The effect of carbohydrates and osmoticum on storage reserve accumulation and germination of Norway spruce somatic embryos

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Received 10 December 2012; revised 22 January 2013

Somatic embryogenesis (SE) represents a useful experimental system for studying the regulatory mechanisms of embryo development. In this study, the effect of carbohydrates and osmoticum on storage reserve accumulation and germination of Norway spruce (*Picea abies* (L.) Karst) somatic embryos were investigated. Using time lapse photography, we monitored development from proliferation of proembryogenic masses (PEMs) to maturation of somatic embryos in two *P. abies* cell lines cultured on two maturation treatments. A combination of sugar assays, metabolic and proteomic analyses were used to quantify storage reserves in the mature somatic embryos. The maturation treatment containing a non-permeating osmoticum, polyethylene glycol (PEG, 7.5%) and maltose (3%) as the carbohydrate gave significantly high maturation and low germination frequencies of somatic embryos compared to the treatment with only 3% sucrose. Somatic embryos treated with 3% sucrose contained high levels of sucrose, raffinose and late embryogenesis abundant (LEA) proteins. These compounds are known to be involved in the acquisition of desiccation tolerance during seed development and maturation. In addition the sucrose treatment significantly increased the content of starch in the somatic embryos while the maltose and PEG treatment resulted in somatic embryos with a high content of storage proteins. The high levels of sucrose, raffinose and LEA proteins in the embryos treated with 3% sucrose suggest that sucrose may improve the germination of somatic embryos by promoting the acquisition of desiccation tolerance.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ppl.12039

Abbreviations – ACN, acetonitrile; DW, dry weight; ESI, electrospray ionization; FW, fresh weight; FA, formic acid; GC/TOFMS, gas chromatograph time-of-flight mass spectrometer; LEA, late embryogenesis abundant; NCBI, national center for biotechnology information; NIST, national institute of standards and technology; OPLSDA, orthogonal projections to latent structures discriminant analysis; PCA, principal component analysis; PEG, polyethylene glycol; PEMs, proembryogenic masses; PGRs, plant growth regulators; SE, somatic embryogenesis; TFA, trifluoroacetic acid.

Introduction

Norway spruce is the dominant native species in Sweden accounting for 42% of the standing volume of forest trees and 50% of forest products (Statistical Yearbook of Forestry 2011). Traditionally, Norway spruce is regenerated using seeds and vegetatively propagated using cuttings (Svobodova et al. 1999). Somatic embryogenesis represents an important method for clonal propagation of economically important conifers including Norway spruce (Nehra et al. 2005, Bonga et al. 2010) and provides an *in vitro* experimental system for studying regulatory mechanisms of embryo development (Businge et al. 2012). In conifers, SE consists of sequential steps starting with; induction of embryogenic cultures followed by proliferation of PEMs, embryo maturation and germination (Bozhkov et al. 2002, Pullman et al. 2003). Induction and proliferation occur with auxin and cytokinin as the plant growth regulators (PGRs) while withdrawal of both PGRs prompts differentiation of early somatic embryos from PEMs. To promote further development and maturation, early embryos are transferred to medium supplemented with ABA (Bozhkov et al. 2002).

Besides the PGRs, additional components of the medium include; amino acids, carbohydrates, osmoticum, NH_4^+ and NO_3^- (von Arnold and Eriksson 1977). L-glutamine is commonly used as the organic nitrogen source (Franklin and Dixon 1994). Glutamine provides nitrogen in a reduced form that can readily be assimilated using less energy unlike the inorganic nitrogen sources (Canovas et al. 2007). Ammonium (NH_4^+) and nitrate (NO_3^-) are used as inorganic nitrogen sources and for regulating the pH of the medium (Ivanova and Staden 2009). The importance of nitrogen metabolism during embryo development has previously been demonstrated. In White spruce (*Picea glauca*), a shift in amino acid metabolism during somatic embryo development was shown to involve increments in glutamine, glutamate and arginine which are the major components of seed storage proteins in conifers (Joy et al. 1997). Additionally, a mutation in the *Arabidopsis thaliana* nitrate transporter gene (*NRT1.6*) resulted in development abnormalities in the form of excessive cell division and loss of turgidity in the suspensor cells of embryos (Almagro et al. 2008).

Carbohydrates such as sucrose are mostly used as exogenous sources of carbon during proliferation and maturation of somatic embryos in conifers (Schuller and Reuther 1993, Lipavská and Konrádová 2004). Existing evidence suggests that carbohydrates are involved in the regulation of gene expression and osmotic environment during somatic embryo maturation. Somatic embryos of oil palm (*Elaeis guineensis*), cultured in the presence of sucrose were found to have increased transcript levels of the *GLO7A* gene which encodes a 7S globulin storage protein (Morcillo et al. 2001). Moreover, osmotic pressure induced by 6% sucrose resulted in the increase of mature somatic embryos in Black spruce (Tremblay and Tremblay 1995). During maturation, ABA and osmotic stress are used to prevent precocious germination and promote the deposition of storage reserves in the somatic embryos (Kermode 1990). ABA prevents cleavage polyembryony permitting development of normal somatic embryos with separated cotyledons (Gupta et al. 1991). Osmotic stress is commonly created by increasing the osmotic concentration of medium using permeating osmoticum such as sucrose or non-permeating osmoticum such as polyethylene glycol (PEG) (Attree et al. 1991). Osmoticum has been shown to induce important changes in gene expression during somatic embryo development. In *P. glauca*, somatic embryos treated with PEG, were found to have high transcript levels of glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 1.4.1.13) genes, which are involved in nitrogen metabolism and deposition of storage protein (Joy et al. 1997, Stasolla et al. 2003).

Carbohydrates and osmoticum are important compounds for maturation of somatic embryos in both gymnosperms and angiosperms (Morcillo et al. 2001, Stasolla et al. 2003). During maturation, embryos accumulate storage reserves including; carbohydrates, proteins and lipids. The reserves are used for inducing desiccation tolerance and providing nutrients during embryo germination and plantlet growth (Crowe et al. 1992, Coelho and Benedito 2008). In the present study, we have investigated the biochemical effects from carbohydrates and osmoticum present during early embryo development to the subsequent accumulation of storage reserves and germination. Two cell lines of *P. abies* cultured on two maturation treatments containing: (i) 3% sucrose; (ii) 3% maltose and 7.5% PEG were monitored by manual time lapse photography to monitor development from proliferation of PEMs to maturation of somatic embryos. A combination of sugar assays, metabolic and proteomic analyses were used to quantify storage reserves in the mature somatic embryos. The results suggest that sucrose improves the germination of somatic embryos by promoting the acquisition of desiccation tolerance.

Materials and methods

Plant material and culturing conditions

Two embryogenic cell lines; 09.77.17 and 09.77.03 of *P. abies* were used in this study. Both cell lines were established in 2009 from seeds of elite crossings at the Forest Research Institute in Sweden (Skogforsk). For each cell line, 0.15 g of embryogenic tissue were sub-cultured with a two week interval on solidified half-strength LP medium supplemented with 9.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4 μM N^6-benzyladenine (von Arnold and Eriksson 1981). Differentiation of early somatic embryos from PEMs was stimulated by transferring the cultures to half-strength LP medium lacking PGRs for one week. Promotion of late embryo development and maturation was done by transferring the cultures to DKM medium (Krogstrup 1986) supplemented with ABA (29.0 μM) for eight weeks. The effect of carbohydrates and osmoticum on storage reserve accumulation and germination of somatic embryos were investigated using two maturation treatments that will be referred to as I and II: (I) 3% (w/v) sucrose; (II) 3% (w/v) maltose and 7.5% (w/v) PEG. Somatic embryo development was monitored using Zeiss STEMI 2000-C and Zeiss AX10 microscopes (Carl Zeiss, Göttingen, Germany).

Somatic embryo number, desiccation and germination

Mature somatic embryos on each embryogenic clump were counted, isolated, flash frozen in liquid nitrogen and stored at –80°C for storage reserve analysis. Additionally, thirty somatic embryos per cell line were transferred to a 60 mm petri dish. The dish was placed inside a 90 mm petri dish containing 1 ml of water and the whole set up was sealed with Para film and left in the dark for desiccation. After three weeks, somatic embryos were transferred to DKM rooting medium (without NH_4NO_3) solidified with 3.5% (w/v) phytagel (Sigma-Aldrich, St. Louis, USA). Two weeks later, the somatic embryos were transferred to quarter-strength SH (Schenk and Hildebrandt 1972) medium solidified with 3% gelrite (Duchefa Biochemie BV, Harlem, The Netherlands).

Extraction and quantification of somatic embryo storage reserves

Sugars

Somatic embryos were freeze dried and homogenized by shaking with a stainless steel bead (7 mm diameter) using a bead mill (MM400, Retsch GmbH, Germany) set to a frequency of 30 Hz s^{-1} for 2 min. Soluble sugars were extracted and analyzed following the enzymatic digestion method described by Stitt et al. (1989). Approximately 5 mg DW each of the homogenized samples was boiled with 250 μl of 80% ethanol at 95°C in a heating block for 30 min. Sample extracts were centrifuged for 10 min at 13 000 *g* and each supernatant (S1) was transferred to a new tube. To recover residual soluble sugars, the pellet was consecutively boiled with 80% ethanol (150 μl) and 50% ethanol (250 μl) at 95°C for 30 min. Each of the resultant supernatants S2 and S3 were collected in new tubes after centrifugation.

Forty microliters of each total ethanolic extract (S1, S2 and S3) and four glucose standards of 0, 0.25, 0.5 and 1 mM were pipetted into a 96 well micro plate (Sarstedt, Nümbrecht, Germany) and mixed with 160 μl of reaction mix: 100 mM Hepes/KOH, pH 7, containing 3 mM $MgCl_2$, 3 mM ATP, 1.3 mM NADP and 0.6 U Glucose-6-phosphate dehydrogenase (Roche Diagnostics GmbH, Mannheim, Germany). Glucose, fructose and sucrose were assayed by adding 1 μl each of hexokinase (0.9 U μl^{-1}) (Roche), phosphoglucose isomerase (1.0 U μl^{-1}) (Roche) and excess amount of invertase

(Sigma) to the samples after 30 min intervals respectively. The sugars released from the ethanolic extracts were determined using an Epoch micro plate spectrophotometer (Bio Tek Instrument Inc., Vermont, USA). Sample absorbance maxima at 340 nm were converted to μg mg^{-1} DW of sugar using the glucose standard curve.

Starch

Starch was extracted and analyzed following the enzymatic digestion method described by Hendriks et al. (2003) and Smith and Zeeman (2006). The residual pellets from ethanolic sugar extraction were boiled in 400 μl of 0.1 M NaOH at 95°C for 30 min to disrupt the cells and gelatinize starch. Eighty microliters of 0.1 M sodium acetate/NaOH buffer containing 0.5 M HCl was added to the gelatinized samples. Forty microliters of each suspension were transferred to a new cap tube followed by 110 μl of a starch degradation mix containing; α-amyloglucosidase (Roche 0.45U), α-amylase (Roche 0.5U) and 50 mM sodium acetate/ NaOH buffer, pH 4.9. The samples were incubated for two consecutive nights at 37°C and the resultant supernatants overnight (ON1) and two overnights (ON2) collected by centrifugation at 3400 *g* for 5 min. Glucose released from starch hydrolysis after each overnight treatment (ON1 and ON2) was determined as described earlier for the glucose assay with hexokinase.

Metabolite profiling

Metabolites were extracted from 25 mg FW of somatic embryos following the method of Gullberg et al. (2004), with slight modifications as described in Businge et al. (2012). Gas chromatography and mass spectrometry analysis was performed as described in Businge et al. (2012) using a gas chromatograph coupled to a time-of-flight mass spectrometer (GC/TOFMS) equipped with an Agilent 7683 autosampler (Agilent, Atlanta, GA, USA) and a silica capillary DB 5-MS column coupled to a Pegasus III GC/TOFMS via an electron impact source (Leco Corporation, St. Joseph, MI, USA). All non-processed mass spectra (MS) files from metabolic analyses were exported into MATLAB 7.11.0 (R2010b) (MathWorks, Natick, MA, USA) in which all data pre-treatment procedures such as baseline correction, chromatograph alignment and hierarchical multivariate curve resolution were performed using custom scripts (Jonsson et al. 2005). All manual integrations were performed using ChromaTOF 4.30 software (Leco) or custom scripts. Detected peaks were identified by a database search, based on MS and chromatographic retention index, using NIST MS-Search v.2.0 with the in-house MS library database established by Umeå Plant Science Center, the MS library maintained by Max Planck Institute in Golm (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) or the NIST98 MS library.

Sample weights and *t1* scores obtained from principal component analysis (PCA) of internal standard peak areas were used to normalize the data before multivariate analysis. Additionally metabolite data were mean centered and UV scaled. In order to determine the metabolites in somatic embryos of each cell line, the orthogonal projections to latent structures discriminant analysis (OPLS-

DA) approach was used. Orthogonal projection to latent structures is a supervised multivariate regression method that can be used to model covariance among a Y- (response or class) and X-matrix (e.g., metabolites). The benefit of OPLS lies in its ability to separate variation related (Y-predictive) and unrelated (Y-orthogonal) to the X-matrix into separate components. Consequently, variation related to X (Y-predictive) is revealed by the loadings along the predictive component, which makes model interpretation simpler (Trygg and Wold 2002, Bylesjö et al. 2006, Wiklund et al. 2008).

Metabolites distinguishing the samples were identified using OPLS-DA loading plots and *t*-test of the respective metabolite peak areas. In all cases, models were judged for quality using the goodness of fit (*R2X*) and goodness of prediction (*Q2X*) parameters. All multivariate analyses were performed using SIMCA-P + 12.0.1 (Umetrics AB, Umeå, Sweden). The relative abundance of raffinose (peak area) was calculated using normalized sample data. The maximal peak area of raffinose was set as 100% and abundance was calculated by dividing each sample peak area by the maximal peak area and multiplying by 100.

Proteins

Proteins were extracted from 60 mg FW of somatic embryos following the method described by Stabel et al. (1990). The samples were ground to a fine powder and extracted in sodium dodecyl sulfate (SDS) extraction buffer: pH 8.5, containing 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (w/v) glycerol, 10 µg ml^{-1} Leupeptin (Sigma) and 100 mM Tris-HCl. Sample extracts were boiled at 95°C for 3 min and the insoluble material was removed by centrifugation at 14 900 *g* for 10 min. Two milligrams of protamine sulphate (Sigma) was added to each milliliter of supernatant to remove excess nucleic acids. The suspensions were then incubated on a shaker at room temperature for 5 min and centrifuged at 14 900 *g* for 10 min. Total proteins were quantified using the DC-Protein assay (Bio-Rad Laboratories AB, Sundbyberg, Sweden). The supernatants containing total protein were used for sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE).

The SDS-PAGE procedure was performed according to the method described by Laemmli (1970). Protein extracts in sample buffer: containing 50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.02% bromophenol blue and 1% 2-mercaptoethanol were boiled at 95°C for 3 min. Proteins were separated using a 12.5% separating and 4% stacking polyacrylamide gel. After electrophoresis, proteins were visualized with Coomassie Brilliant blue R-250 and gel profiles were photographed using a digital single-lens reflex (SLR) camera (Nikon Inc, Melville, USA). Protein weights were estimated by co-electrophoresis with a Precision plus protein molecular weight standard (Bio-Rad) ranging from 250 to 10 kDa.

Protein digestion, mass spectrometry, and data analysis

Somatic embryo total proteins were in-gel digested with trypsin and cleaned using a C18 STAGE-tip as described by Pandey et al. (2000) and Rappsilber et al. (2003). The proteins were eluted with 50%

acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA), dried and resuspended in 0.1% TFA. Proteins were run in triplicate and separated on a nanoACQUITY^{TM} UPLC system (Waters, Massachusetts, USA) solvent A (0.1% formic acid (FA) in water), solvent B (0.1% FA in ACN) equipped with a C18 75-µm × 100 mm reverse phase column (Waters) using a gradient of 1–30% solvent B over 90 min with a flow rate of 300 nl min^{-1}. The mass spectrometer (Waters Synapt^{TM} G2 HDMS) equipped with a nanoflow electrospray ionization (ESI) interface was operated in positive ionization mode with a minimal resolution of 20.000. All data were collected in continuum mode and mass-corrected using Glu-fibrinopeptide B. The data were processed with Protein Lynx Global Server v.2.5.2 (Waters) and the resulting spectra were searched against the Picea family sequences from the National Center for Biotechnology Information (NCBI) non-redundant database along with sequences for human keratin and rabbit Glycogen phosphorylase. The database search settings were; enzyme-specific cleavage with one miss cleavage allowed, oxidized methionine and protein N-terminal acetylation as variable modifications. Peptide and fragment tolerance were set to 10 ppm and 25 ppm respectively with a false positive rate of 1%. Protein quantification was performed relative to 100 fmol of glycogen phosphorylase. Protein abundance (fmol) was converted to average abundance (fmol µg^{-1} total protein) in somatic embryos of cell line 09.77.17 and 09.77.03 cultured on treatment I and II. Overview of the protein samples was performed using principal component analysis (PCA).

Statistical analysis

All statistical analyses including calculation of sample average, mean, standard error of mean (SEM) and two tailed non-paired Student's *t*-tests were performed using the algorithm embedded into Microsoft Excel (Microsoft Corporation, Seattle, USA). The term significant is only used when the change in question is confirmed to be significant ($P < 0.05$) with the *t*-test. Significantly different parameters are indicated using an asterisk (*) on the figures.

Results

Development of Norway spruce somatic embryos

Somatic embryos differed in size depending on the maturation treatment. Somatic embryos cultured on treatment I were generally short and exhibited swollen hypocotyls (Fig. 1A, C). In contrast, embryos cultured on treatment II, were elongated with smaller hypocotyls (Fig. 1B, D). Treatment II yielded the highest number of somatic embryos for cell line 09.77.17 (Fig. 1B) and cell line 09.77.03 (Fig. 1D). The increment in somatic embryos with treatment II was 13 and 14 fold for cell line 09.77.17 and 09.77.03 respectively (Fig. 1E). After six weeks on germination medium, desiccated somatic embryos from treatment I exhibited normal germination and root development (Fig. 2A, C). In contrast, somatic embryos from treatment II exhibited repressed germination and root development (Fig. 2B, D). From treatment I, 83 and 70% of the somatic embryos of cell line 09.77.17 and 09.77.03 germinated respectively (Fig. 2E). In contrast, only 33 and 30% of the somatic embryos from treatment II germinated per cell line respectively (Fig. 2E). The germination frequency was significantly higher ($P = 0.000$) for somatic embryos from treatment I.

Effect of carbon source and osmoticum on accumulation of storage reserves

Sugars and starch

Mature somatic embryos of cell line 09.77.17 and 09.77.03 isolated after eight weeks of culture on treatment I and II, predominantly contained sucrose. However, sucrose content was significantly higher in somatic embryos cultured on treatment I (Fig. 3A, B). Fructose was significantly higher in somatic embryos cultured on treatment I and undetectable in embryos cultured on treatment II (Fig. 3A, B). Glucose contents were comparable in the somatic embryos of both cell lines on the two maturation treatments (Fig. 3A, B). In cell line 09.77.17, somatic embryos cultured on treatment I contained slightly more starch compared to embryos cultured on treatment II (Fig. 3A). However, for cell line 09.77.03, somatic embryos cultured on treatment I, contained significantly ($P = 0.00$) more starch compared to embryos cultured on treatment II (Fig. 3B).

Metabolite profiling

Somatic embryo metabolite profiles were compiled using GC/MS analysis. Analysis of 32 embryo samples yielded a collection of 45 compounds. Of these, metabolite identities could be assigned to 42, leaving three as unknown compounds (Table S1). In the OPLS-DA score plots (Fig. S1A, B), somatic embryo samples separated according to maturation treatment and metabolites distinguishing the samples could be identified from the corresponding loading plots. Somatic embryos cultured on treatment I contained relatively more raffinose compared to embryos cultured on treatment II (Fig. 4). Raffinose is a product of the first step in the biosynthesis of raffinose family oligosaccharides (RFOs) (Peterbauer and Richter 2001).

Proteins

Quantification and SDS-PAGE profiles of somatic embryo total proteins are shown in Figure 5. Quantitatively, somatic embryos of cell line 09.77.17 cultured on treatment I contained significantly more total protein compared to embryos cultured on treatment II (Fig. 5A). In contrast, for cell line 09.77.03, somatic embryos cultured on treatment II contained slightly more total protein compared to embryos cultured on treatment I (Fig. 5A). In both cell lines, the major proteins bands in the somatic embryo total proteins were 75, 50, 42, 33 and 14 kDa in weight (Fig. 5B, arrows). Faint bands were also visible within the 14 to 33 kDa range for both cell lines.

To gain further understanding about the effect of carbon source and osmoticum on the somatic embryo proteome, we performed mass spectrometry analysis of total proteins from cell line 09.77.17 and 09.77.03. Principal component analysis (Fig. S2) revealed clustering of somatic embryo samples according to maturation treatment. A total of 149 proteins (Table S2) were identified and categorized into three groups on functional basis (Fig. 6). The first group (3.4%) included storage proteins while the second group (6.7%) consisted of proteins involved in stress response (Fig. 6). The third and largest group (89.9%) included proteins with other biological functions (Fig. 6).

The storage proteins included a 2S (20 kDa), vicilin (50 and 51 kDa), legumin (57 kDa) and seed storage (19 kDa) proteins (Table 1). The 2S, vicilin and legumin proteins are members of the 2S albumin, 7S and 11S globulin families of seed storage proteins (Shewry et al. 1995). All the storage proteins were mostly abundant in somatic embryos cultured on treatment II (Fig. 7).

Several proteins involved in stress response were also detected in somatic embryos of cell line 09.77.17 and 09.77.03. The proteins included; a dehydrin, late embryogenesis abundant proteins (LEA), heat shock proteins (HSPs) and small heat shock proteins (sHSPs) (Table 1). The dehydrin (19 kDa), LEA (11, 25 and 17 kDa), HSP (17 kDa) and sHSP (17 kDa) were only detected in somatic embryos cultured on treatment I (Table 1 and Fig. 8). Furthermore, LEA proteins (14 and 17 kDa) and HSP (23 kDa) were mostly abundant in somatic embryos cultured on treatment I. One sHSP (23 kDa) was mostly abundant in somatic embryos cultured on treatment II (Table 1 and Fig. 8).

Discussion

The maturation process in conifer somatic embryos has been shown to be stimulated by carbohydrates and osmoticum. In Loblolly pine (*Pinus taeda*) (Pullman et al. 2003), hybrid fir (*Abies alba* × *Abies numidica*) (Salaj et al. 2004), a combination of maltose and PEG was shown to give the best stimulation on embryo maturation frequency and quality of embryo maturation. Similarly, the presence of PEG and maltose significantly stimulated the maturation-response compared to sucrose in *P. abies* (this study). In the present study, we investigated the effect of carbohydrates and osmoticum on accumulation of storage reserves and germination of somatic embryos in two cell lines of *P. abies* cultured on two maturation treatments represented by the control conditions where sucrose is present, and the test conditions where maltose and PEG are present. The cell lines; 09.77.17 and 09.77.03, are type A cell lines, which have previously been characterized and found to have the capability to form normal somatic embryos when treated with ABA (Egertsdotter and von Arnold 1993). For both cell lines, mature embryos contained varying contents of sucrose and raffinose depending on the maturation treatment. Raffinose belongs to the raffinose family of oligosaccharides (RFOs) which are considered as seed storage reserves and to be involved in the acquisition of desiccation tolerance (Obendorf 1997, Muzquiz et al. 1999). Raffinose is synthesized by addition of galactosyl units to sucrose in a reaction catalyzed by raffinose synthase (EC 2.4.1.82) (Peterbauer and Richter 2001). Existing evidence suggests that the content of sucrose affects the accumulation of RFOs. In barley (*Hordeum vulgare*), seeds with high levels of sucrose were found to have increased levels of RFOs (Karner et al. 2004). From our results, we could speculate that on treatment II, the low levels of sucrose in the somatic embryos affected raffinose accumulation.

In hybrid larch (*Larix* × *leuptoeuropaea*), desiccation of somatic embryos has been shown to reduce the endogenous content of ABA and reorient metabolism from maturation to germination (Sandrine et al. 1997). Accordingly, ABA promotes the acquisition of desiccation tolerance during somatic embryo maturation and this requires high levels of sucrose, RFOs and LEA proteins (Blackman et al. 1992, Thomas 1993). Generally, desiccation causes a loss of hydration-water and hydrophilic interactions which maintain molecular structure in cells. As a result, sucrose and RFOs induce desiccation tolerance through the water replacement hypothesis (Koster and Leopold 1988, Crowe et al. 1992). Additionally, sucrose can form a protective intracellular glass which is maintained by interaction with RFOs (Bruni and Leopold 1992a, Obendorf 1997). The LEA proteins induce tolerance to desiccation by interacting with RFOs or by covering molecules with a cohesive layer of water (Blackman et al. 1992, Cuming, 1999). The low content of accumulated sucrose, LEA proteins and raffinose along with the low germination frequency of somatic embryos cultured on treatment II suggests that the embryos were susceptible to damage during desiccation. Furthermore, the results complement the recent findings in somatic embryos of *P. taeda,* that substitution of desiccation with a washing and cold conditioning treatment improved the germination capability of embryos treated with maltose and PEG (Clark et al. 2010).

Embryo maturation involves the deposition of storage reserves which are used during germination and seedling growth (Lai and McKersie 1994). Starch is the major form of storage carbon and is a substrate for biosynthesis of lipids and free sugars such as sucrose (Leprince et al. 1990, Luthra et al. 1991). As the main form of transported carbon and temporary storage in plants, sucrose can readily be converted to starch through the enzymes; sucrose synthase (EC 2.4.1.13), ADP glucose pyrophosphorylase (AGPase) (EC 2.7.7.27) and starch synthase (EC; 2.4.1.21) (Geigenberger and Stitt 1993, Fernie et al. 2002). In support of the hypothesis that PEG restricted the accumulation of sucrose and starch in somatic embryos cultured on treatment II, detached ear experiments in wheat (*Triticum aestivum* var. Cardena) revealed that water stress induced by 6% PEG reduced the activity of sucrose synthase, AGPase and starch synthase associated with a marked reduction in sucrose and starch content in *T. aestivum* grains (Ahmadi and Baker 2001). These findings lead up to the hypothesis that water stress induced by PEG restricts starch accumulation by altering the allosteric properties through which AGPase regulates starch biosynthesis (Ahmadi and Baker 2001).

One dimensional SDS-PAGE revealed identical protein profiles in the somatic embryos cultured on treatment I and II, including the presence of 42, 33 and 14 kDa polypeptides likely corresponding to some of the major storage polypeptides in zygotic and somatic embryos of *P. abies* (Hakman et al. 1990, Stabel et al. 1990). However, additional major storage polypeptides of 22 and 28 kDa (Hakman et al. 1990) were either absent or under-expressed in somatic embryos of our cell lines. Storage proteins are the primary source of amino acids and nitrogen during germination and seedling growth (Shewry et al. 1995). From mass spectrometry analysis of the somatic embryo total proteins, the detected storage proteins were identified as vicilin, legumin and 2S proteins which are also the major storage proteins in gymnosperms. Legumins are synthesized in precursor form during maturation and later broken down into mature globulins while vicilins are oligomeric proteins characterized by a distinctive lack of cysteine residues (Shewry et al. 1995, Shutov et al. 1995). The 2S albumins are water soluble proteins with a high content of arginine, glutamine and asparagine (Youle and Huang 1981). *In P. glauca,* somatic embryos treated with PEG contained three times more storage proteins compared to untreated embryos (Misra et al. 1993). Interestingly, we found that all the identified storage proteins were mostly abundant in somatic embryos cultured on treatment II. The abundance of storage proteins in PEG treated somatic has previously been correlated with changes in gene expression. In *P. glauca*, somatic embryos treated with PEG were found to have elevated transcript levels of glutamine synthetase and glutamate synthase unlike the untreated somatic embryos (Stasolla et al. 2003). Glutamate synthase catalyzes the conversion of glutamate to glutamine while glutamine synthetase catalyzes the conversion of glutamate and ammonia to glutamine. Thus, up regulation of both enzymes in PEG treated somatic embryos, suggests that they augment the synthesis of glutamine which is one of the predominant amino acids in storage proteins of conifers (Joy et al. 1997, Stasolla et al. 2003).

Manual time lapse photography revealed that embryos cultured on treatment II were small in size similar to what has been reported for somatic embryos of *P. abies* cultured on PEG (Find et al. 1997, Bozhkov et al. 1998). Additionally, treatment II resulted in the highest maturation frequency of embryos consistent with previous findings in somatic embryos of *P. taeda* cultured on PEG and maltose (Li et al. 1998, Pullman et al. 2003, Clark et al. 2010). The increment in maturation frequency has been linked to the non-permeating nature of PEG as an osmoticum and nutrient stress in the form of low hexose sugar levels due to slow hydrolysis of maltose (Attree and Fowke 1993, Blanc et al. 2002). In the present study, more hexose sugars were detected in embryos cultured on treatment I consistent with what has been reported for SE in *P. abies*, that sucrose in media is rapidly hydrolyzed to fructose and glucose (Find et al. 1998). In conclusion, the aim of the study was to investigate the effect of carbohydrates and osmoticum on accumulation of storage reserves and subsequent embryo development in somatic embryos of *P. abies*. The maturation treatment containing 3% maltose and 7.5% PEG consistently gave high maturation and low germination frequencies of somatic embryos. The present study demonstrates that a maturation treatment containing maltose and PEG restricts the accumulation of LEA proteins, sucrose, and raffinose in somatic embryos of *P. abies*. The results suggest that maltose and PEG affect the desiccation tolerance of the somatic embryos and thereby reduce the germination frequency.

Acknowledgements – We are grateful to Krister Lundgren and Inga-Britt Carlsson for expert assistance with GC/MS analysis. We thank Junko Takahashi for help with sugar and starch analysis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Identified metabolites in somatic embryos of cell line 09.77.17 and 09.77.03 with retention indicies.

Table S2 Identified proteins in somatic embryos of cell line 09.77.17 and 09.77.03.

Fig. S1 Multivariate analysis of somatic embryo metabolite samples. OPLS-DA score scatters; (A) Cell line 09.77.17 (B) Cell line 09.77.03

Fig. S2 Principal component analysis score scatter for somatic embryo total protein samples.

Edited by Y. Helariutta

Figure legends

Fig. 1. Effect of carbon source and osmoticum on maturation of Norway spruce somatic embryos. Embryogenic cultures: cell lines 09.77.17 (top row), 09.77.03 (bottom row). (A, C) Fully mature cotyledonary embryos after eight weeks of culture on treatment I supplemented with ABA. (B, D) Fully mature cotyledonary embryos after eight weeks of culture on treatment II supplemented with ABA. Bars, 100 µm. (E) Maturation frequency of somatic embryos (data represent mean of thirty independent replicates ± SEM).

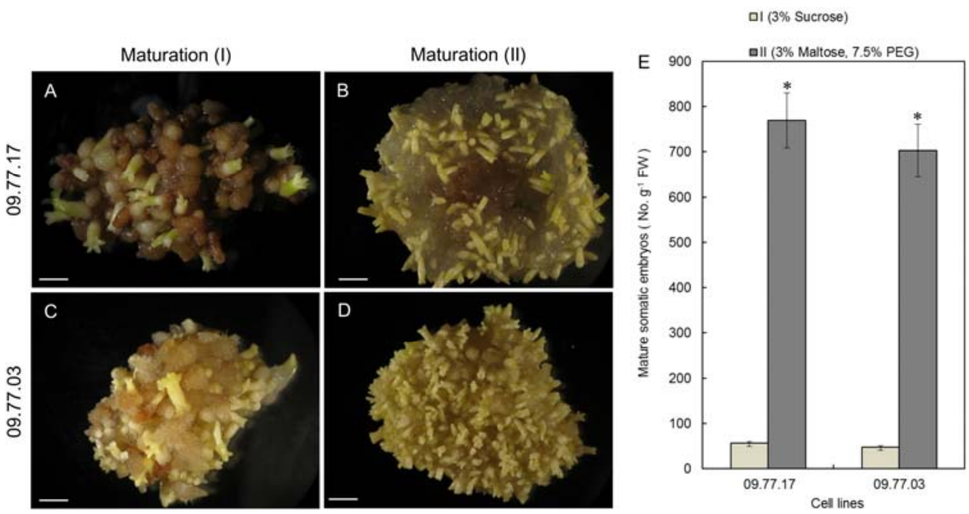

Fig. 2. Effect of carbon source and osmoticum on germination of Norway spruce somatic embryos. Somatic embryos: cell lines 09.77.17 (top row), 09.77.03 (bottom row). (A, C) Somatic embryos with normal germination after six weeks on germination medium. (B, D) Somatic embryos with suppressed germination after six weeks on germination medium. (E) Somatic embryo germination frequency (data represent mean of thirty independent replicates ± SEM).

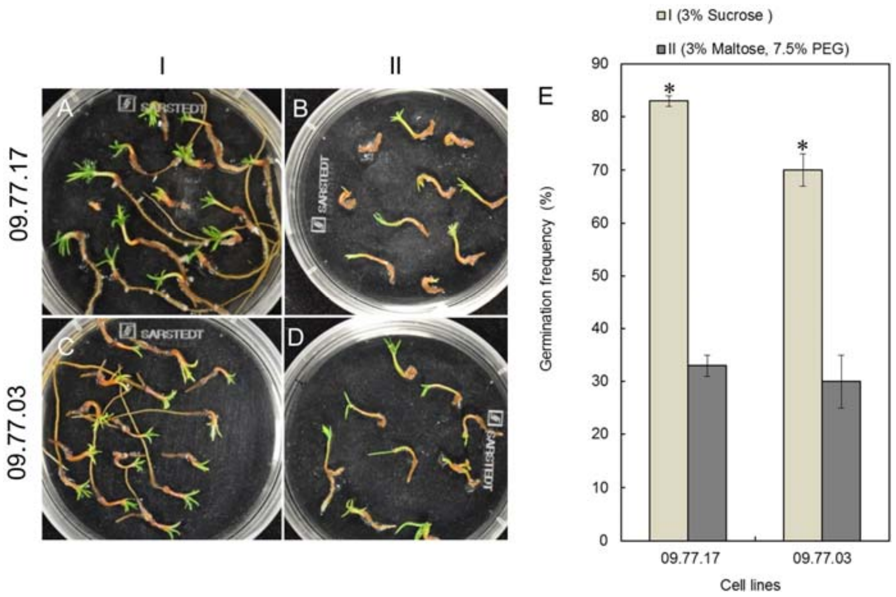

Fig. 3. Effect of carbon source and osmoticum on accumulation of carbohydrates in somatic embryos of Norway spruce. (A) Cell line 09.77.17 (B) Cell line 09.77.03. Data represent mean of five independent replicates ± SEM.

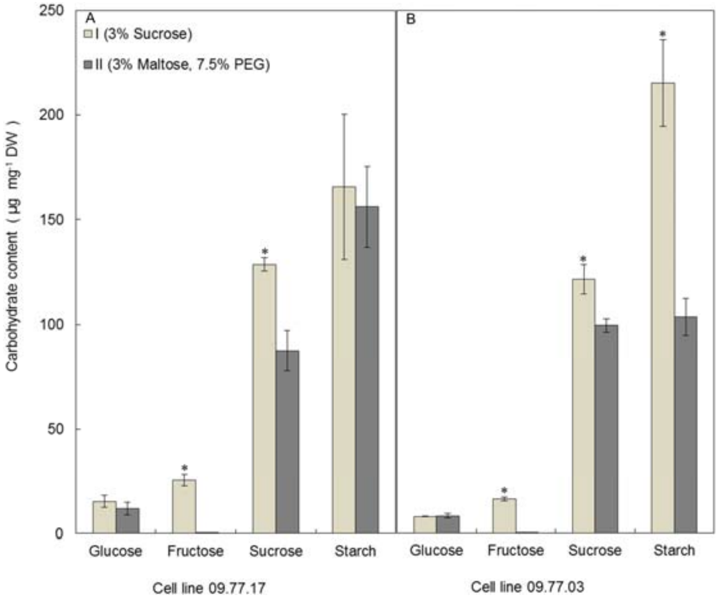

Fig. 4. Effect of carbon source and osmoticum on accumulation of raffinose in somatic embryos of Norway spruce. Data represent mean of eight independent replicates ± SEM.

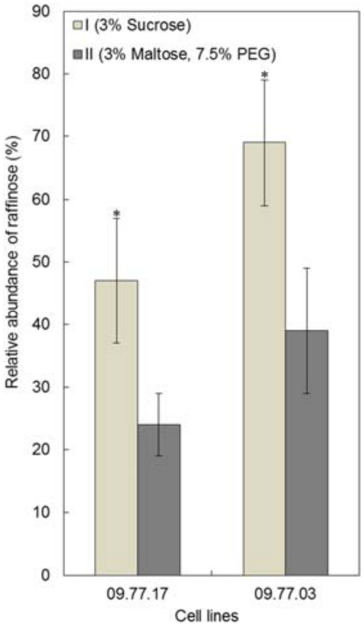

Fig. 5. Effect of carbon source and osmoticum on accumulation of proteins in somatic embryos of Norway spruce. (A) Total protein content. Data represent mean of three independent replicates ± SEM. (B) SDS-PAGE profiles of Coomassie stained total proteins. The I and II in parenthesis represent maturation treatment. 10 µg of proteins were loaded per lane.

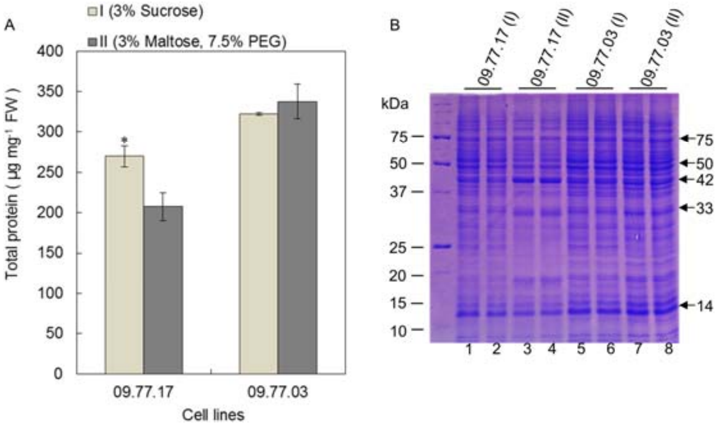

Fig. 6. Classification of identified proteins in somatic embryos of Norway spruce cell lines 09.77.17 and 09.77.03. The proteins in Table S2 were categorized into three groups based on functionality.

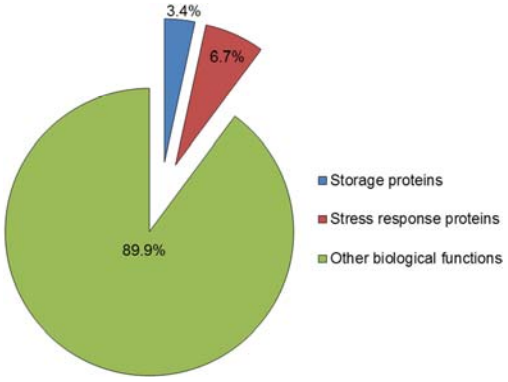

Fig. 7. Effect of carbon source and osmoticum on accumulation of storage proteins in somatic embryos of Norway spruce. The data represents the average abundance ±SEM of proteins in somatic embryos of cell line 09.77.17 and 09.77.03. For each protein, the number in parenthesis represents the NCBI accession number.

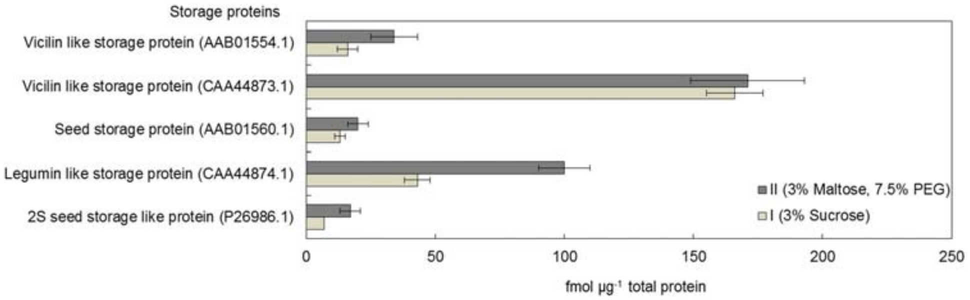

Fig. 8. Effect of carbon source and osmoticum on accumulation of stress response proteins in somatic embryos of Norway spruce. The data represents the average abundance ±SEM of proteins in somatic embryos of cell line 09.77.17 and 09.77.03. For each protein, the number in parenthesis represents the NCBI accession number.

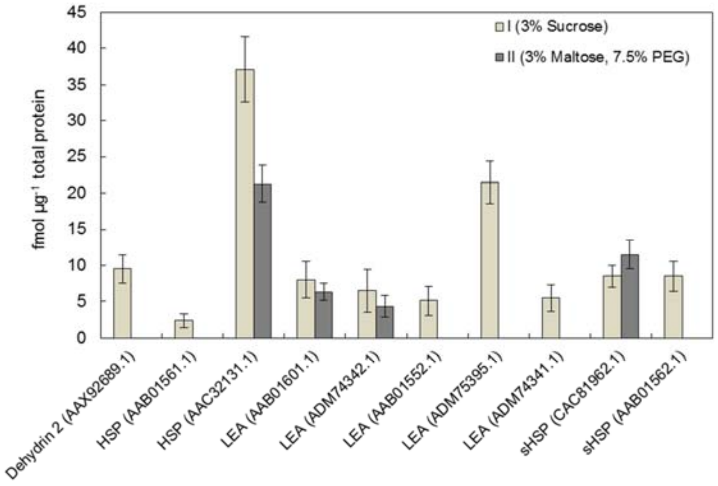

Table 1. Identified storage and stress response proteins across all somatic embryo samples of cell line 09.77.17 and 09.77.03. Acc #, accession number; Exp., Experimental; MM, molecular mass. The + and – signs indicate presence and absence of the proteins in the somatic embryos cultured on treatment I and II respectively.

				Protein Expression	
Protein Class	NCBI Acc #	Protein Description	Exp. MM (kDa)	I	II
Storage					
	P26986.1	2S seed storage like protein	20	+	+
	CAA44874.1	Legumin like storage protein	57	+	+
	AAB01560.1	Seed storage protein	19	+	+
	CAA44873.1	Vicilin like storage protein	50	+	+
	AAB01554.1	Vicilin like storage protein	51	+	+
Stress					
	AAX92689.1	Dehydrin 2	19	+	–
	AAB01561.1	Heat shock protein	17	+	–
	AAC32131.1	Heat shock protein	23	+	+
	AAB01601.1	Late embryogenesis abundant protein	14	+	+
	ADM74342.1	Late embryogenesis abundant like protein	17	+	+
	AAB01552.1	Late embryogenesis abundant protein	11	+	–
	ADM75395.1	Late embryogenesis abundant like protein	25	+	–
	ADM74341.1	Late embryogenesis abundant like protein	17	+	–
	CAC81962.1	Small heat shock protein	23	+	+
	AAB01562.1	Small heat shock protein	17	+	–