



Biotechnological applications of plant freezing associated proteins

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Abstract. Plants use a wide array of proteins to protect themselves against low temperature and freezing conditions. The identification of these freezing tolerance associated proteins and the elucidation of their cryoprotective functions will have important applications in several fields. Genes encoding structural proteins, osmolyte producing enzymes, oxidative stress scavenging enzymes, lipid desaturases and gene regulators have been used to produce transgenic plants. These studies have revealed the potential capacity of different genes to protect against temperature related stresses. In some cases, transgenic plants with significant cold tolerance have been produced. Furthermore, the biochemical characterization of the cold induced antifreeze proteins and dehydrins reveals many applications in the food and the medical industries. These proteins are being considered as food additives to improve the quality and shelf-life of frozen foods, as cryoprotective agents for organ and cell cryopreservation, and as a chemical adjuvants in cancer cryosurgery.

Keywords: antifreeze proteins, cold acclimation, COR proteins, cryoprotection, cryosurgery, dehydrins, freezing tolerance, frozen food, genetic engineering, organ preservation, osmoprotectants, osmotic stress, oxidative stress, plants, regulators, transgenic plants.

Introduction

Life is virtually impossible without the simple water molecule. Its high polarity, lesser density of its solid state compared to its liquid state and the relative stability of its hydrogen bond make it a unique molecule. The water molecules in their liquid state form a large hydrogen-bonded network that ensures the structural stability of biological molecules such as proteins, nucleic acids and the fluid bilayer structure of membranes. This property plays a key role in preserving the functional integrity of living cells. However, as liquid water changes to its solid state at the freezing point of a cell, ice crystals grow through the cells, destroying them and releasing their contents. Like all isothermic organisms, humans can generate enough metabolic heat to enjoy the luxury of the high temperature associated with the liquid state of water. This phenomenon seems so natural to us that we often associate heat with life and cold with death. However, many organisms can adapt and survive temperature extremes. Some of these organisms have the ability to tolerate freezing temperatures as low as -50°C [1].

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Non-isothermic animals like fishes, insects and frogs have developed many ways to face winter. Some hide and avoid freezing in deep water or soil while those that live at the surface adopt different mechanisms. Some try to keep their internal fluid liquid at all cost and others prepare themselves for winter by regulating gradually the ice formation until complete freezing of their internal fluids [2]. Surface animals synthesize antifreeze protein (AFP) and/or accumulate osmoprotectants such as the sugar alcohol glycerol. Unicellular organisms which are almost immobile like bacteria and algae have also developed efficient strategies to live in cold winter conditions. Cold tolerant prokaryotes are usually classified as mesophilic and psychrophilic. The adaptation of mesophilic bacteria to cold is mainly through physiological means whereas psychrobacteria have adapted to low temperature (LT) environments through evolution [3–5].

Plants are multicellular organisms that, unlike animals, cannot escape when confronted by stressful conditions. They either adapt to the new environment or simply die. To ensure survival, several plants have evolved efficient strategies that help them to tolerate extreme winter conditions. These mechanisms are genetically controlled and induced upon exposure to LT [6,7]. This response, termed cold acclimation, is associated with the development of freezing tolerance (FT). Plants avoid freezing damage by using two strategies [1]. The first is freezing avoidance and involves supercooling of some cells and tissues to temperatures as low as -40°C . Supercooling occurs because of a lack of nucleating substances necessary for ice initiation and by the use of barriers for ice growth in some tissues. Although whole plants do not supercool to this extent, the few degrees of protection afforded by supercooling is a significant avoidance mechanism. The most prevalent strategy in overwintering plants is the development of tolerance to freezing [1]. At high freezing temperatures, ice nucleation is initiated in extracellular spaces because of a lower solute concentration [6]. This causes water migration from the cell to the extracellular ice crystal resulting in the loss of vital intracellular water. The degree of cellular dehydration increases with decreasing temperature to a point where dehydration and/or ice crystal formation is sufficient to kill the cells. There exists a great diversity in the capacities of plants to acclimate and survive freezing temperatures, for example, spring cereals, peas and potatoes withstand a few degrees of frost, while winter cereals and cabbage tolerate temperatures to about -33°C , and certain woody plants can survive temperatures lower than -50°C [1,7]. Understanding how these plants survive such freezing temperatures is not only a scientific challenge, but also has important economic applications. Different aspects of plant responses to LT have been reviewed in detail elsewhere and will not be covered here [1,6,8]. The goal of this review is to discuss the different biotechnological applications developed from the basic research in the area of LT tolerance in plants.





Molecular genetics and improvements of low temperature tolerance in plants

Winter survival and crop productivity are influenced by many different winter stresses [9–11] such as absolute freezing temperature, length of freezing period, ice encasement, flooding and oxidative stress caused by LT induced photoinhibition. Exposure of plants to LT produces morphological, biochemical, and physiological changes that are often highly correlated with plant FT and winter survival. This suggests that the phenotypic expression of FT is controlled by a large number of genes with complex interactions. The complexity of the LT response has made it difficult to separate genes responsible for LT acclimation and cold hardiness from those associated with metabolic adjustments to LT. Our inability to effectively characterize LT responses emphasizes the need for a better understanding of winter stresses, the LT responsive genes responsible for adaptation to winter stresses and their interaction with the environment [9]. Because of this complexity, progress in improving cold tolerance by traditional breeding of important crop species has been slow. To overcome this problem, considerable attention has been given to the potential use of genetic engineering to improve this important trait. Identification of genes contributing to LT tolerance and the understanding of their regulation would provide a previously unavailable opportunity for coupling traditional plant breeding methods with biotechnology for crop improvement. Analysis of the literature reveals a growing list of cold-regulated (COR) genes, with at least 50 now identified in different species [8].

The identification of a large number of COR genes makes it difficult to select the right gene for genetic engineering of LT tolerance in crop species that are hard to transform. Thus transgenic studies with model species are an attractive approach to evaluate the potential of individual genes before proceeding with the transformation of crop species. Although these studies have had a limited success in improving FT, they provide valuable information on the mechanisms underlying this important trait. These studies have been classified into three groups based on the presumed function of the gene that was evaluated. The first represents genes encoding structural proteins that might be involved in protecting the cell during LT stress (Table 1). The second represents genes encoding enzymes involved in the biosynthesis of different osmoprotectants, desaturation of lipids, and those in the antioxidative response (Tables 2 to 5). The third represents regulatory genes that control the overall mechanism of LT responses. These studies also revealed the potential and limitations of introducing single genes to modify LT tolerance in plants. In addition, they improved our knowledge of the different metabolic pathways associated with LT tolerance in plants. Many important factors need to be considered for a successful transformation [12]. These include, the transformation techniques, the choice of plant species, the inducibility and tissue specificity of the promoter used, organellar targeting signal, post-translational modification, the availability of prosthetic group and precursors, the microenvironment, and the possible side-reactions of the new product.





Transgenic plants with structural proteins

Antifreeze proteins

AFPs were first identified in fishes as the causative agents of serum freezing point depression [13]. These proteins are unique in that they cause a freezing point depression far greater than would be predicted from colligative properties alone. Later studies demonstrated that the AFP lowers the freezing point by interacting directly with the ice surface and inhibiting the binding of additional water molecules to the ice crystal lattice. This property is known as thermal hysteresis [14,15]. AFPs are also known to inhibit the recrystallization of ice, which is the growth of larger ice crystals at the expense of smaller ice crystals [16]. Larger ice crystals increase the possibility of physical damage within frozen tissues. The inhibition of the recrystallization of ice occurs at very low AFP concentrations (nM) and may be the function of AFPs in freezing tolerant organisms [17]. In addition, they may be involved in protecting cell membranes from cold-induced damage [18]. AFPs have been isolated and characterized from several species. The proteins for which sequence or structural information has been obtained include the five types of fish AFPs, two types from insects, three AFPs from rye plants and one from carrot [19–21]. These AFPs are unrelated in composition and structure. The levels of thermal hysteresis range from 0.2–0.6°C for plant AFPs [19], 1–2°C for fish AFPs [20] to 5–10°C for insect AFPs [22]. Because the properties of these proteins suggested that they may be useful in protecting plants against cold temperatures, many transgenic studies were initiated to evaluate their potential (Table 1).

Historically, it was demonstrated in 1989 that vacuum infiltration of fish AFP into plant leaves produced a depression of the freezing temperature of the tissue [23]. This result led Hightower et al. [24] to transform tomato and tobacco plants with a synthetic AFP based on type I AFP from winter flounder and containing three, alanine-rich, 11 amino acid repeats. Transgenic plants accumulated the corresponding mRNAs, but did not show any antifreeze activity as measured by ice recrystallization inhibition. However, when fusion proteins containing five repeats and targeted to the cytoplasm or the apoplast were expressed in tomato, the transgenic lines were shown to accumulate the chimeric proteins to 0.1% of total soluble leaf proteins and to possess antifreeze activity. Unfortunately, the effects of transgene expression on cold tolerance of tissues were not examined. A synthetic fusion protein containing 5 repeats was also expressed in yeast [25]. The expressed protein was shown to inhibit the recrystallization of ice *in vitro*. Yeast cells expressing this AFP showed a two fold increase in survival after rapid freezing compared to non-transformed cells. In another study, tobacco plants transformed with the type I AFP from winter flounder expressed the AFP mRNA but not the protein under normal growth conditions [26]. However, AFP accumulated to detectable levels only after exposure of the plants to cold, possibly due to a higher stability of the α -helical protein at LT. Whether or not this amount of protein had any antifreeze activity was not determined. Recently, Wallis et al. [27] used codon



Table 1. Transgenic plants expressing different structural proteins.

Class	Source and Protein	Target organism and Location	Effects on cold tolerance	Refs.
AFP	Fish AFP I <i>afa 3, afa5</i>	Tomato (cytoplasm and apoplast) Tobacco	Not tested	24
	Fish AFP I <i>afa5</i>	Yeast	Yes	25
	Fish AFP I	Tobacco	Not tested	26
	Fish AFP I (with potato codon usage)	Potato (apoplast)	Yes	27
	Fish AFP II	Tobacco (cytoplasm and apoplast)	No	28
	Carrot AFP	Arabidopsis	Not tested	29
	Carrot AFP	Tobacco	Not tested	21
Ice nucleator	<i>S. commersonii</i>	Tobacco	Not tested	37,38
	INAZ	Potato		
COR/LEA	<i>C. plantagineum</i>	Tobacco	No	48
	LEA group II			
	Spinach CAP85	Tobacco	Yes	49
	LEA group II			
	Spinach CAP160 novel	Tobacco	Yes	49
	Barley HVA1	Rice	Not tested	50
	LEA group III			
	Wheat Em	Yeast	No	51
	LEA group I			
	Tomato LE25	Yeast	Yes	52
	LEA group IV			
Arabidopsis COR15a	Arabidopsis (chloroplast)	Yes	53	
Barley COR14b	Arabidopsis	Not tested	55	

usage frequencies observed in higher plants to construct a synthetic AFP gene based on type I AFP and added a signal peptide directing it to the extracellular space. A transgenic potato line that expressed this protein to 0.6% of total leaf proteins showed a significant decrease of electrolyte leakage after freezing at -2°C compared to control plants. These results provided the first evidence of the capacity of AFPs to prevent freezing injuries in plant tissues. More recently, a chimeric construct containing the type II AFP gene from fish fused to the plant signal sequence directing secretion to the apoplast was introduced into tobacco [28]. The AFPs that accumulated to 2% of total apoplast proteins were shown to be active in both thermal hysteresis and the inhibition of ice recrystallization tests. However, visual evaluation of plants in the field following light frosts (-0.1 to -3°C) did not reveal any difference between transgenic and wild type plants. This study also reported that the production of plants that accumulate the protein in the cytosol was not successful. The transgenic plants were sterile and only expressed the transcript





for type II AFP. It has been proposed that the extensively disulfide-bonded protein is toxic when targeted to this compartment [28]. Transgenic tobacco and Arabidopsis plants expressing an active carrot AFP have been produced [21,29]. However, the cold tolerance of the transgenic plants was not assessed.

To date, the efforts to improve plant cold tolerance with AFPs have not been successful. Factors such as the sensitivity of the plant to freezing, the low levels of expression and subcellular localization can be responsible, in part, for such results. Future engineering studies with AFPs will also have to examine other considerations to design realistic transformation strategies. The possibility that AFPs may interact and stabilize cell membranes at temperatures above freezing [18] may be used to protect membranes in chilling sensitive plants. Further characterization is needed to define AFPs that possess this property and which of these AFPs can be expressed in plants to high levels without harmful effects. In addition, the identification of insect AFPs with very high levels of thermal hysteresis offers a way to promote supercooling of tissues in freezing sensitive plants. If these proteins can be made to accumulate to high levels in different compartments of the cell, a freezing point depression of a couple degrees could have an important role in frost protection of plants that cannot survive freeze-induced dehydration of the protoplasm. Finally, the identification of plant AFPs that possess enzymatic activities corresponding to three classes of pathogenesis-related proteins suggest that these proteins could be used to engineer freezing tolerant plants with both increased antifreeze activity in the apoplast and disease resistance. These proteins were shown to inhibit the recrystallization of ice which is known to take place most rapidly at temperatures just below freezing and when environmental temperatures fluctuate within the subzero range [30]. Because this activity is mediated by low concentrations of AFPs, targeting high levels of accumulation in the apoplast is realistic and can confer some protection to freezing tolerant plants. Because of the many potential applications of this group of proteins, the molecular and biochemical characterization of these proteins and their evaluation in transgenic studies needs to be pursued.

Ice nucleators

Frost-hardy plants may increase their FT by initiating intercellular ice formation at high subfreezing temperatures [31]. Propagation of intercellular ice at subzero temperatures close to 0°C promotes gradual dehydration of cells, depressing both the cellular freezing and ice nucleation point. Siminovitch and Scarth [32] reported that lethal intracellular freezing occurs in acclimated plants that are allowed to supercool, and that initiation of ice formation at high subfreezing temperatures favoured cell survival. However, many hardy and non-hardy plant species can supercool extensively because they have no internal ice-nucleating agents active at temperatures higher than -5°C [33]. In these plants, supercooling allows a degree of protection during short-term exposure to high freezing temperatures but does not eliminate the risk of spontaneous intracellular freezing that causes cell death. On the other hand, ice nucleation active bacteria can catalyze ice formation





at temperatures as warm as -2°C [34]. Genes encoding ice nucleation proteins from bacteria have been cloned and structurally characterized [35]. A similar high temperature ice nucleation activity has been detected within freezing tolerant insects and animals [36]. To determine if bacterial ice nucleation proteins can function in plant cells, the ice nucleation gene (*inaZ*) from *Pseudomonas syringae* was introduced into tobacco, a freezing sensitive species, and *Solanum commersonii*, a freezing tolerant species [37] (Table 1). Transformants of both species showed increased ice nucleation activity over untransformed controls. The temperature of the warmest freezing initiation in intact leaves was increased from approximately -7°C in the untransformed controls to -4°C in the transformants. It was also shown that 48 hours of LT are required for the maximum ice nucleation activity [38]. Similarly, LT exposure is required for maximum ice nucleation activity in bacteria [39]. The warmest ice nucleation temperature detected in transgenic plants was lower than that conferred by this gene in *P. syringae* (-2°C). This difference is probably due to a lower amount of INAZ protein or an inappropriate localization. In bacteria, these proteins are located in the outer membrane where they form large aggregates that serve as templates for high temperature ice nucleation [35]. Therefore, it remains to be determined if the INAZ protein can be targeted and assembled correctly in a compartment such as the apoplast where it would be most beneficial. These studies demonstrate the feasibility of expressing the bacterial gene *inaZ* in higher plants and open the door to engineering plants capable of initiating ice formation at higher subzero temperatures to improve survival. An alternative strategy to the use of bacterial ice nucleators may one day be the use of plant endogenous ice nucleators. Although they are less active, studies with rye have shown that ice-nucleating activity in cold acclimated plants may be mediated to some extent by a protein component [40]. The identification of this gene and the characterization of the encoded product will ultimately reveal how it functions and consequently its potential in high temperature ice nucleation.

COR/LEA proteins

During exposure of plants to freezing temperatures, the extracellular water freezes before the intracellular water because of its higher freezing point. The resulting lower water potential in the apoplast will draw water out of the cell, leading to dehydration of the protoplasm. It is well established that freeze-induced membrane damage results primarily from the severe dehydration associated with freezing [41,42]. Furthermore, the dehydration stress that occurs during freezing is likely to be similar to the one occurring during drought and salt stress, and so is expected to trigger some common genetic and physiological responses. The identification and characterization of COR genes in many plant species has revealed that many of them encode proteins with unknown functions [8]. A partial list of these genes is found in Tables 2 and 3 of Thomashow's recent review [8]. Analysis of these tables reveals that at least two novel genes encode hydrophobic polypeptides, 9 novel genes encode hydrophilic polypeptides, while other genes encode the hydrophilic





homologs of groups II and III of Late Embryogenesis Abundant (LEA) proteins. LEA proteins were initially found to be induced during late embryogenesis, prior to seed desiccation and in plants during water stress. They were divided into different groups based on conserved structural features [43,44].

The hydrophilic COR proteins of unknown functions are referred to here as COR/LEA proteins. They are unusually hydrophilic, boiling soluble and many are composed largely of repeated amino acid sequence motifs. Sequence analysis reveals that many contain regions capable of forming amphipathic α -helices. Based on these properties, it has been suggested that these proteins may increase tolerance to freezing and dehydration by stabilizing proteins and membranes during dehydrative conditions [8,45–47]. Although many COR/LEA genes have been identified, only a handful have so far been evaluated in transgenic studies (Table 1).

Of all the genes induced during cold acclimation, the most widely surveyed encode proteins belonging to the group II LEA family [8]. A cDNA encoding a member of this group from the resurrection plant *Craterostigma plantagineum* was introduced into tobacco [48]. Transgenic plants that expressed this protein did not show any changes in the tolerance to osmotic stress as measured by the ion-leakage test. In another study, two genes, *cap85* (LEA group II) and *cap160* (novel gene) from spinach, were constitutively expressed in tobacco [49]. Plants expressing the proteins, individually or together, were indistinguishable from wild type plants as assessed by the FT of detached leaves. However, when a kinetic study of freezing at -2°C was carried out, transgenic plants expressing the spinach proteins had slightly lower rates of electrolyte leakage indicating a reduction of freezing stress injury. The potential of overexpressing a group III LEA gene from barley was assessed in rice [50]. Transgenic rice plants showed an increased tolerance to water deficit and salinity as reflected by their higher growth rates, delayed development of symptoms and improved recovery. However, the effect of cold stress on the performance of these transgenic plants was not measured. Although other groups of LEA proteins were not yet shown to be regulated by cold temperatures, they have been evaluated in transgenic studies. For example, the group I LEA protein from wheat was overexpressed in yeast cells and shown to attenuate the growth inhibition normally observed in high osmolarity media [51]. However, no correlation was established between the expression of this protein and increased survival after overnight freezing. Overexpression of a class IV LEA protein from tomato in yeast conferred a small but significant increase in FT [52]. After 24h at -20°C , 8% of the transformed yeast strain survived compared to 2% for the control strain.

Genes encoding novel proteins have also been expressed in plants. The COR15a gene from *Arabidopsis* which encodes a protein that is targeted to the stromal compartment of chloroplasts was constitutively expressed in transgenic *Arabidopsis* plants [53]. Non-acclimated transgenic plants showed an increase of 1 to 2°C in the FT of both chloroplasts frozen *in situ* and isolated leaf protoplasts frozen *in vitro* over the temperature range of -4 to -8°C . Subsequent investigations have indicated that COR15a may increase FT by decreasing the propensity of membranes to form





hexagonal II phase lipids, a major cause of membrane damage in non-acclimated plants over the temperature range of about -4 to -8°C [54]. These results lead this group to propose that proteins that contain possible amphipathic α -helices such as COR15a may alter the intrinsic curvature of membrane monolayers thus leading to a shift in the lamellar-to-hexagonal II phase transition temperature [8,54]. Another novel gene, COR14b from barley was introduced into *Arabidopsis* plants [55]. This gene also encodes a protein that is targeted to the stromal compartment of chloroplasts. However, the effect of its expression on the FT of chloroplasts or whole plants was not measured.

Because FT is considered to be a multigenic trait, it is not surprising that in the studies described above, the expression of one or two COR/LEA proteins does not significantly alter the tolerance to freezing. However, the demonstration that an amphipathic α -helix containing protein defers the occurrence of the freeze-induced formation of the hexagonal II phase to lower temperatures is significant. Many COR/LEA proteins contain such regions and have been localized to cellular compartments such as the nucleus [56], the cytoplasm [56], the chloroplast [57] and the paramural space [47]. If these proteins are found to function in a manner similar to COR15a, they may be used to cryoprotect different membranes. A modest increase of FT by 1 or 2 $^{\circ}\text{C}$ could have a tremendous impact on freezing tolerant crops in early fall and late spring or in freezing sensitive species that suffer membrane injuries due to the formation of the hexagonal II phase [54]. However, newer strategies to improve FT will have to consider that alteration of membrane lipid composition [42] and the accumulation of sugars and compatible solutes [58–59] also contribute to decreasing membrane injuries caused by freezing.

Metabolic engineering to improve cold tolerance

When plants are exposed to abiotic stresses such as low and freezing temperatures, membrane fluidity and membrane bound processes are perturbed [60]. In addition, when extracellular ice forms, there is a flux of water from the cells causing both a dehydration of the protoplast and an increase in the concentration of intracellular solutes, thus putting a strain on membranes and macromolecules [6]. Under conditions in which chloroplasts are exposed to excess excitation energy generated by the interaction of light and LT, there is an increase in photoreduction of oxygen and concomitant production of reactive oxygen intermediates, such as superoxides and peroxides, which can damage membranes and enzymes [12]. Plants react to these stresses by inducing various responses. They synthesize compatible solutes and sugars needed for osmotic adjustment and osmoprotection [6]. They modify the lipid species and their degree of unsaturation to preserve membrane fluidity and prevent freeze induced membrane damage [60,61]. In addition, they activate antioxidant enzymes such as peroxidase and superoxide dismutase, that scavenge reactive oxygen intermediates [62,63]. Knowledge generated from these studies was used to design strategies to improve stress tolerance [58–59,64,65]. Among these approaches are: 1) the introduction of genes encoding enzymes that catalyze





the conversion of naturally occurring substrates into products with osmoprotective properties, 2) genes encoding lipid-modifying enzymes, and 3) genes encoding enzymes involved in the antioxidative response.

Amino acid and derived osmoprotectants

Osmoprotectants (also termed compatible solutes) occur in all organisms from archaeobacteria to higher plants and animals [64,66]. They are highly soluble compounds that carry no net charge at physiological pH and are non-toxic at high concentrations. Osmoprotectants serve to raise osmotic pressure in the cytoplasm and can also stabilize proteins and membranes when salt levels or temperatures are unfavourable [59,64]. Osmoprotectants therefore play important roles in the adaptation of cells to various adverse environmental conditions [67]. There are many amino acid derived osmoprotectants that have been identified in plants under stressful conditions like proline [68–73], 3-dimethylsulfoniopropionate [64], polyamines [74,75], trigonelline [76] and betaines [64,68,77,78].

Although there are many examples of amino acid derived osmoprotectants, metabolic engineering studies to date have concentrated on increasing proline and betaine accumulation [12,64] (Table 2). Proline biosynthesis from the amino acid glutamate can be achieved by using the p5cs gene from mothbean [*Vigna aconitifolia*] encoding a bifunctional enzyme containing the catalytic activities of γ -glutamyl kinase and glutamic- γ -semialdehyde dehydrogenase [12]. Two studies have used this gene to produce transgenic plants with increased proline production [79,80] but tolerance to LT was not measured. However, transgenic tobacco plants with increased proline content [79] were shown to be more osmotolerant than control plants. In the second study, this gene was placed under the control of an ABA inducible promoter thus leading to overproduction of proline only under stress conditions [80]. Transgenic rice plants that accumulated proline to high levels during salt and water stresses showed an enhanced biomass production compared to non-transformed control plants.

Glycine betaine is synthesized in plants via a two-step oxidation of choline which is derived from serine [64,81]. In spinach, the oxidation steps are carried out by the two chloroplastic enzymes choline mono-oxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). The first reaction is ferredoxin-dependent and oxidizes choline to betaine aldehyde. The second reaction is NAD-dependent and converts betaine aldehyde to betaine [64]. This pathway is not the only one in plants because studies with transgenic tobacco plants expressing the barley BADH showed that this enzyme was targeted to peroxisomes. This suggests that in monocots BADH may be mostly localized in peroxisomes [82]. Since there is no reduced ferredoxin in the peroxisomes [64], it is unlikely that the choline-oxidizing enzyme in monocots is CMO. In the bacteria *E. coli*, the conversion of choline to betaine occurs via the two choline dehydrogenases (BET A and BET B) and in the soil bacterium *Arthrobacter globiformis* by the one-step choline oxidase (COD A) [64,77].

To date, only Murata et al. have studied the effects of betaine accumulation on





Table 2. Transgenic plants accumulating amino acid related osmoprotectants.

Class	Source and Protein	Target organism and Location	Effects on cold tolerance	Refs.
Proline	Mothbean P5CS	Tobacco	Not tested	79
	Mothbean P5CS	Rice	Not tested	80
Betaine	<i>A.globiformis</i> COD A	Synechococcus	Yes	83
	<i>A.globiformis</i> COD A	Arabidopsis (chloroplast)	Yes	84-88
	<i>A.globiformis</i> COD A	Rice [cytosol and chloroplast]	Yes	89
	Barley BADH	Tobacco	Not tested	82
	Spinach BADH	Tobacco	Not tested	90
	Spinach CMO	Tobacco	Not tested	91
	<i>E. coli</i> BADH (BET B)	Tobacco	Not tested	92
	<i>E. coli</i> (BET A)	Tobacco	Not tested	93
	<i>E. coli</i> (BET A and BET B)	<i>Synechococcus</i>	Not tested	94

LT tolerance. They transferred the *codA* gene into several species such as the cyanobacterium *Synechococcus sp.* Strain PCC 7942 [83], *Arabidopsis thaliana* [84–88] and rice [89]. Compared to control cells, transformed cyanobacteria cells expressing *codA* showed an enhanced ability to grow at the usually non permissive temperature of 20°C in a medium supplemented with 1mM choline chloride [83]. Furthermore, photosynthesis in transformed cells was more resistant to LT-induced photoinhibition. These effects were attributed to betaine accumulation mediated by the *codA* gene [83]. *Arabidopsis* plants expressing *codA* showed an enhanced growth, germination and survival in the presence of NaCl [85]. When plants were exposed to 5°C under continuous light followed by a recovery period at 22°C, leaves of wild type plants exhibited symptoms of chlorosis whereas those of transformed plants did not. The activity of photosystem II in transgenic plants remained at a higher level than in wild type plants under LT, high light and salt stresses [85,88]. Furthermore, transgenic *Arabidopsis* plants had a greater tolerance to LT, heat stress and high salt during germination and early growth [84,86,87]. Wild-type seedlings germinating at LT were morphologically distorted while transgenic plants were not [86]. Cold sensitive rice plants engineered to express the *codA* protein in the chloroplast or cytosol, showed a faster recovery rate compared to wild type plants after exposure to salt stress [89]. This study also showed that accumulation of betaine in chloroplasts confers a higher degree of tolerance to salt- and LT-induced photoinhibition [89].

In spite of the encouraging results regarding the effect of betaine accumulation on LT tolerance in transgenic plants, poor accumulation of betaine has been observed due to a limited choline supply [91]. These low levels of accumulation are not enough for betaine to function as an osmotic adjustment agent. Therefore, the





observed effects in these studies are probably due to the stabilization of protein structure and function [89]. Research must be continued to further control the flux of choline in transgenic plants and may require additional engineering steps [91]. Studies aimed at improving betaine production in plants are worthwhile because it was recently shown that exogenous application of betaine could improve FT by more than 5°C in both non-acclimated and cold acclimated wheat plants [95]. This increase in tolerance is the highest reported for plants treated with exogenous chemicals or for stably transformed plants. In addition, *in vitro* cryoprotection assays have shown that betaine can protect liposomes, bacteria and plant tissue during freezing [96–99].

Sugar related osmoprotectants

Accumulation of sugar related compounds such as sucrose, raffinose, sorbitol and fructans during cold acclimation of plants has been observed repeatedly [100–109]. Many of these compounds are believed to stabilize membranes and proteins during the dehydrative conditions induced by freezing or drought [6]. According to Crowe et al. [110], sugars can replace water in the maintenance of dehydrated proteins or membranes in a physical state similar to that seen in the presence of water. In addition, an alternative function has been proposed for the disaccharides sucrose and trehalose which can form a glass phase in the dry state [111]. A glass is a liquid of such high viscosity that it is capable of slowing chemical reactions. It was proposed that glass formation (vitrification) could lead to long-term stability of a living system [112]. Transgenic plants expressing different sugar biosynthetic enzymes have been produced with the primary objective to study carbohydrate metabolism and not to evaluate their potential effects on tolerance to LT or other stresses [113–116]. However, some recent advances have furthered our understanding of carbohydrate metabolism and are noteworthy to mention because they could be used in molecular engineering strategies (Table 3).

Trehalose accumulation has long been detected during the onset of desiccation tolerance of organisms such as yeast and resurrection plant [117,118] but its presence was never observed in tissues of other plants, probably due to the presence of high levels of trehalase activity. A most unexpected finding in recent research was the discovery that the trehalose biosynthetic pathway is ubiquitous in plants [117]. Transgenic tobacco plants overexpressing the yeast trehalose phosphate synthase gene (*tps1*) showed a significant tolerance to drought [119,120]. However, these trehalose-accumulating plants exhibited reduced growth under non-stressful conditions [119], and multiple phenotypic alterations [120]. Transgenic tobacco and potato plants were also produced which express the *E. coli* trehalose-6-phosphate synthase and trehalase-6-phosphatase genes [*tps1* and *tpp*] [121,122]. These plants had an improved growth performance during drought but also showed some morphological alterations. Because these alterations may be caused by some as yet unidentified function of trehalose in plants [117], the negative effects on plant development may in the future be alleviated by using a promoter sequence





Table 3. Transgenic plants expressing enzymes involved in the biosynthesis of sugar related osmoprotectants.

Class	Source and Protein	Target organism and Location	Effects on cold tolerance	Refs.
Trehalose	Yeast TPS1	Tobacco	Not tested	119
	Yeast TPS1	Tobacco	Not tested	120
	<i>E. coli</i> TPS1 AND TPP	Tobacco	Not tested	121,122
	Yeast TPS1	Potato Yeast	Yes	124
	Fructans	<i>B. subtilis</i> Fructosyl transferase	Tobacco	Not tested
Mannitol	<i>E. coli</i> MTLDH	Tobacco (cytoplasm and chloroplast)	Not tested	130,131
	<i>E. coli</i> MTLDH	<i>Arabidopsis</i>	Not tested	132
	<i>E. coli</i> MTLDH	Tobacco	Not tested	133
	<i>E. coli</i> MTLDH	Tobacco	Not tested	134
	D-ononitol	Ice plant IMT1	Tobacco	Not tested
Inositol	<i>S. polyrrhiza</i> INS3PS	<i>Arabidopsis</i>	Not tested	137
	Apple S6PDH	Tobacco	Not tested	138
l	Apple S6PDH	Tobacco	Not tested	139

that drives expression in specific cell types or under stress conditions [121]. A general observation from these studies is that trehalose accumulates to low levels in transgenic plants. It was shown that this problem could be circumvented by inhibiting endogenous trehalase, an enzyme involved in trehalose turnover [121]. Furthermore, it was recently shown that expressing trehalose phosphate synthase or neutralizing trehalase could improve FT in yeast [123,124]. Based on these results, attempts to engineer trehalose accumulation will undoubtedly be pursued.

Several flowering plant species accumulate as their carbohydrate storage pool, the linear and branched polymers of fructose called fructans [115]. The degree of polymerisation of plant fructans varies greatly but generally, the fructosyl chain is about 30 to 50 fructosyl residues long. The global distribution of fructan-accumulating plants shows that they are especially abundant in temperate climate zones with seasonal drought or frost, but that they are almost absent in tropical regions [125]. The physiological role of fructans is not fully understood. They are thought to lower the cellular sucrose concentration to prevent sugar-induced feedback inhibition of photosynthesis [126] and also be involved in drought





and cold tolerance [127]. Unlike starch which is insoluble and accumulates in amyoplasts, fructans are soluble molecules that accumulate preferentially in vacuoles. A bacterial fructan biosynthetic enzyme was used to transform tobacco plants [128,129]. The fructan-producing transgenic plants were found to be more resistant to PEG-induced drought stress, as determined by their growth properties and biomass accumulation. In this study, the use of a bacterial enzyme resulted in the production of fructans with a degree of polymerisation of more than 100000 fructosyl residues [129]. Because such molecules are not normally present in plants, they may themselves induce a stress response that affects the drought tolerance of these plants [115]. The availability of plant fructan biosynthetic genes will allow the detailed study using transgenic plants of the effects of fructan accumulation on stress tolerance [115].

Polyols such as mannitol [130–134], D-ononitol [135,136], inositol [137] and sorbitol [138–139] form another class of compounds with osmoprotective activities that have been the targets of molecular engineering studies. These sugar alcohols are closely related to sugars, as they represent the chemically reduced form of an aldose or ketose sugar. It has been suggested that sugar alcohols, because of their water-like hydroxyl groups, may mimic the structure of water and maintain an artificial sphere of hydration around macromolecules [58,65,114]. While transgenic *Arabidopsis* plants accumulating inositol did not show any significant difference in stress tolerance, the transgenic plants accumulating mannitol, D-ononitol and sorbitol did show some enhanced tolerance to drought or salt stress. These studies are particularly instructive because they show that engineering a new pathway for sorbitol accumulation in tobacco resulted in growth defects and necrosis [139]. This was attributed, in part, to interference with inositol biosynthesis and alterations in carbohydrate allocation and transport. Furthermore, engineering a new pathway for D-ononitol accumulation in tobacco makes D-ononitol production stress inducible because its precursor inositol is also stress inducible in this plant [136]. These studies highlight the importance of metabolic analyses of different transgenic and stressed plants to estimate *in vivo* pathway fluxes and intermediate pool sizes [77]. Once available, such data could be incorporated into metabolic models and used to predict the impact of inserting a transgene on pathway flux.

Lipid modifications

In cold sensitive plants, temperatures ranging from 0°C to about 12°C restrict growth and may even result in the plant's death [140]. A widely held view was that the high level of disaturated species of phosphatidylglycerol (PG) in the chloroplast membranes was at the base of the chilling sensitivity of these plants [60]. Many enzymes capable of lipid desaturation in plants and other organisms have been identified [141,142], and some were used to produce transgenic plants to investigate the effects of changing lipid unsaturation on chilling sensitivity [60,143] (Table 4). The first direct evidence of a link between the level of saturated lipids and chilling sensitivity was provided by Murata and colleagues [144]. They cloned





Table 4. Transgenic plants expressing lipid modifying enzymes.

Class	Source and Protein	Target organism and Location	Effects on cold tolerance	Refs.
Acyltransferase	<i>Arabidopsis</i> G3PAT	Tobacco	Yes	144
	Squash G3PAT		No increase	
	<i>E. coli</i> G3PAT	<i>Arabidopsis</i> (chloroplast)	No increase	146
Desaturase	<i>Arabidopsis</i> G3PAT	Rice	Yes	145
	<i>Arabidopsis fad7</i>	Tobacco	Yes	148,149
	<i>Arabidopsis fad3</i>	Tobacco	No	150-152
	<i>A.nidulans</i>	Tobacco	Yes	153
	$\Delta 9$ -desaturase	(chloroplast)		

the cDNAs for the plastid glycerol-3-phosphate acyltransferase from squash, a chilling-sensitive species, and from *Arabidopsis*, a chilling-resistant species. The enzyme from squash preferentially transfers saturated lipid species to the *sn-1* position of glycerol-3-phosphate while the *Arabidopsis* enzyme selectively transfers unsaturated lipid species. Transgenic tobacco plants expressing the squash cDNA contained elevated levels of disaturated PG (76%), whereas plants expressing the *Arabidopsis* cDNA contained slightly reduced levels of disaturated PG (28%) compared with wild type plants (36%). Plants expressing the squash enzyme showed severe signs of chilling sensitivity as measured by LT-induced photoinhibition in leaf disks and development of chlorosis in LT-exposed plants. This observation has now been extended to rice plants that have been transformed with the cDNA from *Arabidopsis* [145]. Transgenic rice plants expressing this gene had higher levels of unsaturated species of PG and were found to have higher net photosynthetic rates when exposed to 17°C compared to control plants. Other groups also analysed the effects of increased levels of saturated molecular species of PG by targeting the product of the *plsB* gene of *E. coli* to the chloroplasts of *Arabidopsis* [146] or studying the *fab 1* mutant of *Arabidopsis* [147]. Transgenic plants expressing the *plsB* gene displayed a chilling-sensitive phenotype while *fab 1* mutants did not. However, *fab 1* mutants did show growth retardation after prolonged exposure to LT, and eventually died if exposed for more than 4 weeks. Experiments with tobacco and *Synechocystis* [60] have suggested that higher levels of unsaturated membrane lipids accelerate the recovery process from the LT-induced photoinhibition and may be the determining factor in the chilling-insensitive phenotype.

An alternative approach to modify the level of unsaturation in membrane lipids is to express fatty acid desaturase genes in plants. Transgenic tobacco plants have been produced that overexpress the chloroplastic *fad7* ω -3 desaturase from *Arabidopsis* responsible for the formation of trienoic fatty acids (16:3, 18:3) in leaf tissues [148,149], the microsomal *fad 3* ω -3 desaturase from *Arabidopsis* responsible for the formation of 18:2, 18:3 fatty acids [150–152], and the $\Delta 9$ -desaturase from *Anacystis nidulans* targeted to the chloroplast which introduces a cis-double bond





at the $\Delta 9$ position of both 16 and 18 carbon saturated fatty acids [153]. All transgenic plants contained higher levels of unsaturated fatty acids compared to control plants, but only the *fad 7* and $\Delta 9$ desaturase expressing plants exhibited a significant increase in chilling tolerance. Failure to detect an increase in the LT tolerance of transgenic plants expressing the microsomal *fad 3* gene may indicate that unsaturation of chloroplast lipids may play a more important role in chilling tolerance [142,152]. Results from these transgenic studies indicate that there may exist a realistic potential in alleviating the chilling sensitivity of some species by combining several engineering steps such as decreasing disaturated PG and increasing unsaturation.

Compared to chilling sensitive plants, freezing tolerant plants can grow at low positive temperatures and induce adaptive changes that enable them to withstand subsequent exposures to freezing. In these plants, the primary cause of freezing injury is the destabilization of the plasma membrane during freeze-induced dehydration [41]. Several studies have now documented the subtle changes induced by cold acclimation in the plasma membrane lipid composition of rye [154], *Solanum* species [155], oat [156], and *Arabidopsis* [156]. Changes that have been proposed to increase the cryostability of the plasma membrane during freeze-induced dehydration are an increase in the proportion of di-unsaturated species of phosphatidylcholine (PC) and phosphatidylethanolamine and a decrease in the proportion of cerebrosides and sterol containing lipids [156]. Although membrane-engineering studies [157,158] have shown that increasing the proportion of di-unsaturated species of PC in the plasma membrane decreases membrane injury, no such genetic studies have yet been reported. Manipulating the proportion of certain lipid species in the model plant *Arabidopsis*, containing endogenous mechanisms for frost tolerance, could be a valuable tool in evaluating the potential benefits or limitations of these changes on the cryostability of the plasma membrane during freeze-induced dehydration. However, studies with the unicellular organism *Acholeplasma laislawii* [159] indicate that the proportion of different lipid species may be controlled by the activity of the enzymes that appear to be responsive to the physical state of the membrane in which they operate. If such a feedback mechanism is in operation in freezing tolerant plants during cold acclimation, it may prove difficult to genetically manipulate the relative proportions of lipid species to enhance the cryostability of the plasma membrane. A greater understanding of the mechanisms that bring about changes in lipid species in these freezing tolerant plants will indicate if this genetic engineering approach has potential.

Antioxidative response

Free radicals and other active derivatives of oxygen are inevitable by-products of biological redox reactions. During normal growth conditions, plants are well adapted for minimizing the damage that occurs from misuse of photosynthetic energy transfer. However, perturbation of electron transport under conditions of photoinhibition can lead to the transfer of an excess of electrons to oxygen and





create an oxidative stress. Such conditions are encountered by crops that are exposed to periods of cold or drought during a growing season and this affects plant productivity [62,63]. During environmental stresses, plants increase the synthesis of non-enzymatic antioxidants such as the tripeptide glutathione (GSH) and vitamins C (ascorbate) and E (α -tocopherol), as well as in antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), ascorbate peroxidase (APX) and catalases [160]. However, the degree of enhancement of the antioxidative response in plants is limited by the relatively poor capacity for induction of the enzymes of the defense system [161]. Therefore, to evaluate if tolerance to oxidative stress could be increased, many studies have characterized transgenic plants expressing genes encoding different oxygen radical scavenging enzymes (Table 5). These studies have been extensively reviewed elsewhere [62,161–166].

SOD are enzymes that react with superoxide radicals at almost diffusion-limited rates to produce hydrogen peroxide [167]. Although there has been some inconsistencies in the phenotypes of transgenic plants expressing SOD, the overall observation is that elevated levels of SOD in a variety of cellular compartments can lead to a protection from oxidative stresses caused by the herbicide methyl viologen (MV), high light intensity and low and freezing temperatures [163–165]. The conflicting results in these studies have been attributed to the sensitivity of Cu/Zn SOD to peroxide compared to Mn SOD, the suborganellar association of Cu/Zn SOD with PSI, and possible induction of an antioxidative response by the peroxide produced [161,163–165]. Preliminary studies with transgenic plants that overexpress APX or GR show that they had reduced damage following either MV or photooxidative treatment [164,165]. Furthermore, the expression of a combination of antioxidant enzymes, GR and SOD, in transgenic tobacco plants was shown to have synergistic effects on stress tolerance [168]. Further characterization of transgenic plants overexpressing GR [169] and different forms of SOD [170–173] have been reported. The justification of such studies was recently demonstrated in yeast by showing the importance of the cytoplasmic SOD for resistance to freeze-thaw stress [174]. The overexpression of bacterial GR in the chloroplasts of *L. esculentum* did not lower its chilling sensitivity [169] while transgenic maize plants that overproduced Fe SOD or MnSOD in chloroplasts displayed better growth rates during chilling and enhanced tolerance to MV [171,172,175]. Field trials of two elite alfalfa plants transformed with either a Mn SOD targeted to the mitochondrion or the chloroplast show that, after one winter, most transgenic plants had higher survival rates and herbage yield than control plants [173]. This extends the previous observation that overexpression of Mn SOD in transgenic alfalfa plants often improves the winter survival of this crop [176,177].

Recent studies have also evaluated the potential of increasing resistance to oxidative stress by using novel approaches. Mannitol, a compatible solute, was previously shown to act as a hydroxyl radical scavenger *in vitro* [178]. The targeting of mannitol biosynthesis to chloroplasts in transgenic tobacco plants resulted in an increased resistance to MV-induced oxidative stress [133]. Another promising



Table 5. Examples of transgenic plants expressing enzymes involved in the antioxidative response.

Class	Source and Protein	Target organism and Location	Effects on cold tolerance	Refs.
SOD	Petunia	Tobacco (chloroplast)	Not tested	191,192
	Cu/Zn SOD (chloroplastic)	Tomato (chloroplast)	Yes	
	Pea	Tobacco	Not tested	193
	Cu/Zn SOD (chloroplastic)	(cytoplasm and chloroplast)		
	Tomato	Potato	Not tested	194
	Cu/Zn SOD	(cytoplasm and chloroplast)		
	Pea	Tobacco	Yes	195,196
	Cu/Zn SOD	(chloroplast)		
	Tobacco	Alfalfa	Yes	173,176
	Mn SOD	(mitochondria and chloroplast)		177
	Tobacco	Tobacco	Not tested	197-199
	Mn SOD	(mitochondria and chloroplast)		
	Tobacco	Maize	Yes	171,175
	Mn SOD	(chloroplast)		
<i>Arabidopsis</i>	Fe SOD	Tobacco (chloroplast)	Yes	200
	<i>Arabidopsis</i>	Poplar	No	170
	Fe SOD	(chloroplast)		
<i>Arabidopsis</i>	Fe SOD	Maize (chloroplast)	Not tested	172
GR	<i>E. coli</i>	Tobacco	Not tested	168,
	GR	(cytoplasm and chloroplast)		204,205
	<i>E. coli</i>	Poplar	Not tested	201-203,208
	GR and GS	(chloroplast)		
	Pea	Tobacco	Not tested	206,207
γ -ECS	GR	(cytoplasm and chloroplast)		
	<i>E. coli</i>	Poplar	Not tested	209-211
	γ -ECS	(cytoplasm and chloroplast)		
	<i>E. coli</i>	Tobacco	Not tested	190
OTHERS	γ -ECS	(chloroplast)		
	Pea	Tobacco	Not tested	212
	APX	(chloroplast)		
	<i>E. coli</i>	Tobacco	Not tested	182
	Catalase	(chloroplast)		
	<i>E. coli</i>	Tobacco	Yes	181
	GST/GPX			
	Tobacco	Tobacco	Not tested	185
AOX				
<i>B. juncea</i>	Tobacco	Not tested	187	
	Glyoxalase I			

approach is to use enzymes with both glutathione S-transferase and glutathione peroxidase activities (GST/GPX) that are believed to play an important role in the scavenging of the toxic by-products of lipid peroxidation in plants [179,180]. The expression of a cDNA encoding a GST/GPX enzyme conferred in young



transgenic tobacco seedlings a growth advantage during chilling and salt stress [181]. However, this advantage was transient because after 2 to 3 weeks no differences were discernable. Another group has characterized the overexpression of a bacterial catalase in chloroplasts of tobacco [182]. Photosynthesis of transgenic plants was more tolerant to high irradiance under drought conditions compared to wild-type plants. However, the severity of the stress resulted in the complete inactivation of endogenous ascorbate peroxidase in transgenic and wild-type plants. These results suggest that under such conditions qualitative modifications of the antioxidative machinery may be more important than quantitative modifications. Another enzyme that has been proposed to function in plants by limiting formation of reactive oxygen species is alternative oxidase (AOX) localized in mitochondria [183,184]. Transgenic cultured tobacco cells with increased levels of AOX had lower levels of formation of reactive oxygen species in mitochondria [185]. Limiting oxidative stress in mitochondria may find applications because recent work with maize [186] has shown that chilling involves other subcellular organelles in the production of peroxide such as the mitochondria. Another group has studied the potential of overexpressing an enzyme of the glyoxalase system in transgenic tobacco plants [187]. The glyoxalase system is involved in the protection against methylglyoxal toxicity, generated at low levels spontaneously or enzymatically from glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [188,189]. Glyoxalase I, the enzyme used in their study [187], catalyses the formation of S-D-lactoylglutathione from the hemithioacetal formed non-enzymatically from methylglyoxal and reduced GSH [188]. Glyoxalase II catalyses the hydrolysis of the product to D-lactic acid and regenerates the reduced GSH. Transgenic plants overexpressing glyoxalase I showed a significant improvement in tolerance to methylglyoxal and high salt, as tested in a detached leaf disc senescence assay [187]. These results suggest that a potential advantage of overexpressing enzymes of the glyoxalase system would be in reducing the level of methylglyoxal under stress conditions and enabling regeneration of GSH. Another approach that was attempted is to engineer an increased GSH biosynthesis capacity [190], a major non-enzymatic antioxidant that is perceived to be particularly important in protecting chloroplasts from oxidative damage. Increased GSH biosynthetic capacity in the chloroplasts of transgenic tobacco plants resulted in greatly enhanced oxidative stress, which was manifested as light intensity-dependent chlorosis or necrosis. Because foliar pools of both GSH and γ -glutamylcysteine (the immediate precursor to GSH) were altered, it was proposed that transgenic plants suffered continuous oxidative damage caused by a failure or the redox-sensing process in the chloroplast [190].

Studies in this field have demonstrated that some components of the antioxidative response show a potential in protecting plants against oxidative stress. However, further knowledge is needed on the level of oxidative stress that cellular compartments experience under different abiotic stresses. Such information would allow the development of strategies to compensate for the different responses observed in various species or under different abiotic stresses. Moreover, the





increased expression of a single antioxidative response component may not have a drastic effect on the tolerance to a particular abiotic stress but may still have an impact on biomass production during a growing season. A case that illustrates this point is the study of transgenic alfalfa plants expressing MnSOD in the chloroplast or mitochondria [173]. This study also showed that in some independent transgenic plants, winter survival and yield actually decreased and that the transformation event had a greater influence on survival and yield than the subcellular site of SOD targeting. These observations stress the importance that a reliable evaluation of a transgene's potential in alleviating antioxidative or other stresses may require the analysis of many independent transgenic plants to detect a tendency. Furthermore, this study did not reveal any differences in cellular viability during freezing between transgenic and control plants. Therefore, field survival of transgenic plants may be mediated by the beneficial effect of increased SOD activity under other field stress conditions such as ice encasement, flooding, or long-term freezing [173]. The use of various transgenic plants is indispensable to evaluate the potential of components of the antioxidant response system for increased field survival and productivity during abiotic stresses.

Global regulators (Master switch)

Increasing evidence provided by genetic and molecular analyses have revealed that the cold-induced increase in FT is regulated by a multisignal transduction network [213,214]. The current working model proposes that cold is first sensed by either the plasma membrane or cytoplasmic and chloroplastic molecular complexes which transduce the signal via a cascade of kinases and phosphatases, leading to the activation of specific transcription factors which in turn activate the transcription of the plethora of COR genes. This differential gene expression is responsible for both the metabolic adjustment of growth at LT and the development of FT. Understanding the regulation of these molecular events is the goal of many laboratories. This knowledge may provide means to produce, by genetic transformation, transgenic plants in which all the genes needed for FT are expressed. Groundbreaking results have been obtained thus far by identifying and overexpressing two signal transduction molecular targets, a transcription factor and a kinase.

The recent cloning of the transcription factors CBF1 and DREB1A by the groups led by Michael Thomashow and Kazuo Shinozaki marked an important milestone in the field of molecular genetics of cold acclimation [215,216]. These proteins and related members bind to the *cis*-acting element CCGAC present in the promoter region of COR genes [215–217]. The genes encoding these proteins were expressed in transgenic *Arabidopsis* plants. The results were striking: not only were COR genes expressed at higher levels, but most importantly, the plants showed significant increases in FT [216,218,219]. These results undoubtedly represent the most significant advance in the field of FT improvement, as was outlined in some recent reviews [220,221]. It remains to be determined, however, if CBF/DREB homologs exist in other species particularly in wheat and rye, the two cultivated crops showing





the best FT. Although the molecular components involved in regulating LT gene expression in these species have yet to be identified, recent studies with cereals have revealed the chromosome that has a major positive impact on cold tolerance and gene expression [46]. Studies with the Cheyenne/Chinese Spring chromosome substitution series have shown that chromosome 5A positively regulates the expression of at least four COR gene families correlated with tolerance. This chromosome was also the one most frequently found to have the greatest effect on tolerance in wheat and barley. These findings suggest that chromosome 5A contains gene(s) that constitute the 'master switch' governing the expression of COR genes in cereals (Fig. 1). While the exact nature and number of genes that compose the master switch on chromosome 5 are not known, these results suggest that more realistic transformation strategies could also be used in cereals for the production of more tolerant crops.

In wheat, as many as 15 out of 21 chromosomes have been shown to influence cold tolerance [222]. Thus, it is unlikely that single regulators like CBF/DREB in *Arabidopsis* or the master switch in cereals would be the sole determinants of COR gene expression, especially since so many genes show an increased expression at LT [8,223]. In addition, several genes possibly involved in FT (*sfr*) in *Arabidopsis* remain to be identified or cloned [224], and it is likely that some of them will be cold inducible but that their promoter region will lack the CCGAC element bound by the CBF/DREB proteins. An example is the *WCS19* gene from wheat, which is cold inducible but shows no CCGAC element in its promoter region [225, Ouellet and Sarhan, unpublished results]. It is therefore likely that other transcription factors involved in the activation of COR genes exist and must be identified so as to increase the tools at our disposal for elaborating strategies for engineering cold tolerance.

Another promising approach for the improvement of stress tolerance in plants was recently shown by the transformation of yeast and tobacco cells with *At-DBF2*, a serine/threonine protein kinase [226]. This gene was isolated from *Arabidopsis* and is the homologue of the yeast *Dbf2* gene, which encodes a protein kinase present in the multisubunit CCR4 general transcriptional complex. *At-DBF2* was isolated by screening for osmotolerance in yeast, after transformation with an *Arabidopsis* cDNA library prepared from siliques. The rationale behind the screen was that siliques contain the maturing seeds where is triggered a developmental program that will lead to the acquisition of desiccation (hence osmotic) tolerance. When *At-DBF2* or *DBF2* were overexpressed in yeast, the transformants showed increased tolerance to osmotic, salt, cold and heat stresses. When the tobacco BY2 cells were transformed with *At-DBF2*, they showed increased tolerance to salt, osmotic, cold and heat stresses, but not to oxidative stress. In addition, the authors showed that the expression of the *At-DBF2* gene is upregulated by the same stresses for which the protein confers tolerance. These results provide evidence that *At-DBF2* is involved positively in a general osmotic stress response pathway. Thus, transformation of crops with *At-DBF2* could provide the benefit of improving the tolerance to many stresses, including the major causes of crop losses like drought,



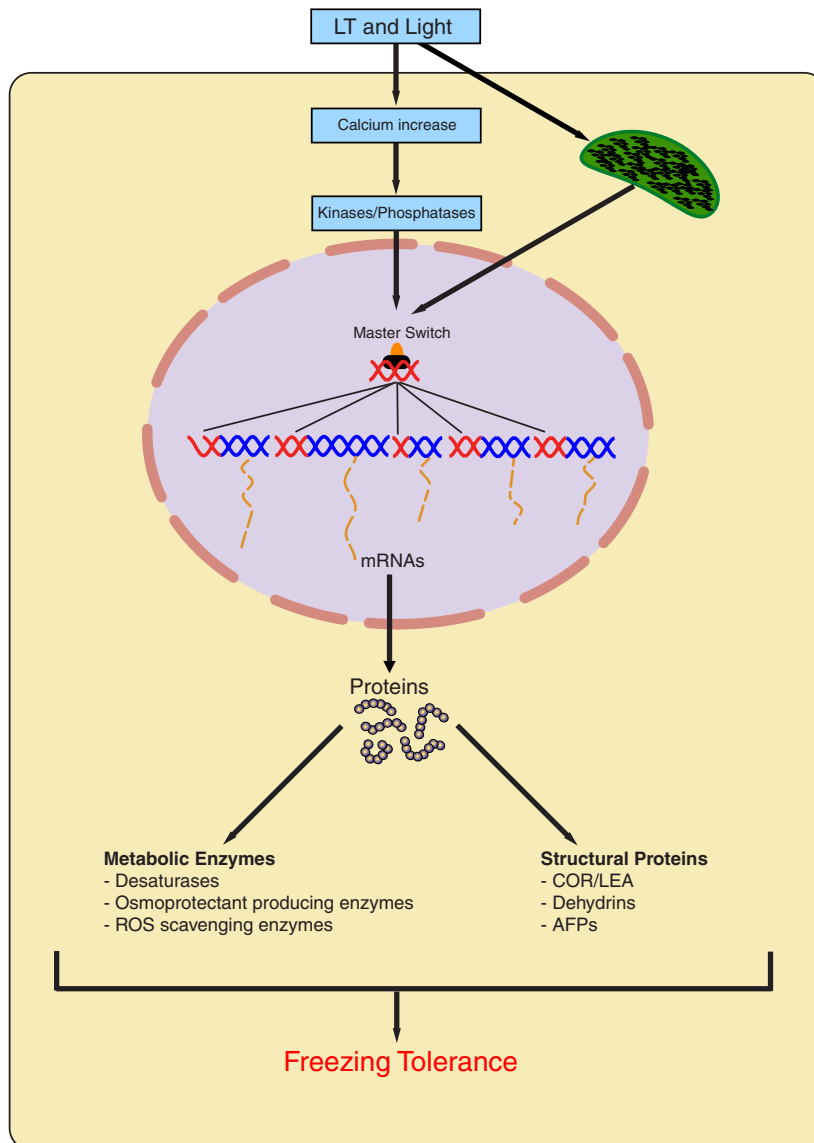


Fig. 1. Master switch hypothesis: Hypothetical model describing how low temperature in association with light regulate the expression of cold responsive genes. Low temperature is first perceived by putative membrane associated receptor(s) and/or chloroplast redox sensing mechanisms, and then transmitted to the nucleus. This triggers the global regulator (master switch). These transcription factors activate the promoters (red helix) of cold-regulated genes (blue helix). The transcribed mRNAs are translated to different proteins that contribute to the development of freezing tolerance.





cold and salinity.

An important factor that must be considered when planning the genetic engineering of plants is the selection of an appropriate promoter to drive the expression of the transgene. Very often, plants modified to overexpress genes of interest have been transformed with constructs bearing the gene under the control of a constitutive promoter. The most widely used promoters are those of the maize ubiquitin gene and of the CaMV35S gene, for monocots and dicots respectively. However, the overexpression of a gene resulting from the use of a constitutive promoter is not necessarily an objective that is desirable in all cases. Indeed, few studies have focused on the physiological consequences related to the constitutive expression of genes that are normally inducible, and thus expressed only when needed. In some cases, chimeric promoter regions have been constructed to combine specific characteristics for expression. Zhu et al. [80] fused an ABA-responsive element from the barley *Hva22* gene, a rice actin 1 minimal promoter, and an *Hva22* intron to obtain a stress-inducible promoter. This element was used to increase the level of Δ^1 -pyrroline-5-carboxylate synthetase in transgenic rice plants, which lead to a drought and salt-inducible increase of the proline content and a concomitant increase in tolerance to those stresses. The activity of the promoter at LT was not reported. Another example of a chimeric promoter is discussed below.

Detrimental effects of constitutive expression of normally tightly regulated genes can occur. Plants transformed to express the DREB1A gene constitutively (from the CaMV35S promoter) showed better FT, but also severe growth retardation [219]. This detrimental effect was not reported for the homologous CBF1 overexpression [218]. When the expression of DREB1A was regulated by the cold-inducible *rd29A* promoter, plants achieved an even higher FT but most importantly, minimal effects on growth were noted [219]. These results provide convincing evidence that inducible expression may represent the best strategy to be used for crop improvement. In this respect, the promoter of the cold-inducible *wcs120* gene from wheat should also prove to be a useful tool for regulation of transgene expression [227].

Another factor to consider is the species specificity of the promoter used. Towards this end, some studies have focused on the identification of promoters that are more efficient in different plants. For example, several constructs bearing, in different combinations, fragments of the CaMV35S promoter, an intron of the bean phaseolin gene, the Ω sequence of TMV, and termination sequences from the CaMV 35S or nopaline synthase genes have been tested in rice (monocot) and tobacco (dicot) [228]. It was shown that the most efficient constructs for rice were not the same as for tobacco, suggesting differences in the specificity of gene expression between monocot and dicot plants. Schenk et al. [229] reported on the activity of a promoter from the sugarcane badnavirus in transient and transgenic expression. They showed that the promoter was active in several monocot and dicot species, to a level similar to that of the maize ubiquitin and CaMV 35S promoters. The viral promoter conferred constitutive expression of the transgene. It could thus be a useful tool for the high level expression of foreign genes in both monocot and dicot transgenic





plants. To our knowledge, only one study reported on the activity of a cold-responsive promoter in different species [227]. The *wcs120* promoter was found to be active and, most of all, cold inducible in both monocot and dicot species in *in vitro* transient assays, whether they are cold tolerant or not. This characteristic makes it a very important tool to exploit for crop improvement of cold tolerance. The use of a broad range, cold-inducible promoter such as that of the *wcs120* gene would allow the expression of genes only when the plant is under cold stress conditions. It would also alleviate the need to develop different constructs for different species. However, the effectiveness of this promoter in different transgenic plants remains to be evaluated.

Molecular markers

Another important application of the knowledge gained from basic studies of COR genes is the development of cold tolerance markers. The expression of several genes such as the *wcs120*, *wcor 410* and *wcs 719* was found to be highly correlated with the capacity of plants and tissues to develop FT [47,230,231]. Using a number of wheat and rye cultivars differing in their acclimation capacity, it was found that the accumulation of these proteins correlates well with the capacity of each cultivar to develop FT. In addition, a near perfect correlation was also observed between the accumulation of the WCS120 protein and the increase in LT_{50} of both winter and spring wheat. These data support the concept that the anti-WCOR410, 120 and 719 antibodies may represent useful tools for breeders to select for FT in cereals. In a similar study, Lim et al. [232] reported that a 25-kDa member of LEA group II proteins could serve as a genetic marker to distinguish between cold-hardy and less hardy genotypes of the woody plant *Rhododendron* [232]. The relative level of this protein within a genotype can also serve as a physiological indicator of FT status under a range of phenological (acclimation) or developmental (age) conditions. The use of molecular markers is easy, rapid, inexpensive and non-destructive compared to invasive, time consuming methods currently used to evaluate the LT_{50} . These antibodies are currently used to assess the segregation of FT in different crosses and to study the inheritance of their respected genes. The use of these molecular markers was instrumental in mapping and regulation studies. Using a combination of Southern and western analyses on the ditelocentric series of Chinese Spring wheat, in which one homologous pair of chromosome arms is missing in each line, several COR genes were mapped to specific chromosome arms [233]. In addition, the chromosome that regulates cold tolerance and gene expression was also identified using these molecular markers. These studies provide the basis of the master switch hypothesis discussed in the previous section.

Potential applications to food products

Freezing is a widely used technique for food preservation during storage and transportation. It reduces spoilage by inhibiting microbial growth and helps in





extending shelf life of a wide variety of foods. However, there are many examples of food stuffs that cannot be frozen without unacceptable changes in quality, particularly the high water containing tissues, such as fruits, vegetables, fish and meats [24]. It is known that extracellular and intracellular ice formation results in undesirable changes in taste and texture due to cellular dehydration, concentration of solutes, and mechanical factors that can collectively lead to destruction of the plasma membrane [234,235]. Ice formation in frozen foods is influenced by both rate of freezing and storage temperature. In foods that are frozen slowly, ice crystals are large and randomly distributed and ice fills the extracellular space causing dehydration of the cell and forcing cells apart. When plant tissues are frozen rapidly, water does not translocate across the plasma membrane and small uniformly distributed ice crystals are formed within the cell. Although foods are usually flash frozen to produce small ice crystals, these ice crystals may grow larger over time through a process known as recrystallization. Recrystallization occurs in frozen foods because temperature gradients form within the products during freezing or thawing, or because temperatures fluctuate during storage or when products are in transit. For example, home frost-free freezer temperatures may rise to near 0°C daily during defrost cycles, resulting in extensive recrystallisation and limiting the length of time that foods can be stored without a dramatic loss of quality. These freeze/thaw cycles cause membrane damage and produce a loss in the liquid-retaining properties of cells, thereby causing meat, vegetable or fruit tissues to lack turgor upon thawing. Furthermore, cellulose dehydration in fruits and vegetables during freezing may account for changes in textural qualities [234]. These changes are usually associated with the degradation of chlorophylls and the oxidation of other pigments that reduce the quality and the nutritional value of thawed fruits and vegetables. Studies of frozen beef have shown that recrystallization increases with higher storage temperature and damages cellular membranes, causing greater protein denaturation and exudation from the tissue during thawing [236]. The recrystallization of ice is also a problem in foods that are eaten while frozen. For example, ice cream stored for long periods develops a gritty texture due to the growth of individual ice crystals. Low-fat frozen dessert formulations often contain more water, thus increasing problems associated with ice recrystallization.

Presently, cryoprotectants such as glycerol, sugars and dimethyl sulfoxide (DMSO) are used to control ice crystal formation in biological tissues. However, these compounds function colligatively and must be present at relatively high concentrations (usually >0.5M) to be effective. At these concentrations, cryoprotectants have adverse effects on the taste, texture and even toxicity of frozen foods. For example, the addition of sugars may reduce the direct injury to fruit caused by ice formation, but these sugars may also prevent reabsorption of water during thawing and actually increase the loss of water and nutrients from the thawed fruit. It is also possible to decrease recrystallization using high molecular weight polymers that act as stabilizers [237–239]. For example, gelatin at concentrations up to 0.5 mg mL⁻¹ reduces the recrystallization of ice during freezing and thawing





[240]. On the other hand, polymers such as xanthan and guar gums may alter crystal growth indirectly by increasing solution viscosity and lowering the nucleation temperature [239]. However, these polymers are also used at relatively high concentrations (0.5% w/w), which may affect the consistency of the final product and prohibit their use as additives in all types of frozen foods.

Antifreeze proteins

To find an alternative to the currently available cryoprotectants, scientists have long considered using substances made by organisms that survive freezing conditions. The first substance, with properties to protect an organism from freezing, was discovered in fish of the southern polar ocean [241]. The characterization of these substances led to the identification of AFPs. Recently, proteins with similar functions were found in cold tolerant plants and insects [19,22]. AFPs are distinct from other modifiers of ice crystal growth known to date because they inhibit the growth of ice noncolligatively, by adsorbing onto the crystal surface and inhibiting the further addition of water molecules [15]. AFPs are effective in modifying crystal growth at concentrations 200 to 500 times lower than solutes that act colligatively [242]. AFPs also inhibit the recrystallization of ice at concentrations as low as 25 µg of protein per liter [243]. At these very low concentrations, AFPs are unlikely to directly affect the taste or consistency of frozen foods. Thus, AFPs are attracting the attention of food technologists interested in controlling the way ice crystals grow in frozen foods. They are considered as prime candidates for food additives and even for incorporation into the genomes of the raw-commodity [244,245].

The use of AFPs in frozen foods has been studied previously using type I AFPs and AFGPs (antifreeze glycoproteins) isolated from fish caught in polar oceans. In studies of frozen meat, Payne et al [246] demonstrated that beef and lamb soaked in solutions containing 0.1 mg AFP or AFGP ml⁻¹ for 4.5 to 6 hr, then frozen to -20°C for 2 to 3 days, had smaller ice crystals than controls when the meat was examined by light and scanning electron microscopy. Moreover, meat treated with AFGPs before freezing and then stored frozen for 8 weeks also exhibited less drip with no detrimental effect on flavour, texture, tenderness, juiciness, and overall quality [247]. Likewise, the flesh of fishes naturally containing AFP exhibits significantly less drip and is juicier in sensory trials compared with flesh of fish that do not produce AFPs [248]. In studies involving frozen desserts that are eaten while frozen, synthetic AFPs similar to type I AFPs from fish have been shown to inhibit ice recrystallization in popsicles and ice cream [249]. The presence of AFPs in these products presumably inhibits ice recrystallization and preserves the smooth, creamy texture of a high quality product.

Although preliminary studies provided good evidence that fish AFPs may be useful additives to frozen foods, the widespread use of these proteins in frozen foods has been limited by both ethical and marketing considerations. First of all, the production of transgenic plants harbouring genes encoding fish AFPs was considered to be unethical because vegetarians may not wish to eat plant products





containing fish proteins. Secondly, the addition of fish AFPs to frozen foods such as ice cream was shown to be unacceptable to consumers, who think the food may taste “fishy”. Rather than abandoning the idea of using AFPs in frozen foods, food producers have sought an alternate source of the proteins. The recent discovery of AFPs in plants provided this alternative, and renewed the interest of using AFPs to reduce freezing damages and to improve the overall quality of frozen plant products. The most studied AFPs from plants are those from rye [19]. These AFPs accumulate only during cold acclimation of cereals and were found to be similar to pathogenesis related (PR) proteins such as endoglucanases, endochitinases and thaumatin. The dual activities of these proteins could serve two functions as additives in frozen foods by inhibiting the recrystallization of ice and by reducing microbial activity within the food products [30]. This will undoubtedly have a tremendous impact on the quality and shelf life of both frozen foods and fresh produce that needs to be stored at low positive temperatures. The use of plant AFPs as food additives is very attractive due to its potentially easier acceptance by consumers, its relatively low production cost and its presence in renewable resources. There is a strong possibility that we will see plant AFPs as a new class of frozen food additives in the near future.

Dehydrins

The other group of plant proteins that may become the target of new biotechnological applications in the food and cosmetic industries is the dehydrin group. This protein family is synonymous with LEA group II proteins or the D-11 family. Dehydrins are the most conspicuous of soluble proteins induced by dehydrative stresses. A literature survey revealed that they have been detected in well over 100 independent studies of drought stress, cold acclimation, salinity stress, and embryo development [45,250]. They have a broad size range, show no compelling similarity in amino acid sequence to any known enzyme or protein of known function and accumulate to levels in excess of 1% of total soluble proteins. Dehydrins can be classified by their content in different amino acid segments named K, S and Y, which are usually repeated several times. The K segments are predicted to form amphipathic α -helices. Dehydrins are also peculiar by their high hydrophilicity and boiling solubility which renders their purification very easy. Several lines of evidence are consistent with a role of dehydrins in membrane interactions including immunolocalization, plasma membrane association and adoption of an α -helical structure by several cereal dehydrins in the presence of SDS [47,251]. It was also shown that dehydrins could protect enzymes from freezing damage *in vitro*. For example, the WCS120 protein at 0.2 μ M has the same efficiency as 250 mM sucrose in *in vitro* cryoprotection assays of lactate dehydrogenase, meaning that WCS120 is over a million-fold more efficient than sucrose on a molar basis [56]. Similar results have been obtained with other dehydrins [252,253]. It was proposed that dehydrins, acting synergistically with compatible solutes, may replace water for the “solvation” of membranes and proteins, thus alleviating the dehydration-induced membrane destabilization





and protein coagulation [45]. Second, these proteins may reduce the incidence of events leading to the formation of non-bilayer structures between membranes. This can be achieved by the proteins abundant association with different lipid species of membranes, thus causing a steric hindrance to the interaction between membrane bilayers or preventing lipid demixing that occurs during dehydration. Finally, dehydrins may also function in counteracting the irreversibly damaging effects of the increasing ionic concentration during dehydration. High ionic concentration decreases interbilayer repulsion due to charge screening and leads to the interaction between bilayers and the induction of non-bilayer structures.

As plants are the only multicellular organisms able to tolerate freezing and dehydration stresses (plant seeds lose 80% of their water content during maturation and remain viable) and because these processes are highly correlated with dehydrin accumulation, these specific plant proteins may find very innovative applications in the future. For example, as a complement to AFP additives, dehydrins may further protect frozen foods against storage shrink and textural degradation due to ice recrystallization during temperature fluctuations, and by protecting membranes prevent water loss and ion leakage. As another example, dehydrins may be used in ointment against frostbite because ointments have to date been shown to give at best only a negligible protection against frostbite of the face and ears [254,255]. Frostbite involves exactly the same phenomenon as plant cell freezing: ice formation, increasing osmolality of the extracellular fluid and intracellular dehydration [256,257].

Applications in medicine

Freeze-associated proteins have also been considered for potential applications in medicine. So far, AFPs have been used in studies on cryodestruction of undesirable cells such as cancer cells [258] and in the protection of vital molecules, tissues and organs [259].

Cryodestruction of tumour cells

In the case of cryosurgery, cooled probes inserted into tumorigenic tissue with intra-operative monitoring produce local freezing, to destroy the unwanted tissues without affecting the surrounding healthy ones. However, recent ultrasound studies have shown that the volume of tissue which later became necrotic is only 70 to 80% of the size of the frozen volume [260,261]. This can lead to complications, such as recurrence of cancer in these peripheral regions. The use of concentrated AFPs was found to enhance the destruction of cells or tissues frozen *in vitro* under these fast and highly hypothermic conditions [262,263]. These encouraging results led Pham et al. [264] to test AFPs *in vivo*. In a study with nude mice containing subcutaneous metastatic prostate tumours, they showed that the preoperative injection of a 10 mg/ml AFP solution into the tumour prior to freezing enhanced the destruction of cancerous cells under thermal conditions which normally yield





cell survival. At concentrations higher than about 5 mg/ml of AFP, independently of the thermal conditions, the ice crystals that form develop a microsize highly destructive spicular shape. These findings suggest that the adjunctive use of AFPs in cryosurgery could reduce cancerous cell survival in the periphery of the cryogenic lesions and thus prevent or reduce the complication from undesirable tissues that survive freezing. It is important, however, to conduct clinical trials to evaluate the potential and efficiency of this technique in humans.

Cryoprotection

The ability to cryopreserve human organs and tissues would have an important impact in medicine, particularly in organ transplantation and reproduction [259]. Up till now, chemical substances, like dimethyl sulfoxide (DMSO), glycerol and sucrose, are used as cryoprotective agents. Because these substances are used at high concentrations that may cause toxicity and side effects, AFPs, especially from plants, could be an interesting alternative. These proteins are natural products and have proven effects in organisms that survive freezing. Prospects for cryopreservation are numerous, ranging from oocytes and platelets to whole organs and bodies.

Assisted reproduction relies on embryo cryopreservation which involves many ethical considerations. Oocyte cryopreservation may provide a good alternative and allow the banking of supernumerary oocytes following superovulation techniques [265,266]. Although promising results have been obtained with cryopreserved oocytes [267,268], variability has precluded routine application of the technology in clinical practice [269]. Exposure to cryoprotective agents and LT has been reported to disrupt the oocyte cytoskeleton, and harden zona pellucida, rendering the oocyte impenetrable to sperm. To circumvent this problem, the potential of using AFPs to protect oocytes during the freezing process has been evaluated. Fish AFGPs have been shown to protect immature pig oocytes during hypothermia [270], and cryopreservation [271–274]. Further studies will indicate if they can be used routinely for the cryopreservation of oocytes.

Another potential application of AFPs is the cryopreservation of human platelets. Stored platelets can be kept only for five days at room temperature before microbial contamination and progressive aging induced activation [275]. Cooling below 5°C increases ion leakage and platelet activation by calcium entry. A preliminary study has shown that AFGP can prevent ion leakage and thereby improved the survival of platelets at temperatures as low as 4°C [275]. Certainly, more research is required to determine the most appropriate AFP that is needed to increase the membrane stability of the platelets and hence storage time. The recent identification of many plant stress associated proteins may provide additional tools that can be used to tackle this problem. The development of an efficient technique to prolong the storage of human platelets would have enormous health benefits. Human platelets are essential for blood clotting, and transfusions of platelets are routinely given to burn victims, chemotherapy patients and patients afflicted with thrombocytopenia,





a side effect of many invasive surgeries such as cardiopulmonary bypass or bone marrow transplantation [275].

Cryopreservation of complex tissues and organs is much more difficult than single cell preservation. These consist of different cell types, whose membranes are in physical contact. Since the cooling rate and the lowest permissive temperature seem to be specific for each cell type, the challenge to cryopreserve whole tissues and organs is far from being resolved [276,277]. It may seem obvious that the solution is to avoid or prevent ice formation and may be achieved by vitrification [111]. However, the highly concentrated vitrification solution that is needed has undesirable toxic effects. To circumvent this problem, Rubinsky et al. [278] added AFPs to the vitrification solution used to preserve mammalian livers. After six hours at -3°C , the liver was still able of bile production in a culture dish when thawed to 37°C . Microscopic analysis of tissue slices also showed that the damage to the structural integrity of the liver was relatively small and that the hepatocytes appeared mostly intact. These results led the authors to conclude that AFPs have a protective effect on the whole liver during freezing.

Although these results are encouraging for the future of cryobiology, further research must be pursued to study also the cryopreservation of other organs. For example, a heart stays viable for only four to eight hours after it is removed from its donor [279]. Longer preservation times may save thousands of lives each week. Studies with fish AFP have shown that they can sometimes exacerbate the damage during freezing of rat cardiac explants [280] and cardiomyocytes [281]. If the negative effect of these AFPs is of structural nature, the problem may be circumvented by using plant AFPs or other freezing associated proteins like dehydrins which are completely different in structure and may have a complementary function [19]. In a near future, the characterization of cold and freezing associated proteins from organisms that survive freezing temperatures, particularly plants that survive temperatures as low as -50°C , may lead to novel applications. These include near freezing point long-term surgeries, frostbite protection, organ and platelet preservation and whole body cryostorage for deep space travel.

Acknowledgments

Research in our laboratory has been supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche of the Ministère de l'Éducation du Québec.

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