Cold-induced changes affect survival after exposure to vitrification solution during cryopreservation in the south-west Australian Mediterranean climate species *Lomandra sonderi* (Asparagaceae).

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Abstract

There is limited knowledge of the effects of exposure to low temperatures in the unique Mediterranean climate plant species of Western Australia. We have thus investigated the effect of low temperature on cryogenic tolerance in *Lomandra sonderi*, an endemic perennial species of southwest Western Australia. *Lomandra sonderi* plants were preconditioned in tissue culture at constant 23 °C (12h light/dark cycle) or alternating 20/-1 °C (16h light and 8h dark cycle). Shoot tips from both conditions were analysed for their phospholipid, sterol and soluble sugar compositions. Shoot tips were also cryoexposed *via* a droplet-vitrification protocol. Survival in both preconditioning regimes for cryoexposed and non-cryoexposed samples was the same, but plants from the 20/-1 °C regime displayed an improved tolerance to the overall cryopreservation process in both cryoexposed and non-cryoexposed samples, thereby eliminating exposure to liquid nitrogen as a primary cause of reduced post-cryogenic viability. Preconditioning of *in vitro* shoots of *L. sonderi* at 20/-1 °C induced significant increases in phosphatidylcholine (from 7.30 ± 3.46 to 22.2 ± 7.80 ng mg^{-1} FW) and increases in several soluble sugars (fructose, galactose, glucose, sucrose) compared to shoots incubated at 23 °C - changes consistent with known cold acclimation responses in plant species generally - but sterol content remained largely unchanged. Analysis of electrolyte leakage in shoot tips from both preconditioning regimes generated a significantly lower LT_{50} value in the 20/-1 °C samples (-5.45 ± 0.53 °C) over the 23 °C samples (-2.5 ± 0.08 °C). Increased tolerance to cryoexposure in *L. sonderi* appears to lie mainly with acclimation-induced changes in membrane composition and promotion of membrane stability and hence increased resistance to freeze damage.

Keywords

Lomandra sonderi, phospholipid, sugar, cold acclimation, electrolyte leakage, cryopreservation

Introduction

Continuous maintenance of valuable germplasm material of propagation-recalcitrant or rare and endangered plants requires considerable resource allocation on the part of conservation and restoration practitioners. Such material requires reliable long-term storage methods while awaiting utilisation in conservation and restoration projects. Plants exhibiting low seed quality, or poor seed germination due to complex seed dormancy are often propagated *via in vitro* cultures of plantlets at considerable expense. Consequently, the ongoing protection of each genetic line in culture is paramount. The high level of manual input involved in a continuous micropropagation enterprise is prone to accidental contamination through operator error and/or equipment failure, while other less common (but potentially catastrophic) hazards such as fire may result in losses of irreplaceable material (Offord et al. 2009). Cryopreservation is an efficient and ultimately safer alternative for the long-term storage and biosecurity of valuable culture lines of plants (coupled with off-site storage). Agricultural, horticultural and endangered plants with low-seed production, complex dormancy or desiccation-intolerance properties that cannot otherwise be stored in conventional seed banks can all benefit from cryogenic storage technology (Kaczmarczyk et al. 2011; Kaczmarczyk et al. 2012; Food and Agriculture Organisation of the United Nations [FAO] 2013).

Cryopreservation involves the storage of a range of plant tissues (meristems, excised zygotic embryos, cell suspensions or callus tissues) at ultra-low temperatures in liquid nitrogen (LN) either at -135 °C in the vapour phase or -196 °C in the liquid phase (Kaczmarczyk et al. 2012). This process halts all cellular metabolic processes and preserves viability over extended periods of time (Kaczmarczyk et al. 2012). However, cryopreservation is still limited by a lack of knowledge of how different plant species respond to the cryoprotective agents (CPAs) or any other pre-cryopreservation treatment (Kaczmarczyk et al. 2012). Each step in the cryopreservation process can potentially impact on the survival of the cryopreserved material for better or worse, depending on the cryo-capability of each species, particularly as regards desiccation tolerance and ability to tolerate CPA toxicity (Arakawa et al. 1990; Sakai et al. 1990; Nishizawa et al. 1993).

The cell membrane is the primary site of cryo-injury when plant tissues are exposed to sub zero temperatures (Steponkus 1984). In addition to intracellular ice formation and the mechanical damage caused by this process, expansion-induced lysis, loss of osmotic responsiveness, membrane fusion and altered osmotic behaviour are other types of low temperature injury related to the cell membrane (Steponkus 1984; Wolfe and Bryant 1999). The preconditioning of plants by exposure to low non-freezing temperatures has been shown to improve survival following cryostorage in various plant species, with studies focusing on agriculturally important species or those native to subtropical or temperate climates, regardless of their ability to cold acclimate under natural conditions (Chang et al. 2000; Leunufna and Keller 2005; Kaczmarczyk et al. 2008; Kushnarenko et al. 2009). Additionally, sub-lethal

exposure to low temperature has been shown to cause changes in the chemical composition of cell membranes that coincides with improved resistance to cryo-injury (Steponkus 1984; Palta et al. 1993; Uemura et al. 1995). However, the cold acclimation ability of endemic Australian plants, such as those from the hot-summer Mediterranean climate regions of southwest Western Australia, has seldom been investigated to the same extent (Funnekotter et al. 2013). Plant species in this region of Australia can indeed be difficult to cryopreserve successfully (Kaczmarczyk et al. 2013).

A commonly used method of determining the relative stability or permeability state of the cell membrane is by the measurement of electrolyte leakage as a consequence of low temperature cellular damage (Campos et al. 2003; Mancuso et al. 2004). Low, lethal temperatures cause a change in the semi-permeable nature of the cell membrane, which results in a large, irreversible loss of electrolytes from plant tissues (Campos et al. 2003; Mancuso et al. 2004). Plants able to acclimate *via* changes that stabilize the cell membrane, can therefore tolerate lower temperatures by making their cell membranes "less leaky", a response that can be easily measured and quantified using a conductivity probe and meter (Maier et al. 1994; Campos et al. 2003). The cell membrane typically consists of a lipid bilayer made up primarily of phospholipids (PLs). The type of PL headgroup and fatty acid (diglyceride) chains, as well as the level of unsaturation in these chains affect the stability, fluidity and permeability of the membrane (van Meer et al. 2008). Major PL headgroup types found in plants include phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidic acid (PA) (Uemura and Steponkus 1994; Uemura et al. 1995). Cold acclimation has shown to increase the concentration of PE and PC with unsaturated fatty acids as well as increased total PL levels, which reduce the occurrence of deleterious membrane phase transitions and improve freeze tolerance (Uemura and Steponkus 1994; Uemura et al. 1995).

Sterols or steroid alcohols are also an integral component of cell membranes that restrict the motion of the PL fatty acyl chains and thus control the fluidity and mobility of the membrane (Hartmann 1998; Dufourc 2008); this also reduces the likelihood of phase separation occurring in the PLs (Dufourc 2008). An increase in membrane sterol concentrations can also bring PL molecules closer together, thus reducing the permeability of the membranes (Demel and De Kruyff 1976). The concentration of different types of sterols also has an effect on cell membranes. β-sitosterol has a greater effect on membrane permeability and ordering than stigmasterol (Schuler et al. 1991; Hartmann 1998). Increased chill tolerance in banana meristems and mung beans has been correlated to an increase in the β-sitosterol to stigmasterol ratio (Guye 1989; Zhu et al. 2006). Intracellular sugars or sugar alcohols also have a stabilising effect on cell membranes (Crowe et al. 1988; Wolfe and Bryant 1999; Turner et al. 2001). Soluble sugars reduce the gel phase to fluid phase transition temperature (T_m) during dehydration of cellular membranes (Wolfe and Bryant 1999; Koster et al. 2000; Lenné et al. 2010). These are known to

occur *via* osmotic and volumetric effects of the sugars on membranes (Lenné et al. 2010). In addition, high intracellular sugar concentrations promote a vitrified state of water during cooling due to the highly viscous nature of their aqueous solutions (Wolfe and Bryant 1999).

Using the south-west Australian endemic *Lomandra sonderi* as a model species (Menon et al. 2012) the overall aim of this study was to understand how low temperature preconditioning treatment might induce cold acclimation and potentially improve post-cryogenic survival in a Mediterranean species that might be expected to have a limited capacity to respond to low temperature acclimation. In this respect, changes to the temperature at which freeze damage occurs as well as the overall membrane PL and sterol and intracellular sugar compositions were determined during this study. These attributes were selected as indicators of cold acclimation in response to low temperature preconditioning due to their role in membrane stability and freeze damage mitigation. We discuss how the changes in composition observed relate to changes in post-cryogenic survival.

Methods

Plant material

In vitro plants of *Lomandra sonderi* were made available from the tissue culture collection at Kings Park and Botanic Garden, Western Australia (originally sourced from BHP Billiton Worsley Alumina Pty. Ltd., Boddington, Western Australia). Shoots of *L. sonderii* were maintained on a basal tissue culture medium (BM), which consisted of half strength Murashige and Skoog (MS; Murashige and Skoog 1962) macro- and micro-elements with 500 µM myo-inositol, 500 µM 4-morpholineethanesulfonic acid, 100 µM NaFeEDTA, 4 µM niacin, 3 µM thiamine-HCl, 2.5 µM pyridoxine-HCl, 0.06 M sucrose, 0.8% (w/v) agar and pH adjusted to 6.5 prior to autoclaving (20 min at 121 °C). BM with 0.2 µM 6-benzylaminopurine (BAP) was used for shoot maintenance and multiplication, and as a pre-culture treatment just prior to cryopreservation. Heat labile plant hormones including gibberellic acid (GA_3) and zeatin (Z) [6-(4-hydroxy-3-methylbut-2-enylamino) purine] were filter-sterilised and added to BM following autoclaving to prepare recovery medium (RM). Maintenance medium (BM + 0.2 µM BAP) was dispensed into 250 ml polycarbonate containers (~40-50ml per container) with polypropylene lids (each with 1 x 9 mm diam. vent covered with 0.2 micron micropore filter) then autoclaved; while RM was autoclaved in 500 ml media bottles and dispensed into sterile 55 mm diam. Petri plates at ~ 10 ml per plate. All media plate pouring and manipulations of *in vitro* plant material (for maintenance and cryopreservation) were carried out under sterile conditions in a laminar flow cabinet.

Temperature preconditioning

L. sonderi plants were preconditioned *in* vitro on BM (+ 0.2 µM BAP) for 3-week incubation

periods using two temperature regimes: 20/-1 °C alternating (20°C during 16 h light period and -1°C during 8 h dark period) and constant 23 °C with a 12 h photoperiod, prior to any cryoexposure experiments. Preconditioning treatments were carried out in temperature controlled incubators with lighting supplied by 36 W cool white fluorescent tubes (Philips Alto; photon flux density of ~30 μmol m^{-2} s^{-1}).

Cryoexposure

Lomandra sonderi shoot tips were extracted from *in vitro* grown shoots and cryostored using the method previously developed by Menon et al. (2012). Shoot tips from the two preconditioning regimes (20/-1 °C and 23 °C) were transferred onto BM containing 0.2 μM BAP for 48 h as a pre-culture step, prior to loading treatment. Shoot tips were placed in ~ 10 ml of a loading solution (LS) comprised of 2 M glycerol and 0.4 M sucrose in liquid BM (as described above, without agar) in a 55 mm sterile plate for 20 min, then treated in PVS2 [30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) in liquid BM with 0.4 M sucrose; (Sakai et al. 1990)] for 10 min at 23 °C. Each shoot tip was individually transferred to a 1 μL droplet of PVS2 on a sterilised aluminium foil strip (~3 mm wide x 20 mm long; 5 shoot tips per strip). Two foil strips were placed in an empty, pre-chilled cryovial, plunged into LN and stored for a minimum of one hour.

Rewarming and recovery

Cryovials containing *L. sonderi* shoot tips were rewarmed by immersion in a 40 °C water bath for 10 seconds. The foil strips with shoot tips were then quickly removed from vials and shoot tips released into washing solution (1M sucrose in liquid BM) and incubated for 20 min at ~23°C. Control shoot tips were transferred directly after PVS2 treatment to this washing solution (no immersion in LN). All shoot tips were then placed onto a recovery medium (solid BM containing 1.0 μM Z and 0.5 μM GA_3) for two weeks in darkness at ~25 °C, and then transferred to 23 °C/12 h photoperiod (as previously described). The survival of shoot tips on RM was determined using visual indicators 6 weeks after the date of rewarming. Green shoot tips showing leaf growth and increase in size were considered to be alive. Other shoot tips that did not show any signs of greening or growth after six weeks were considered non-viable. Percentage survival rates of the shoot tips were then determined.

Electrolyte leakage and LT_{50} calculation following staged cooling

Approximately 2 g of *L. sonderi* leaf material from the two temperature preconditioning regimes were excised per sample. The material per sample was placed in a glass test tube, and 200 μL of deionised water was added to it. A total of 8 samples per preconditioning regime were placed into a cooling bath

(Model 9512 Refrigerating/Heating Circulator, PolySciences, Inc.), preset at -1 °C. The control samples were placed directly on ice. After one hour at -1 °C, the temperature of the cooling bath was gradually reduced at the rate of -1.5 °C h^{-1} to -10 °C, with samples taken out every hour and stored on ice (8 hour procedure). 500 µL of deionised water was added to each sample before storage overnight at 4 °C. After 24 h, 5 mL of deionised water was added to each sample before transferring to a shaker at 100 rpm overnight. 1 mL of each sample was transferred to 10 mL of deionised water and the conductivity of the diluted solution was measured (C_1) using a 900-C Conductivity Meter (TPS Australia). The samples were boiled at 100 °C for 30 min. 1 mL of each boiled sample was then transferred to 10 mL of deionised water and the conductivity of the diluted solution was measured (C_2). The electrolyte leakage during cooling was calculated as a percentage of total electrolyte concentration via the following formula:

$$\% \text{ loss of electrolytes} = (C_1/C_2) * 100$$

To account for the loss of electrolytes for reasons other than freeze damage, this value was then zero corrected via the following formula:

$$\text{Zero correction} = \% \text{ leakage of sample} - \% \text{ leakage of control}$$

These values were then normalized to the highest % electrolyte leakage value, to prevent percentage values above 100%, using the following formula:

$$\text{Normalized value} = ([\text{zero correction}]/[\text{highest \% leakage value}]) * 100$$

The normalized values of percentage electrolyte leakage were used to construct a sigmoidal curve from which the LT_{50} was calculated (the temperature at which 50% of total ion leakage occurs) using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA.

Extraction of sterols and phospholipids

Sterols and phospholipids were analysed as described by Funnekotter et al. (2013). *L. sonderi* shoot tips from the two temperature preconditioning regimes were isolated (9-11 mg, ~12 shoot tips). Shoot tips were homogenized in LN and 1 µg cholestanol and 1 ng 14:0/14:0-PC and 14:0/14:0-PG were added as sterol and PL internal standards. In order to dissolve the samples, 400 µL of methanol was added followed by incubation at 75°C for 20 min. 400 µL of chloroform and 400 µL of water were added to each followed by centrifugation at 10,000 rpm for 3 min. The chloroform phase containing the lipids was

239 then extracted. Solid phase extraction using Supelclean^{TM} LC-Si 1 ml columns (Supelco, Bellefonte,
240 USA) was performed on the chloroform phase. Neutral lipids were extracted using chloroform,
241 glycolipids were extracted using acetone and the PLs were extracted using methanol. The neutral lipid
242 and PL phases were dried under nitrogen gas, and the glycolipid phase was discarded.
243
244 *Derivatisation and gas chromatography mass spectrometry (GC/MS) of sterol phase*
245 A solution of 10% potassium hydroxide (KOH) dissolved in methanol (500 µl) was used to
246 dissolve the extracted neutral lipid phase. This was followed by incubation at 75°C for one hour and then
247 followed by a triple hexane extraction. Sterols were derivatised by incubation in 20 µL methoxypyridine
248 at 65°C with shaking at 750 rpm for one hour and then incubation in 30 µL bis-
249 (trimethylsilyl)trifluoroacetamide (BSTFA) at 65°C with shaking for one hour. An Agilent 7890A GC
250 system coupled to an Agilent 5975C inertXL MSD (Agilent Technologies; Palo Alto, CA, USA) was
251 utilised for the identification of sterols. A Varian VF-5MS column (30 m x 250 µm x 0.25 µm; Agilent
252 Technologies) was used for separation of the samples. A 1 µL splitless injection was used to load the
253 derivatised sterol extracts. The pressure of helium gas was 21.28 psi with a flow rate of 2.34 mol min^{-1}
254 within the column. Temperature of the column was increased from 70 °C to 325 °C. Agilent Technologies
255 MSD ChemStation G1701EA E.02.00.493 was used for identification and quantitation of sterol species.
256
257 *Liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS-MS) of*
258 *phospholipids*
259 100 µL of 50% v/v acetonitrile (in water) was used to dissolve the PL samples obtained from the
260 solid phase extraction. An Agilent ZORBAX 300 Extended-C18 capillary column attached to an Agilent
261 6340 ion trap LC/MS was used for the separation of PLs. A model 1200 capillary pump system from
262 Agilent Technologies (Palo Alto, CA, USA) was used to inject the samples into the column. The method
263 from Funnekotter et al. (2013) modified from Kim et al. (2009) and Min et al. (2010) was used for the
264 runs. The solvents components of the mobile binary phase used were 50% v/v acetonitrile in water (A)
265 and 90:10 (v/v) isopropanol/acetonitrile (B). The solvents contained 0.1% (v/v) formic acid and 0.05%
266 ammonia for positive ion and negative ion detection, respectively. A separation gradient was used to
267 separate the PL species. The gradient equilibrated to 55% solvent B as the column was loaded with the
268 sample. The gradient ramped to 70% solvent B over 40 min and was held at 70% solvent B for an
269 additional 5 min. Re-equilibration occurred *via* a 10 min post-run. A capillary voltage of 3500 V with a
270 nebulizer pressure of 15 psi and dry nitrogen gas flow of 7 L min^{-1} at 325 °C was used for electrospray
271 ionisation. The ion scanning range for the tandem MS runs was 170-860 amu and 220-950 amu for the
272 positive and negative modes, respectively. The scanning range for the quantitative MS runs was 620-860

amu for both the positive and negative modes. Agilent Technologies Data Analysis for 6300 Series Ion Trap LC/MS v3.4 (Build 175) was used for identification and quantification of PL species. Calibration standards of 18:0/18:0-PA, 18:0/18:0-PC, 18:0/18:0-PE, 18:0/18:0-PG, 18:1/18:1-PI and 16:0/16:0-PS (Avanti Polar Lipids, Alabaster, AL, USA) were used to determine the quantities of species identified.

Double-bond Index (DBI)

The DBI was calculated for the fatty acyl chains of the PLs detected, with a formula modified from (Quartacci et al. 2001):

$$DBI = \frac{[(1 \times \%\text{monoenes}) + (2 \times \%\text{dienes}) + (3 \times \%\text{trienes}) + (4 \times \%\text{tetraenes} + (5 \times \%\text{pentaenes})]}{\Sigma\% \text{ saturated fatty acids}}$$

Extraction of sugars

L. sonderi shoot tips from the two temperature preconditioning regimes were isolated (9-11 mg). Shoot tips were homogenised in LN and sugars were extracted in 500 µL 20:2:0.2 methanol/water/ribitol solution. Ribitol was used as an internal standard with a concentration of 0.2 mg mL^{-1}. Samples were then incubated at 75°C for 20 min. 100 µL of the samples were then transferred to GC vials and dried in a Speed Vac®.

Derivatisation and GC/MS of sugars

Derivatisation of sugars was also conducted in methoxypyridine and BSTFA as per the sterols. Sugars were also identified using the Agilent 7890A GC system coupled to the Agilent 5975C inert XL MSD as per the sterols. Flow rate of helium gas in the column was modified to 1 mol min^{-1} at 8.8085 psi. All other parameters remained unchanged from the sterol runs. Fructose, galactose, sucrose, glucose, sorbitol, trehalose and raffinose (Sigma-Aldrich, St. Louis, MO, USA) were used as calibration standards for the quantification of data. Agilent Technologies MSD ChemStation G1701EA E.02.00.493 was used for identification and quantitation of sugar species.

Statistics

The cryoexposure experiments were performed using six replicates (6 x 15 shoot tips) for each treatment. The cooling bath experiments were performed with three replicates per treatment. The sterol and sugar experiments were performed with three replicates each (three times ~12 shoot tips) per treatment. PL experiments were performed with four replicates (four times ~12 shoot tips) per treatment. The Shapiro-Wilk test was used to check normality of data. No transformations were applied if data was

306 statistically normal. Statistical comparisons were made using independent t-tests for two-condition
307 comparisons using SigmaPlot for Windows Version 11.0 Build 11.0.0.77 (Systat Software, Inc. Chicago,
308 IL, USA).
309
310 **Results**
311 *Cryoexposure*
312 As previously determined (Menon et al. 2012), there was a significant difference in post-
313 cryoexposure survival of shoot tips derived from the two preconditioning temperatures investigated (23
314 and 20/-1 °C). Plants that had undergone the 20/-1 °C preconditioning had shoot tip survival of ~32.2%
315 for samples not exposed to LN (-LN) and samples exposed to LN (+LN). Samples from the 23 °C
316 preconditioning regime showed only ~14.4% survival for both -LN and +LN samples (Menon et al.
317 2012). However, within each regime, there was no significant difference between -LN and +LN samples
318 (Menon et al. 2012).
319
320 LT_{50}
321 Alternating temperature preconditioning of plants resulted in altered electrolyte leakage readings
322 from shoot samples, indicating that a change in cell membrane characteristics had occurred. The mean
323 LT_{50} value of shoot cultures incubated at 23°C was **-2.50 ± 0.08** °C compared to **-5.45 ± 0.53** °C for
324 shoots incubated at 20/-1 °C.
325
326 *Phospholipid analysis*
327 PC and PE accounted for the highest content of PLs in both preconditioning regimes (over 97%).
328 The remaining PL classes (PA, PG, PI and PS) were less abundant, being less than 3% of the total PLs
329 detected. There was a significant increase ($P < 0.05$) in the amount of PC in shoot tips from the 20/-1 °C
330 preconditioning regime compared to those from the 23 °C preconditioning regime. The amounts of all the
331 other PLs detected, particularly PE, did not significantly change between temperature treatments (Fig. 1).
332 Due to the large increase in PC content, the total PL content in shoot tips from the 20/-1 °C
333 preconditioning (25.0 ng mg^{-1} FW) was significantly higher ($P < 0.05$) than in those from the 23 °C
334 preconditioning (9.8 ng mg^{-1} FW). See Table S1 in Supporting Information for content of individual PL
335 classes measured.
336 A total of 51 different PL species were identified and quantified (see Table S2 in Supporting
337 Information). The widest range of PL species was detected in PC and PE, with only one type of PA
338 species detected. Table 1 reports the amounts of each individual fatty acyl chain type detected in the PLs.
339 Across both preconditioning regimes, palmitoleic acid (16:1) was the most common fatty acid detected,

constituting 20.3% and 18.7% of all fatty acyl chains detected in the 23 and 20/-1 °C samples, respectively (Table 1). The other fatty acyl chains found in high amounts in the 23 °C samples were oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3), all of which were present in significantly higher amounts in the 20/-1 °C samples (Table 1). Large, significant increases were also seen in fatty acyl chains 16:0, 18:0, 18:4, 20:2, 20:3 and 20:4 in the 20/-1 °C samples. The amount of C16 chains of the PLs had the largest increase after preconditioning, rising by 333% (Table 1).

Table 2 shows a comparison of the degrees of saturation and unsaturation in fatty acids between preconditioning regimes. There were large, significant increases in both the saturated and the unsaturated monoenes, dienes, trienes and tetraenes in the 20/-1 °C samples (Table 2). There was also an increase in pentaenes but this was not statistically significant. The associated DBIs were computed, revealing a large (50%), significant drop in DBI after preconditioning at 20/-1 °C (9.93 ± 0.81) compared to the 23 °C regime (20.32 ± 1.23).

Sterol Analysis

Cholesterol, stigmaterol and β-sitosterol were the three sterols detected. Any other sterol types (such as campesterol) were either present in trace quantities or non-existent. β-sitosterol accounted for the highest content of sterols in both preconditioning regimes. There was no significant difference in the content of any of the three sterol types detected between the two temperature pre-treatments.

The total content of sterols measured for the 23 °C samples (383.9 ± 120.6 ng mg^{-1} FW) was ~8.5% higher than the 20/-1 °C samples (353.7 ± 80.6 ng mg^{-1} FW), but was not significantly different. See Table S3 in Supporting Information for content of individual sterol types measured.

Soluble Sugar Analysis

There were seven types of sugar identified in the shoot tips. Fructose, galactose, sucrose and glucose were the main sugars present (>30 ng mg^{-1} FW) and their concentrations were significantly different between the two preconditioning regimes (Fig. 2). The other three sugars identified (sorbitol, trehalose and raffinose) were detected in significantly smaller amounts (<10 ng mg^{-1} FW) (Fig. 2). See Table S4 in Supporting Information for content of individual sugar types measured.

The total sugar contents in the two preconditioning regimes investigated showed large variations ($P < 0.001$). The total sugar content of the 20/-1 °C samples (310.1 ± 65.3 ng mg^{-1} FW) was ~15 times higher than that of the 23 °C samples (20.6 ± 8.4 ng mg^{-1} FW).

Discussion

Overall, the preconditioning treatment resulted in substantial increases in sugar levels and PC content of shoot tissues, accompanied by a two-fold improvement in the ability of shoot tips to survive desiccation. In a previous study following preconditioning at 20/-1 °C shoot tips of *L. sonderi* showed an optimal mean survival of 32.2 ± 11.5% following cryopreservation (Menon et al. 2012). In comparison, only 14.4 ± 2.7% of shoot tips derived from the 23 °C preconditioning treatment survived after the same cryogenic treatment (Menon et al. 2012). The mean survival rates of the -LN controls were very similar to the corresponding mean +LN survival rates in shoot tips with the same preconditioning regime (Menon et al. 2012), suggesting that factors other than the cryopreservation protocol used (which was identical apart from the LN step) are responsible for the differences in survival observed. As previously determined (Menon et al. 2012), the lack of difference in survival between +LN and -LN samples could be attributed to the effective elimination of ice formation through the use of CPAs. Any reduction in survival would have therefore not been directly attributable to the LN immersion step, but rather to other processes such as toxic or desiccation stresses experienced by the plant material during the preparatory steps leading to cryopreservation. To temper these deleterious effects, cold acclimation stimulates plants to develop tolerance mechanisms that enhance their capacity to survive adverse conditions such as desiccation, to which plant tissues are subject during the cryogenic steps leading up to LN immersion (Thomashow 1999). These tolerance mechanisms arise primarily *via* improved stability of cell membranes and accumulation of sugars (Thomashow 1999). Sugar accumulation is one of the mechanisms of desiccation tolerance in 'resurrection plants', such as *Craterostigma plantagineum,* as well as changes to phospholipid metabolism due to differential activation of phospholipase D genes (Bartels and Salamini 2001). Similar families of genes responsible for related processes, such as sugar transporters, sugar synthases and LEA proteins are upregulated as a response to both cold and desiccation stresses (Ingram and Bartels 1996; Thomashow 1999; Chinnusamy et al. 2007). It is therefore reasonable to postulate that improving the tolerance of a plant to one kind of stress, i.e. cold stresses, *via* preconditioning, would also improve tolerance to other stresses such as desiccation.

The LT_{50} values for the 23 °C and 20/-1 °C preconditioning regimes differed significantly. Similar findings were reported by Mancuso et al. (2004), who investigated the freezing tolerance of the Australian genera *Grevillea* and *Callistemon* using a similar electrolyte leakage method and determined that plants that were acclimated naturally to cold winter conditions exhibited significantly lower LT_{50} values. According to the plant hardiness map of Australia, divided into seven zones (Dawson 1991), the two *Grevillea* species studied by Mancuso et al. (2004; Table 3) grow in Northwest Western Australia (near Shark Bay), i.e. zone 5, with minimum temperatures during winter from 5 °C to 0 °C, and the four *Callistemon* species grow in Eastern New South Wales, a zone 3 region, with minimum temperatures

during winter from 0 °C to -5 °C. *Lomandra sonderi* naturally grows in southwest Western Australia, a zone 3 region, with similar winter conditions to the *Callistemon* species. Nevertheless, a major difference between both studies is that *L. sonderi* cultures were acclimated *in vitro* under controlled laboratory conditions intended to simulate natural average winter conditions, as opposed to the acclimation of pot-grown plants tested by Mancuso et al. (2004), which were maintained in greenhouses during winter. Regardless of the differences in methodology, Table 3 shows that the LT_{50} values of non-acclimated and acclimated *L. sonderi* plant material are not greatly different from either *Callistemon* or *Grevillea* species, as described by Mancuso et al. (2004).

Cold acclimation is, however, known to be more effective in species that naturally grow in low average temperature climates than those from warmer origins (Flint 1972). For instance, the electrolyte leakage analysis of xylem tissue of evergreen Peach (*Prunus persica* [L.] Batsch) sampled in May (i.e. non-acclimated or deacclimated) generated an LT_{50} value of -3.0 compared to samples taken in January, (i.e. cold acclimated) which had an LT_{50} value of -17.0 °C (Arora et al. 1992). The plants were grown in Kearneysville, West Virginia, USA, which has an average annual minimum temperature of -17.8 °C to -20.6 °C (USDA 2012). This indicates that although cold acclimation can occur in species from warmer Mediterranean climates, the degree of acclimation possible appears to be far lower than in species from much colder climatic regions. Our findings with *L. sonderi* tend to reflect this difference.

The lower LT_{50} values for *L. sonderi* in the 20/-1 °C regime are strong indicators of reduced membrane permeability and improved membrane stability, which may partly explain the improved cryogenic tolerance of shoot tips following a suitable acclimation treatment, as found for other species (Flint et al. 1967; Thomashow 1999; Campos et al. 2003; Mancuso et al. 2004).

PLs are the major component of the plasma membrane (van Meer et al. 2008). The increase measured in the total PL content in the 20/-1 °C preconditioning samples was largely due to the large (two-fold) increase in the amount of PC (Fig. 1). Funnekotter et al. (2013) observed a similar trend in the PC content of the Australian species they studied where it rose by 77% in *Grevillea scapigera* and by 65% in *Loxocarya cinerea*, after preconditioning at 20/10 °C for three weeks. This suggests that preconditioning using low temperature regimes seems to increase PC content to stabilise cell membranes as a mechanism of chill tolerance. It is known that strong hydrating PL classes like PC are more likely to retain a lamellar phase in the cell membrane as compared to PL classes like PE, which are less hydrating and tend to form hexagonal II phases instead (Sen and Hui 1988). The total amount of PE, which is substantially lower than that of PC, remained unchanged in the two preconditioning regimes. These findings agree with the model of strongly hydrating PL classes, such as PC, which are more likely to maintain a functional liquid crystalline (lamellar) phase during cooling. Membranes containing higher amounts of PC are therefore more likely to withstand the stresses experienced during the entire

cryopreservation process, which is reflected in the increase observed in both cryopreserved (+LN) and control (-LN) shoot tip survival.

The gel-to-liquid crystalline transition temperature, i.e. the temperature at which the gel phase of a PL bilayer melts to form a lamellar phase, is affected by the fluidity of the membrane, which in turn affects its stability. The strength of attractive van der Waals forces between lipid molecules largely controls the phase behaviour of lipid bilayers. These forces are affected by the length and saturation of fatty acid tails of PLs. Longer acyl chains result in higher transition temperatures and reduced fluidity; conversely, higher degrees of unsaturation result in lower transition temperatures and increased fluidity (Silvius 1982). Interestingly, recent studies have revealed that increases in unsaturation in model cell membranes increase resistance to the deleterious effects of CPAs (Hughes et al. 2012; Malajczuk et al. 2013) We would, therefore, expect to see a rearrangement in the composition of fatty acyl chains of PLs after 20/-1 °C preconditioning, as the plant attempts to increase the fluidity (and, hence, lower the transition temperature) of its cell membranes as a stress response, either by accumulating more short-tailed fatty acids or by increasing the degree of unsaturation. Indeed, the largest increase was seen in 16C chains in the PLs detected (increased by 333%), followed by 18C chains (increased by 195%) and then 20C chains (increased by 150%; no significant increases were detected in 20:0, 20:1 and 20:5 fatty acyl chains). It would appear that an accumulation of PLs containing shorter fatty acyl chains is favoured during the cold acclimation of *L. sonderi*.

The DBI is a measure of the overall degree of unsaturation in fatty acyl chains. Increases in DBI indicate a higher degree of unsaturation, which in turn mean increased disorder in the PL bilayer structure due to the increase in the number of kinks in the chains, which consequently leads to increased fluidity (and reduced transition temperature) (Silvius 1982; Nishida and Murata 1996). Higher DBI has been related to stress tolerance in other plants (Nishida and Murata 1996). However, in the case of *L. sonderi* a large 50% *decrease* in DBI was observed after preconditioning at 20/-1 °C (9.93 ± 0.81) compared to preconditioning at 23 °C (20.32 ± 1.23). This trend was similarly observed by Funnekotter et al. (2013) in *Loxocarya cinerea*, which had a significantly lower DBI after preconditioning using an alternating 20/10 °C regime, compared to preconditioning using a constant 23 °C regime. Zhu et al. (2006), who utilised a sucrose-pretreatment regime to improve cryoexposure tolerance in different banana varieties, found a significant decrease of DBI in PL fatty acids in the pretreated samples and simultaneously observed significantly improved cryogenic survival as a result of this pretreatment. It becomes clear that the loss of fluidity due to the decrease in DBI is counteracted by the accumulation of short, saturated fatty acyl chain PLs, in particular the more strongly hydrating PC, as discussed above. The level of contribution of each factor to membrane fluidity is, however, unknown and merits further research.

475 Membrane sterols are known to improve membrane stability *via* ordering of PLs (Demel and De
476 Kruyff 1976), which can also protect membranes against damage by cryosolvents such as DMSO and
477 polyols (Hughes and Mancera 2013; Hughes et al. 2013). The sterols identified in shoot tips of *L. sonderi*
478 include stigmasterol, cholesterol and β-sitosterol. There was no statistically significant change measured
479 in total sterol content across the two preconditioning regimes. Similarly, O'Neill et al. (1981) and
480 Funnekotter et al. (2013) detected no changes in free sterols in *Fragraria virginiana* and *Arabidopsis*
481 *thaliana,* in their respective studies, after cold acclimation. This may not hold true for all plant species,
482 however, as Funnekotter et al. (2013) also found that *G. scapigera* and *L. cinerea* showed significantly
483 higher amounts of sterols after preconditioning at 20/10 °C (69% and 88% increases, respectively). This
484 suggests that, in the case of *L. sonderi*, membrane stability and/or permeability is not necessarily
485 regulated exclusively by or reliant on changes in sterol content, at least in response to the temperature
486 range treatments in this study.
487 Sugars are known to have a stabilising effect on cell membranes and promote the vitrification of
488 water at low temperature (Wolfe and Bryant 1999). Preconditioning at 20/-1 °C largely increased the total
489 content of sugar species in shoot tips of *L. sonderi* compared to the total content of sugars after 23 °C
490 preconditioning. This may be due to the physiological responses to cold stress when exposed to low
491 temperature in the 20/-1 °C preconditioning regimes. Increased intracellular sugar content may be a stress
492 response that favours the vitrification of water (and hence the reduction of ice formation). In addition, the
493 increased solute potential within the cells as a consequence of the increased sugar concentration would
494 greatly reduce the desiccation effects of the cryopreservation processes. This can be associated with the
495 greater survival rates that were recorded in both the cryopreserved (+LN) and control (-LN) shoot tips
496 exposed to 20/-1 °C preconditioning. The large change in total sugar content was caused primarily by
497 significant increases in glucose, fructose, galactose and sucrose, with minor, insignificant changes in
498 trehalose, raffinose and sorbitol (which were detected in relatively small amounts). These changes
499 indicate a robust response to the low temperature preconditioning regimes. This was similarly observed
500 by Funnekotter et al. (2013) in *G. scapigera* and *L. cinerea*, which showed increases in total sugar content
501 after preconditioning at 20/10 °C. These observations relate to previous findings, which showed an
502 increase in sugar content as a survival response to abiotic stresses (Sasaki et al. 1996, Zhu et al. 2006).
503 The preconditioning regimes used in this study caused several significant changes to the plant
504 shoot tip cells that are likely to be related to the improvement in post-cryoexposure survival. Changes to
505 membrane composition, and by extension, its stability, as demonstrated by electrolyte leakage and
506 PL/sterol analysis, appear to be involved in the reduction of freeze, osmotic and toxic stresses (Menon et
507 al. 2012).

Studies describing acclimation effects on cryostorage success have been published with temperate and sub-tropical species (Chang et al. 2000; Leunufna and Keller 2005; Kaczmarczyk et al. 2008; Kushnarenko et al. 2009), however the number of studies on Mediterranean spp. especially Australian taxa is very limited by comparison. Indeed, given that five of the 34 internationally recognised biodiversity hotspots are found in small but floristically diverse Mediterranean climate regions including southwest Western Australia, South Africa's Cape floristic region, the European Mediterranean basin, the Californian Floristic Province and the Chilean Matorral it is imperative the underlying physiology of plants from these environments be better understood as a means to aid and enhance current conservation measures (Myers et al. 2000; Mittermeier et al. 2005). It might be argued that temperate or subtropical species are 'related enough' to Mediterranean species in terms of climatic extremes (particularly temperature) to void claims of any discernable physiological differences, however in the context of south-west Australian taxa the type of hot-summer Mediterranean climate is not directly comparable to classical temperate or sub-tropical climes. In particular, summer temperatures can be extreme and drought frequent, unlike most 'temperate' climates; with short cool wet winters this is again quite different from sub-tropical climates with warm dry 'winters' and hot wet summers (McKnight and Hess 2000). Therefore it is logical to expect that the adaptive physiology of south-west Australian Mediterranean plants would also express a different range (and possibly intensity) of responses to acclimation treatments compared to temperate or sub-tropical taxa. From this perspective while the current study reflects some subtle differences in adaptive physiology for acclimated *L. sonderi* plants, it also illustrates several key similarities to other studies on cold acclimation in species from more temperate northerly regions, suggesting that basic acclimation responses are conserved but adaptations are likely to have evolved in response to unique local environmental stresses.

The information gained on the effects of preconditioning with respect to changes in the membrane PL and cell sugar contents provide an excellent framework for further research on membrane stability and its relation to improved stress tolerance. Overall this study provides valuable insight into the underlying processes governing effects of low temperature preconditioning on post-cryogenic survival of native Australian plant species and enhances our current cryogenic approaches for the long term storage of valuable and endangered plant germplasm, as well as other plant material currently maintained under tissue culture conditions used for post-mining restoration.

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706 **Supporting Information**

707

708 **Table S1.** Phospholipids (ng mg^{-1} FW) detected in *Lomandra sonderi* shoot tips after temperature
709 preconditioning at 23 °C and 20/-1 °C for 3 weeks.
710 **Table S2.** Identified phospholipid species (ng mg^{-1} fresh weight ± SD) found in *Lomandra sonderi* shoot
711 tips after preconditioning at 23 °C or 20/-1 °C for 3 weeks.
712 **Table S3.** Sterols (ng mg^{-1} FW) detected in *Lomandra sonderi* shoot tips after temperature
713 preconditioning at 23 °C and 20/-1 °C for 3 weeks.
714 **Table S4.** Sugars (ng mg^{-1} FW) detected in *Lomandra sonderi* shoot tips after temperature
715 preconditioning at 23 °C and 20/-1 °C for 3 weeks.
716

717 **Figure Legends**
718 **Fig. 1** Content of phospholipid (PL) classes (ng mg^{-1} fresh weight) analysed in *Lomandra sonderi* shoot
719 tips exposed to three-week preconditioning at 23 °C or 20/-1 °C. Bars represent average ± SE of four
720 replicates per treatment assessed. Bars labelled with different letters differ significantly when compared
721 across treatments ($P < 0.05$). PC - phosphatidylcholine, PE - phosphatidylethanolamine, PA -
722 phosphatidic acid PG - phosphatidylglycerol, PI - phosphatidylinositol, PS - phosphatidylserine
723

724 **Fig. 2** Content of each sugar (ng mg^{-1} fresh weight) analysed in *Lomandra sonderi* shoot tips exposed to
725 three-week preconditioning at 23 °C and 20/-1 °C. Bars represent average ± SE of three replicates of
726 treatments assessed. Bars labelled with different letters differ significantly when compared across
727 treatments ($P < 0.05$)
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740 **Figure 1.**

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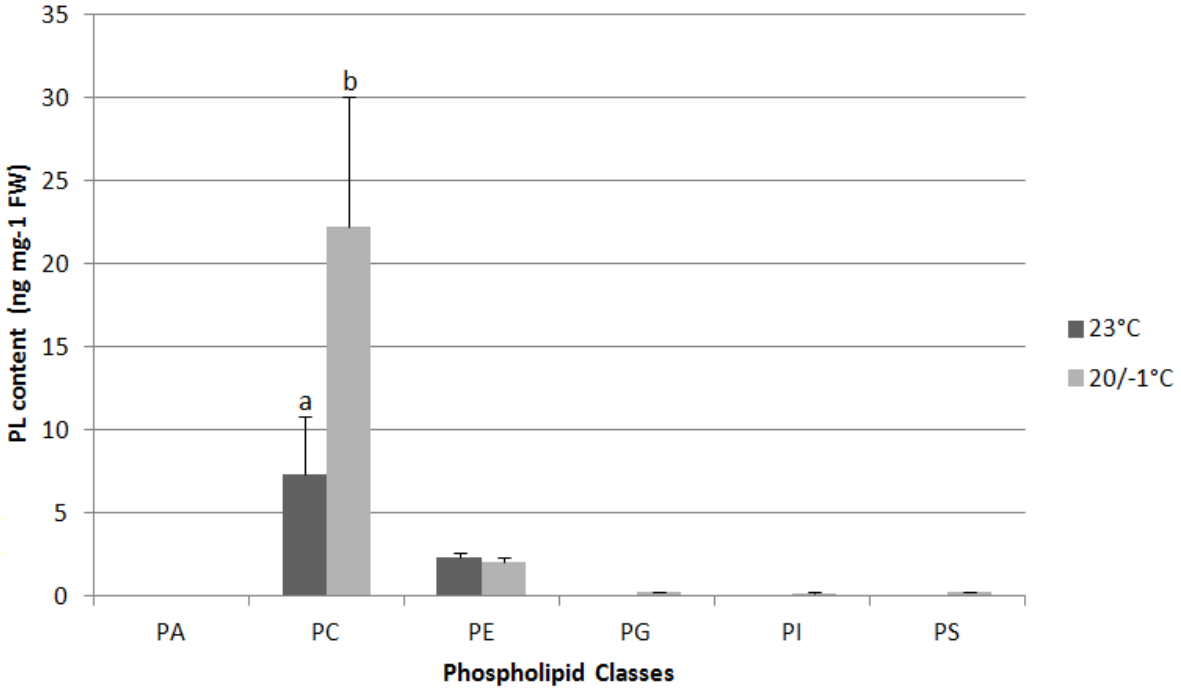

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744 **Figure 2.**

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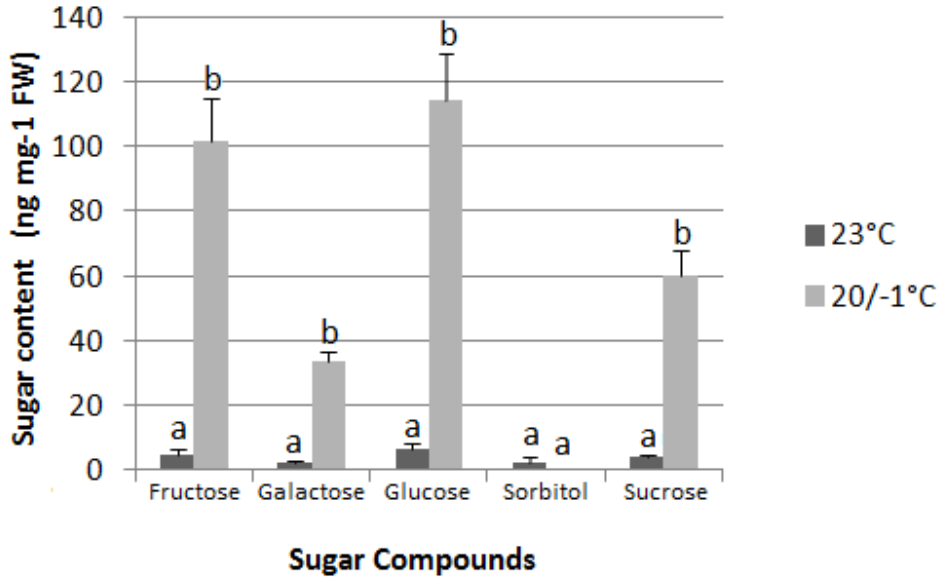

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