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Cryoprotectin: a plant lipid-transfer protein homologue that stabilizes membranes during freezing

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Plants from temperate and cold climates are able to increase their freezing tolerance during exposure to low non-freezing temperatures. It has been shown that several genes are induced in a coordinated manner during this process of cold acclimation. The functional role of most of the corresponding cold-regulated proteins is not yet known. We summarize our knowledge of those cold-regulated proteins that are able to stabilize membranes during a freeze–thaw cycle. Special emphasis is placed on cryoprotectin, a lipid-transfer protein homologue that was isolated from cold-acclimated cabbage leaves and that protects isolated chloroplast thylakoid membranes from freeze–thaw damage.

Keywords: cold acclimation; cold-regulated (COR) proteins; cryoprotection; freezing tolerance; plant membranes

1. INTRODUCTION

Plant freezing tolerance is one of the major determinants of agricultural productivity in areas where freezing occurs, even sporadically. In addition, in many areas the natural distribution of plant species is determined by their ability to survive freezing events. Therefore, the mechanisms underlying plant freezing tolerance and freezing damage have been the subject of scientific research for many years (for comprehensive reviews see Levitt 1980; Steponkus 1984). Especially in plant species from temperate climates, winter survival is greatly influenced by the ability of plants to cold acclimate, i.e. to increase their freezing tolerance during a period of exposure to low, non-freezing temperatures in the autumn. In the laboratory, cold acclimation can be induced by transferring plants for several days or weeks, to a constant growth temperature between 5 and 0 °C.

In spite of intensive research, the molecular basis of cold acclimation remains largely unknown (Xin & Browse 2000). It has been shown that cold acclimation is accompanied by distinct changes in gene expression (see Guy 1990; Hughes & Dunn 1996; Thomashow 1998, 1999, for reviews). The induction of a subset of these genes in transgenic *Arabidopsis thaliana* plants, constitutively expressing cold-regulated transcription factors, proved the vital role of at least some of the proteins encoded by cold-induced genes in plant freezing tolerance (Jaglo-Ottosen *et al.* 1998; Kasuga *et al.* 1999; Gilmour *et al.* 2000).

Information about the actual protective function of such proteins, however, is rare. There are reports about the ability of cold-induced 'late embryogenesis abundant' proteins (Kazuoka & Oeda 1994; Wisniewski *et al.* 1999) and the cold-induced *Arabidopsis* protein COR15 (Lin & Thomashow 1992*a*) to stabilize lactate dehydrogenase from animal muscle against freeze-induced inactivation. However, as it has been found that, in general, plant survival during freezing is limited by the stability of cellular membranes and not soluble proteins (Steponkus 1984; Xin & Browse 2000), the relevance of such findings for plant freezing tolerance seems at least doubtful.

Consequently, research into the functional components of plant cold acclimation and freezing tolerance has concentrated on identifying cryoprotective plant substances that are able to stabilize membranes under stress conditions (for reviews see Hincha *et al.* 1996; Oliver *et al.* 2002). The plasma membrane and chloroplast thylakoid membranes are the best characterized model systems for this type of investigation. We have used thylakoids from spinach to identify and characterize cryoprotective proteins.

Plasma membrane and thylakoids are damaged during a freeze–thaw cycle, both *in vivo* and *in vitro* (see for reviews Steponkus 1984; Krause *et al.* 1988; Hincha & Schmitt 1992*a*). Thylakoids lose their ability for electron transport and photophosphorylation. This is accompanied by the loss of internal soluble proteins and osmotically active solutes, leading to vesicle collapse (see for reviews Hincha & Schmitt 1992*b*; Hincha *et al.* 1996). Freeze–thaw damage to thylakoids can be quantified as the release of the soluble internal electron transport protein plastocyanin, or as a reduction in thylakoid volume. Damage *in vivo* is deferred to lower temperatures during cold acclimation, in the case of the plasma membrane (figure 1; electrolyte leakage) and thylakoids (figure 1; plastocyanin leakage).

This shows that thylakoids are a relevant model system to study the molecular basis of freezing tolerance *in vitro*. We will give a short overview of the cryoprotective plant proteins currently identified in different systems, with a focus on proteins that stabilize thylakoids *in vitro*.

One contribution of 15 to a Discussion Meeting Issue 'Coping with the cold: the molecular and structural biology of cold stress survivors'.

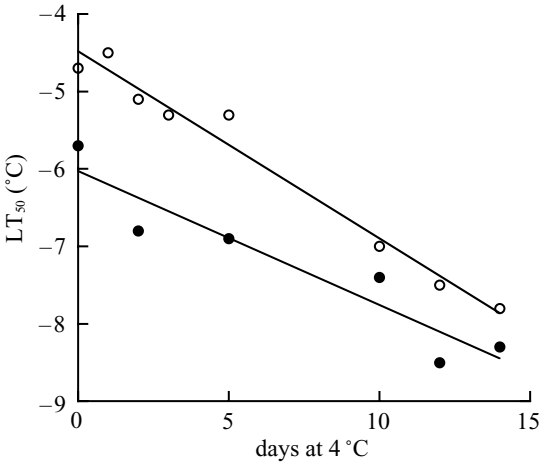

Figure 1. Cold acclimation increases freezing tolerance of plant leaf cells. The damage to spinach leaves was measured as the damage to the plasma membrane (electrolyte leakage) or the chloroplast thylakoids (plastocyanin leakage) after leaves had been exposed to freeze–thaw cycles through different minimum temperatures. Electrolyte (Hincha 1994) and plastocyanin (Hincha *et al.* 1987) leakage were determined as described in previous publications. From the resulting curves, the temperature at which 50% of the leaf electrolytes or plastocyanin had been released (LT_{50}) was determined. Plants were grown under non-acclimating conditions (12 L : 12 D; 25 °C : 15 °C) and were transferred to cold-acclimating conditions (4 °C constant temperature) at day 0. Black circles represent plastocyanin leakage ($r = 0.94$) and open circles represent electrolyte leakage ($r = 0.99$).

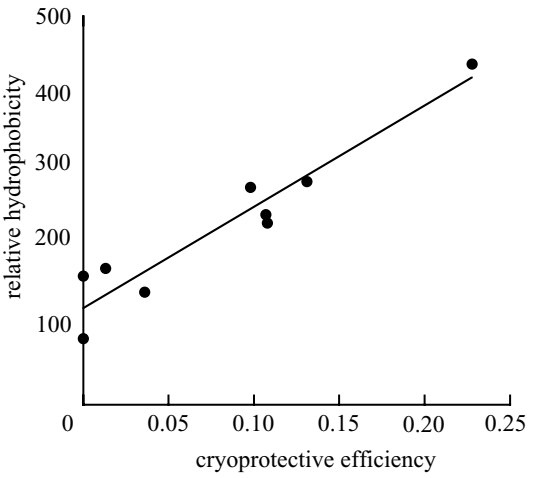

Figure 2. Correlation between the cryoprotective efficiency of different lectins and their relative hydrophobicity. The cryoprotective efficiency is the negative slope of the lines that were obtained by plotting freeze–thaw damage as a function of protein concentration (Hincha *et al.* 1993). The relative hydrophobicity of the lectins was determined in titration experiments using the hydrophobicity-sensitive fluorescent dye 2-*p*-toluidinylnaphthalene-6-sulphonic acid. ($r = 0.95$.) (Redrawn from data published in Hincha *et al.* 1993, 1997*c*.)

2. CRYOPROTECTIVE PLANT PROTEINS

(a) *COR15*

The involvement of the cold-regulated protein COR15am from *Arabidopsis thaliana* in stabilizing the plasma membrane and chloroplasts of leaf cells has been established in transgenic *A. thaliana* plants, which constitutively express the corresponding gene (Artus *et al.* 1996; Steponkus *et al.* 1998). COR15am is a soluble protein that is localized in the chloroplast stroma (Lin & Thomashow 1992*b*). It is thought to stabilize the inner chloroplast-envelope membrane against fusion with the plasma membrane under conditions of freeze-induced dehydration. The protein is able to stabilize the bilayer phase of model membranes containing non-bilayer lipids such as phosphatidylethanolamine or MGDG (Steponkus *et al.* 1998), a glycolipid that is present in high concentrations in thylakoids and the inner chloroplast-envelope membrane (Uemura & Steponkus 1997). The mechanism by which this is achieved, and whether it involves binding of COR15am to membranes, is not yet known (Thomashow 1999). It is, however, clear that COR15am does not protect isolated thylakoids during freezing, which also contain a large fraction of MGDG (Artus *et al.* 1996).

(b) *Osmotin-like protein*

A protein from bittersweet nightshade (*Solanum dulcamara*) has recently been shown to stabilize isolated protoplasts from kale (*Brassica oleracea*) during a freeze–thaw cycle (Newton & Duman 2000). Protein sequencing revealed that it belongs to the osmotin family of pathogenesis-related proteins. There is currently no information about the physical mechanism of protection or a possible role of this protein in cold acclimation and freezing tolerance *in vivo*.

(c) *β-1,3-glucanase*

Like osmotin, glucanases belong to the group of pathogenesis-related proteins. A class I β-1,3-glucanase from tobacco has cryoprotective activity for thylakoid membranes, and immunologically related proteins are accumulated in cabbage and spinach during cold acclimation under natural conditions (Hincha *et al.* 1997*b*). In contrast to other cold-regulated proteins, the glucanases are not induced at 4 °C but probably require lower temperatures and/or freezing. The mode of action of the β-1,3-glucanase has not been investigated in detail. It has, however, been shown that the protein is able to reduce solute loading of thylakoids during freezing and the resulting osmotic rupture of the membrane vesicles during thawing (Hincha *et al.* 1997*b*).

(d) *Lectins as cryoprotective model proteins*

Lectins are sugar-binding proteins, which are classified according to their specificity for different monosaccharides. Since thylakoids contain galactolipids (Webb & Green 1991), galactose-specific lectins were investigated for their potential membrane stabilizing properties. They were found to bind to thylakoid membranes, and some exert a cryoprotective effect (Hincha *et al.* 1993, 1997*c*). Similar to the case of the β-1,3-glucanase, the cryoprotective lectins reduce solute loading of thylakoids during freezing (Hincha *et al.* 1993). Cryoprotection requires binding to digalactolipid headgroups, but although all investigated lectins bind to thylakoids, the magnitude of cryoprotection is highly variable between the proteins. We found that the cryoprotective efficiency of the different lectins is linearly related to their relative hydrophobicity (figure 2). A proposed mechanism of cryoprotection

Table 1. Cryoprotective activity in plants is cold induced.
(Crude cryoprotectin fractions were isolated from cabbage or spinach leaves of plants grown either under non-acclimating conditions in a greenhouse, or after cold acclimation under natural conditions during winter. Cryoprotection was determined with thylakoids isolated from the leaves of non-acclimated spinach plants using the plastocyanin leakage assay (compare text for details). Data were taken from Hincha et al. (1990).)

protein source	concentration ($\mu g\ ml^{-1}$)	cryoprotection (%)
cabbage, cold acclimated	200	75
cabbage, non-acclimated	200	8
spinach, cold acclimated	230	63
spinach, non-acclimated	230	0
bovine serum albumin	500	10

involves binding of the lectins to the galactolipid headgroups, and hydrophobic interactions with membrane lipids, which leads to reduced fluidity at the membrane surface, resulting in reduced solute permeability (Hincha *et al.* 1997*a*). This reduces solute diffusion during freezing and therefore osmotic membrane rupture during thawing, which was measured as reduced plastocyanin release.

Most lectins that have been assayed are commercially available seed lectins (Hincha *et al.* 1993), which obviously cannot play a role in leaf frost hardiness. However, two cryoprotective lectins from mistletoe (*Viscum album* L.) leaves showed increased concentrations during the winter months, when leaf frost hardiness is high, and low concentrations in the summer, when the leaves are susceptible to freezing damage (Hincha *et al.* 1997*c*). Another mistletoe leaf lectin, which is not cryoprotective *in vitro*, shows no seasonal variation in concentration. Therefore, in addition to their usefulness as a model system for protective protein–membrane interactions lectins may, in some cases, also play a role in plant freezing tolerance and cold acclimation.

(e) *Cryoprotectin*

Plant leaf proteins that protect isolated thylakoid membranes from freeze–thaw damage were first reported by Heber and co-workers (Heber & Kempfle 1970; Volger & Heber 1975). It was later demonstrated that this cryoprotective activity is cold induced in both cabbage and spinach leaves, and is not due to non-specific effects caused by the presence of soluble proteins (table 1). In addition, treatment of the extracts with a protease confirmed that the cryoprotective activity is indeed due to the presence of protein and not to the presence of contaminants such as sugars (Hincha *et al.* 1990).

Protection of thylakoid membranes during a freeze–thaw cycle could be shown as both retention of plastocyanin and the preservation of thylakoid volume (Hincha & Schmitt 1992*b*). Similar to the cases of β-1,3-glucanase and cryoprotective lectins (see above), protection is achieved, at least in part, through a reduction in solute loading during freezing. Protein fractions that completely suppress plastocyanin leakage during freezing result in a volumetric behaviour that is indistinguishable from non-frozen controls (figure 3). A detailed analysis of the physical mechanism of the cryoprotective activity of cryoprotectin is currently underway.

We have purified a cryoprotective protein from cold-acclimated cabbage leaves to electrophoretical homogeneity and called this protein cryoprotectin (Sieg *et al.* 1996). Although this purified fraction showed only one band in a silver-stained SDS–PAGE gel, it contained more than one homologous polypeptide, as revealed by protein sequencing (Hincha *et al.* 2001). All peptides belong to the family of WAX9 proteins (Pyee *et al.* 1994; Pyee & Kolattukudy 1995), which show a high degree of sequence homology to plant LTPs (figure 4). WAX9 E, purified from the wax layer of cabbage leaves, has lipid transfer activity between liposomal membranes but no cryoprotective activity. In contrast, cryoprotectin has cryoprotective activity but no lipid transfer activity. This led us to the hypothesis that cryoprotectin may be a specialized LTP homologue that protects membranes during freezing, due to a binding mechanism that would preclude lipid transfer activity (Hincha *et al.* 2001). Stable binding of cryoprotectin to thylakoid membranes could be shown in recent experiments (H. A. M. Sror, G. Tischendorf, J. M. Schmitt, D. K. Hincha, unpublished data). Unfortunately, we have so far not been able to determine which of the four remaining WAX9 proteins is responsible for the cryoprotective activity. Functional expression of other LTP genes in bacteria or yeast has been performed successfully (Masuta *et al.* 1992; Klein *et al.* 1998; Lullien-Pellerin *et al.* 1999) and will be used to identify cryoprotective WAX9 proteins.

In general, LTPs are characterized by their small molecular mass (between 7 and 10 kDa) and eight conserved Cys residues (figure 4) that form four disulphide bridges in a highly conserved pattern (Douliez *et al.* 2001). LTPs, including cryoprotectin, are also characterized by their high stability at high temperatures and in the presence of chemical denaturants (Watanabe & Yamada 1986; Terras *et al.* 1992; Cammue *et al.* 1995; Hincha *et al.* 2001; Lindorff-Larsen & Winther 2001). This has been attributed to the stabilizing influence of four disulphide bridges in such small proteins (Lindorff-Larsen & Winther 2001), although it should be noted that the influence of disulphide bridges on protein stability is far from straightforward (Zavodsky *et al.* 2001).

LTPs have been identified in several plant species and are usually present in gene families with several closely related members (Kader 1996). In *A. thaliana*, for instance, a genome-wide search revealed the presence of at least 15 LTP genes (Arondel *et al.* 2000). Some of these genes are expressed in specific tissues (Clark & Bohnert 1999). The physiological function of LTP in plants is not clear. Because some LTPs are excreted into the medium from cultured plant cells (Sterk *et al.* 1991), and the LTP WAX9 has been found in the wax layer of cabbage and broccoli leaves (Pyee *et al.* 1994; Hincha *et al.* 2001), it has been proposed that some LTPs may be involved in the transport of cutin molecules from the cell to their extracellular destination. Some LTPs are thought to play a role in pathogen defence, as they possess antibiotic properties that are induced in plants in response to pathogen attack (Garcia-Olmedo *et al.* 1995). In addition, induction of several LTP genes has been shown to occur due to environmental stresses such as cold (Hughes *et al.*

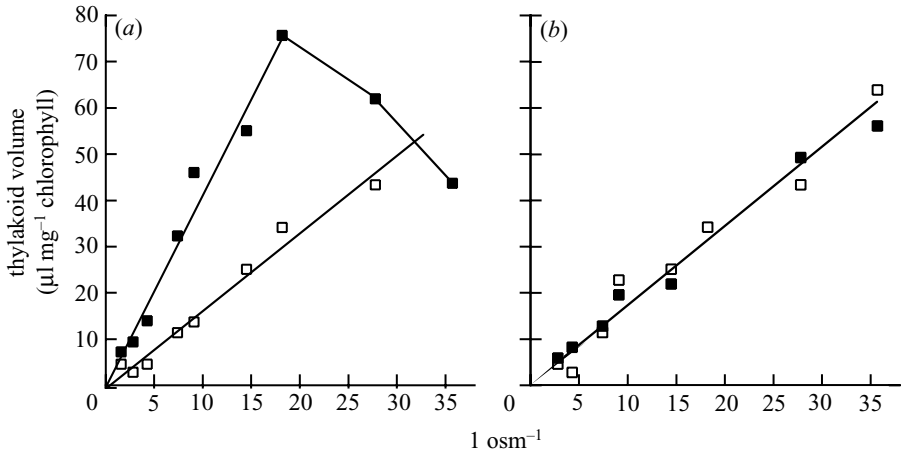

Figure 3. Effects of a freeze–thaw cycle on the volume of isolated thylakoids. Thylakoid volume is plotted as a function of reciprocal osmolality of the sucrose solutions used to suspend the membranes (Boyle–van't Hoff plot; see Bakaltcheva *et al.* 1992; Hincha 1986, for details). Thylakoids were isolated from the leaves of non-acclimated spinach plants. Samples contained 2.5 mM NaCl and sucrose at concentrations between 20 and 500 mM, and were incubated for 3 h at 0 °C (open squares) or −20 °C (black squares). Samples in (*a*) contained no additional protein, while samples in (*b*) contained cryoprotectin at a concentration sufficient for complete cryoprotection. (Figure redrawn from data presented in Hincha *et al.* 1990.)

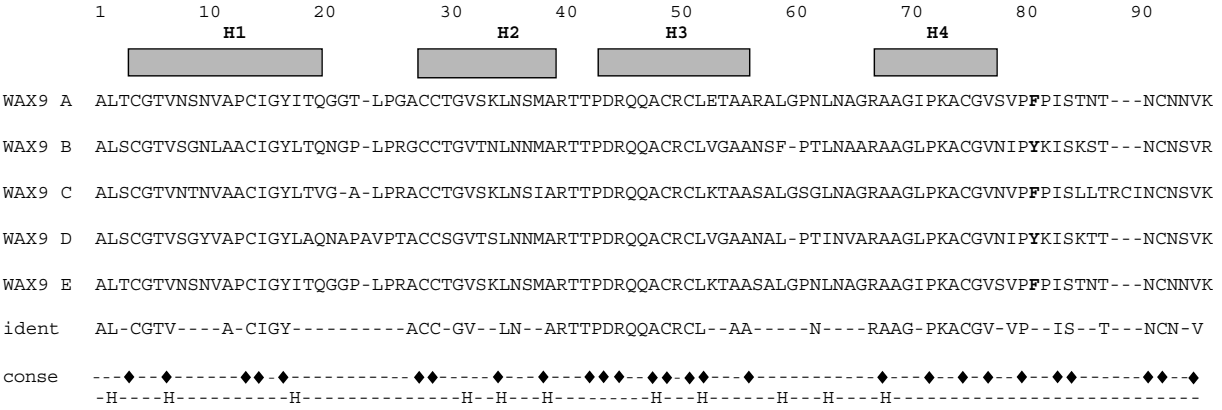

Figure 4. Amino-acid sequences of the five non-specific LTPs WAX9 A, B, C, D and E. The protein sequences were deduced from the sequences of cloned cDNAs isolated from broccoli (Pyee & Kolattukudy 1995) and cabbage (Hincha *et al.* 2001), which are both varieties of the same species, *Brassica oleracea* L. H1–H4 denote the sequences involved in the formation of the four consensus α-helices (Poznanski *et al.* 1999). Amino acids in the line 'ident' are identical in all WAX9 proteins, while diamonds in the line 'conse' denote amino acids found to be conserved in all WAX9 proteins and in 36 other LTP sequences compared recently (Poznanski *et al.* 1999). In addition, conserved hydrophobic residues are indicated by an H in the appropriate position, and the conserved aromatic residue near position 80 is indicated in bold.

1992; White *et al.* 1994; Molina *et al.* 1996), desiccation (Dunn *et al.* 1991; Hughes *et al.* 1992; White *et al.* 1994; Trevino & O'Connell 1998) or salt stress (Torres-Schumann *et al.* 1992; Soufleri *et al.* 1996).

Cryoprotectin is the first LTP for which a functional role in membrane stabilization under stress could be shown. Interestingly, this is connected with a loss in lipid transfer activity (Hincha *et al.* 2001). The same loss of transfer activity has also been found for an antimicrobial LTP from onion seeds (Cammue *et al.* 1995), which, however, showed membrane-destabilizing effects with liposomes (Tassin *et al.* 1998). This leads to the interesting question of structure–function relationships, to understand how small changes in the amino-acid sequences of these extremely conserved small proteins (figure 4) can lead to such dramatic changes in biochemical activity. LTPs are a very promising group of proteins for such studies. In addition to the fact that they are small and highly conserved in sequence, their tertiary structure is also highly conserved, as revealed by X-ray and NMR studies.

High-resolution X-ray structures of crystallized LTP have been reported for maize (Shin *et al.* 1995; Han *et al.* 2001), wheat (Charvolin *et al.* 1999) and rice (Lee *et al.* 1998). Solution structures obtained by NMR spectroscopy have been reported for maize (Petit *et al.* 1994; Gomar *et al.* 1996; Lerche & Poulsen 1998), wheat (Simorre *et al.* 1991; Gincel *et al.* 1994; Sodano *et al.* 1997; Tassin-Moindrot *et al.* 2000), barley (Heinemann *et al.* 1996; Lerche *et al.* 1997; Lerche & Poulsen 1998), rice (Poznanski *et al.* 1999) and onion (Tassin *et al.* 1998). In addition, molecular modelling has been used to infer the lipid-binding properties of a cold-induced leaf LTP (Keresztessy & Hughes 1998) and a seed LTP from barley (Douliez *et al.* 2001). There were only minor structural

differences when the same maize LTP was investigated in the crystallized form by X-ray diffraction and in solution by NMR (Gomar *et al.* 1998).

The overall folding of all investigated LTP is formed by a bundle of four α-helices (denoted H1–H4 in figure 4) connected by three loops, and a C-terminal fragment without an apparent secondary structure. The helices H1, H2 and H3 are arranged in a regular and consecutive up–down–up motif, while H4 is almost perpendicular to the other three helices. This fold is stabilized by the four disulphide bridges formed by the eight Cys residues. This structure forms a hydrophobic cavity that runs the whole length of the molecule. Solution and crystal structures, in the presence of various free fatty acids (Shin *et al.* 1995; Lerche & Poulsen 1998; Han *et al.* 2001), lysolipids (Gomar *et al.* 1996; Charvolin *et al.* 1999) and other hydrophobic molecules (Lerche *et al.* 1997; Tassin-Moindrot *et al.* 2000), have shown that LTPs bind acyl chains in this cavity and thereby shield them from contact with the aqueous medium. In the antimicrobial onion LTP, the replacement of two hydrophobic amino acids by aromatic amino acids blocks the binding cavity, and is presumably the reason for the observed lack of lipid transfer activity (Tassin *et al.* 1998). Whether this leads to membrane binding of the protein, however, has not been reported.

In the future, site-directed mutagenesis studies will be needed, along with molecular modelling and structural investigations, to understand how LTPs perform the different tasks for which they have been selected during evolution, and to enable us to create optimal proteins for specific technical applications.

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Discussion

C. Gerday (*Laboratory of Biochemistry, University of Liege, Liege, Belgium*). Have you got any idea about the affinity of your cryoprotecting protein for calcium? My question is related to another point you mentioned. It is the inhibitory effect of the glucose sulphate. It is maybe due to a sequestration of calcium?

D. K. Hincha. Okay, but would galactose or a digalactose be inhibitory? Would that sequester the calcium?

C. Gerday. Yes. Solubility products of calcium sulphate are low, so maybe there would be a similar effect with the EDTA.

D. K. Hincha. That suggests a chelating effect. It is a good idea, we might check that.

P. L. Davies (*Department of Biochemistry, Queen's University, Ontario, Canada*). I am still a little confused about the relationship of cryoprotectant to the WAX9 proteins. Are these homologues or do they have sequence identity?

D. K. Hincha. As far as we have identified them, the sequences are identical. There are no differences.

P. L. Davies. Are they truncated at the N-terminus?

D. K. Hincha. Yes they are. That is one of the problems that we have, that all the genes that have been cloned have this N-terminal extension. The proteins that we sequenced do not have that extension. What I did not show you is that we have good evidence from immunogold labelling electron microscopy that the protein is actually present inside the cells. We find it in the chloroplast, we find it in the cytosol. So, for some reason it is not exported. Why that is so, I do not know.

P. L. Davies. Are these functional as monomers or is there any suggestion that they might form multimers?

D. K. Hincha. No. They are functional as monomers, at least as far as lipid transfer goes. The lipid-transfer proteins are very well characterized. A number of them have been characterized by X-ray and by high-resolution NMR in solution. They have a very interesting fold. They have a long hydrophobic cavity where the fatty acid binds and this is very strongly conserved in all the proteins that have been looked at. So the way people think they transport lipids is with one fatty acid in this hydrophobic cavity and hydrogen bonding between the protein and the lipid head group. In lipid transfer it is very clear that they are active as monomers. If that is true for cryoprotective activity, of course we do not know because we do not know the molecular interaction.

C. Gerday. Do these proteins have a general cryoprotective activity in different membranes or they are specific for those membranes of thylakoid?

D. K. Hincha. We do not know yet. We have been trying to set up an assay system with liposomes that has not been entirely successful. It has only been a half-hearted approach. Apparently you have to purify the protein much further to get an effect in liposomes compared with thylakoids and I presumed that there are things like lipases or other enzymes that damage the liposomes. This has nothing to do with cryoprotectants, so we are still trying to work that out.

C. Gerday. Their expression in *E. coli* seems to be cytosolic. They are not going to the membrane of *E. coli*?

D. K. Hincha. No. They are basically soluble proteins. When we purify them from cabbage, we just make an aqueous extract. We just homogenize the leaves and then centrifuge everything out. So, the majority of them are soluble. We do have evidence that they are partly bound to the membrane. If we make an extract and treat that with EDTA we get low protein in the supernatant.

C. Gerday. But this is not a cold acclimation where they go to the membrane after a cold shock?

D. K. Hincha. No. Before cold acclimation the proteins are not there. They only come up during cold acclimation.

M. Knight (*Department of Plant Sciences, University of Oxford, Oxford, UK*). Would your volumetric assay be extendable to protoplasts? Obviously you would have to centrifuge at a much lower speed, but if one was interested in looking at the effects of various additives to freezing tolerance of the protoplast, could you use that assay?

D. K. Hincha. In principle, it should be but I am not sure. Protoplasts are a lot more fragile than thylakoids, so I am not sure if they would survive the centrifugation in these glass capillaries.

M. Knight. But presumably the thylakoids experiment does not need centrifugation. If you had lots of time, you could just wait for them to sediment out? You could do the same for protoplasts.

D. K. Hincha. You need a certain amount of packing and I do not know. The problem is if you wait for too long you just get biological degradation.

M. Knight. So, if you were interested in looking at effects on the plasma membrane you would be down to counting protoplasts?

D. K. Hincha. Yes, just staining or something like that.

M. Smallwood (*Department of Biology, University of York, York, UK*). I wonder how much protein to lipid you need to see this activity. Have you done those response studies?

D. K. Hincha. We have not really made that calculation. What we know is that we need very little protein. Usually you get away with something below one microgram per millilitre but we have not really calculated what the molar ratio would be.

M. Smallwood. The reason I ask is that in your model it binds to the surfaces of membranes and alters the mobility of the head groups. For that model, you would need quite a lot of protein.

D. K. Hincha. What we find is that in the absence of freezing we do not see a big effect of the protein, for instance on permeability. Freezing of course concentrates everything and probably just pushes more of the protein into the membrane. I think that the whole thing rests on the question of how the protein is oriented on the membrane. If the protein is spread out on the membrane and really not inserting into it, you probably do not need that much. You probably just get an ordering effect on the surface of the membrane, without having every lipid molecule attached to a protein.

G. Creissen (*Department of Applied Genetics, John Innes Centre, Norwich, UK*). You have already alluded to the fact that these proteins are found in various parts of the cell: protected thylakoid membranes and perhaps protoplast plasmalema. It is no good protecting one membrane group if you do not protect all of them. The import machinery for proteins across chloroplast membranes and mitochondrial membranes is quite different. Do you have evidence that there is a big gene family of these proteins? Perhaps all the different leader sequences allow the chanelling of these proteins to the different membranes they need to protect?

D. K. Hincha. The whole business of intracellular localization, I must admit, is a pure mystery to me. All the leader sequences that have been found so far clearly point to ER processing and export. There are no similarities to mitochondrial or chloroplast import sequences at all. We have five genes, but the leader sequences are all very similar. Just from looking at those sequences, you would not expect any of these proteins to end up in the thylakoids or in the chloroplast. At least WAX9 E we know is exported, and the leader sequence of WAX9 E is extremely similar. Between the different WAX9 genes the leader sequences have the highest homology to each other. I have no idea how that works and we do not know if different proteins end up in different compartments. All I can say is, when we isolate intact chloroplasts from cold-acclimated spinach, we find cryoprotectant inside the chloroplasts, and when we do immunogold labelling of cabbage leaf slices we find gold label on the chloroplasts, in the cytoplasm and in the nucleus. How it gets there I have no idea.

A. Tanghe (*Laboratory of Molecular Cell Biology, University of Leuven, Leuven, Belgium*). Have you an idea about the influence of cryoprotectant on the water permeability of the membrane?

D. K. Hincha. No, we have not looked at that. If you get a reduction in surface fluidity, it should influence the permeability of the membrane, because normally it is considered that the thermodynamic threshold is for molecules to get out of the bulk water phase into the lipid phase, and that is hindered if the fluidity of the membrane is reduced. In the kind of experiments we are doing here it probably does not have a big impact, because water permeability is, by many orders of magnitude, faster than the permeability of everything else, so even if that is slowed down I do not think that would have a major influence.

A. Tanghe. The reason I ask this is that I am doing research on baker's yeast, and freeze resistance of this baker's yeast, and I recently found that expression of aquaporins of water channels is related to freeze resistance. If I overexpress water channels I get a larger freeze resistance, and the hypothesis we are working with is that if you can have a rapid outflow of water upon freezing you reduce the intracellular ice crystal formation and the damage of the cell.

D. K. Hincha. I am aware of that train of thought but this really rests on your experimental conditions. That is, if you do very fast freezing then the water permeability may be limiting. If you do slow freezing there is always enough time for the cell to be in equilibrium and then it should not matter.

G. Warren (*School of Biological Sciences, Royal Holloway, University of London, Egham, UK*). I think we should note that aquaporins are among the cold-induced proteins in many plants. Do you know how general the presence of this type of protein is?

D. K. Hincha. We have not done a systematic study. Basically, just using the antibodies, we know that it is present in *Arabidopsis* and, if I remember it correctly, it seems to be present in cucumbers after low-temperature treatment, which is very weird because cucumbers are not freezing resistant at all. They are cold sensitive and chilling sensitive.

GLOSSARY

LTP: lipid-transfer protein
MGDG: monogalactosyldiacylglycerol
NMR: nuclear magnetic resonance