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Caroline Teyssier, Stéphane Maury, Martine Beaufour, Cécile Grondin, Alain Delaunay, et al.. In search of markers for somatic embryo maturation in hybrid larch (*Larix x eurolepis*): global DNA methylation and proteomic analyses. *Physiologia Plantarum*, 2014, 150 (2), pp.271-291. 10.1111/ppl.12081 . hal-02087600

HAL Id: hal-02087600

<https://hal.science/hal-02087600v1>

Submitted on 27 May 2020

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In search of markers for somatic embryo maturation in hybrid larch (*Larix x eurolepis*): global DNA methylation and proteomic analyses.

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Abstract

A global DNA methylation and proteomics approach was used to investigate somatic embryo maturation in hybrid larch. Each developmental step during somatic embryogenesis was associated with a distinct and significantly different global DNA methylation level: from 45.8% mC for undifferentiated somatic embryos (1 week proliferation) to 61.5% mC for immature somatic embryos (1 week maturation), while maturation was associated with a decrease in DNA methylation to 53.4% for mature cotyledonary somatic embryos (8 weeks maturation). The presence of 5-azacytidine (hypo-methylating agent) or hydroxyurea (hyper-methylating agent) in the maturation medium altered the global DNA methylation status of the embryogenic cultures, and significantly reduced both their relative growth rate and embryogenic potential, suggesting an important role for DNA methylation in embryogenesis. Maturation was also assessed by examining changes in the total protein profile. Storage proteins, identified as legumin-like and vicilin-like, appeared at the precotyledonary stage. In the proteomic study, total soluble proteins were extracted from embryos after 1 and 8 weeks of maturation, and separated by two-dimensional gel electrophoresis. There were 147 spots which showed significant differences between the stages of maturation; they were found to be involved mainly in primary metabolism and the stabilization of the resulting metabolites. This indicated that the somatic embryo was still metabolically active at 8 weeks of maturation. This is the first report of analyses of global DNA methylation (including the effects of hyper- and hypo- treatments) and proteome during somatic embryogenesis in hybrid larch, and thus provides novel insights into maturation of conifer somatic embryos.

Abbreviations

5-aza, 5-azacytidine; ABA, abscisic acid; mC, methylcytosine; EMs, embryonal masses; Glc, Glucose; Frc, fructose; FW, fresh weight; HU, hydroxyurea; SE, somatic embryos; ZE, zygotic embryos;

Introduction

Somatic embryogenesis in hybrid larch species has been studied at INRA, in France for over two decades. The results have facilitated the development of an improved protocol leading to the routine production of plants (emblings). Recently, this protocol was applied for propagation of the new hybrid variety REVE-VERT (*Larix x eurolepis* [Lelu-Walter and Pâques, 2009](#)). Somatic embryogenesis in hybrid larch is a model system for studying somatic embryo (SE) differentiation in gymnosperms. In this context, research has mainly been conducted either to improve the methodology for larch somatic embryogenesis ([Gutmann et al. 1996](#); [Lelu-Walter and Pâques, 2009](#)) or to describe physiological aspects of embryogenesis ([Label and Lelu, 2000](#); [von Aderkas et al. 2002](#)).

Ontogenetic development in trees is characterized by successive phase changes which are accompanied by morphological and physiological modifications (Poethig, 1990). There are marked differences in organogenetic capacity between young and adult stages: loss of morphogenetic competence reflects the transition towards a mature state. Among the mechanisms involved in the regulation of plant development, DNA methylation is one of the key factors controlling gene transcription (Feng et al. 2010; Miguel and Marum, 2011). This methylation is an epigenetic mechanism, which causes changes in gene expression without modifying the DNA sequence. These changes are correlated with stages in the plant life cycle, such as age in *Sequoiadendron giganteum* (Monteuuis et al. 2008) and maturation in *Pinus radiata* (Valledor et al. 2010). However, these correlations do not prove that there is a causal role for methylation in mediating specific phase changes. *In vitro* tissue culture methods and chemical treatments are known to induce modifications in DNA methylation, which may ultimately affect the phenotype and/or the potential for regeneration in cultures (Lambé et al. 1997; Valledor et al. 2007; Rodríguez López et al. 2010).

Recent reports on cell lines at distinct stages of differentiation have suggested causal relationships between DNA methylation levels and morphogenetic competence *in vitro* (Causevic et al. 2005), and have led to the cloning of morphogenesis markers exhibiting variations in DNA methylation (Causevic et al. 2006) and the identification of epialleles that are conserved between parental lines during shoot regeneration *in vitro* (Maury et al. 2012). In Gymnosperms, the only available methylation data related to SE development are recent and still very fragmented. They are from studies on *Pinus pinaster* (Klimaszewska et al. 2009), *Pinus nigra* (Noceda et al. 2009) and *Picea omorika* (Lejcek-Levanic et al. 2009). To improve our understanding of this mechanism, we aim to describe the global DNA methylation status of embryogenic cultures of hybrid larch at various SE stages. We also seek to determine whether the global DNA methylation status of embryogenic cultures can be altered and, if so, whether such an alteration could cause a change in the morphogenetic competence of hybrid larch with respect to SE development. To this end, embryonal masses were exposed to a hypo-methylating agent (5-azacytidine, 5-aza; Lambé et al. 1997; Causevic et al. 2005; Causevic et al. 2006), or a hyper-methylating agent (hydroxyurea, HU; Causevic et al. 2005; Causevic et al. 2006).

Proteins are often considered to be useful markers for maturity and/or quality of SEs (Klimaszewska et al. 2007; Tereso et al. 2007). Accumulation of total protein has been used to follow maturation of SEs in conifers (Sallandrouze et al. 1999; Lippert et al. 2005). More specifically, accumulation of storage proteins has been monitored (Gösslová et al. 2001; Brownfield et al. 2007). All these studies described the deposition of storage proteins during maturation but made assumptions about the identity of the proteins based on gel retention times. However, in somatic embryos of *Pinus strobus*, vicilin-like and legumin-like proteins, which constitute the main storage protein in this conifer, were identified by mass spectrometry; their presence is in agreement with the previous hypothesis (Klimaszewska et al. 2004). Recently, efforts have been made to describe somatic embryogenesis at the molecular level, and current techniques for proteomic studies combine the resolution of 2D-gel electrophoresis with a high

degree of accuracy in protein identification by mass spectrometry. As for methylation data, in conifers, proteomic datasets during somatic embryogenesis are limited. They are currently available only for *Cupressus sempervirens* (Sallandrouze et al. 1999) and *Picea glauca* (Lippert et al. 2005). These studies have led to advances in the identification of proteins involved in the embryogenesis process, and some of these proteins may be candidates for further investigation in terms of biology and functional relevance.

Our objective was to clarify the involvement of individual proteins in the maturation process and to identify the wide range of proteins that are differentially regulated in the embryos during embryo maturation. We used 1D and 2D gels to quantitatively assess the expression patterns of proteins throughout the process of hybrid larch SE maturation.

These global DNA methylation and proteomic analyses are novel for hybrid larch and represent new insights into SE maturation. They provide the first molecular data on the biochemistry of embryonal mass as it relates to embryogenic potential.

Material and methods

Plant material

Experiments were conducted with an embryogenic line (N23) of *Larix x eurolepis* (*L. decidua* x *L. kaempferi*). Embryonal masses (EMs) were induced from immature zygotic embryos (ZE) of variety REVE-VERT, which was produced crossing one European larch clone with a polymix of 12 Japanese clones (Lelu-Walter and Pâques, 2009). Hybrid seeds stored at -20°C were dissected to excise ZE from the surrounding megagametophyte tissue. Samples (ZE, megagametophyte) for protein assay were immediately frozen in liquid nitrogen prior to storage at -80°C.

Embryonal mass multiplication

Multiplication medium consisted of basal MSG medium (Becwar et al. 1990) containing 1.45 gL⁻¹ glutamine (SIGMA) supplemented with 9 µM 2,4-dichlorophenoxyacetic acid, 2.3 µM 6-benzyladenine and 60 mM sucrose, solidified with 4 gL⁻¹ gellan gum (Phytigel™, SIGMA). EM was cultured according to Lelu-Walter and Pâques (2009). Briefly, approximately 300 mg fresh weight (FW) EM was suspended in 5 ml liquid medium and poured over a filter paper disc (Whatman N° 2, 7.5 cm diameter) in a Büchner funnel. The filter paper with dissociated EM was placed on multiplication medium for one week. Relative growth rate was determined as ((FW_{n+1} – FW_n)/ FW_n).

SE maturation

SE were matured according to Lelu-Walter and Pâques (2009). Briefly, approximately 200 mg FW proliferating EM was suspended in liquid MSG medium without plant growth regulator, and distributed over a filter paper disc as described earlier. Each filter paper disc with dissociated EM was

incubated for 1 week on plant growth regulator free medium supplemented with activated charcoal (10 gL⁻¹, SIGMA) and 0.1 M sucrose. Relative growth rate, as defined above, was measured throughout the period of culture on charcoal medium. Filters were then transferred onto MSG medium containing 0.2 M sucrose, 1 µM indolebutyric acid, and 60 µM *cis-trans* (±) abscisic acid (ABA) for 8 weeks. Maturation was conducted in darkness at 25°C ± 1°C. After 8 weeks of culture, cotyledonary SE were counted and the embryogenic potential (number of SE per g FW) was estimated.

DNA methylation pattern

Effect of hypo-methylating and hyper-methylating drugs

Maturation was performed as described above (= control) and in the presence of either 5-aza (hypo-methylating drug) or HU (hyper-methylating drug). Each drug was added (100 µM) to all the media (proliferation, charcoal, ABA), which were prepared a few hours before the experiments. Samples were collected at the following stages of SE development ([von Aderkas et al. 2001](#)): after one week of culture on proliferation medium (undifferentiated SE, i.e. late stage of early embryogeny), and after one week of culture on charcoal medium, corresponding to immature SE (i.e. early stage of late embryogeny). At these two stages SE were collected with the surrounding tissue, whereas after 8 weeks of culture in the presence of ABA, cotyledonary SE were collected separately. Samples (about 400 mg FW) were frozen in liquid nitrogen before being stored at -80°C for subsequent DNA methylation analysis. For each collection date, three replicates were analysed, and experiments were repeated three times.

Genomic DNA extraction and HPLC determination of DNA methylation percentage

Genomic DNA was extracted from 400 mg samples of frozen hybrid larch *in vitro* cultures ground into a fine powder with liquid N₂. Extraction buffer was composed of 0.2 M Tris-HCl and 0.2 M EDTA (pH 9.0), supplemented with 1.5% (w/v) SDS and 10 mM β-mercaptoethanol ([Causevic et al. 2005](#)). The suspension was digested successively with 100 U ribonuclease A (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 1 h at 37°C, and with 1 U proteinase K (Sigma-Aldrich) for 1 h at 60°C. Extraction was done twice with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and twice with chloroform/isoamyl alcohol (24:1, v/v). Precipitation was achieved by the addition of 0.1 M NaCl and two volumes of ethanol at -20°C. DNA was washed with 70% ethanol and resuspended in ultrapure water. DNA quantification was carried out by measuring absorption at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA).

About 5 µg of pure genomic DNA were enzymically hydrolysed into nucleosides and analysed by High Performance Liquid Chromatography (HPLC) according to Gourcilleau et al. (2010). A hydrophobic column, Gemini™ (150 x 4.6 mm, 5µm, Phenomenex, Le Pecq, France), was used. The isocratic mobile phase was composed of 0.5% methanol (v/v) and 5mM glacial acetic acid in water. The flow rate was 1.5 mL.min⁻¹. Nucleosides were identified on the basis of comigration with commercial stand-

ards (Sigma-Aldrich) under the same HPLC conditions. Methylcytosine (mC) percentages were calculated using the following formula: $\%mC = (mC/(C + mC)) \times 100$, where C and mC represent 2'-deoxycytidine content and 5-methyl-2'-deoxycytidine content, respectively. At least five independent analyses and three replicates were performed for each measurement. In order to test for RNA contamination, the corresponding nucleoside standards (with retention times distinct from those of DNA nucleosides) were also injected. To calibrate the method, methylcytosine percentages of genomic DNA extracted from poplar were calculated and found to be similar to published data for this species (Gourcilleau et al. 2010). This method has been recently re-validated by realizing analysis of DNA methylation in two different laboratories with another procedure (Zhu et al. 2013).

Proteomic analysis

Total protein assay

Maturation was performed as described above (= control). On ABA medium, SE were collected after 1 (globular shape), 3 (pre-cotyledonary), 6 (cotyledons initiated) and 8 weeks (cotyledonary SE). At the two later stages, SE were collected separately from surrounding tissue. Total protein extracts were prepared (five replicates) from each type of sample (SE, ZE, megagametophyte). From 25 to 50 mg FW of frozen SE were homogenized with 0.5 ml of lysis buffer (10% (v/v) glycerol; 2% (w/v) SDS; 5% (v/v) β -mercaptoethanol; 2% (w/v) poly(vinyl)polypyrrolidone; 50 mM Tris pH 6.8). The extracted samples were incubated for 5 min at 95°C, and then centrifuged at 17000 rpm. Supernatants were transferred to new tubes and pellets were re-extracted once. The two supernatants were pooled. Total protein concentrations were determined using the Bradford assay with bovine serum albumin as a standard.

1D gel electrophoresis

Extracted proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a gradient gel (12-18%) with stacking gel (4%) following standard protocols. The gel was stained for proteins with colloidal Coomassie Brilliant Blue G-250 (CBB-G). The main bands observed in the megagametophyte lane and the corresponding bands in the lanes for ZE and SE (8 weeks old) were cut from the gel and subjected to LC-MS/MS identification (see below).

2D gel electrophoresis

Maturation was performed as described above (= control). Protein extracts were prepared in triplicate from SE matured for 1 and 8 weeks on maturation medium. About 400 mg of frozen sample was placed in a pre-chilled mortar, and ground in liquid N₂ for 5 min to produce a fine powder. The frozen powder was transferred to a microcentrifuge tube and proteins were extracted and precipitated with TCA-acetone as described in Teyssier et al. (2011). The protein concentration of each preparation was

determined using a modified Bradford assay as described in Teyssier et al. (2011) with bovine serum albumin as a standard.

First dimension separation was performed with 300 µg protein loaded onto 24 cm IPG strips, pH 4-7 (Protean IEF Cell system, Bio-Rad, France) and subjected to isoelectric focusing (IEF) at 25°C for 80 kVh using an IPGphor system (GE Healthcare Europe, France). Prior to the second dimension separation, strips were equilibrated according to Teyssier et al. (2011). Then 2D PAGE was performed using an Ettan DALTsix unit (GE Healthcare Europe, France) with 11% polyacrylamide gels, overnight at a constant 110 V and 80 mA, until the bromophenol blue front reached the end of the gel. Four to five biological replicates were analysed for each sample. Gels were stained with colloidal CBB-G according to Gion et al. (2005), then images were captured with a transmission densitometer (ImageScanner, GE Healthcare, France) at a resolution of 600 dpi, digitized and analysed using Progenesis software (Nonlinear Dynamics, United Kingdom). Gels were aligned. In order to compensate for slight variations in sample loading and gel staining, the volume (product of the area of the spot and its intensity) of each spot detected was normalized relative to the total volume of the spots on the gel. Every spot detected automatically was manually checked. A threshold of 2500, calculated on the basis of the ratio of normalized volume / area of the spot, was applied to the normalized volume of the spot to determine the number of spots present at each developmental stage.

Nanospray LC-MS/MS and data analysis

Spots of interest were manually excised and individually transferred to microcentrifuge tubes. Gel spots were destained for 1 h 30 min at 4°C using acetonitrile mixed with 100 mM ammonium bicarbonate (45/55, v/v). After spot washing and in-gel trypsin (EC:3.4.21.4) proteolysis of proteins, the peptides produced were extracted onto Poros beads and purified with ZipTips (Millipore, USA) as previously described ([Beaufour et al. 2012](#)).

Liquid chromatographic separations of the peptide products of proteins separated on 1D gels were performed on an UltiMate 3000 nano-ultraLC system (Dionex, USA) using preconcentration on a 25 cm reverse-phase column (Acclaim PepMap RSLC C18, 75 µm ID, 2 µm particles, 100 Å pore size, Dionex) at 450 nL/min with a 15 min linear gradient of 2-35% solvent B; solvent A consisted of 0.1% aqueous formic acid and solvent B consisted of 0.1% FA in acetonitrile. Online MS and MS/MS were performed using a high resolution maXis quadrupole-time-of-flight spectrometer (Bruker, Germany) at an ESI voltage of 4500 V. Peaks with the three highest intensities and a minimum of 400 ion counts were selected for CID MS/MS fragmentation using an isolation window of 3-9 Da depending on the *m/z* value.

Conditions for the analysis of spots from 2D gels by liquid chromatography coupled to mass spectrometry were as described in Beaufour et al. (2012).

Protein identification by database sequence assignment

In the case of proteins separated on 1D gels, the MS/MS spectra acquired were searched against the UniProtKB database covering the plant kingdom (version downloaded on July 7, 2011 from www.uniprot.org) using the Mascot identification engine (version 2.3, Matrix Science, UK). The spectra were filtered with a minimum intensity of 50 and deconvoluted (charge 1+ to 5+). The search was conducted allowing for a maximum of 2 missed cleavages, 5 ppm Da tolerance for precursor ions and 0.04 Da for fragment ions, respectively. The confidence threshold was set to 95% ($p < 0.05$). Methionine oxidation and formation of β -mercaptoethanol adduct with cysteines were allowed. Only proteins having a minimum of three peptides with individual peptide scores greater than 20 were retained for further analysis. Using these parameters, protein scores were all greater than 35, corresponding to a positive identification threshold at $p < 0.05$.

In the case of proteins separated on 2D gels-, MS/MS spectra were searched against the DFCI *Pinus* genus tentative consensus sequence database (<http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=pine>, release 8.0 April 22, 2010) using PEAKS Studio software (version 5.2, Bioinformatics Solutions Inc., Canada). The data were converted to the mzXML file format and preprocessed by charge attribution on precursor ions (1+ to 3+) and filtering of spectra (quality factor = 0.65), followed by centroiding, deconvolution and deisotoping of MS/MS peaks. Data were merged within a 0.5 min window. Assignment of fragment ions to peptide sequences was performed using 2 maximum missed cleavages, with a 2.0 or 0.5 Da tolerance for precursor ions and fragment ions, respectively. The modifications allowed were variable methionine oxidation and fixed carbamidomethylation. Only proteins having peptide scores greater than 60% and protein scores greater than 88%, corresponding to a 95% true positive rate, were retained. With these thresholds, all proteins that were found with PEAKS Studio had a minimum of two unique identified peptide sequences. For all identifications, protein lists for each spot were curated manually to remove redundancies.

Statistical analyses

Statistical analysis was carried out with R software (version 2.13.1, R Development Core Team 2011). One-way ANOVA was performed on the results for mass growth during the multiplication and active charcoal steps, as well as for embryogenic potential after maturation. Student's *t*-test and general linear hypothesis tests ([Hothorn et al. 2008](#)) were performed to test the significance of the effects of different treatments on mass growth and embryogenic potential.

Effects of embryo development on total protein content were evaluated using one-way ANOVA. Variations in total protein content during maturation were analysed with multiple comparisons of means ([Hothorn et al. 2008](#)) with Tukey contrasts ($p < 0.05$). The intensity change for each spot in the proteomic study was analysed with Student's *t*-test on the basis of the normalized spot volume ($p < 0.01$). Any spot with a *p*-value < 0.01 according to Students' *t*-test, with a ratio of 1.7 between the two stages and a minimum normalized volume of $2 \cdot 10^6$ for at least one of the two stages, was deemed to have changed significantly between stages and was subsequently excised for further analysis.

Results

Variations in global DNA methylation and effects of hypo-methylating and hyper-methylating drugs during somatic embryogenesis in *Larix x eurolepis*

During maturation of SE, significant variations (one way ANOVA, $p < 0.05$) in global DNA methylation (% mC) were observed between undifferentiated SE ($45.8 \pm 3.8\%$ mC; mean \pm SD; Fig. 1A) and immature SE ($61.5 \pm 3.1\%$; Fig. 1B), but not with cotyledonary SE ($53.4 \pm 7.8\%$; Fig. 1C). The presence of drugs in the culture media affected global DNA methylation levels (Fig. 1A, 2A, 3A), growth (Fig. 1B, 2B) and embryogenic capacity of embryonal masses (Fig. 3B). Compared to the control conditions, the global DNA methylation after 1 week of multiplication decreased slightly from $45.8 \pm 3.8\%$ to $41.3 \pm 5.9\%$ in the presence of 5-aza whereas it significantly increased (to $73.5 \pm 6.1\%$) in the presence of HU (Fig. 1A). The relative growth rate of EM was significantly reduced from 6.3 ± 0.9 to 1.8 ± 0.8 in the presence of 5-aza and 2.7 ± 1.4 in the presence of HU (Fig. 1B), and in both cases the EM had a brownish appearance with necrotic areas. Drugs also affected both global DNA methylation and proliferation after 1 week of maturation. At this stage, global DNA methylation significantly decreased or increased in the presence of 5-aza or HU, respectively (Fig. 2A), compared to control conditions. Embryonal mass growth rate was drastically reduced from 4 ± 1.0 to 0.1 ± 0.2 (2.5% of residual growth rate) in the presence of 5-aza and to 2.7 ± 1.1 in the presence of HU (Fig. 2B). Finally, after 8 weeks of maturation, the global DNA methylation of the cotyledonary SE decreased from $53.4 \pm 7.8\%$ in control conditions to $48.6 \pm 3.1\%$ in the presence of 5-aza and significantly increased to $82.0 \pm 3.4\%$ (Fig. 3A) in the presence of HU. Recovery of cotyledonary SE was significantly affected by presence of the drugs, both of which reduced the embryogenic potential (Fig. 3B). On average, under control conditions line N23 produced 577 ± 60.6 embryos per g fresh weight, but its embryogenic potential was reduced to 174 ± 50.5 in the presence of 5-aza and drastically decreased to 34 ± 47 (5.8% of the control embryogenic potential) in the presence of HU.

Total protein assay

Accumulation of proteins in the developing SE

Total protein extracts, isolated from samples collected at various times during somatic development, were determined by the Bradford assay (Table 1). A gradual increase in protein content could be observed through the different stages of somatic embryogenesis, whether expressed per mg of FW or per number of embryo units (ANOVA associated probability $< 2.2 \cdot 10^{-16}$). By 6 weeks of maturation, the maximum level of protein ($63.4 \pm 8 \mu\text{g} \cdot \text{mg}^{-1}$ FW) was reached in the embryos, and no significant change was observed from then on. At 8 weeks of maturation, SE had similar protein level to ZE (78.6 ± 17.2 and $98.3 \pm 11.1 \mu\text{g} \cdot \text{unit}^{-1}$ respectively).

1-D electrophoresis analysis

Total protein extracts were prepared from embryos harvested at different maturation times, and separated by SDS-PAGE under denaturing conditions (Fig. 4). The equivalent extracts from ZE and megagametophytes of dehydrated seeds were loaded as controls. Protein profiles showed changes during maturation, leading to the presence of major bands in extracts of 6 and 8 weeks maturation SE; they are numbered from 1 to 7 in Fig. 4. This pattern was similar to those observed with ZE and megagametophyte protein extracts. All these major bands could be seed storage proteins, on the basis of their apparent molecular weights. Proteins in the seven bands from 8 weeks maturation SE, ZE and megagametophyte were identified by LC-MS/MS (Table 2 and Appendix S1 in Supporting Information). Vicilin-like protein and/or legumin-type storage protein was found in all these bands, except in band 7. In band 2 from the SE at 8 weeks (Table 2, sample SE2), no storage protein could be identified from any of the replicate mass spectrometry analyses.

2D gel electrophoresis

Changes in protein profiles of SE were investigated for 2 maturation stages (1 and 8 weeks) using 2D gel separation. It was possible to detect 1188 reproducible spots in gels stained with colloidal CBB (Fig. 5). After normalization of spot volume and background filtering there were 891 detectable spots in 1-week maturing SE and 1188 spots in 8 weeks mature SE. The differences in each normalized spot volume between 1 and 8 weeks of maturation were tested statistically using a *t*-test ($p < 0.01$) and spots showing significantly different changes of at least 1.7 fold were kept for further analysis. Of the differentially expressed proteins, spots having a normalized spot volume of at least 2.10^6 at either 1 or 8 weeks of maturation were selected to ensure that enough material was available for further LC-MS/MS analysis and protein identification (Table 3). This yielded 147 differentially expressed and detectable proteins, which are shown as numbered spots on the 2D gel (Fig. 5). Of these spots, 55 and 92 were up-regulated in SE after 1 and 8 weeks maturation, respectively. The *m/z* values of peptide ion fragments were matched with protein sequences present in the DFCI database. For all but 5 spots, LC-MS/MS identification produced an assignment to a single protein. In the remaining 5 analyses, multiple proteins per spot were identified with scores above the significance threshold. These spots were removed from the dataset and not subjected to further analysis (see Appendix S1 in Supporting Information); from this point on in the text, strict equivalence between a spot and a protein is assumed. Overall, 131 different proteins were identified and assigned to functional categories based on protein orthology (Table 4); of these 131 proteins, 17 could not be classified. Annotated proteins fell mainly into the “Metabolism” (41%) or “Genetic information processing” (24%) categories.

Discussion

Global DNA methylation varied during hybrid larch somatic embryogenesis and could not be artificially modified without decreasing or abolishing the growth and embryogenic potential of embryonal mass

Organogenesis is achieved in three steps: (1) the acquisition of organogenic competence by explant cells, (2) the division and determination of quiescent cells to form organ primordia and (3) the morphogenesis of organs (Sugiyama, 1999). The way in which cells remodel their gene expression program is central to this process, and in Angiosperms it has been shown to involve large-scale chromatin reorganization during the acquisition of cell pluripotency in protoplast systems (Zhao et al. 2001; Tessadori et al. 2007). Further studies have identified genes targeted for DNA methylation during this process; for example, some members of the NAC-domain family (transcription factors involved in developmental processes) displayed hypo-methylation-dependent upregulation of expression (Avivi et al. 2004; Berdasco et al. 2008; Miguel and Marum, 2011). Many Angiosperm species show variations in the levels of DNA methylation during somatic embryogenesis and embryogenic lines generally exhibit lower global DNA methylation levels than non-embryogenic lines (LoSchiavo et al. 1989; Miguel and Marum, 2011; Maury et al. 2012). Our objective in the present study was to test the extent and possible role of DNA methylation during somatic embryogenesis in Gymnosperms.

Given that the differentiation status of hybrid larch tissues increases during the successive steps required for SE, our results suggest that global DNA hyper-methylation in the embryonal mass is associated with somatic embryo differentiation in hybrid larch: methylation increased from 45.8% mC for undifferentiated somatic embryos to 61.5% mC for immature somatic embryos while morphogenesis and maturation were associated with a final decrease in DNA methylation to 53.4% for mature cotyledonary SE. Such variations in DNA methylation levels (and a similar range of values) and morphogenetic properties have previously been reported in plants using HPLC or HPCE methods (Lambé et al. 1997; Fraga et al. 2002; Causevic et al. 2005; Causevic et al. 2006; Hasbun et al. 2008; Noceda et al. 2009; Fraga et al. 2012; Maury et al. 2012), but never for the entire process of somatic embryogenesis in a gymnosperm. Indeed, Fraga et al. (2012) reported levels of DNA methylation from 22.6% to 44.3% in two accessions of *Acca sellowiana* during 30 days of culture of SE. Treatment with 5-Aza (50 or 100 μ M) and 2,4-D-free resulted in a marked decrease in methylation for both accessions, ranging from 37.6% to 20.8% (a final range variation of 16.8%) in agreement with our data in larch SE. Different embryogenic lines of maritime pine with high or low embryogenic capacity were shown to display similar global DNA methylation levels (about 18%). However, methylation-sensitive amplification analyses of polymorphism revealed specific variations in methylation between young and aged embryogenic lines (Klimaszewska et al. 2009). In *Pinus nigra*, Noceda et al. (2009) reported a clear negative correlation between global DNA methylation level and embryogenic potential. They showed that the global DNA methylation level of the genome varied from 18 to 30% for embryogenic and non-embryogenic lines respectively. Thus, hypo-methylation of the genome

appears to be a characteristic of embryogenic lines that are able to regenerate whole plants ([Noceda et al. 2009](#)). However, these authors did not use hypo- or hyper-methylating agents to test the causality of the relationship.

In order to assess the impact of modifications in global DNA methylation on embryogenic capacity, hybrid larch embryonal masses were cultured in the presence of a hypo-methylating agent (5-aza) or a hyper-methylating agent (HU), as previously reported for other plant cell systems *in vitro* ([Lambé et al. 1997](#); [Causevic et al. 2005](#); [Yamamoto et al. 2005](#); [Causevic et al. 2006](#); [Fraga et al., 2012](#)). The range of concentration used for 5-aza is variable (from 0.4 to 1000 μM) in previous studies ([LoSchiavo et al. 1989](#); [Causevic et al. 2005](#); [Yamamoto et al. 2005](#); [Klimaszewska et al. 2009](#); [Fraga et al., 2012](#)) and showed a dose effect response with a possible cell toxicity. This is in agreement with the brownish appearance with necrotic areas of larch EM. Nevertheless, [Klimaszewska et al. \(2009\)](#) did not obtain significant change in DNA methylation using only 40 μM of 5-aza in agreement with data from [Causevic et al 2005](#) leading us to test a 100 μM of 5-aza concentration. Indeed, [Fraga et al. \(2012\)](#) using 50 to 100 μM 5-aza has recently shown an increasing number of somatic embryos of *Acca sellowiana* by combining 2,4D and 5-aza. As expected, it was possible to modify the global DNA methylation status of hybrid larch embryogenic lines, but the extent of modification was dependent on the stage of somatic embryogenesis and on the drug. It is noteworthy that 5-aza induced the greatest hypo-methylation in immature somatic embryos, which developmentally are hyper-methylated compared to EM, while HU was most effective in cotyledonary somatic embryos, which are developmentally hypo-methylated compared to immature SE. In addition, the greatest impacts on the growth and embryogenic potential of EM were found with these two treatments. These observations are in agreement with the data of [LoSchiavo et al. \(1989\)](#) and [Yamamoto et al. \(2005\)](#), who showed that artificially altering DNA methylation immediately and irreversibly stopped embryogenesis and the expression of embryo-related functions in carrot cell suspension cultures. Embryos are arrested at the globular stage in media containing 2,4-dichlorophenoxyacetic acid, suggesting that cells capable of initiating differentiation are inhibited (undergo no further division) under these conditions ([Lambé et al. 1997](#)). Another example was reported for maritime pine, where embryogenic masses cultured in the presence of low concentration of the hypo-methylating agent 5-aza did not affect the global DNA methylation level but showed alterations in their type of DNA methylation (demethylation of mCC sites for example) using Methylation-sensitive amplification polymorphism (MSAP) analysis ([Klimaszewska et al. 2009](#)). This was depending on drug concentration and duration of treatment and was followed by a slight increase of EM treated with 10 and 15 μM of 5-aza. In hybrid larch SE, both methylation-modifying agents dramatically decreased EM growth as well as embryogenic potential; this could be due to the toxicity of the agents when used at 100 μM . The effect was particularly noticeable for 5-aza treatments of undifferentiated SE and cotyledonary SE, where a slight DNA hypo-methylation is associated with a

marked decrease in EM growth or embryogenic potential as previously reported (Yamamoto et al. 2005; [Klimaszewska et al. 2009](#)). Overall, our data show that each developmental step during somatic embryogenesis in hybrid larch is associated with a different level of global DNA methylation. No causal relationship between global DNA methylation and somatic embryogenic capacity has yet been clearly established for trees ([Klimaszewska et al. 2009](#); [Leljak-Levanic et al. 2009](#); [Noceda et al. 2009](#)), but our data have shown that artificial modifications of DNA methylation (hyper- and hypo-), particularly those that counteract the normal developmental variations, result in the abolition of morphogenesis. Nevertheless, it can be excluded that combinations of pulses of hypo- or hyper-methylating drugs at various concentrations with phytohormonal treatments at specific stages of larch somatic embryogenesis could help to improve this process in larch as it has been reported in *Acca sellowiana* (Fraga et al., 2012).

The identification of storage proteins during SE maturation was in accordance with existing hypothesis; their abundance did not appear to change during the cotyledonary stage

During ZE development, cellular expansion happens after the morphogenesis phase. It includes accumulation of storage compounds and acquisition of desiccation tolerance. Storage material can accumulate in different forms, one of which is protein. Our results show an increase in protein content during SE maturation, reaching a maximum at the cotyledonary stage, i.e. after 6 to 8 weeks. Quantitatively and qualitatively the total protein contents are very similar in ZE and in matured SE. In our work, most of the major protein bands present in ZE, mature SE and megagametophyte were identified by LC-MS/MS as legumin-like and vicilin-like proteins. The same protein classes are present in the same types of tissues in *Pinus strobus* ([Klimaszewska et al. 2004](#)); and their presence is suspected in various studies on other conifers ([Brownfield et al. 2007](#)). Legumin-like and vicilin-like proteins are typical storage proteins, belonging to the globulin protein family ([Shewry et al. 1995](#)). Under denaturing and reducing conditions, the subunits of the 11S-legumin-like protein are separated, producing specific bands. On the basis of their apparent molecular weights determined from denaturing gel electrophoresis (Fig. 4), band 6 could be the small subunit of 11S-legumin-like protein whereas bands 2 to 4 could be the large subunit. This is consistent with the corresponding peptide sequence data from mass spectrometric analysis (data not shown). Because of the multigenic character of the 11S-legumin-like protein family ([Shi et al. 2010](#)), such proteins could be present in different isoforms, producing multi-banded patterns like those that we observed. Although vicilin-like proteins are generally encoded by a large number of genes, our results show a single band (band 1) on the gel which is correlated with SE maturation state, thus confirming that it represents a potential SE maturation marker for hybrid larch, as has already been proposed for spruce ([Lippert et al. 2005](#)).

Separation on 2D gels identified 147 proteins that were differentially expressed during SE maturation

We found 1188 reproducible protein spots that could be detected in SE whatever the stage. Most of these were not expressed differentially between 1 and 8 weeks (w) of maturation (data not shown), and they corresponded to “housekeeping” proteins involved in basic metabolism (Finnie et al. 2002). Among the differentially-expressed spots, 147 were unambiguously identified as representing differentially expressed proteins. In 55 cases, proteins were more abundant in immature SE (1w) while in 92 cases they were more abundant in mature SE (8w). Keeping in mind the fact that the same amount of total soluble protein was loaded on each 2D gel, the changes between SE maturation stages could be explained by cell differentiation. At the cotyledonary stage, we might have expected a decrease in the number of different spots due to the accumulation of the storage proteins generally found in mature embryos. This would have been consistent with the presence of a small number of dominant bands in the protein profile at the mature stage. Although storage proteins are easily detectable by means of classical electrophoresis (Lelu-Walter et al. 2008), this is not the case in 2D proteomic studies (Lippert et al. 2005 ; Shi et al. 2010). These apparent discrepancies originate from the fact that the isoelectric point of most storage proteins we have identified is beyond the pH range used in the first dimension of our 2D gel.

Among the proteins identified in the 147 significant spots, 4 were present in multiple spots (chalcone synthase: spots 1, 30, 33, 35, 110; HSP70: spots 3, 11, 26, 41, 103, 108, 125; HSP90-1: spots 19, 32, 121; RuBisCO large subunit binding protein subunit beta: spots 20, 114). Similar spot redundancy was found in other conifers (Gion et al. 2005; Lippert et al. 2005; Balbuena et al. 2009). Multiple spots for a protein may often result from post-translational modifications, but can also be attributed to gene duplication, differentially spliced forms, or allelic variants. These assignments of multiple spots to single proteins may artificially increase the representation of some functional classes at different SE maturation stages. The case of a differentially-expressed protein annotated as Glucose-6-phosphate (Glc-6-P) isomerase (spots 12 and 112) is typical of our results. This enzyme is a key isomerase catalyzing reversible flipping between fructose-6-phosphate (Frc-6-P) and Glc-6-P. Spot 12 is more abundant in 8w SE whereas spot 112 is more abundant in 1w SE. But their position on the 2D gel (Fig. 3) shows that the two spots are very close to each other, indicating that there are extremely small differences in pI and apparent size. These differences may be due to the existence of isomeric forms showing specific activation patterns during SE maturation.

Differentially expressed proteins in mature SE (8w) are mainly involved in primary metabolism

Primary metabolism is highly represented (39 out of 141; 27%) in the annotations of proteins expressed differentially during hybrid larch SE maturation. These protein annotations relate to three pathways: glucose metabolism (2,3-bisphosphoglycerate-independent phosphoglycerate mutase, dihydrolipoyl dehydrogenase, enolase 1, Frc-bisphosphate aldolase, Glc-6-P isomerase, glyceraldehyde 3-P dehydrogenase, phosphoglycerate kinase, pyruvate decarboxylase, pyruvate dehydrogenase, pyruvate kinase); starch synthesis (fructokinase 2, Glc-1-P adenylyltransferase small

subunit, Glc-6-P isomerase, granule-bound starch synthase 1); and pentose metabolism (6-phosphogluconate dehydrogenase family protein, Frc-bisphosphate aldolase, Glc-6-P isomerase, transketolase, UTP--Glc-1-P uridylyltransferase 1). Out of these 39 soluble proteins with functions in primary metabolism, 8 and 31 are present in immature (1w) and mature (8w) SE, respectively, suggesting that mature SE are undergoing active metabolic processes that require co-activation of several enzymes from these three pathways. In *Picea mariana*, it has been shown that sucrose from the culture medium can be initially metabolized into Glc and Frc outside the tissues by enzymes excreted into the medium (Tremblay and Tremblay, 1995). As discussed above, two forms of Glc-6-P isomerase are abundant at each maturation stage. This key enzyme produces Glc-6-P or Frc-6-P from free sugars. Our result represents a starting point for a hypothetical reconstruction of what may be happening in hybrid larch mature SE (Fig. 6). Most of the annotated proteins from the starch pathway (excepted for fructokinase 2) were more abundant in the mature SE. This suggests enhanced starch metabolism in mature SE, which has been already observed in hybrid larch in our laboratory (unpublished data) but also in *Pinus pinaster* (Tereso et al. 2007) and *Picea mariana* as well as *Picea glauca* (Iraqi and Tremblay, 2001). As a Glc turnover process, the pentose phosphate pathway produces reducing equivalents in the form of NADPH and pentoses which are involved in nucleotide metabolism. Pentose phosphate metabolism appears to be slightly more active (Fig. 6) in mature SE (6P-glucuronate dehydrogenase, Frc 1,6-bisphosphatase, Frc biphosphate aldolase; Glc 6P isomerase and transketolase are upregulated at this stage). According to our results, glycolysis is enhanced in 8w SE, as indicated by the greater abundance of pyruvate kinase and pyruvate decarboxylase. Thus, either via glycolysis or for the synthesis of storage carbohydrates, there may be sinks for Glc and Frc in mature SE. Interestingly, in *Picea abies* mature SE contained predominantly sucrose, with low amounts of Glc and Frc (Lipavská et al. 2000), while in ZE these two simple hexoses disappeared two weeks after cotyledon formation (Gösslová et al. 2001). Unfortunately, none of these studies investigated the glycolytic activities of pyruvate kinase and pyruvate decarboxylase.

Mature SE (8w) show an increase in proteins involved in amino-acid metabolism

Based on the increased abundance of eleven proteins in mature SE, amino acid synthesis appears to be up-regulated at this stage. The observation of active protein synthesis in late embryogenesis is common in proteomic studies performed on conifers (Balbuena et al. 2009; Shi et al. 2010; Zhen et al. 2012). An increase in amino acid synthesis is consistent with the results of our assays of total protein content and with the observation that protein bodies increase in size and number during embryo development in hybrid larch (data not shown) and in *Cupressus sempervirens* (Sallandrouze et al. 2002). These protein bodies are the site of accumulation of storage proteins. However, we cannot exclude the possibility that these amino acids were supplying metabolic pathways other than protein synthesis. The enzymes adenosylhomocysteinase and bifunctional methylthioribulose-1-P dehydratase / enolase-phosphatase E1 are involved in the synthesis of methionine (Met), but the presence of S-

adenosylmethionine synthetase supports the use of Met as a precursor of S-adenosylmethionine (Ado-Met), the main methyl group donor in cells. Ado-Met is also a source of amino-alkyl and amino groups used for the biosynthesis of polyamines and ethylene (Bouvier et al. 2006), and a source of methyl groups for DNA methylation. There have been reports that Met metabolism has an important role in seed development and germination, as illustrated by proteomic analyses of developing *Medicago truncatula* seeds (Gallardo et al. 2007) or Chinese fir seeds (Shi et al. 2010).

Pathways for protein folding and stabilization appear to be enhanced in mature SE

Our results for proteins differentially expressed during hybrid larch maturation (Table 3) also show 21 proteins annotated as HSPs or related to protein folding, namely HSP70, cognate HSP70, stromal HSP70 related protein, HSP90, HSP101, luminal binding protein, RuBisCO large subunit binding protein (also known as chloroplastic chaperonin subunit beta and annotated as belonging to the HSP60 family), endoplasmic homolog, calreticuline and HSP60. Of these 21 proteins, 13 were more abundant at the 8w mature SE stage. While HSPs and related proteins are best known for stabilizing proteins and assisting in protein refolding under stress conditions, some of them are responsible for protein folding, assembly translocation and degradation (Sung et al. 2001; Wang et al. 2004). Many studies suggest that their presence is required throughout embryogenesis. For instance, in Douglas fir, luminal binding protein (a member of the HSP70 family) was shown to be regulated during seed development and early seedling growth (Forward and Misra, 2000). Moreover, HSPs are reported to play a role in SE induction in *Brassica napus* and *Daucus carota* (Binarova et al. 1997; Kitamiya et al. 2000) in *Picea glauca* and *Brassica napus* SE maturation (Reddy et al. 1998). However, our results show that some isoforms of HSPs are enhanced at either an early or a late stage of SE maturation (HSP70, HSP90, RuBisCO large subunit binding protein subunit beta). This has also been observed in *Cunninghamia lanceolata* (Shi et al. 2010). In our study, the HSPs were present in higher quantities at the late stage than at the early stage, probably in order to prepare the embryos for desiccation; this result is consistent with the high level of proteins synthesized at this stage. The HSPs could also have a protective function in response to the stress conditions that characterize *in vitro* growth.

Conclusions and perspectives:

In our search for biochemical determinants of SE maturation in hybrid larch (*Larix x eurolepis*) we have now identified many differences between SE at 1w and 8w of maturation. Global DNA methylation levels undergo variations throughout the stages of somatic embryogenesis, and they cannot be altered without reducing or even completely abolishing the process, suggesting an important role for DNA methylation. It is now well established that DNA methylation plays a role in gene expression and mobilization of transposons (Berdasco et al. 2008; Feng et al. 2010; Miguel and Marum, 2011). The potential toxicity of methylation-modifying agent cannot be excluded.

Nevertheless, it seems now relevant to go further and identify epialleles during this developmental process in order to demonstrate the role of DNA methylation. Recent reports describe the use of several different approaches for the identification of epialleles during plant culture *in vitro* ([Berdasco et al. 2008](#); [Miguel and Marum, 2011](#); [Maury et al. 2012](#)). The use of high-throughput genome technologies will accelerate the release of complete genome sequences for trees such as hybrid larch, and this may lead to a better understanding of biological processes as well as the validation of global DNA methylation variations reported in this study and the identification of epigenetic markers ([Gentil and Maury, 2007](#); [Johannes et al. 2009](#) ; [Miguel and Marum, 2011](#)). Studying the latter aspect is particularly relevant, since cell-to-cell variation in methylation has already been observed in callus, the epiallelic variants being transmitted to regenerated plants and fixed (Krisova et al. 2009; [Jaligot et al. 2011](#)). DNA methylation can control gene expression and thus indirectly impact on protein biosynthesis. The data presented here therefore also describe the maturation of SE in terms of protein composition. Generally accepted assumptions about the identities of the storage proteins have been validated. An accurate assessment of the state of maturation can now be made on the basis of the accumulation of genetic markers. Several specific proteins, mainly involved in primary metabolism, have been identified during this work; they clearly show the active status of mature cotyledonary SE, and will be further evaluated as potential genetic markers of SE maturation in the production of hybrid larch plants.

This is the first report of global DNA methylation (including the effects of hyper- and hypo-treatments) and proteomic expression profiles during somatic embryogenesis in hybrid larch, and these data make a major contribution to the otherwise scarce molecular information related to SE maturation in conifers.

Acknowledgments

We would like to acknowledge the financial support provided for this work by INRA, department EFPA (Ecologie des Forêts, Prairies et milieux Aquatiques, Projet innovant 2009). Proteomics was supported in part through a Région Centre/FEDER grant for high-resolution mass spectrometry (SyMBioMS, #2699-33931).

We thank Cyril Colas (FR2708) for expert assistance with ultra high-resolution mass spectrometry",

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Supporting information:

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

Appendix S1 Identification of proteins in gel bands according to band number and sample type.

Appendix table S2: Spots containing more than one putatively identified protein.

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Version définitive du manuscrit publié dans / Final version of the manuscript published in :
Physiologia Plantarum, 2014, 150(2), 271-291 <http://dx.doi.org/10.1111/ppl.12081>

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