular chaperones, antifreeze proteins, and others with a presumed function in tolerance to dehydration caused by freezing (Guy 1990, Thomashow 1999 and Mohapatra et al. 1989). The change in the gene expression occur in plant during cold acclimatization a developmental process that results in increased tolerance (Guy, 1990 and Steponkus et al. 1993). Since then, it has repeatedly been speculated that certain COR (cold regulated) genes might have role in freezing tolerance. To test this notion investigators have turned to isolating the characterizing genes that are expressed in response to low temperature. These efforts have led to the identification of a number of genes such as the COR 15a KINI LTI 78 fad 7 genes of Arabidopsis thaliana.

It is well known to farmers and scientists that low temperatures can kill plants. Low temperature stress is a major environmental factor that not only limits where crops can be grown but also reduces yields depending on the weather in a particular growing season. In addition to exceptionally stressful years that cause significant yield reductions, less extreme stress almost certainly causes smaller losses over large areas to produce comparable yield reductions every year. Even in cases when freezing stress does not result in yield loses. It often results in crop quality reduction. Each year, worldwide losses in crop production due to low temperature damage amount to approximately $ 2

Billion. Some of the major losses include the 1995 early fall frosts in the US which caused losses of over $1 billion to corn and soyabeans. The occasional freezes in Florida have shifted the citrus belt further south, and California sustained $650M of damage in 1998 to the citrus crop due to a winter freeze. The inherent cold hardiness of the crop determines in which agricultural areas it can be grown. Crops that are more resistant to freezing stress would allow some geographical regions to grow more profitable and productive crops with less environmental risks. However despite continued efforts, traditional breeding has had only limited success in imparting crop plants with better freezing tolerance due to very little was known about the mechanisms that regulate chilling and freezing tolerance. With the advent of molecular genetics and biotechnology, it is now possible to genetically engineer plants to be more tolerant to many environmental adversities, including low temperature.

Conclusion

Applications of genomic approaches and gene knockout strategies are progressing to accelerate efforts to assess systematically and understand complex quantitative traits such as acquired tolerance to temperature extremes. By using genetic and molecular approaches, a number of relevant genes have been identified and new information continually emerges to enrich the CBF cold-responsive pathway. Thus, the CBF/DREB1 genes are thought to be activators that integrate several components of the cold acclimation response by which plants increase their tolerance to low temperatures after exposure to nonfreezing conditions.

However, the results of the transcriptome study demonstrate the highly complex nature of plant adaptation to low temperature. To overcome this problem a transgenic approach to promoting cold tolerance has been widely adopted, with some success. For example, increasing the accumulation of two compatible solutes, i.e., glycinebetaine and trehalose, in transgenic rice by overexpressing either E. coli choline oxidase, or trehalose-6-phosphate synthase fused to trehalose-6-phosphate phosphatase, enhanced tolerance to both salt and cold. In fact a large number of genes identified in different studies have currently annotated with ‘‘unknown function’’ and involve new genes and new pathways indicates that our knowledge of the transcriptional control of the low temperature response is limited, and the regulation of these transcriptional responses is far more complex than previously believed. Information on the low-temperature transcriptome, proteome and metabolome is expected to continue to increase in the near future. This information is necessary for our understanding of the complex network of molecular changes that are important for chilling and freezing tolerance in crop plants. Finally, a well focused approach combining the molecular, physiological and metabolic aspects of cold stress tolerance is required for bridging the knowledge gaps between short- and long-term effects of the genes and their products, and between the molecular or cellular expression of the genes and the whole plant phenotype under stress.

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