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Proline and the Cryopreservation of Plant Tissues: Functions and Practical Applications

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1. Introduction

Cryopreservation has been proven to be an effective technology for the cost-effective, long-term preservation of genetic material. A wide range of plant material including cultured cells, tissues, embryos, meristems, pollen and seeds can be effectively preserved for extended periods of time and, when thawed, can be used to rapidly produce stock plants, with good preservation of genetic and physiological characteristics. Numerous protocols including controlled rate cooling, PVS2 vitrification, encapsulation-vitrification, and encapsulation-dehydration have been developed that allow the cryopreservation of a wide range of plant germplasm (Burritt, 2008), but irrespective of the protocol used each step in a cryopreservation protocol has the potential to impose a stress on plant cells. Low temperatures that lead to freezing can impose stress on cells and tissues in two ways, by the direct effects of low temperatures on cell function and integrity or by the cellular dehydration that occurs when the cells water freezes. Several of the mechanisms by which these two forms of stress can damage plant cells are shown in Figure 1.

Numerous studies have shown that cold temperatures induce the accumulation of metabolites, including low-molecular-weight carbohydrates such as fructose, glucose, maltose and raffinose, and amino acids such as proline and glutamine (Taji et al., 2002; Cook et al., 2004). These metabolites play important protective roles in freezing tolerance in whole plants (Kaplan and Guy, 2004) and this has lead to their extensive use in the protocols developed for the cryopreservation of isolated plant cells and tissues (Burritt, 2008). In particular, the amino acid proline has been found to help confer freezing tolerance in a wide variety of both animal and plant cells, and is often added to cryoprotective solutions or is used for preconditioning plants or pretreating isolated cells or tissues prior to cryopreservation (Burritt, 2008). Despite its widespread use, little is known of the mechanisms via which proline protects cells during cryopreservation.

This chapter gives an overview of proline synthesis and metabolic regulation in plants and the changes in proline metabolism associated with desiccation and freezing tolerance, which are both of importance for the successful cryopreservation of plants cells and tissues. The

use of proline as a cryoprotectant or pre-growth additive for the cryopreservation of plant cells and tissues is then overviewed and the potential mechanisms via which proline can protect plant cells is critically evaluated. Future research needs are then discussed.

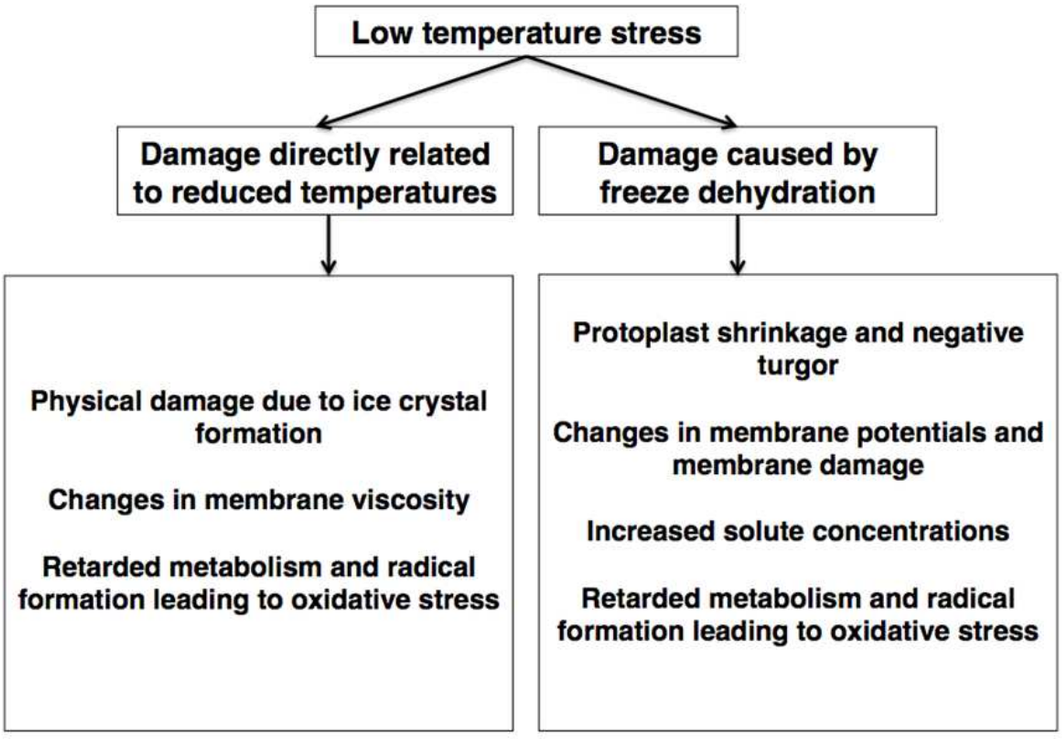

Fig. 1. Potential damage caused by the stresses associated with exposure of plant cells to low temperatures.

2. Proline and plants

2.1 The function of proline in plants

Essential for primary metabolism, both as a free amino acid and as a component of proteins, proline is distinctive among the proteinogenic amino acids as it contains a secondary amino group and a distinctive cyclic structure (Lehmann et al., 2010). The cyclic structure of proline causes exceptional conformational rigidity, compared to other amino acids, as proline's side chain locks its φ backbone dihedral angle at approximately -75° and this determines the arrangement of the peptide chain and can lead to the stabilization or destabilization of secondary protein structures.

As well as being important for primary metabolism proline appears to have numerous other functions in plants. Research has clearly demonstrated that proline levels show significant fluctuations in response to environmental stress (Bohnert et al., 1995), but the precise mode of action of proline remains largely a matter of speculation. In addition to its role in environmental stress tolerance, recent research has provided evidence that proline may also play important roles in plant development both as a metabolite and as a signal molecule (Mattioli et al., 2009). Studies have shown that proline could play important roles in embryo and seed development, stem elongation, and the transition from vegetative growth to flowering (Mattioli et al., 2008; Mattioli et al., 2009)

2.3 Proline biosynthesis and catabolism

The proposed pathways for proline biosynthesis and catabolism in plants are outlined in Figure 2. In plants proline can be synthesized from glutamate or ornithine, however under most conditions proline is mainly synthesized from glutamate rather than from ornithine, as the enzyme ornithine-δ –aminotransferase (dOAT) is down regulated (Szabados & Savoure, 2009). Two enzymes are required for the synthesis of proline from glutamate. The first enzyme, Δ1- pyrroline-5-carboxylate synthase (P5CS) is a bifunctional enzyme that phosphorylates and reduces glutamate to glutamyl-5- semialdehyde (G5SA) that then spontaneously converts to Δ1- pyrroline-carboxylate (P5C). The second enzyme, Δ1-pyrroline-carboxylate reductase (P5CR) further reduces the P5C intermediate to proline (Delauney & Verma, 1993). P5CS has been found to be encoded by 2 genes in most plants, while P5C is encoded by only a single gene (Szekely et al. 2008; Strizhov et al., 1997) The rate-limiting step in the above pathway is the γ -glutamyl kinase activity of P5CS, which is sensitive to feedback inhibition by the presence of relatively low cellular proline levels (Zhang et al., 1995). Alternatively proline can be synthesized from ornithine by dOAT, which converts ornithine and α-ketoglutarate to P5C and glutamate by transamination (Stranska et al., 2008). Funck et al., 2008, in a study of *Arabidopsis thaliana,* found that mutant plants which lacked dOAT activity could not mobilize nitrogen from arginine or ornithine, but could accumulate proline and so suggested the main role for dOAT was arginine degradation. They also suggested that as dOAT is localized in the mitochondria and that it would be unlikely that P5CR could directly utilize dOAT-generated P5C, as P5CR is localized in the cytosol or in plastids.

Proline degradation in plants takes place in mitochondria and so is by in large separated from the biosynthetic pathway. The first step in proline catabolism is the oxidation of proline to P5C by proline dehydrogenase (PDH), which in Arabidopsis and tobacco is encoded by two homologous genes (Mani et al., 2002; Ribarits et al., 2007; Verbruggen & Hermans 2008). The P5C generated is then converted to glutamate by pyrroline-5-carboxylate dehydrogenase (P5CDH), which is thought to be encoded by a single gene in all of the plant species analysed to date (Ayliffe et al. 2005; Mitchell et al. 2006). However, biochemical analysis P5CDH in *Nicotiana plumbaginifolia* and *Zea mays* has revealed two slightly different enzyme activities that may arise from a single gene, or a second P5CDH gene may be present (Elthon & Stewart 1982; Forlani et al. 1997). In plants under stress, the accumulation of proline is thought to be due not only to increased synthesis, but also to inactivation of degradation pathways (Delauney & Verma, 1993)).

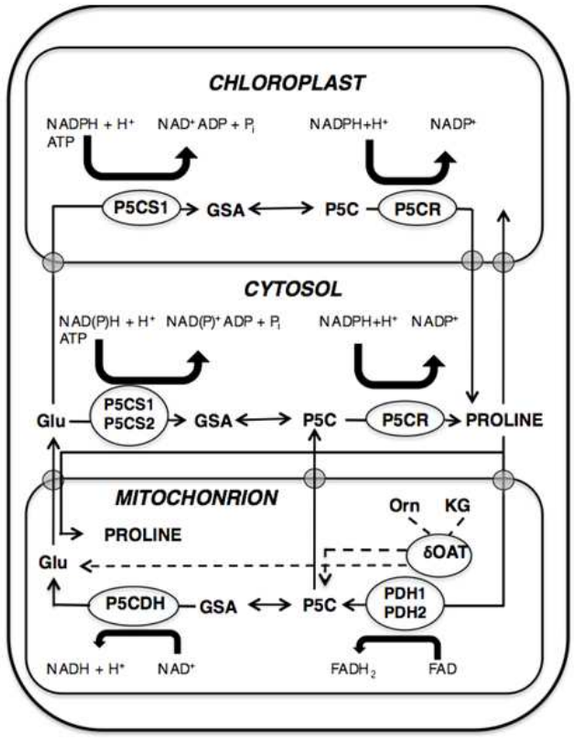

Fig. 2. Proposed model for proline metabolism in higher plants (adapted from Lehmann et al. 2010). Glu glutamate, Orn ornithine, P5C pyrroline-5-carboxylate, GSA glutamic-γ-semialdehyde, KG α-ketoglutarate. P5CS P5C synthetase, P5CR P5C reductase, PDH proline dehydrogenase, P5CDH P5C dehydrogenase, δOAT ornithine-δdaminotransferase. Transporters and potential transporters are shown as grey circles.

3. Proline in plant cells under stress

3.1 Proline accumulation in higher plants

Stress has been shown to induce proline accumulation in a wide range of organisms including eubacteria, protozoa, invertebrates and plants (Verbruggen & Hermans, 2008; Kostal et al., 2011) and proline accumulation is believed to be very important as part of the physiological adaptation of plants to stress. In plants a wide range of abiotic and biotic stressors have been shown to induce proline accumulation including, salt, drought, high temperatures, low temperatures, heavy metals, anaerobiosis, nutrient deficiency, organic pollutants, ultraviolet (UV) radiation and pathogen infection (Chu et al., 1978; Alia & Saradhi, 1991; Saradhi et al., 1995; Hare et al., 1999; Siripornadulsil et al., 2002). The level of proline that accumulates in plants in response to stress varies greatly and is highly dependent on the plant species, with increase of up to 100 fold compared to controls reported in the literature (Verbruggen & Hermans, 2008).

With respect to cryopreservation, numerous studies have demonstrated the importance of proline for plant cold tolerance (Swaaij, Jacobsen & Feenstra 1985, Swaaij et al. 1986; Duncan & Widholm 1987; Ait-Barka & Audran 1997; Hoffman et al., 2010; Javadian et al., 2010; Burbulis et al., 2011). Studies on plants relatively insensitive to chilling, such as barley (Chu

et al. 1978), rye (Koster & Lynch 1992), winter wheat (Dorffling et al. 1997), and *Arabidopsis thaliana* (Xin & Browse 1998; Nanjo et al. 1999) have demonstrated significant positive correlations between cellular proline accumulation and improved cold tolerance.

In addition, plant cells under dehydrating conditions, which are often a consequnce of cryopreservation, undergo osmotic adjustment by accumulating one or several low molecular weight organic solutes, which are often referred to as compatible osmolytes and/or osmoprotectants. These molecules play a critical role in counteracting the effect of osmotic stress in plants at the cellular level (Yoshiba et al., 1997). In plants under dehydrating conditions such as drought or high salinity, proline is one of the most common compatible osmolytes and while several amino acids are known to accumulate in response to osmotic stress, proline appears to be the preferred organic osmoticum in many plants and may have a specific protective role in the adaptation of plant cells to dehydration. For example, in a study of *Triticum aestivum* L. (durum wheat) under salinity stress, Poustini et al. (2007) found a positive correlation between proline levels and osmotic potential, and concluded that proline is an important osmolyte for osmotic adjustment in wheat under water stress. In addition, it has been demonstrated that transgenic tobacco plants with elevated levels of proline biosynthesis show increased tolerance to hyperosmotic stress (Kavi Kishot et. al., 1995), providing further evidence of a cause-and-effect relationship between proline levels and osmotic tolerance. Proline normally accumulates in the cytosol, where it contributes to the cytoplasmic osmotic adjustment in response to water loss without interfering with normal cellular processes and biochemical reactions (Ashraf & Foolad, 2007).

3.2 Proline and cryopreservation

During cryopreservation, plant cells encounter similar problems to those they encounter under freezing conditions in the field. They under go changes in the spatial organization of biological membranes, biochemical and chemical reactions can be retarded, and the status and availability of water can be altered. For these reasons proline is likely to be an effective cryoprotectant for cryopreserved plant cells and tissues.

4. The use of proline as a cryoprotectant

Proline has been used for many years in numerous cryoprotection protocals for the preservation of a wide range of both animal and plant cells and tissues. For example, Li et. al. (2003) investigated the effects of addition of proline, glutamine, and glycine to the Tes-Tris-egg yolk (TTE) freezing medium used for cryopreservation of cynomolgus monkey (*Macaca fascicularis*) spermatozoa. They found that the addition of 5 mM proline, 10 mM glutamine, and 10 or 20 mM glycine to TTE significantly improved post-thaw sperm motility and membrane integrity compared to controls without an amino acid. Of the three amino acids tested proline was effective at the lowest concentration.

Proline has also been found to be useful for the cryopreservation of plant cells, meristems and embryos. Jain et al. (1996) included proline in the cryoprotectant solution as part of a protocol that was used to successfully cryopreserve embryogenic suspension cells of two commercially cultivated aromatic Indica rice varieties using a simple one-step freezing procedure that did not require a controlled-rate freezer. Brison et al. (1995) used a preculture medium enriched with dimethylsulfoxide and proline prior to the cropreservation of *in vitro* grown interspecific Prunus rootstock, Fereley-Jaspi (R). In a study to develop a cryoprotection protocol for highly

freezing sensitive *Begonia* species, Burritt (2008) found that adventitious shoots of the rhizomatous begonia, *Begonia x erythrophylla* were sensitive to dehydration and very sensitive to freezing. While pre-treatment with 0.75 M sucrose significantly increased the percentage of encapsulated shoots surviving dehydration, pre-treatment with sucrose did not afford cryoprotection without prior dehydration.

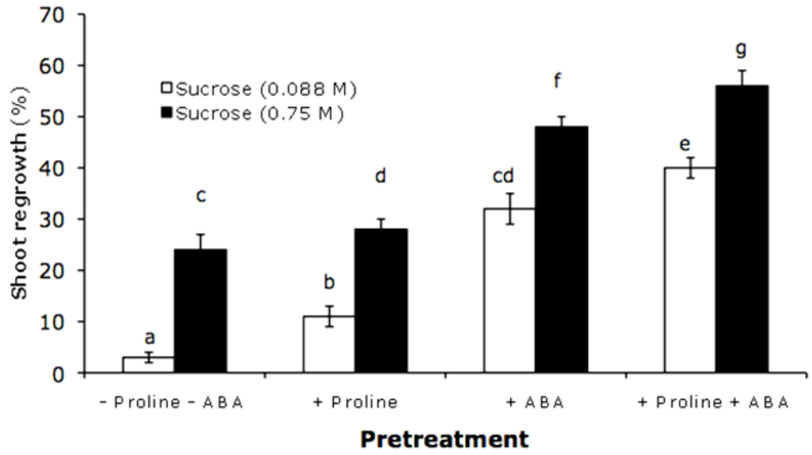

Fig. 3. The percentage of *Begonia x erythrophylla* shoots surviving pre-treatment with ABA (3.8 uM) and/or proline (2.15 mM). The different letters indicate statistically different values at $p < 0.05$ (modified from Burritt 2008).

Addition of abscisic acid (ABA) and proline to the pre-treatment medium significantly improved the percentage of shoots surviving freezing. Pre-treatment of shoots with a medium containing, 0.75M sucrose, 3.8 µM ABA and 2.15 mM proline resulted in greater than 50% of shoots surviving freezing (Figure 3).

Christianson (1998) used a 3-4 day preconditioning treatment using a tissue culture medium supplemented with 10^{-5} M ABA and 100 mM proline to greatly increase survival rates and simplify a protocol for moss cryopreservation. Pretreatment with the combination of proline and ABA was used as part of a cryopreservation protocol that could be used for *Ceratodon purpureus*, *Funaria hygrometrica*, *Physcomitrella patens*, and two species of *Sphagnum*. Cryopreserved cultures remained viable at least one year at -80°C.

In addition to both animal and plant cells, proline has been found to be particularily useful for the cryopreservation of algal cells. Kuwano et al. (2004) found the gametophytic cells of six species of Laminariales, *Laminaria japonica* Areschoug, *L. longissima* Miyabe, *Kjellmaniella crassifolia* Miyabe, *Ecklonia stolonifera* Okamura, *E. kurome* Okamura, and *Undaria pinnatifida* (Harvey) Suringar could be cropreserved using a cryoprotective solution containing ethylene glycol and proline. The cells were suspended in a mixture of ethylene glycol and proline, and slowly cooled to -40°C over a period of 4 h. After a cooling step, the cells were immediately immersed in liquid nitrogen. Viabilities ranged from 36.2% to 67.2%. Nanb et al. (2009) developed a cryopreservation protocol for gametophyte strains of the edible macroalgae *Undaria pinnatifida* (Harvey). Following a pretreatment involving exposure of male and female gametophytes to low levels of light, they used a two-step cooling method with a mixture of cryoprotectants including 10% L-proline and 10% glycerol, before freezing in liquid nitrogen. Gametophyte survival rates were high, ranging from 43-60% for females

and 64-100% for males. The morphology of the sporophytes formed from the cryopreserved gametophytes appeared normal and the authors suggested that this cryopreservation method could be used to preserve culture stocks of *U. pinnatifida* for mariculture.

5. Proline a multifunctional cryoprotectant?

5.1 Possible mechanisms of protection

Because of its ability to act as an osmoprotectant without interfering with normal cellular processes and biochemical reactions proline has been used in a range of different cryopreservation protocols both for animal and plants cells and tissues, however the exact mode by which protection is achieved is still a matter of considerable debate in the scientific literature. Proline could potentially acting as storage reserve of carbon and nitrogen, a compatible osmolyte, a buffer for cytosolic pH, a scavenger of reactive oxygen species (ROS) and as an aid to balancing cellular redox status (Smirnoff & Cumbes 1989; Hare & Cress, 1997). It has also been proposed that proline could act as a molecular chaperone, helping to stabilize the structure of proteins, and as part of the signal transduction chain alerting plant cells to the presence of a stressor and hence triggering adaptive responses (Maggio et al. 2002).

5.2 Proline as an osmolite

The osmoregulatory role of proline in plant cells exposed to hyperosmotic stress has been the subject of numerous studies and under environmental conditions that result in cellular dehydration such as drought, freezing or extreme salinity, it is widely accepted that proline accumulates and acts as a compatible solute helping to protect cells from damage (Heur, 1994). Accumulation of cytoplasmic osmolytes, such as proline, is thought to aid in reducing the cellular water potential to a level below the external water potential, this enables water to move into the cell and be maintained there, while at the same time minimising potentially deleteriously increases in ionic strength. However, there is some debate in the published literature as to whether increased cytosolic levels of free proline has any direct adaptive value (Heur, 1994). While there are many reports of positive correlations between the capacity for proline accumulation and dehydration and cold tolerance (see section 3.1), some researchers still challenge the value of the ability of plant cells to accumulate proline as a positive index for osmotic stress resistance (Heur, 1994 & references therein).

5.3 Proline as precursor for other molecules

It has been suggested that stress-induced accumulation of amino acids like proline may not only have an osmoregulatory role, but that they could also be a mechanism to provide cells with a pool of the precursors required to synthesis other molecules known to be involved in biotic and abiotic stress responses (Sanchez et al., 2008). For example polyamines can be synthesized from arginine or ornithine and ornithine from glutamate, hence the pathways for proline and polyamine biosynthesis are interlinked, and both groups of molecules are important in plant stress responses (Groppa & Benavides 2008). Little is known about the roles of polyamine metabolism in the process of cryopreservation, but Ramon et al. (2002) reported that an increase in putrescine content was positively correlated with the survival rate after simple freezing or after vitrification of banana meristem cultures.

Stored amino acids could also be useful during the recovery process following stress. The accumulation of large cellular pools of amino acids could allow the rapid synthesis of enzymes and the repair of structural proteins, allowing a more rapid recovery of cells following cryopreservation, but this possibility has yet to investigated.

5.4 Proline as an antioxidant

Reactive oxygen species, such as the superoxide anion (O_2•), hydrogen peroxide (H_2O_2), and the extremely reactive hydroxyl radical (•OH) are produced within cells as a consequence of normal metabolic processes, but the production of ROS often increases when cells are under stress (Smirnoff, 1993; Halliwell & Gutteridge, 1999). When ROS are produced at levels high enough to overcome the antioxidant defences that normally control cellular ROS levels, oxidation of DNA, proteins and membrane fatty acids occurs, the latter can result in lipid peroxidation and loss of membrane function (Halliwell & Gutteridge, 1999). Such damage is commonly referred to as oxidative stress (Lesser, 2006; Burritt & MacKenzie 2003; Burritt, 2008). Cryopreservation protocols comprise a number of steps, each of which has the potential to cause stress that could increase ROS production. Recent studies have shown that dehydration and freezing can both lead to increased ROS production and lead to oxidative stress (Feck et al., 2000; Roach et al., 2008). A recent study on oxidative stress and antioxidant metabolism during the cryopreservation of olive somatic embryos demonstrated the importance of oxidative stress and antioxidant metabolism for the successful cryopreservation of plant cells (Lynch et al., 2011).

As mentioned in Section 4 Burritt (2008) found that addition of ABA and proline to the pre-treatment medium significantly improved the percentage of *Begonia x erythrophylla* shoots surviving freezing, this increase in percentage survival was accompanied by a decrease in levels of hydrogen peroxide (Figure 4) and oxidative damage, measured as the levels of lipid peroxides, observed in the shoots immediately following thawing (Figure 5).

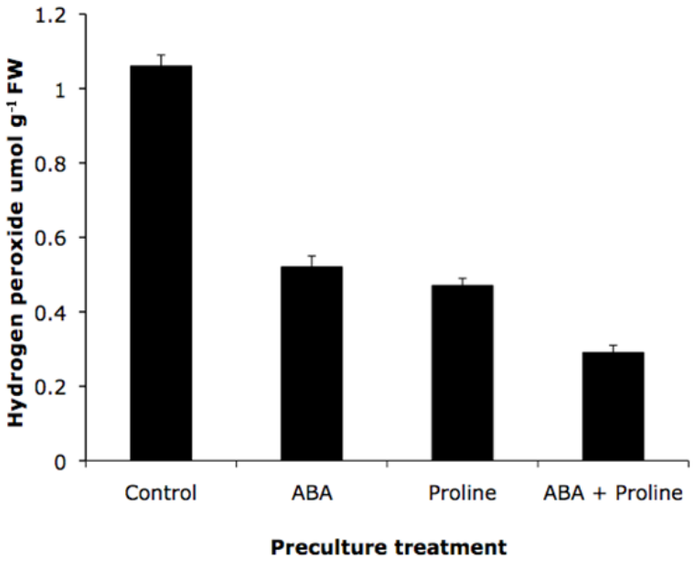

Fig. 4. The influence of pre-treatment with ABA (3.8 uM) and/or proline (2.15 mM) on hydrogen peroxide levels, as determined by Chesseman (2006), in post thaw *Begonia x erythrophylla* shoots cryopreserved as described by Burritt (2008).

Because of its chemical properties proline has a high capacity to quench singlet oxygen and hydroxyl radicals. Pyrrolidine, which forms the 5- membered ring of proline, has a low IP and so proline is able to form a charge-transfer complex, enabling it to quench singlet oxygen effectively. Proline can also react with hydroxyl radicals under hydrogen abstraction forming a stable radical (Matylsik, 2002). Therefore the accumulation of proline to high levels in plant cells under stress or plants cells treated with exogenous proline as part of a cryopreservation protocol could greatly increase the ROS scavenging capacity of said cells and reduce the potential for oxidative damage. In particular, as proline has the potential to reduce ROS levels it could help reduce oxidative damage to vital cellular macromolecules and hence stabilize proteins (Anjum, 2000),) DNA (Iakobashvil, 1999) and lipid membranes (Alia, 1991). The accumulation of proline-rich proteins and particularly proline residues in cellular proteins is thought to provide additional protection against oxidative stress (Matylsik, 2002). The increase in ROS scavenging capacity brought about by increased intracellular proline levels could be a key mechanism by which proline helps reduce the freezing and dehydration associated cellular damage associated with most cryopreservation protocols.

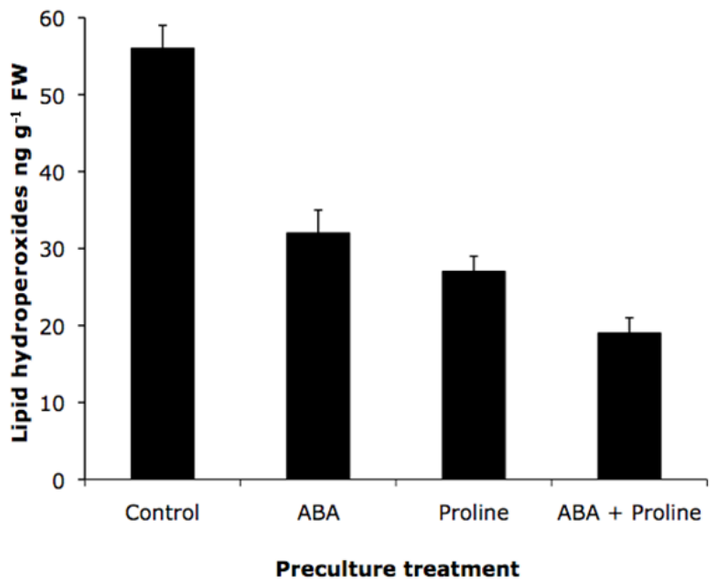

Fig. 5. The influence of pre-treatment with ABA (3.8 uM) and/or proline (2.15 mM) on lipid hydroperoxide levels, as determined by Mihaljevic et al. (1996), in post thaw *Begonia x erythrophylla* shoots cryopreserved as described by Burritt (2008).

Interestingly ABA combined with proline reduced hydrogen peroxide production and oxidative damage, measured as lipid peroxidation, more effectively in post thaw *Begonia x erythrophylla* shoots than ABA or proline alone. Christianson (1998) also found that ABA and proline in combination improved the survival of moss gametophytes following cryopreservation. These results suggest a possible interaction between ABA and proline may exist.

5.5 Is there an inaction between ABA and proline?

Studies have shown a relationship between proline and ABA with respect to cold tolerance (Xi & Li, 1993; Lou & Reid, 1997). In a recent study of maize suspension-cultured cells Chen and Li (2002) showed that an ABA treatment at warm temperatures improved the tolerance of cells to subsequent chilling, and that both ABA-treated and untreated maize cells accumulated proline in response to chilling. Chen and Li also found that ABA-treated cells showed less lipid peroxidation during chilling and unlike untreated cells were able to retain the accumulated proline intracellularly.

In post thaw *Begonia x erythrophylla* shoots ABA combined with proline resulted in much higher shoot survival than pretreatment with ABA or proline alone. Interestingly ABA combined with proline resulted in far higher intracellular proline concentrations (Figure 6). The greater concentrations of proline seen in the combined treatment could be due increased endogenous synthesis of proline, induced by exposure to ABA, combined with uptake of exogenous proline during the pretreatment phase and/or to an ABA induced mechanism that helps reduce proline leakage, but further investigations are required to determine how the combined application of ABA and proline increase shoot survival after cryopreservation.

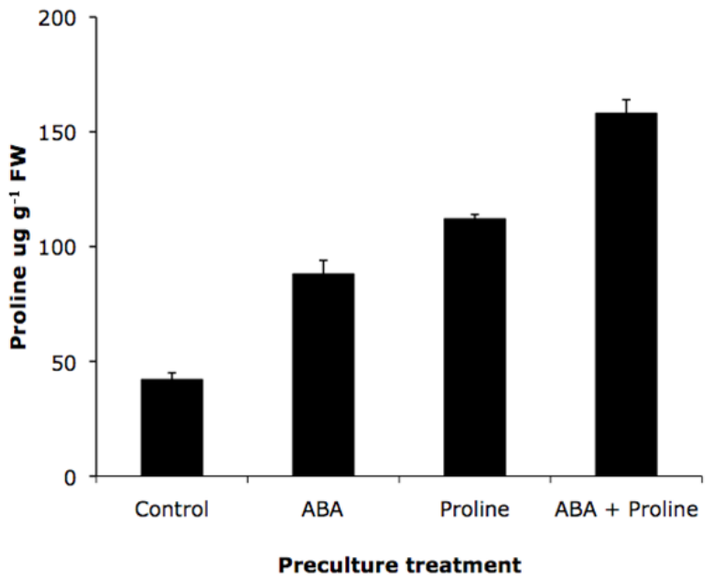

Fig. 6. The influence of pre-treatment with ABA (3.8 uM) and/or proline (2.15 mM) on proline levels, in post thaw *Begonia x erythrophylla* shoots cryopreserved as described by Burritt (2008).

5.5 Proline and direct macromolecule protection

As well as the potential protective mechanisms detailed above, proline has been shown to directly protect key cellular macromolecules, in particular lipid membranes and proteins such as enzymes (Verbruggen & Hermans, 2008). Proline molecules can intercalate between the head groups of membrane phospholipids during freeze-dehydration helping to reduce mechanical stresses in the membranes, or alter the physical properties of membranes

making them less prone to a liquid crystalline-to-gel transition (Hoekstra et al., 2001). It has also been suggested that proline molecules can directly replace missing water molecules between the phospholipids headgroups (Rudolph et al., 1986).

In addition, according to the preferential exclusion hypothesis, proline is one of a group of solutes that, when in aqueous solution, are excluded from contact with the surfaces of proteins and phospholipid bilayers (Arakawa & Timasheff, 1983). Accordingly addition of proline to a solution stabilizes the native structure of protein monomers and protects oligomeric protein complexes from denaturation and dissociation. Rudolph et al. (1986) demonstrated that the activity of the enzyme lactate dehydrogenase could be protected in part during freeze-thaw cycles by increasing the concentration of proline from 0 to 200 mM in the buffer in which the enzyme was solubilised.

5.6 Other mechanisms

There are several other mechanisms via which proline could contribute to over coming the stresses associated with cryopreservation. For example, the accumulation of proline could also be a mechanism to store energy as the oxidation of a single proline molecule can produce up to 30 ATP equivalents (Atkinson, 1971). Replenishment of NADP+ and redox cycling have also been sugested as potential mechanisms associated with stress tolerance, (Hare & Cress 1999), as has a role in stress signal transdution (Hare et al., 1997).

6. Conclusions

While numerous studies have demonstrated that proline can be used to improve the survival of plant cells and organs following cryopreservation, there is little definitive evidence as to the mode of action of proline. More research is required to determine how proline protects plant cells at the cellular level and to determine how other treatments that confer cryotolerance, such as ABA pretreatments interact with proline metabolism and could hence improve the cryotolerance of plant cells. However, despite our lack of knowledge with respect to the mode of action of proline, this amino acid continues to be of great value as a cryoprotectant that can be used with a wide range of cell types from many different organisms.

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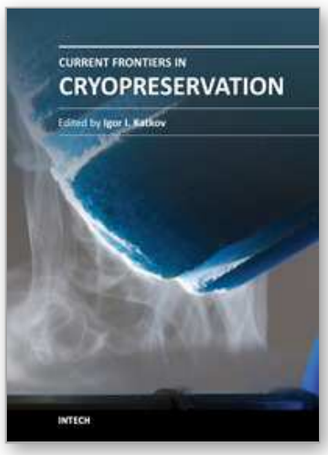

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Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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