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Increased gelling agent concentration promotes somatic embryo maturation in hybrid larch (*Larix* × *eurolepsis*): a 2-DE proteomic analysis

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An integrated physiological and proteomic approach was used to investigate the effects of high gellan gum concentration in the medium during maturation of somatic embryos (SE) of hybrid larch, by comparing embryos incubated in media with a high gellan gum concentration (8 g l^{-1}) and the standard concentration (4 g l^{-1}) after 1, 3, 6 and 8 weeks of maturation. Because of the reduced availability of water in the 8 g l^{-1} medium, the cultured embryos had a lower osmotic water potential ($\Psi\pi$) and water contents, but higher dry weights (DWs), at 8 weeks compared with embryos cultured on the standard medium. The high gellan gum concentration induced a desiccation that is characteristic in zygotic embryo maturation. Total soluble proteins were extracted from SE with trichloroacetic acid (TCA)–acetone after 1 and 8 weeks of maturation on media with 4 and 8 g l^{-1} of gellan gum, and separated by two-dimensional gel electrophoresis (2-DE) at pH 4–7. More than 1100 proteins were reproducibly detected on each gel. At 1 and 8 weeks respectively, the abundances of 62 and 49 spots detected in analyses of embryos matured at the two gellan gum concentrations, significantly differed. Among 62 significantly differing spots at 1 week of maturation, the corresponding proteins of 56 were reliably identified by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), and were found to be mainly involved in 'carbohydrate metabolism', 'genetic information processing' or 'environmental information processing' according to KEGG taxonomy. Both physiological parameters and the proteins identified suggested that the embryos were stressed when they were cultured on 4 g l^{-1} of gellan gum.

Introduction

Advances in biotechnology offer attractive new opportunities for propagating conifers. Clonal propagation methods, such as somatic embryogenesis, have potentially numerous applications and advantages over conventional rooted cuttings (Klimaszewska et al. 2007). The speed with which new material can be produced

Abbreviations – ABA, abscisic acid; ACN, acetonitrile; 2-DE, two-dimensional gel electrophoresis; EMs, embryonal masses; DW, dry weight; FW, fresh weight; HSP, heat shock proteins; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; MS/MS, tandem mass spectrometry; PGR, plant growth regulator; SE, somatic embryos; TCA, trichloroacetic acid; $\Psi\pi$, osmotic water potential.

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and the high potential for clonal multiplication make somatic embryogenesis a powerful and flexible tool for the release of improved varieties. Since the 1990s, INRA has carried out a research on somatic embryogenesis in larch species, specifically in hybrid larch *Larix* × *eurolepis* (*L. decidua* × *L. kaempferi*), which is known for its remarkable vigour, together with superior stem straightness and adaptation to a range of sites, compared with the parental species (Pâques 1992).

One of the critical steps in somatic embryogenesis is the production of high quality somatic embryos (SE) and the conversion of embryos to plants. SE maturation is a complex process that is influenced by many factors. Hence, to optimize maturation in order to exploit the embryogenic potential of various hybrid lines to its full potential, it is necessary to optimize the process. Key factors in maturation of conifer SE include the plant hormone abscisic acid (ABA) and the osmotic water potential ($\Psi\pi$) of the medium. Hence, culture media with 60 μ*M* ABA and 0.2 *M* sucrose as well as polyethylene glycol (PEG) have been routinely used to promote SE maturation in larch and spruce species (Attree et al. 1995, Lelu and Label 1994). In addition, partial or full desiccation treatments applied to SE promoted plantlet development (Lelu et al. 1995). In plants, most seeds become desiccated during the later stages of zygotic embryo development; a process that plays an important role in the transition between the embryo maturation and germination (Kermode 1990). Furthermore, SE subjected to desiccation for a week reportedly showed transient increases in their endogenous ABA contents, within 6–24 h (Dronne et al. 1997). Thus, SE appears to be capable of adapting and responding very rapidly to environmental changes.

The maturation of SE of various pine species is routinely promoted by using media with gellan gum concentrations exceeding the standard 4 g l^{-1} (up to 12 g l^{-1}, as reviewed by Klimaszewska et al. 2007). This effect was also recently observed in hybrid larch, for which an increase in gel strength from 4 to 8 g l^{-1} greatly enhanced the recovery of well-developed cotyledonary SE that were able to germinate and develop into plantlets at a high rate (Lelu-Walter and Pâques 2009). It has been demonstrated that increased gel strength resulted in a reduction in availability of water for the cultured cells (Klimaszewska et al. 2000), a critical factor for pine SE maturation. Osmotic conditions also appear to control seed development in vivo (reviewed in Bradford 1994), yet a few osmotic measurements have been reported for conifer SE (Klimaszewska et al. 2000). Therefore, the first objective of this study was to characterize the $\Psi\pi$ and water content of hybrid larch SE during maturation on media with different gellan gum concentrations.

In hybrid larch, efforts have been made to describe SE maturation at the molecular level (Guillaumot et al. 2008, Mathieu et al. 2006). As a further and complementary step to gene expression analyses, studies at the proteomic level may also help to develop new in vitro culture strategies for plant propagation by identifying protein markers of optimal or stressed culture conditions. Qualitative or semi-quantitative techniques have been used to characterize the protein contents during maturation of SE of hybrid larch (Gutmann et al. 1996) and several other conifer species, such as spruce (Hakman et al. 1990, Roberts et al. 1990) and pines (Klimaszewska et al. 2004, Lelu-Walter et al. 2006). However, two-dimensional gel electrophoresis (2-DE), a powerful tool for the separation, simultaneous display and quantification of large numbers of proteins has only been applied in conifer somatic embryogenesis studies to examine changes in protein profiles during the development of SE of *Picea abies* (Hakman et al. 1990), *Cupressus sempervirens* (Sallandrouze et al. 1999) and *Picea glauca* (Lippert et al. 2005) but under one maturation treatment. Therefore, the second objective was to identify proteins that are differentially regulated during embryo maturation in the presence of high (8 g l^{-1}) or low concentration (4 g l^{-1}) of gellan gum. As SE subjected to drying treatment may rapidly react to environmental change (Dronne et al. 1997), this study was conducted after 1 week of maturation. The 2-DE patterns obtained under the two experimental conditions were compared, and proteins displaying consistent variation between the conditions were identified by means of tandem mass spectrometry (MS/MS) associated with liquid chromatography.

We report here results that describe for the first time a 2-DE proteomic analysis of the maturing hybrid larch SE on media with two gelling agent concentrations. The protein expression profiles provide novel insights into the process of SE maturation, and a basis for practical application of this knowledge.

Materials and methods

Culture of embryonal masses

Experiments were conducted with the N23 line of hybrid larch *Larix* × *eurolepis* (*L. decidua* × *L. kaempferi*) obtained in 2003 at INRA Orléans, France, from an immature zygotic embryo (Lelu-Walter and Pâques 2009). Embryonal masses (EMs) were subcultured in clumps every 2 weeks onto fresh proliferation medium, which consisted of basal MSG medium (Becwar et al. 1990) and supplemented with 9 μ*M* 2,4-dichlorophenoxyacetic acid, 2.3 μ*M* 6-benzyladenine

and 60 m*M* sucrose, solidified with 4 g l^{-1} gellan gum (Phytagel, Sigma Chemical Co., St Louis, MO). EMs were multiplied according to the proliferation method previously developed for *Pinus pinaster* and *Pinus sylvestris* (Lelu-Walter et al. 2006, 2008). One-week-old proliferating EMs clumps were collected and suspended in 5 ml of liquid proliferation medium, vigorously shaken to break them up into a fine suspension, and poured as a thin layer onto a filter paper disc (Whatman No. 2) in a Büchner funnel. A low-pressure pulse was applied to drain the liquid, and the filter paper with attached cells was placed on the surface of fresh proliferation medium. The density of EMs was approximately 300 mg fresh weight (FW) per filter. Maturation of SE was carried out according to Lelu-Walter and Pâques (2009). Briefly, 1-week-old EMs actively growing on filter paper were weighed, dispersed into liquid MSG medium with no plant growth regulator (PGR), and distributed onto a filter paper disc as described earlier. Filter paper discs with dissociated EMs (approximately 200 mg FW) were incubated for 1 week on PGR-free MSG medium supplemented with activated charcoal (10 g l^{-1}, Sigma), 0.1 *M* sucrose and 4 g l^{-1} gellan gum. Filters were then transferred onto an MSG medium containing 0.2 *M* sucrose, 1 μ*M* indolebutyric acid, 60 μ*M* cis-trans (±)-ABA (Sigma) and either 4 or 8 g l^{-1} gellan gum. Maturation was conducted in darkness. After transfer onto the ABA media, SE underwent maturation. During early stages, after 1, 3 and 6 weeks (von Aderkas et al. 2001) SE were too small to be collected without surrounding suspensor parts, but after 8 weeks they were already cotyledonary and could be easily isolated from the rest of the culture. Biological replicates were obtained by harvesting SE from different Petri dishes containing material at the same maturation stage.

Harvested SE were directly frozen in liquid N_2 and stored at −80°C until required for $\Psi\pi$ measurement and proteomic analysis. The samples destined for water content determinations were used immediately after the harvest.

Dry weight and water content of the samples

At each collection date (after 1, 3 , 6 or 8 weeks of maturation), the samples (about 200 mg) were weighed immediately after harvest to determine their FW, and their dry weight (DW) was determined after oven-drying at 70°C for 6 h. The percentage DW was calculated by multiplying their DW to FW ratio by 100, and their water content, expressed as g H_2O/g DW (Dronne et al., 1997), was calculated as follows: water content = (FW − DW)/DW. Nine samples were used for each developmental stage.

Water availability

Water availability of the maturation medium was determined from the water content in the filter paper disc after incubation on the medium, as previously described (Klimaszewska et al. 2000). An autoclaved filter paper disc (Whatman No. 2) was placed on the medium with 4 or 8 g l^{-1} gellan gum. The Petri dish was sealed with Parafilm and incubated for 48 h under the same conditions as for the maturation of EMs. The filter paper disc was subsequently weighed, and its DW was obtained after oven-drying at 40°C for 2 h. The amount of water absorbed by the filter paper disc was normalized by the ratio between the mean weight of 10 filter paper discs and the weight of the individual paper disc. The assay was repeated with five filter paper discs per medium with no culture on it, to determine the initial water availability.

Osmotic water potential ($\Psi\pi$)

The $\Psi\pi$ of samples of SE harvested at each collection date was determined as follows: the samples (200 mg FW) were ground using a pestle directly in the tube and centrifuged for 2 min at 13 000 *g*, then the $\Psi\pi$ of 100 μl of the supernatant was measured using a Hermann Roebling type 13/13 DR automatic pressure micro-osmometer (Messtechnik, Berlin, Germany). Values in osmolarity units (mosmol kg^{-1} water) were converted to MPa using the Van't Hoff equation, $\Psi\pi = -CiRT$: where C is the osmolarity value in mol kg^{-1}, i is an ionization constant assumed to be equal to unity, R is the gas constant (0.00831 kg MPa mol^{-1} K^{-1}), and T is absolute temperature (in Kelvin). The $\Psi\pi$ of the maturation media, and (as negative controls) maturation media maintained under the same conditions without any culture, were also measured with the same protocol than with samples (200 mg of fresh medium). The assay was repeated 10 times for each sample type (embryos or media with either 4 or 8 g l^{-1} of gellan gum).

Protein extraction for 2D gels

Frozen sample (400 mg) of each type to be analyzed was placed in a prechilled mortar, and ground in liquid N_2 for 5 min to a fine powder. The frozen powder was transferred to a 2 ml microtube and proteins were extracted with trichloroacetic acid (TCA)–acetone precipitation as described by Damerval et al. (1986). Briefly, the powder was homogenized in 1.8 ml of precipitation solution

[10% (w/v) TCA, 0.07% (v/v) β-mercaptoethanol in cold acetone], and proteins were allowed to precipitate for 2 h at −20°C. The mixture was then centrifuged and the resulting pellet was washed three times with 0.07% (v/v) β-mercaptoethanol in cold acetone (centrifuging each time at 14 000 *g* for 15 min at 4°C). The extract was completely dried before resolubilization in 7 *M* urea, 0.4% (v/v) Triton®X100 (Sigma, France), 4% (w/v) CHAPS, 2 *M* thiourea, 10 m*M* DL-dithiothreitol and 1% (v/v) IPG buffer. The protein concentration in each preparation was determined using a modified Bradford assay described by Ramagli and Rodriguez (1985) with bovine serum albumin as a standard.

2-D PAGE analyses

For the first dimensional separation, samples prepared as described earlier containing 300 µg protein were loaded onto 24-cm IPG strips, pH 4–7 (Protean IEF Cell system, Biorad, Marnes-La-Coquette, France) and subjected to isoelectric focusing (IEF) at 25°C and 60 kVh using an IPGphor system (GE healthcare Orsay, France). Prior to the second dimensional separation, strips were equilibrated twice for 10 min under gentle shaking at room temperature in equilibration solution [50 m*M* Tris HCl pH 8.8; 6 *M* urea; 30% (v/v) glycerol; 2% (v/v) SDS; 0.002% (w/v) BBP] containing either 1% (w/v) DL-dithithreitol or 2.5% (w/v) iodoacetamide. Then 2-D PAGE was performed using an Ettan Dalt six unit (GE, France) with 11% polyacrylamide gels, overnight at constant 110 V and 80 mA. Four biological replicates were analyzed for each sample. Gels were stained with colloidal CBB-G according to Gion et al. (2005), then images were captured with a transmission densitometer (Image Scanner, GE Healthcare, France) at 600-dpi resolution, digitized and analyzed using Progenesis software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom). The volume of each spot detected was normalized relative to the total volume of the spots on the gel. Every spot detected automatically was manually checked.

Nanospray LC-MS/MS and data analysis

In-gel digests of the excised spots were carried out using trypsin according to the procedure described by Plomion et al. (2006). Peptides were analyzed by online capillary HPLC coupled to a nanospray LCQ Deca XP Ion Trap mass spectrometer (Thermo-Finnigan, San Jose, CA). The peptides were eluted from the trap column onto an analytical 75-µm id × 15-cm C18 PepMap™ column (LC Packings, Amsterdam, The Netherlands) with a 5–40% linear gradient of solvent B over 35 min [where solvent A was 0.1% formic acid in 5% acetonitrile (ACN), and solvent B was 0.1% formic acid in 80% ACN]. The separation flow rate was set at 200 nl min^{-1}. The mass spectrometer was operated in positive ion mode with a 2-kV needle voltage and a 3-V capillary voltage. Data were acquired in a data-dependent mode alternating between an MS scan survey over the range m/z 150–2000, a zoom scan on the most intense ion and its MS/MS spectrum using a 2 m/z units ion isolation window and a 35% relative collision energy. After mass spectrometric analyses, all data were searched using the SEQUEST algorithm through the Bioworks 3.3.1 interface (Thermo-Finnigan, Torrance, CA) against 355 326 entries in the DFCI Pine Gene Index release 7.0 (http:// compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine; July 2008). DTA files were generated for the MS/MS spectra that reached both a minimal intensity (5.10^{-4}) and a sufficient number of ions (15). The DTA generation allowed the averaging of several MS/MS spectra corresponding to the same precursor ion with a tolerance of 1.4 Da. The spectra from precursor ions with molecular masses >3500 or <600 Da were rejected. The search parameters were as follows: mass accuracy for the peptide precursor and peptide fragments was set to 2 and 1 Da, respectively. Only b- and y-ions were considered for mass calculation. Oxidations of methionines (+16 Da) and carbamidomethylation of cysteines (+57 Da) were considered as differential modifications. Two missed trypsin cleavages were allowed. Only peptides with Xcorr higher than 1.9 (single charge), 2.2 (double charge) and 3.75 (triple charge) were retained. In all cases, ΔCn had to be >0.1 and the peptide *P*-value $<10^{-3}$. Proteins identified by a unique peptide were rejected. Proteins were classified into groups based on their functional categories using the KEGG orthology database (http://www.genome.jp/kegg/kegg2.html).

Statistical analyses

Statistical analysis was carried out with R software (version 2.8.0; R Development Core Team, 2008. R: *A language and environment for statistical computing*. R Foundation for Statistical Computing; Vienna, Austria). Effects of the treatments on the DW, water content and $\Psi\pi$ measurements were evaluated using one-way ANOVA. Variations of these parameters during maturation in relation to the gellan gum level in the medium were analyzed with multiple comparisons of means with Tukey contrasts ($P < 0.001$ or $P < 0.05$). The effects of gellan gum on $\Psi\pi$ at 8 weeks were evaluated by Student's *t*-test.

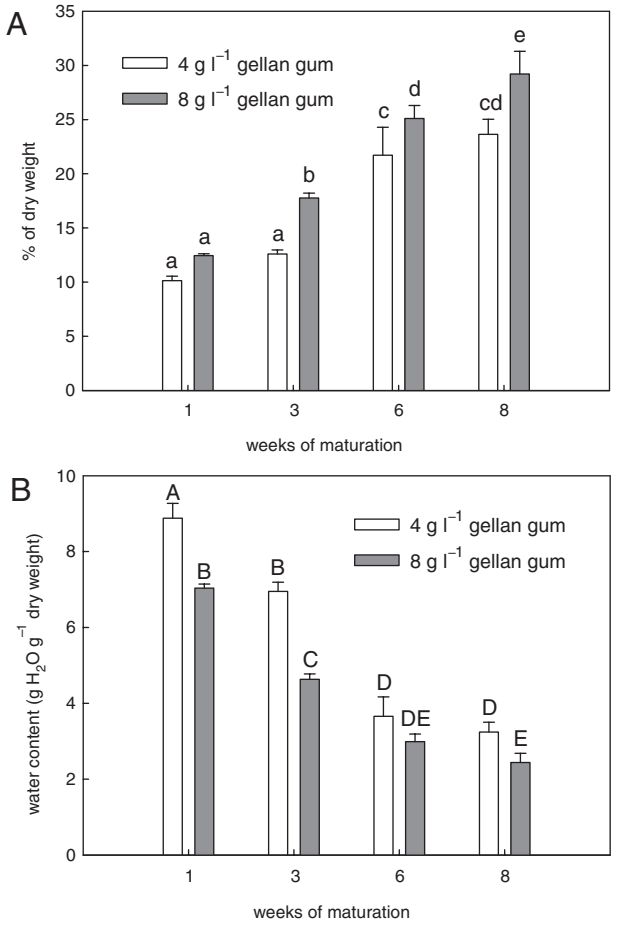

Fig. 1. Percentage of DW (A) and water content (B) in SE during maturation according to gellan gum concentration. Bars represent standard errors (n = 9). Significantly different groups are indicated by different letters ($P = 0.05$).

The pattern of each spot in the proteomic study was analyzed by Student's *t*-test on the basis of the normalized spot volume ($P < 0.05$). Any spot with a *P*-value of <0.05 according to Student's *t*-test associated with the gellan gum treatment was deemed to have changed significantly between treatments and was subsequently excised. Sixty-two spots were identified for excision by this process.

Results

Water status of SE cultures

To characterize the water status of the SE during maturation, the water content and DW of the embryos on both types of media were followed throughout their development (Fig. 1A, B). Compared to embryos cultured on medium with 4 g l^{-1} gellan gum, embryos transferred to medium with 8 g l^{-1} gum showed a significant increase in DW ($P < 0.007$, Fig. 1A) and reduced water content ($P < 0.007$, Fig. 1B). In addition, the two parameters, DW and water content, varied with both the age of the culture ($P < 2.2.10^{-16}$ for both parameters) and the gellan gum concentration ($P < 5.71.10^{-16}$ and $P < 5.16.10^{-13}$ respectively), and their maximum DW content coincided with their lowest water content. The between-treatment differences in results could be because of the significant decrease in water availability on 8 g l^{-1} compared with 4 g l^{-1} gellan gum (593.9 ± 4.0 mg vs 620.5 ± 15.2 mg, respectively; n = 5, $P = 0.002$).

The $\Psi\pi$ of the SE, and the respective media, was measured at various maturation stages. The media with no culture showed no variation in $\Psi\pi$, regardless of the gellan gum level or maturation time (data not shown). In contrast, in the presence of SE, at 8 weeks the $\Psi\pi$ of the 8 g l^{-1} gellan gum medium was significantly lower ($P = 0.00107$) than that of the 4 g l^{-1} medium (Fig. 2A, B). The $\Psi\pi$ of SE was lower at all maturation stages compared with media. During SE maturation, no effect of gellan gum concentration on their $\Psi\pi$ was detected ($P = 0.28114$). However at the 8-week stage, $\Psi\pi$ was lower in SE exposed to 8 g l^{-1} gellan gum than in the counterparts exposed to 4 g l^{-1} ($P = 0.0023$).

Protein patterns

Soluble protein profiles of embryos exposed to 4 or 8 g l^{-1} gellan gum in the medium, and sampled at 1 and 8 weeks of maturation, were determined using 2D-gel electrophoresis. Variation in the relative abundance of 1188 consistent spots was evaluated. Their distribution in samples from embryos exposed to 4 and 8 g l^{-1} gellan gum at 1 and 8 weeks of maturation are shown in Fig 3. Sixty-four percent (759/1188) of the proteins were common to all stages, 16% were common to a given maturation time regardless of the gellan gum content, 7.6% were specific to 4 g l^{-1} gellan gum samples, and 6.4% were specific to 8 g l^{-1} gellan gum samples (Fig. 3). The total number of detected spots was similar in samples exposed to both of the gellan gum concentrations. The analysis of the spot pattern revealed that most of the spots did not significantly differ in volume (corresponding to the product between the area and the intensity of the spot) according to the gellan gum concentrations. Sixty-two spots displayed significant differences in their normalized volume at 1 week of maturation, and 49 spots at 8 weeks of maturation. Among these significant spots at stages 1 and 8 weeks of maturation, only three were in common (spots #88, 367 and 962). They were identified as Ras-related protein Rab2B (#88), glucose-regulated

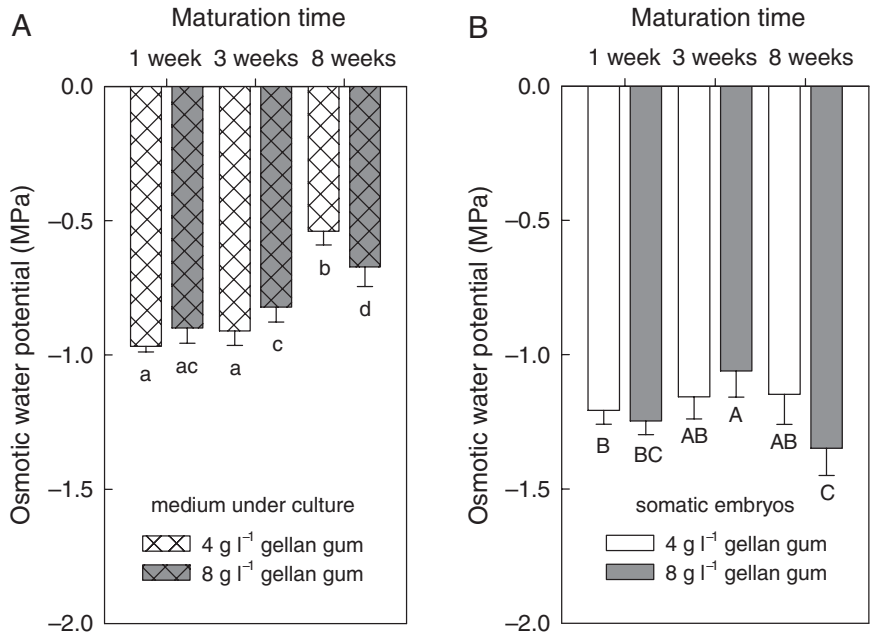

Fig. 2. Change with time in Ψ_π of the culture media (A) and SE (B) during maturation according to gellan gum concentration. Error bars represent standard error (n = 8 for media and n = 10 for tissues). Significantly different groups are indicated by different letters (multiple comparison of means: $P = 0.05$).

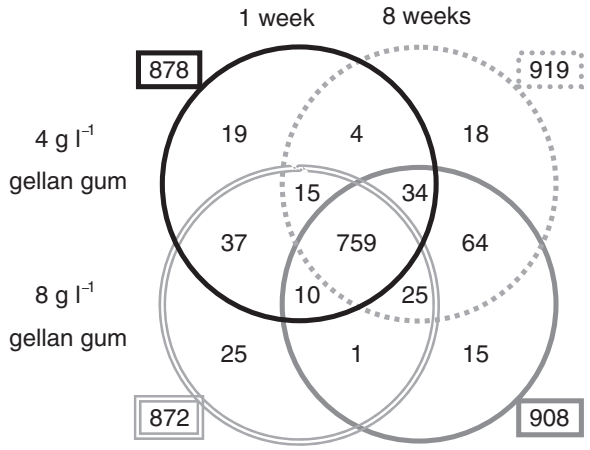

Fig. 3. Venn diagram showing distributions of the total number of spots (1188) between the two stages. Values in rectangular boxes indicate the total number of spots per stage.

protein homolog 4 (#367) and an undocumented protein annotated as chromosome chr5 scaffold-67 (#962).

Among 62 significant spots at 1 week of maturation, 56 were reliably identified using at least two peptides (Table 1 and Fig. 4). Six spots displayed more than one putative protein identity and were discarded from further analysis (see Appendix S1 in Supporting Information).

Two thirds of all identified proteins displayed a significant increase in abundance when the SE were cultured with 4 g l^{-1} gellan gum, and one third when the SE were cultured with 8 g l^{-1} gellan gum. The identified proteins were classified according to their functional categories (Table 2). Most of the proteins fell into the 'metabolism' category (38%). Forty-one percent of these proteins are involved in carbohydrate metabolism, mainly in gluconeogenesis. Proteins involved in environmental information processing were over-expressed only on the 8 g l^{-1} gellan gum medium.

Discussion

Effects of increased gellan gum concentration on physiological parameters

Increased gellan gum concentration in the maturation medium has been found to greatly improve the maturation yield and quality of SE of various pine species (Klimaszewska et al. 2007), including *Pinus strobus* (Klimaszewska and Smith 1997), *Pinus pinaster* and *Pinus sylvestris* (Lelu et al. 1999) and, recently, hybrid larch (Lelu-Walter and Pâques 2009). Increasing the gellan gum concentration from 4 to 8 g l^{-1} greatly enhanced the recovery of hybrid larch cotyledonary SE, which could subsequently germinate and develop into plantlets at a high rate. The results of the present study

Table 1. Identification of proteins according to gellan gum level in the culture medium. [a]Excised spot number, refers to the spot number labeled in Fig. 4. [b]Tentative consensus (TC) given in the DFCI database. [c]Number of matching peptides (# pep.). [d]Ratio of the amino acids in detected peptides to total protein amino acids: coverage (% cov.). [e]Accession number of the corresponding protein in the Uniprot database (http://www.uniprot.org/). [f]Theoretical molecular mass (Mr) and the isoelectric point (pI) computed by the ExPASy website (http://www.expasy.ch/).

Putative proteins more abundant in SE cultured on 4 g l^{-1} gellan gum compared with 8 g l^{-1}

							Theoretical		Experimental	
Spot number[a]	Assignment	TC[b]	# pep.[c]	% cov.[d]	Accession number[e]	Species	M_r (Da)	pI	M_r (Da)	pI
18	Heat shock cognate 70 kDa protein	TC105009	2	6.4	Q9M6R1	*Malus x domestica*	71216	5.2	39101	4.9
	GRP94	TC110737	3	9.3	A7YAU9	*Pinus taeda*	95380	4.8		
50	Argininosuccinate synthase	gi|90042890	3	3.0		*Medicago truncatula*	63733		51840	5.9
	Argininosuccinate synthase	TC95974	1	5.2	A9TX31	*Physcomitrella patens*	52392	6.0		
107	Pyruvate decarboxylase isozyme 2	TC81547	4	4.8	Q9FVF0	*Fragaria x ananassa*	64847	5.9	60204	5.8
	Pyruvate decarboxylase isozyme 2	TC88813	2	9.4	Q84V95	*Lotus corniculatus*	65132	5.9		
113	Glucose-6-phosphate isomerase cytosolic	TC107785	2		Q9FXM5	*Spinacia oleracea*	61754	6.4	59629	5.1
	Glucose-6-phos phate isomerase cytosolic A	TC81691	4	6.9	Q8H8M6	*Vitis vinifera*	62524	6.8		
118	Endoplasmin homolog	TC110737	3	9.3	P35016	*Catharanthus roseus*	93492	4.9	41975	5.0
138	RPT2A; ATPase	gi|90038952	4	10.0		*Arabidopsis thaliana*	54652		51169	5.2
	AAA ATPase; 26S proteasome subunit P45	TC80721	1	3.6	Q2HTA3	*Medicago truncatula*	49488	5.9		
151	Heat shock protein 81-1	TC101959	3	12.7	A2YWQ1	*Oryza sativa*	80194	5.0	44273	5.0
	Heat shock protein 81-3	TC108803	5	8.0	P51818	*Arabidopsis thaliana*	80052	5.0		
	Molecular chaperone Hsp90-1	TC96311	2	12.2	Q6UJX6	*Nicotiana benthamiana*	80105	4.9		
	Hsp90-2-like	TC98348	2	12.3	Q2XTE5	*Solanum tuberosum*	80417	5.1		
165	Pyruvate decarboxylase	TC81547	5	3.5	Q9FVF0	*Fragaria ananassa*	65244	5.9	60044	5.9
	Pyruvate decarboxylase	TC81434	3	4.3	Q84V95	*Lotus corniculatus*	65132	5.9		
294	Enolase 1	TC80765	5	5.4	Q9LEJ0	*Hevea brasiliensis*	47830	5.6	31088	5.3
347	Oligopeptidase A	TC100165	2	3.7	A9YWR9	*Medicago truncatula*	79121	5.4	67068	5.2
352	GRP94	TC101142	3	9.0	A7YAU9	*Pinus taeda*	95380	4.8	54426	4.9
391	Chalconeflavonone isomerase	TC99105	2	9.1	A5HBK6	*Pyrus communis*	23364	5.6	49669	5.8
430	Dihydrolipoyl dehydrogenase 1 mitochondrial	TC99823	2	3.8	Q9LNF3	*Arabidopsis thaliana*	53988	7.0	48487	5.7
461	3-isopropylmalate dehydrogenase	TC83730	7	19.7	A2XK82	*Oryza sativa*	41747	5.3	46412	5.7
486	Heat shock protein 812	TC113528	2	5.1	P55737	*Arabidopsis thaliana*	80064	5.0	37186	5.1
	Heat shock 70 kDa protein mitochondrial	TC114628	2	5.2	Q01899	*Phaseolus vulgaris*	72537	6.0		
	Heat shock 70 kDa protein mitochondrial	TC98916	3	8.7	Q08276	*Solanum tuberosum*	73077	6.4		
535	Phosphoglucomutase	TC102152	4	12.1	A5HSI1	*Bambusa oldhamii*	63412	5.4	62918	5.6
	Phosphoglucomutase	TC87322	2	5.7	A5HSI2	*Bambusa oldhamii*	14993	8.2		
574	Chromosome chr8 scaffold_29	TC93736	4	4.1	A7PT66	*Vitis vinifera*	89589	5.1	46795	5.1
578	Heat shock protein 81-3	TC108803	6	9.1	P51818	*Arabidopsis thaliana*	80052	5.0	46923	5.0
588	Alcohol dehydrogenase	TC81108	3	10.0	Q43023	*Pinus banksiana*	40460	5.7	49030	5.7
	Alcohol dehydrogenase	TC84361	3	10.7	Q43022	*Pinus banksiana*	40554	5.8		
633	Aspartate aminotransferase, chloroplast precursor	TC97755	4	8.2	P46248	*Arabidopsis thaliana*	49831	8.2	46380	6.1

Table 1. Continued.

Putative proteins more abundant in SE cultured on 4 g l^{-1} gellan gum compared with 8 g l^{-1}

Spot number[a]	Assignment	TC[b]	# pep.[c]	% cov.[d]	Accession number[e]	Species	Theoretical M_r (Da)	Theoretical pI	Experimental M_r (Da)	Experimental pI
649	flavanone 3-hydroxylase	TC81214	3	11.9	Q5XPX2	*Ginkgo biloba*	40410	5.6	47593	5.5
657	NAD-dependent sorbitol dehydrogenase	TC94525	4	14.0	Q9MBD7	*Prunus persica*	39145	6.5	47274	5.8
712	Chalcone synthase	TC84952	4	8.6	Q8GUU4	*Pinus pinaster*	43154	6.1	46221	6.1
	Chalcone synthase	TC85748	6	15.2	Q2ENC0	*Picea abies*	43264	6.2		
	Chalcone synthase	TC93000	6	16.4	Q2ENB1	*Abies alba*	43227	6.0		
	Chalcone synthase	TC93671	6	14.4	Q9MBF0	*Pinus densiflora*	43352	5.8		
763	Enolase 1	TC80765	2	5.9	Q9LEJ0	*Hevea brasiliensis*	47830	5.6	45231	6.1
773	1-aminocyclopropane-1-carboxylic acid oxidase	TC81819	2		Q84L58	*Cicer arietinum*	35089	5.0	44848	5.4
785	Fructose-bisphosphate aldolase	TC114376	2	9.9	A7P3F7	*Vitis vinifera*	42894	8.9	41464	5.7
791	Type IIIa membrane protein cp-wap13	TC106627	3	8.0	O24548	*Vigna unguiculata*	39422	6.2	46157	5.6
793	Heat shock protein 81-3	TC108803	3	5.0	P51818	*Arabidopsis thaliana*	80052	5.0	45199	5.0
801	Heat shock protein 101	TC96791	3	4.6	Q6F2Y7	*Oryza sativa*	100896	5.9	44624	5.4
825	Adenosine kinase isoform 2T	TC108369	2	12.0	Q5DKU6	*Nicotiana tabacum*	37524	5.2	45167	5.3
889	Granulebound starch synthase 1 chloroplastic amyloplastic	TC96565	2	3 .6	Q43784	*Manihot esculenta*	66968	8.3	52350	5.0
972	Superoxide dismutase	TC83788	2	5.7	A5JVZ3	*Ginkgo biloba*	25268	8.8	23585	5.2
996	Thiosulfate sulfurtransferase	TC93574	4	9.2	Q9ZPK0	*Datisca glomerata*	41382,8	6.5	39867	5.3

Putative proteins more abundant in SE cultured on 8 g l^{-1} gellan gum compared with 4 g l^{-1}

Spot number[a]	Assignment	TC[b]	# pep.[c]	% cov.[d]	Accession number[e]	Species	Theoretical M_r (Da)	Theoretical pI	Experimental M_r (Da)	Experimental pI
2	Chalcone synthase	TC82329	3	9.9	Q8GUU4	*Pinus pinaster*	43154	6.1	47306	6.0
	Chalcone synthase	TC83662	3	10.7	Q9MBF0	*Pinus densiflora*	43352	5.8		
	Chalcone synthase	TC85748	4	11.0	Q2ENC0	*Picea abies*	43264	6.2		
	Chalcone synthase	TC93000	4	11.9	Q2ENB1	*Abies alba*	43227	6.0		
88	Ras-related protein Rab2B	TC90586	4	24.7	P49104	*Physcomitrella patens*	23061	7.0	20808	6.1
166	Heat shock 70 kDa protein	TC94610	2	3.7	Q96269	*Arabidopsis thaliana*	91618	5.1	66876	5.2
259	Isopentenyl diphosphate isomerase	TC108307	5	9.39	Q078Z5	*Nicotiana langsdorffii x N. sanderae*	33511	6.2	31663	5.1
	Isopentenyl diphosphate isomerase	TC82458	5	9.57	Q1XIS8	*Gentiana lutea*	27216	5.0		
	Isopentenyl diphosphate isomerase	TC94076	5	10.34	Q0QYT2	*Ipomoea batatas*	33770	5.7		
260	Inositol3phosphate synthase	TC114216	5	14.1	Q9FYV1	*Sesamum indicum*	56234	5.6	57427	5.8
292	6-phosphogluconate dehydrogenase, decarboxylating	TC90774	3	4.4	A5BGC9	*Vitis vinifera*	53920	6.2	55671	5.8
332	Ras-related protein Rab11B	TC86043	2	7.5	Q40521	*Nicotiana tabacum*	24259	5.5	24256	5.6
367	Glucose-regulated protein homolog 4	TC85812	3	9.3	Q9AVT8	*Picea abies*	48217	4.8	39325	4.9
374	Acyl-CoA binding family protein	TC88346	4	16.9	Q10AZ9	*Oryza sativa Japonica*	58001	5.2	67738	5.4

Table 1. Continued.

Putative proteins more abundant in SE cultured on 8 g l^{-1} gellan gum compared with 4 g l^{-1}										
							Theoretical		Experimental	
Spot number[a]	Assignment	TC[b]	# pep.[c]	% cov.[d]	Accession number[e]	Species	M_r (Da)	pI	M_r (Da)	pI
375	Proteasome subunit beta type	TC100209	6	17.3	A7P520	*Vitis vinifera*	24915	5.3	22692	5.6
406	Predicted protein	TC81496	2	3.5	A9TZW4	*Physcomitrella patens*	49946	5.8	53755	6.0
410	Proteasome subunit beta type 3	TC87204	6	37.0	O65084	*Picea mariana*	22908	5.5	20329	5.5
	Proteasome subunit beta type-3	TC101700	4	6.5	O65084	*Picea*	22908	5.5		
451	Ras-related protein RIC1	TC82990	3	11.5	P40392	*Oryza sativa*	22476	5.2	19850	5.9
747	UDP-glucose dehydrogenase	TC111185	2	5.1	Q6RK08	*Cinnamomum osmophloeum*	47042	5.9	55160	5.9
	UDP-glucose dehydrogenase	TC88051	2	8.5	Q6RK07	*Cinnamomum osmophloeum*	52933	6.0		
781	Actin7	TC90279	5	16.2	P53495	*Arabidopsis thaliana*	41735	5.3	43571	5.6
788	AcetylCoA acetyltransferase cytosolic 2	TC81432	6	20.5	Q3E8F1	*Arabidopsis thaliana*	43291	6.0	42485	5.7
962	Chromosome chr5 scaffold-67,	TC89652	3	8.8	A7Q9M3	*Vitis vinifera*	37542	6.1	42837	5.8
995	Uncharacterized protein At2g41620 -3E-61	TC87364	2	10.0	Q94CF2	*Arabidopsis thaliana*	96615	6.7	18765	5.9

show that the high gellan gum level (8 g l^{-1}) in a medium resulted in a DW increase in the SE, accompanied by a reduction in their water content throughout maturation. After 8 weeks the water content was 2.44 g H_2O/g DW, very similar to values previously recorded when SE were subjected to a desiccation treatment (Lelu et al. 1995). Therefore, our results suggest that use of 8 g l^{-1} gellan gum, rather than 4 g l^{-1}, enhanced dehydration of the SE.

Accordingly, water availability was lower in filter papers placed on the 8 g l^{-1} medium than in others placed on the 4 g l^{-1} medium, indicating that water could be less readily taken up by the cultures from the medium, and this could be linked to the lower quantity of water found in the SE cultured on the 8 g l^{-1} medium. Nevertheless, those SE accumulated more DW than their counterparts cultured on the 4 g l^{-1} medium. Therefore, the results indicate that the development of SE was enhanced rather than limited by the reduced water availability on the medium with the higher gellan gum concentration.

It should be noted that the amount of water in the two media was almost identical, as only the gellan gum concentration varied, implying that the gel strength differed substantially between them (Klimaszewska et al. 2000) but their water potential was very similar, as observed in the absence of embryo cultures (results not shown). In contrast, when growing SE was present, the 8 g l^{-1} culture medium had a lower $\Psi\pi$ than the 4 g l^{-1} medium. Similarly, cotyledonary SE that developed on the 8 g l^{-1} medium had lower $\Psi\pi$ than counterparts on the 4 g l^{-1} medium. Hence, the results suggest that as water is less available in the medium with 8 g l^{-1} gum, SE might possess mechanisms that adjust their $\Psi\pi$ sufficiently to ensure that transfer of the water from the medium occurs but within physiological limitations. These results are in agreement with observations of similar phenomenon in *Pinus strobus* (Klimaszewska et al. 2000). This hypothetical adjustment of the SE $\Psi\pi$ to water availability could explain the greater dehydration observed in the SE cultured on 8 g l^{-1}.

Effect of increased gellan gum concentration on protein relative abundance

We evaluated and compared the soluble protein patterns in SE cultured on the two media during maturation by 2-D gel analysis, and detected 62 spots corresponding to proteins that appeared to be differentially expressed. The corresponding proteins were identified in 56 of these spots by the MS/MS; a similar success rate (90.3%) to rates obtained in proteomic analyses of other types of plant tissues, e.g. 75, 91.2, 78 and 77%, respectively, in analyses of *Pinus pinaster* wood-forming tissue

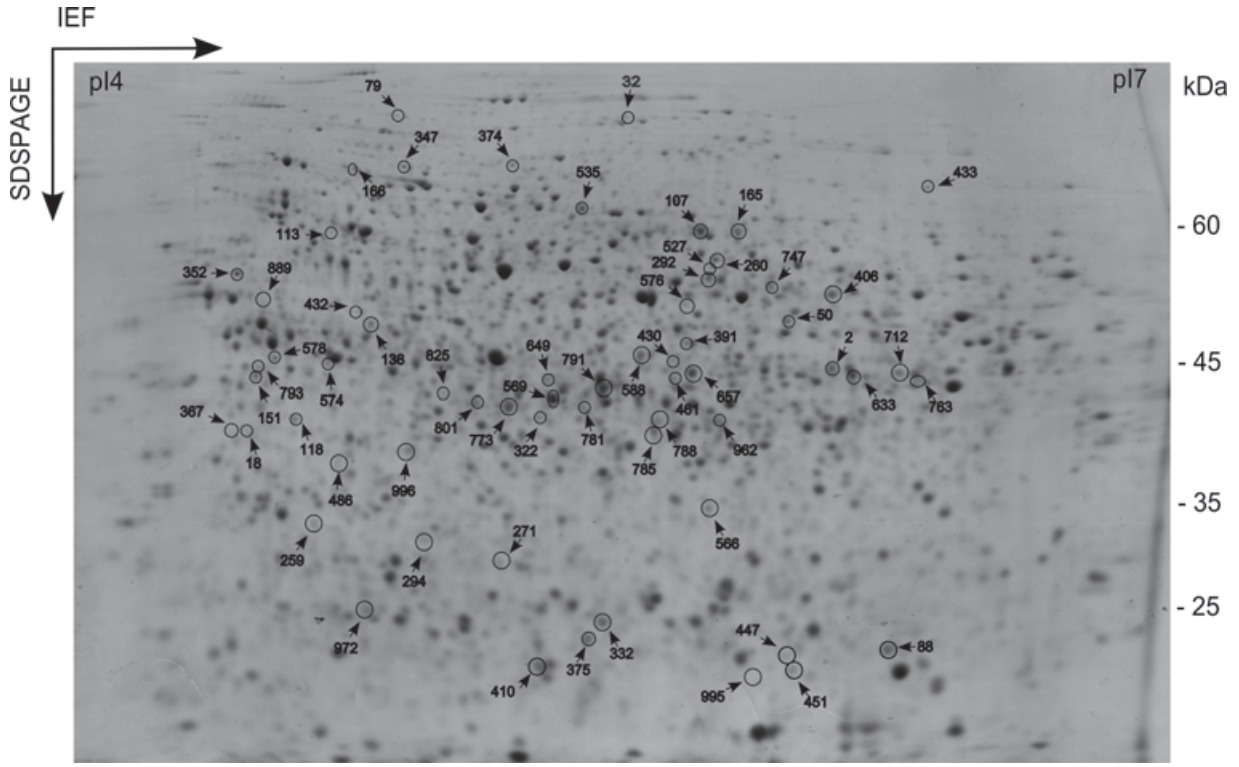

Fig. 4. Representative 2-DE map obtained for SE after 8 weeks of maturation on 8 g l^{-1} gellan gum. Marked spots displayed significant differences in their abundance ($P < 0.05$) between SE cultivated on medium with 4 and 8 g l^{-1} gellan gum.

(Gion et al. 2005), eight *Populus trichocharpa* tissues (Plomion et al. 2006), *Picea abies* seedlings (Valcu et al. 2008) and *Pinus radiata* needles (Valledor et al. 2008). However, levels of coverage and the numbers of identifiable peptide fragments generated per spot were low compared to those generally obtained (Bonhomme et al. 2009a, 2009b, Yang et al. 2007). This can be explained by the limited genomic resources available for conifers, especially *Larix*. It should also be noted that for six spots more than one protein was identified, a phenomenon that has been previously reported by several authors (Gion et al. 2005, Jorge et al. 2005, 2006, Pan et al. 2009, Valledor et al. 2008). In some cases this may be because of degradation products of some proteins such as RuBisCO (spot #271), which are often detected in proteomic studies (Gion et al. 2005, Jorge et al. 2006, Valledor et al. 2008) and may co-migrate with other proteins. In other cases it may be because of the presence of multiple isoforms of a protein, e.g. in the present study two isoforms of enolase, a marker of embryogenic maturation (Lippert et al. 2005), were found to be differentially expressed in SE cultured on the 4 and 8 g l^{-1} gellan gum media (spots #294 and 763). The presence of protein isoforms with different expression patterns during seed maturation has already been reported in barley (Finnie et al. 2006).

In studies of the effects of water deficits in intact plants, decreased levels of proteins involved in carbon metabolism and protein have often been found (Bonhomme et al. 2009a, 2009b, Jorge et al. 2006, Plomion et al. 2006). Accordingly, seven of the proteins identified as being differentially expressed in the present study are involved in the glycolysis/gluconeogenesis pathway (alcohol dehydrogenase, dihydrolipoyl dehydrogenase, enolase, fructose-bisphosphate aldolase, glucose-6-phosphate isomerase, phosphoglucomutase and pyruvate decarboxylase), and all were expressed at a lower level in SE cultured on the medium with 8 g l^{-1} gellan gum. Pyruvate decarboxylase, the only one of these enzymes to catalyze an essentially irreversible reaction, plays an important role in determining how much of the cell's carbon is directed toward catabolism via glycolysis. The presumably accompanying reduction in rates of carbon catabolism may promote the observed increase in DW of the SE cultured on the 8 g l^{-1} medium. Glucose in these SE could participate in the observed increase of $\Psi\pi$. The enzyme fructose-biphosphate aldolase, which was downregulated in the

Table 2. Distributions of the identified putative proteins among functional classes according to culture conditions. For each class, the total number of proteins is indicated in brackets and the corresponding percentage in the first column. Then number of proteins showing higher abundance in one of the culture conditions are listed: 4 > 8: number of proteins with higher abundance in SE cultivated with 4 g l^{-1} gellan gum; 8 > 4: number of proteins with higher abundance in SE cultivated with 8 g l^{-1} gellan gum. The 'metabolism' class is divided into subclasses with the corresponding percentage and number of proteins in each subclass presented (italic values). Proteins involved in more than one process were assigned to more than one categorical group. Hence the sum of proteins in the categories exceeds the total number of proteins.

	%	4 > 8	8 > 4
Metabolism (26)	38%	19	7
carbohydrate metabolism	*41.0%*	*11*	*5*
energy metabolism	*5.1%*	*2*	*0*
lipid metabolism	*7.7%*	*1*	*2*
nucleotide metabolism	*2.6%*	*1*	*0*
amino acid metabolism	*15.4%*	*5*	*1*
metabolism of other amino acids	*2.6%*	*0*	*1*
metabolism of cofactors and vitamins	*2.6%*	*1*	*0*
biosynthesis of secondary metabolites	*17.9%*	*4*	*3*
xenobiotics biodegradation and metabolism	*5.1%*	*1*	*1*
Genetic information processing (16)	23%	11	5
Environmental information processing (6)	9%	0	6
Cellular processes (16)	23%	10	6
Others (5)	7%	3	2

SE cultured on the 8 g l^{-1} medium, is involved in many pathways, including glycolysis/gluconeogenesis, the pentose phosphate pathway, fructose and mannose metabolism and the Calvin cycle. The first three belong to carbohydrate anabolism and the last one belongs to the carbohydrate catabolism. A decrease of activity in the Calvin cycle leads to a decrease in carbohydrate synthesis, while a decrease in fluxes through the pentose phosphate cycle implies a decrease in glycolysis and hence enhanced the production of nucleic acid precursors. The decreased abundance of enzymes involved in glycolysis could be related to the increased DW of SE cultured on the medium with 8 g l^{-1} gum observed in this study.

Modification of the somatic embryogenesis culture medium also induced changes in the abundance of proteins involved in the protection of the cell, including detoxification processes and protection of cellular components. The proteins flavanone 3-hydroxylase and aspartate aminotransferase, which were more abundant in SE cultured on medium with 4 g l^{-1} gellan gum, are involved in secondary metabolism. Secondary metabolism is reportedly more active in plants under stresses that lead to increased production of free radicals (Edreva et al. 2008), which could explain why one of the proteins found to be expressed more strongly in SE cultured with 4 g l^{-1} of gellan gum included superoxide dismutase. Furthermore, several heat shock proteins (HSPs) were found to be differentially expressed (HSP70, HSP81, HSP90, HSP101 and GRP94), all but one of which were more abundant in the SE cultured on the 4 g l^{-1} medium. These proteins could be involved in the assembly and stabilization of newly synthesized proteins during cell division and expansion (Sung et al. 2001, Wang et al. 2004). HSPs are generally more abundant in zygotic than in SE (Sghaier-Hammami et al. 2009), and they are especially abundant in late stages of embryo maturation, their accumulation being induced by seed dehydration. At 1 week, SE still contain about 90% water. As this does not suggest water stress, the presence of HSPs could indicate the presence of other types of abiotic stresses (Lee and Schoffl 1996, Marsoni et al. 2008). Intriguingly, the need for both cell detoxification and stabilization of proteins by HSPs appears to be greater when the maturation of embryos occurs in the presence of 4 g l^{-1} gellan gum than when 8 g l^{-1} is present.

Despite these indications that the SE cultured on the medium with 4 g l^{-1} gellan gum may have been stressed, water availability and $\Psi\pi$ were lower in the medium with 8 g l^{-1} gellan gum, indicating that the latter may have induced drought stress. However, this hypothesis was not supported by the profiles of various proteins that are reportedly induced by drought, either directly or indirectly, including 6-phosphogluconate dehydrogenase (decarboxylating), actin, enolase, fructose phosphate aldolase, phosphoglucomutase and superoxide dismutase (Ali and Komatsu 2006, Costa et al. 1998, Jorge et al. 2006, Plomion et al. 2006, Riccardi et al. 1998, Salekdeh et al. 2002, Tausz et al. 2004). On the basis of observed profiles of these proteins, we therefore cannot infer the presence of such a stress under these conditions. Indeed, they were among the 56 identified differentially expressed proteins that were expressed at a higher level in SE cultured on the 4 g l^{-1} medium. Two of them, 6-phosphogluconate dehydrogenase and fructose phosphate aldolase, are key metabolic enzymes, the first being involved in the pentose phosphate pathway, while superoxide dismutase catalyses conversion of the superoxide radical to H_2O_2 and plays a key role in detoxification processes (Alscher et al. 2002). Overexpression of the corresponding gene has also been shown to enhance tolerance to salt, water and osmotic stresses in tobacco (Badawi et al. 2004). In addition to the increased abundance of HSPs in SE cultured on the 4 g l^{-1} medium, the observed increases in expression of pyruvate decarboxylase (which directs carbon metabolism toward glycolysis) and apparent detoxification capacity (indicated by the increased expression of superoxide dismutase) suggest that maturation on medium containing 4 g l^{-1} gum may induce stress in the SE.

In contrast, observed increases in the abundance of actin and type Ras protein in SE cultured with 8 g l^{-1} gellan gum indicated that cell division was more active on this medium, and this was confirmed for later stages, as the total protein content and DW measurements showed that these SE were more advanced in the maturation process. Furthermore, many studies have shown that the length of the maturation period of SE played an important role in promoting desiccation tolerance (Attree et al. 1992), but also that the partial or full desiccation of coniferous SE had a positive effect on subsequent plantlet development (Attree et al. 1991, Lelu et al. 1995). The qualitative improvement described by Lelu and Paques (2009) could be attributed to the decrease of water content in SE during maturation.

In conclusion, the data presented here describe the physiological effects of an increased gellan gum concentration in the medium used to culture SE of hybrid larch in which we observed a decrease in $\Psi\pi$ and water content, and an increase in DW. The putatively identified differentially abundant proteins suggest that the SE physiological status was better on the medium with the higher gellan gum concentration, as indicated by the reduction in abundance of enzymes involved in the glycolysis pathway and HSPs. These results strengthened our choice to mature hybrid larch embryogenic lines with 8 g l^{-1} gellan gum. We are now investigating this medium modification for other conifer species. This is the first report of a 2-DE proteomic analysis of conifer SE maturation in the presence of gelling agent at high concentration, and the first published proteomic analysis of *Larix*. In general, there is a lack of molecular information related to conifer SE maturation at the protein level, and further studies are required to clarify the involvement of individual proteins in the maturation process.

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Supporting Information

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

Appendix S1. Spots containing more than one putative identified protein, presented according to gellan gum level in the culture medium.

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