BMC Plant Biology

Research article

Accumulation of dehydrin-like proteins in the mitochondria of cereals in response to cold, freezing, drought and ABA treatment

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Published: 11 June 2002

BMC Plant Biology 2002, **2**:5

Received: 7 December 2001
Accepted: 11 June 2002

This article is available from: http://www.biomedcentral.com/1471-2229/2/5

Abstract

Background: Dehydrins are known as Group II late embryogenesis abundant proteins. Their high hydrophilicity and thermostability suggest that they may be structure stabilizers with detergent and chaperone-like properties. They are localised in the nucleus, cytoplasm, and plasma membrane. We have recently found putative dehydrins in the mitochondria of some cereals in response to cold. It is not known whether dehydrin-like proteins accumulate in plant mitochondria in response to stimuli other than cold stress.

Results: We have found five putative dehydrins in the mitochondria of winter wheat, rye and maize seedlings. Two of these polypeptides had the same molecular masses in all three species (63 and 52 kD) and were thermostable. Drought, freezing, cold, and exogenous ABA treatment led to higher accumulation of dehydrin-like protein (dlp) 63 kD in the rye and wheat mitochondria. Protein 52 kD was induced by cold adaptation and ABA. Some accumulation of these proteins in the maize mitochondria was found after cold exposition only. The other three proteins appeared to be heat-sensitive and were either slightly induced or not induced at all by all treatments used.

Conclusions: We have found that, not only cold, but also drought, freezing and exogenous ABA treatment result in accumulation of the thermostable dehydrins in plant mitochondria. Most cryotolerant species such as wheat and rye accumulate more heat-stable dehydrins than cryosensitive species such as maize. It has been supposed that their function is to stabilize proteins in the membrane or in the matrix. Heat-sensitive putative dehydrins probably are not involved in the stress reaction and adaptation of plants.

Background

Plants respond to stress conditions through physiological, morphological, and metabolic processes. A common element in response to many environmental stresses is cellular dehydration. It, in turn, induces biosynthesis of abscisic acid (ABA), which is well known as a stress hormone because of its rapid and massive accumulation under water stress conditions and participating in stress signal transduction pathways [1,2]. Dehydration and ABA cause changes in solute concentration (osmoprotectants), those in membrane fluidity and composition, and induc-

tion of a number of cold-induced proteins, many of which remain unstudied [3–6].

As is known, among the induced proteins, dehydrins (Group II late embryogenesis abundant (LEA) proteins) have been most commonly studied, yet we still have an incomplete knowledge of their fundamental role in the cell. They are evolutionarily conserved among photosynthetic organisms including angiosperms, gymnosperms, ferns, mosses, liverworts, algae and cyanobacteria, as well as in some non-photosynthetic organisms such as yeast [7–10]. Dehydrins have been most extensively studied in relation to drought and cold stresses [1,11–13]. Many of dehydrins have been identified in plants in response to exogenous ABA or varied stresses involved ABA as a regulator [1,15,16]. On the other hand, protein level of some dehydrins is regulated by low temperature only.

In particular, studies of stress-induced accumulation of five dehydrins in *Arabidopsis* revealed that two of them (LTI30 and COR47) accumulated primarily in response to low temperature. The level of another two proteins (ERD14 and LTI29) was up-regulated by ABA and low temperatures, whereas RAB18 was only found in ABA-treated plants [16]. The dehydrin-like protein P-80 (80-kD) from barley accumulated under cold acclimation though drought, ABA or heat stress did not increase the level of P-80 [17]. We suppose that accumulation of protein under a certain stress suggests functional specialization for this protein and clarifies its role in the stress reaction. Reaction to both ABA and a certain stress (drought, freezing, salt ets.) seem to be valuable information for understanding the function of dehydrin in the cell.

As is started, dehydrins are hydrophilic, thermostable and have a high amounts of charged amino acids[1,14]. They are hypothesized to function, stabilizing large-scale hydrophobic interactions such as membranes structures or hydrophobic patches of proteins. Highly-conserved polar regions of dehydrins have been suggested to hydrogen-bond with polar regions of macromolecules, acting essentially as a surfactant, to prevent coagulation under conditions of cellular dehydration or low temperatures [18]. Immunolocalization and subcellular fractionation results have showed that members of the dehydrin family are present in the nucleus, cytoplasm, and plasma membrane [19,20]. Recently we have found that two dehydrin-like proteins (dlps) accumulated in mitochondria of *Triticum aestivum* (L.) (winter wheat), *Secale cereale* (L.) (winter rye), and in *Zea mays* (L.) (corn) in response to cold [21]. We also demonstrated a positive correlation between accumulation of two mitochondrial dlps and cold tolerance of the species studied.

The aim of this study was to determine whether dlps accumulate in plant mitochondria in response to stimuli other than cold treatment (4°C for wheat and rye; 10°C for maize) and whether ABA participates in the processes of their accumulation. Here we report that drought, freezing and exogenous ABA treatment as well as cold result in increase of thermostable dlps contents in plant mitochondria. The most cryotolerant wheat and rye accumulated more heat-stable dehydrins than maize.

Results and discussion

In a previous study we found accumulation of two dehydrin-like proteins in the plant mitochondria under low temperature treatment [21]. Now five dlps have been found even at the control temperature due to a gel density decrease and an increased protein load (Fig. 1). We also re-calculated relative molecular masses of dlps. The bands corresponding to all these proteins were very weak or absolutely absent if we used antibodies blocked by dehydrin peptides (data not shown).

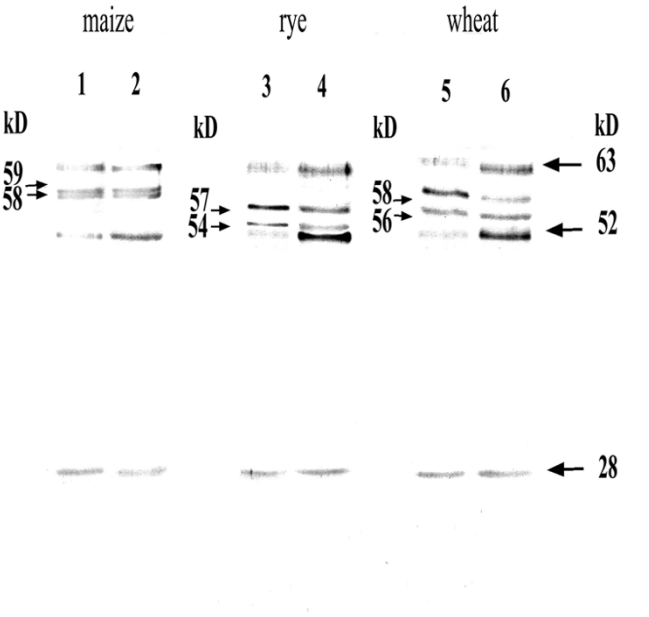

Figure 1
Dehydrin-like proteins (dlps) of maize, wheat and rye mitochondria after cold adaptation. Mitochondria from control (1, 3, 5) and cold-treated (2, 4, 6) seedlings were sonicated for disruption. Mitochondrial protein of maize (1, 2), winter rye (3, 4), and winter wheat (5, 6) were fractionated by 10% SDS-PAGE (25 μg of protein per lane), electroblotted onto the nitrocellulose membrane, and probed with antibody against dehydrin. Molecular masses of dlps common to all the species are indicated on the right side of and the molecular masses of species-specific dlps are given on the left side of the corresponding bands.

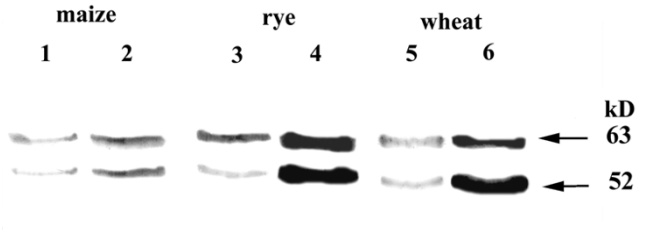

Figure 2
Thermostable mitochondrial dehydrin-like proteins of maize, wheat and rye after cold adaptation. Protein separation and Western blotting were the same as in Fig. 1. Molecular masses of dlps are indicated.

Two of these polypeptides had the same molecular masses in all the species (63 and 52 kD). Analysis of the thermostable fraction revealed that these dlps like most of dlps reported earlier [1,8] were of heat-stable nature (Fig. 2). At the same time, the other three proteins did not appear to be thermostable: one heat-sensitive protein with the molecular mass 28 kD was registered in all three species and the other two had similar, but slightly different masses – 59 and 58 kD, 57 and 54 kD, 58 and 56 kD for maize, rye, wheat, respectively (Fig. 1). The finding that the proteins immunologically related to dehydrins was found to be constitutive and heat-sensitive was unusual. In the literature available we found only one reference concerned: Li with coworkers had described proteins related to dehydrins being constitutive and heat-sensitive, in fucoid algae [9]. It is interesting that all three heat-sensitive mitochondrial dlps from maize, wheat and rye were constitutive too. These proteins were not induced by all the treatments used (Fig. 1, 3, 4, 5). Based on this observation we concluded that these proteins were not involved in the stress reaction and adaptation.

As shown by us earlier [21] and as we show in the present work (Fig. 1,2) the two revealed thermostable dlps accumulated during cold treatment. Earlier we used data based on sonicated mitochondrial samples[21]. At present we study not only sonicated (Fig. 1,2) but also unsonicated ones (Fig. 3, 4, 5), which enables resolution of all mitochondrial dehydrins and not only primarily weakly bound with large fragments of mitochondria or membranes.

Analysis of the unsonicated mitochondrial fraction of total proteins (heat-sensitive and heat-stable) showed that freezing and drought stresses, and ABA treatment as well as cold caused strong accumulation of dlp63 in the wheat (Fig. 5) and rye (Fig. 4) mitochondria and had no effect in the mitochondria of maize (Fig. 3). The dlp52 was induced only by exogenous ABA and cold treatment in the mitochondria of rye and wheat, and in the rye ones also

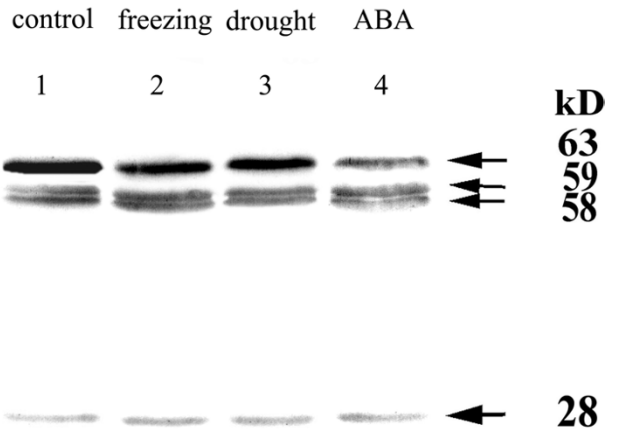

Figure 3
Mitochondrial dehydrin-like proteins from the control (1), freezing (2) and drought (3) stressed and ABA treated (4) maize seedlings. Protein separation and Western blotting were the same as in Fig. 1. Molecular masses of dlps are indicated.

by drought (Figs. 1, 4, 5). At the same time in the thermostable fraction (Fig. 2) there was some increase of these proteins in maize mitochondria as well, although in less degree than in the rye and wheat ones. Apparently, the share of heat-stable protein in the total protein fraction of the same mol. wts was little. It made visualization of heat-stable polypeptides in the total fraction difficult.

Thus cold and ABA treatment resulted in significant accumulation of both heat-stable dlps in the rye and wheat mitochondria. The dlp 63 kD accumulated during all treatments used, but dlp 52 kD during cold and ABA only, but during drought also in the rye organelles. It suggests that dlp52 does not accumulate under freezing and drought conditions or its content is too low, in comparison with dlp63, to detect some changes. In any case it appears that both heat-stable proteins, particularly dlp63, may be associated with the protection of mitochondria against desiccation or other stresses caused by freezing.

The relative abundance of the thermostable dlps was higher in freezing tolerant species (rye or winter wheat) than in the maize as had been shown earlier [21]. At the same time the comparison of dehydrin contents among species based on immunochemical data should be accepted only with caution since we did not estimate binding affinity of antibodies in each species.

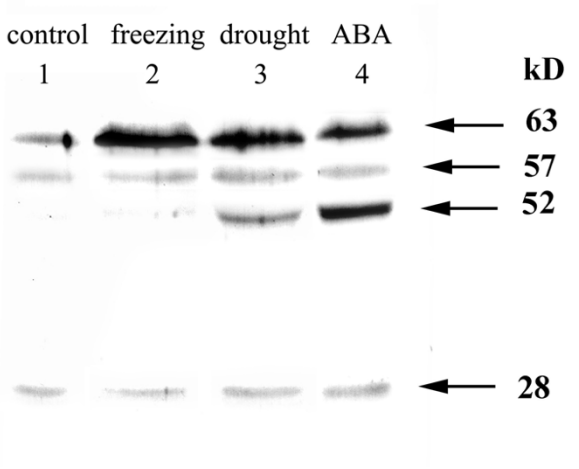

Figure 4
Mitochondrial dehydrin-like proteins from the control (1), freezing (2) and drought (3) stressed and ABA treated (4) winter rye seedlings. Protein separation and Western blotting were the same as in Fig. 1. Molecular masses of dlps are indicated.

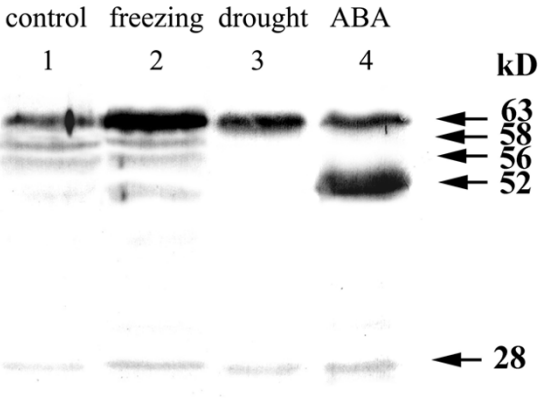

Figure 5
Mitochondrial dehydrin-like proteins from the control (1), freezing (2) and drought (3) stressed and ABA treated (4) winter wheat seedlings. Protein separation and Western blotting were the same as in Fig. 1. Molecular masses of dlps are indicated.

The treatment of exogenous ABA provided some confirmation of ABA participating in induction of heat-stable dlps 63 and 52 kD. It corroborates again an important role of ABA in plant responses to stresses related to dehydration not only at the whole-plant level but also at the subcellular level.

Cold-induced accumulation of the thermostable mitochondrial dlps in all species studied (Fig. 1, 2) was accompanied by increasing of plant cryotolerance. This increase had been shown previously [22]. The quantity of survived seedlings under 24 hours at -8°C rose from zero to 61% after 7 days of acclimation. There are also a number of papers describing cold acclimation of rye at low positive temperatures from 2 to 5 [23]. Though maize is not a freezing-tolerant species, maize seedlings could acclimate at the temperature near low limit of growth [24,25]. The connection between accumulation of the mitochondrial dlps and increase of plant cryotolerance suggests an important role of the dlps in the development of the tolerant state of cryotolerant cereals.

The freezing treatment that we used presumably led to damage of cells or even death due to severe dehydration. It has recently been shown that after freezing of even very sensitive species like bean, tobacco, tomato and cucumber, ice was extracellular. Only corn had combination of extracellular and intracellular damage [26]. The increase of dlp63 in rye and wheat mitochondria under freezing treatment perhaps resulted from very fast association of this protein with organelles under cellular dehydration. This suggests presence of this protein in the cytoplasm before freezing. The absence of dlp63 accumulation in the maize mitochondria may be explained by low contents of this protein in the cytoplasm of maize cells. Thus, the mitochondria of freezing-tolerant cultures such as winter wheat and rye have cryotolerant mechanisms that are different from those of freezing-sensitive species such as maize.

The accumulation of dehydrins during various treatments has been studied in different plant species [1]. The accumulations of dehydrins (WCS120) related to freezing tolerance were demonstrated also on cereal species [20]. In wheat and other cereals the *wcs120* gene family was coordinately regulated by low temperatures and the resulting proteins accumulated in significant amounts in freezing-tolerant members of *Poaceae*. Immunogold labeling indicated that the WCS120 protein family was localized in both the nucleus and cytoplasm of cold acclimated tissues [27]. The presence of these proteins in high amounts in the nucleus indicates that they may protect or stabilize the transcriptional machinery from inactivation [20].

Another LT-responsive dehydrin referred to as WCOR410 was identified [19]. Due to its acidic nature and the absence of the glycine-rich repeat presented in the cold-regulated dehydrin WCS120 [20], WCOR410 is thought to belong to a different subtype of the D-11 protein family, the so-called acidic dehydrin [19]. The WCOR 410 protein

family is preferentially associated with the plasma membrane of cells in the sensitive vascular transition area where freezing-induced dehydration is likely to be more severe. The authors [19] proposed that this dehydrin might play a role in preventing destabilization of the plasma membrane that occurs under dehydrative conditions. These results confirm, at least in part, the previous hypothesis of the authors that dehydrin subtypes can accomplish their function in different compartments [19].

Our data concerning presence and accumulation of dehydrins in the mitochondria of cereals, to our mind, also confirm this hypothesis. Mitochondrial membranes are very permeable for the water which moves out of the cells into the intercellular spaces concomitantly with freezing temperatures decreasing and drought stress increasing. Dehydration may lead to damage of the mitochondria. Apparently there are some tolerance mechanisms in the cell against the injuring effect of dehydration on these organelles. Our results concerning the accumulation of heat-stable dehydrin-like proteins in the mitochondria under the cold treatment, drought and freezing stress suppose that mitochondrial dlps should be involved in the freezing- and dehydrative-tolerance mechanisms. First, as was mentioned before, the revealed heat-stable dehydrins have the K segment. It has been proposed to form an amphipathic α-helix which is responsible for the lipid-associating properties [14]. Possibly, a role of the dehydrin K segment (and respectively of dlps containing this site) is hydrophobic interaction with partially denatured mitochondrial proteins or membranes which stabilizes them. Second, the hydrophilic nature of the dlps is well suited to replace water and stabilize mitochondrial membranes through polar interactions during dehydration.

The higher accumulation of the dlps in winter wheat and rye mitochondria probably reflects stability of the mitochondria, cells and the whole plant under dehydration and freezing. The target of mitochondrial dlps action is unclear. Precise localization of these proteins in the organelles and the finding of targets of it action would give more information about the function of mitochondrial dlps. Further experiments concerning dlp localization in mitochondria are in progress.

Conclusions

We have found that not only cold but also drought, freezing and exogenous ABA treatment also result in accumulation of heat-stable dlps in plant mitochondria. The most tolerant winter wheat and rye accumulate more of the heat-stable dehydrins than maize. The dlps found in the mitochondria could possibly function to prevent destabilization of membranes and/or stabilize proteins in the membrane or in the matrix of these organelles. The exogenous ABA treatment results in high accumulation of thermostable dlps in rye, lesser accumulation in wheat and has no effect in maize. It appears that ABA participates in induction of thermostable mitochondrial dlps in cryotolerant crops. Heat-sensitive putative dehydrins probably are not involved in the stress reaction and adaptation.

Materials and methods

Two-day-old etiolated seedlings of *Triticum aestivum* (L.) (winter wheat) and *Secale cereale* (L.) (winter rye) were grown at 23°C, of *Zea mays* (L.) (maize) were grown at 27°C. Unstressed plants were maintained under growth conditions for one additional day. Cold acclimation was performed by subjecting two-day-old seedlings to the temperature of 4°C for wheat and rye and to the temperature of 10°C for maize for 7 days. The temperature for maize adaptation was chosen on the basis of the data describing the rise of cold tolerance in chilling-sensitive varieties of maize under acclimation at 14°C, as the temperature is near the lower limit of growth [24]. Since we used the chilling-tolerant variety of maize, the seedlings rather fast grew at 14°C (data not shown) and differed from control 4 days-old seedlings, so we used 10°C for acclimation. There were not any visually noticeable necrosis or other injuries on the maize seedlings after 7 days acclimation. The length of the acclimated seedlings corresponded to 4 days-old seedlings growing at 27°C.

Freezing was performed at -10°C for 15–20 min till the ice crystallization on the surface of three-day seedlings started. Such severe treatment allowed us to demonstrate how rapidly the association of dlps with mitochondria under low temperature/dehydration stress occurred. Transferring of two-day-old seedlings on dry filter paper for 1 day under the growth conditions served as a model of drought stress. ABA treatments were made at the control temperature by spraying two-day seedlings with the 1 mM (mixed isomers) ABA (Sigma) solution with 0.1% of Tween-20 (Sigma). This rather high ABA concentration had been earlier applied as a potent inductor of cold tolerance and cold acclimation genes [24,28]. ABA-treated seedlings were harvested the next day after the treatment. The control and treated seedlings were compared at similar growth stages.

Crude mitochondria were isolated from seedlings by a method described in [29] and resuspended in a buffer containing 20 mM MOPS-KOH (pH 7.4), 300 mM sucrose. Further purification was performed on a discontinues Percoll gradient consisting of 18%, 23%, 40% Percoll supplemented with 300 mM sucrose and 10 mM MOPS-KOH (pH 7.4) [30]. Samples layered onto the Percoll gradient were centrifuged at 22,000 g_n for 45 min from which pure mitochondria were collected at the interface of the 23/40 % Percoll layers. Mitochondria were purified from Percoll by diluting the mitochondria in a wash buffer of

10 mM MOPS-KOH (pH 7.4) and 300 mM sucrose and centrifuging at 24,000 g_n for 10 min. The mitochondrial pellet was resuspended in a wash buffer and centrifuged at 24,000 g_n for 5 min. The purity and integrity of mitochondria were determined by measurement of cytochrome c oxidase activity (EC 1.9.3.1) [30].

The mitochondria were sonicated for one minute three times for full disruption in 100 mM Tris-HCl (pH 7.6) containing 1 mM phenylmethylsulfonyl fluoride. Undisrupted mitochondria were precipitated by centrifugation at 17,000 g_n for 5 minutes and then discarded. The supernatant was divided into two fractions: (1) fraction 1 was boiled for 20 minutes to isolate thermostable proteins; and (2) fraction 2 was subjected immediately to protein precipitation. Proteins from fractions 1 and 2 were precipitated with 10 % trichloroacetic acid, washed with cold acetone, and dissolved in a sample loading buffer for SDS-PAGE.

Alternatively, purified mitochondria were dissolved in the sample loading buffer for SDS-PAGE and boiled for 3 min. Non-soluble residue were precipitated by centrifugation at 17,000 g_n for 5 minutes and discarded.

Protein concentrations of samples were determined according to Esen [31].

Proteins were subjected to SDS-PAGE using a mini-Protean PAGE cell (Bio-Rad) according to the manufacturer's instruction. Western blotting and immunodetection were carried out as described by Timmons and Dunbar [32]. Proteins were transferred from the SDS-gel to a nitrocellulose membrane in Towbin buffer. Blots were blocked in a Tris-buffer saline containing nonfat dry milk (5 % w/v) and 0.1 % Tween-20 (Sigma). Blots were then incubated with anti-dehydrin antibodies (1:1,000 dilution) (kindly provided by Dr. T. J. Close). Primary antibodies were detected using secondary antibodies conjugated to alkaline phosphatase (Sigma) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

All experiments were replicated three to four times.

List of abbreviations

dlp – dehydrin-like-protein;

LEA – late embryogenesis abundant;

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Acknowledgements

The research was funded by the Russian Foundation of Basic Research (projects 02-04-48599 and 02-04-48728). We sincerely thank Dr. T. J. Close for his having supplied us with the dehydrin antibody and dehydrin-specific peptide.

References

1. Close TJ: **Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins.** *Physiol Plant* 1996, **97:**795-803
2. Yamaguchi-Shinozaki K, Shinozaki K: **A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress.** *Plant Cell* 1994, **6:**251-264
3. Guy CL: **Cold acclimation and freezing tolerance: role of protein metabolism.** *Ann Rev Plant Physiol Plant Mol Biol* 1990, **41:**187-223
4. Thomashow MF: **Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms.** *Ann Rev Plant Physiol Plant Mol Biol* 1999, **50:**571-599
5. Bohnert HJ, Nelson DE, Jensen RG: **Adaptations to environmental stresses.** *Plant Cell* 1995, **7:**1099-1111
6. Hare PD, Cress WA, Van Staden J: **Dissecting the roles of osmolyte accumulation during stress.** *Plant Cell Environ* 1998, **21:**535-553
7. Close TJ, Lammers PJ: **An osmotic stress protein of cyanobacteria is immunologically related to plant dehydrins.** *Plant Physiol* 1993, **101:**773-779
8. Campbell SA, Close TJ: **Dehydrins: genes, proteins, and associations with phenotypic traits.** *New Phytol* 1997, **137:**61-74
9. Li R, Brawley SH, Close TJ: **Proteins immunologically related to dehydrins in fucoud algae.** *J Phycol* 1998, **34:**642-650
10. Mtwisha L, Brandt W, McCready L, Lindsey GG: **HSP12 is a LEA-like protein in *Saccharomyces cerevisiae*.** *Plant Mol Biol* 1998, **37:**513-521
11. Labhilili M, Joudrier P, Gautier M-F: **Characterization of cDNAs encoding *Triticum durum* dehydrins and their expression patterns in cultivars that differ in drought tolerance.** *Plant Sci* 1995, **112:**219-230
12. Moons A, Bauw G, Prinsen E, Van Montagu M, Straeten DVD: **Molecular and physiological responses to abscisic acid and salts in roots of salt sensitive and salt tolerant Indica rice varieties.** *Plant Physiol* 1995, **107:**177-186
13. Pelah D, Wang W, Altman A, Shoseyov O, Bartels D: **Differential accumulation of water stress-related proteins, sucrose synthase, and soluble sugars in *Populus* species that differ in their water stress response.** *Physiol Plant* 1997, **99:**153-159
14. Close TJ: **Dehydrins: a commonality in the response of plants to dehydration and low temperature.** *Physiol Plant* 1997, **100:**291-296
15. Close TJ, Fenton RD, Moonan F: **A view of plant dehydrins using antibodies specific to the carboxy terminal peptide.** *Plant Mol Biol* 1993, **23:**279-286
16. Nylander M, Svensson J, Palva TE, Welin BV: **Stress-induced accumulation and tissue-specific localization of dehydrins in *Arabidopsis thaliana*.** *Plant Mol Biol* 2001, **45:**263-279
17. Bravo LA, Close TJ, Corcuera LJ, Guy CL: **Characterization of an 80-kDa dehydrin-like protein in barley responsive to cold acclimation.** *Physiol Plant* 1999, **106:**177-183
18. Ismail AM, Hall AE, Close TJ: **Purification and partial characterization of a dehydrin involved in chilling tolerance during seedling emergence of cowpea.** *Plant Physiol* 1999, **120:**237-244
19. Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N, Sarhan F: **Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat.** *Plant Cell* 1998, **10:**623-638
20. Sarhan F, Ouellet F, Vazquez-Tello A: **The wheat wcs120 gene family. a useful model to understand the molecular genetics of freezing tolerance in cereals.** *Physiol Plant* 1997, **101:**439-445
21. Borovskii GB, Stupnikova IV, Antipina AA, Downs CA, Voinikov VK: **Accumulation of dehydrin-like-proteins in the mitochondria of cold-treated plants.** *J Plant Physiol* 2000, **156:**797-800
22. Stupnikova IV, Borovskii GB, Voinikov VK: **Accumulation of heat-stable proteins in winter wheat seedlings during hypothermia.** *Russ J Plant Physiol* 1998, **45:**744-748
23. Webb SM, Uemura M, Steponkus PL: **A comparison of freezing injury in oat and rye: two cereals at the extremes of freezing tolerance.** *Plant Physiol* 1994, **104:**467-478

24. Anderson MC, Prasad TK, Martin BA, Stewart CS: **Differential gene expression in chilling-acclimated maize seedlings and evidence for the involvement of abscisic acid in chilling tolerance.** *Plant Physiol* 1994, **105:**331-339
25. De Santis A, Landi P, Genshi G: **Changes of mitochondrial properties in maize seedlings associated with selection for germination at low temperature: fatty acid composition, cytochrome c oxidase and adenine nucleotide translocase activities.** *Plant Physiol* 1999, **119:**743-754
26. Ashworth EN, Pearce RE: **Extracellular freezing in leaves of freezing-sensitive species.** *Planta* 2002, **214:**798-805
27. Houde M, Daniel C, LaChapelle M, Allard F, Laliberte S, Sarhan F: **Immunolocalization of freezing-tolerance-associated proteins in the cytoplasm and nucleoplasm of wheat crown tissues.** *Plant J* 1995, **8:**583-593
28. Guan L, Scandalios JG: **Two structurally similar maize cytosolic superoxide dismutase genes, Sod4 and Sod4A, respond differentially to abscisic acid and high osmoticum.** *Plant Physiol* 1998, **117:**217-224
29. Voinikov VK, Varakina NN, Pobezhimova TP, Rudikovsky AV: **Reconstruction of energy-producing activity in *in vitro* mitochondria maize seedlings.** *Rus J Plant Physiol* 1991, **38:**530-537
30. Davy De Virville J, Aaron I, Alin MF, Moreau F: **Isolation and properties of mitochondria from *Arabidopsis thaliana* cell suspension cultures.** *Plant Physiol Biochem* 1994, **32:**159-166
31. Esen AA: **A simple method for quantitative, semi-quantitative and qualitative assay of protein.** *Anal Biochem* 1978, **89:**264-273
32. Timmons TM, Dunbar BS: **Protein blotting and immunodetection.** *Methods Enzymol* 1990, **182:**679-688