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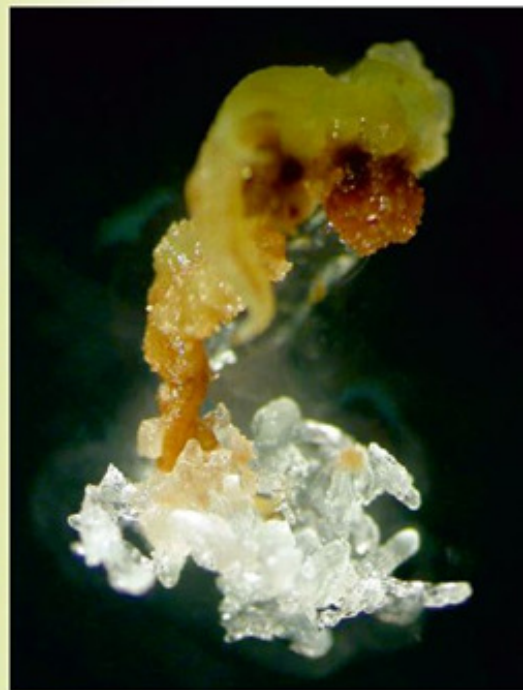
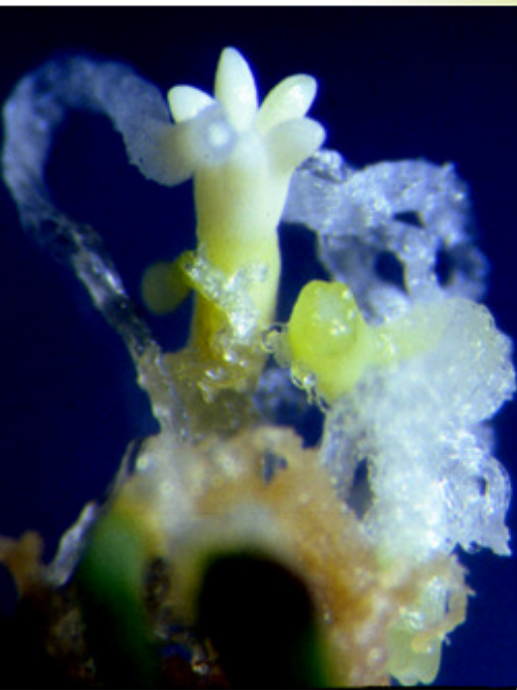
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Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management



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Somatic embryo maturation in maritime pine (*Pinus pinaster*): contribution of a 2-DE proteomic analysis for a better understanding

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Abstract: Somatic embryogenesis has reached application stage for *Picea* and *Larix* species; however it remains to be optimized for pine species. Thus maritime pine maturation needs improvement. To optimize the maturation step, it is necessary to develop markers that can be used to verify or monitor the quality of somatic embryos (SE). Therefore, a proteomic study was performed of two maturation stages to identify protein markers. There were about 100 significantly and differentially expressed proteins (Student's test, $p < 0.001$). They were mainly involved in carbohydrate or lipid metabolism and genetic information processing. In addition, many storage proteins were identified (vicillin-like, legumin-like, LEA proteins); some of them were, surprisingly, observed from the beginning of maturation. Our ultimate goal is to have a better understanding of SE maturation in *Pinus pinaster*. It is expected that this information will help to optimize the process and *in vitro* plant production.

Introduction

Maritime pine is extensively used in south-western Europe for lignocellulose production. Increasing demand for wood from this pine species requires efficient production of improved varieties and this is currently achieved only through seed orchard management. As in most long-lived tree species, variety design and deployment would greatly benefit from setting up an efficient vegetative propagation system of selected seed resources. Somatic embryogenesis is a promising technology for most conifers (Klimaszewska et al., 2007) and significant progress has been made in recent years for maritime pine (Lelu-Walter et al., 2006). However, whether « true » maturity of harvested cotyledonary SE has been achieved is still a debatable question. Cotyledonary SE are currently selected at the end of the maturation phase based on morphological criteria. Embryo maturation is a crucial step, giving rise to the establishment of reserve compounds including specific storage proteins that will ensure optimal germination and subsequent *in situ* plantlet development. A 2D-PAGE proteomic approach has been developed at INRA of Orléans (Teyssier et al., 2011) to define the protein status of developing SE. We aim to achieve a better understanding of the processes acting during the maturation phase of SE.

As a first step towards this goal this work presents our first results in proteomic analysis during SE development. This new knowledge is expected to refine our current methodology for practical application of somatic embryogenesis in maritime pine.

Materials and methods

Experiments were conducted with the embryogenic line AAY06006 initiated in July 2006 by FCBA from immature zygotic embryos harvested from mother clone G1.2631 (Landes origin). Trees were pollinated with a mixture of pollen from Morocco genotypes. Maturation was performed according to Lelu-Walter et al., 2006 with

the following modifications. Basal maturation medium consisted in mLV basal medium (Litvay et al., 1985) that contained 9 g l^{-1} gellan gum (PhytigelTM, SIGMA), $80 \mu\text{M}$ *cis-trans* (\pm) abscisic acid (ABA), and 0.2M sucrose. Cotyledonary SE were harvested after 1 (immature stage) or 12 weeks maturation (cotyledonary stage) for 2DE-proteomic analysis according to Teyssier et al. (2011). Briefly, total soluble proteins were extracted in liquid nitrogen from 400 mg ES (fresh mass) and precipitated with phenol. In the first dimensional separation (IEF), samples containing 450 μg protein were loaded and equilibrated onto 24-cm IPG strips, pH 4–7 (Protean IEF Cell system, Biorad, Marnes-La-Coquette, France). The second dimensional separation was performed in 2-D PAGE. Five biological replicates were analyzed for each sample. Gels were stained with colloidal CBB-G according to Gion et al., (2005), then images were scanned 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 automatically detected was manually checked. Selected spots were analyzed for protein identification by in-gel proteolysis followed by peptide extraction and nanoLC-MS/MS (nano high performance liquid chromatography on Dionex Ultimate coupled to tandem mass spectrometry on a Bruker Esquire HCT Ion Trap) and interrogation of *Pinus* TC databases.

Results and discussion

Statistical analysis and mass spectrometry identification

More than 1000 spots were reproducibly defined from 2D gels. Student's t test was performed on each of the normalized volume spots and more than 10% of all spots have shown a significant volume difference between the 2 tested stages ($P < 0.001$). Figure 1 shows their location on a 2D gel sample after 12 weeks of maturation. The proteins inside spots are defined according to their pI and MW.

The proteins were identified by in-gel proteolysis followed by nanoLC-MS/MS, using current TC DNA sequence databases. Whenever possible, the identified proteins were classified according to their biological function.

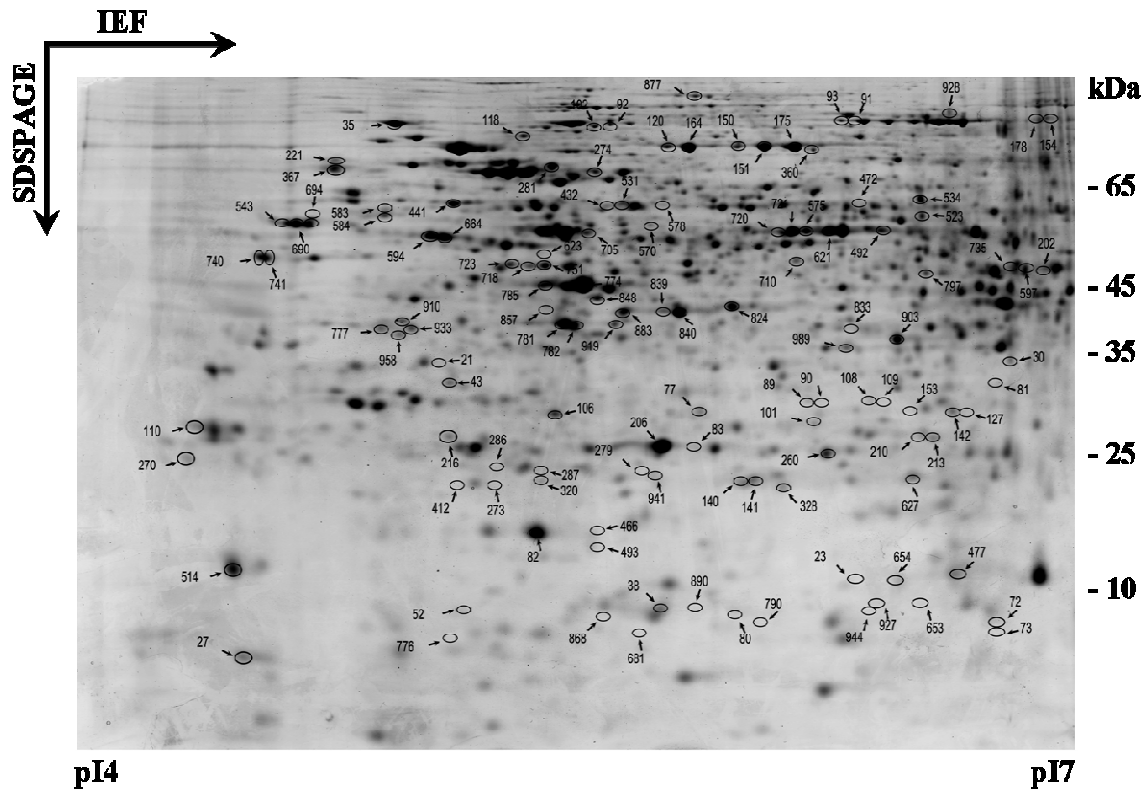


Figure 1. Representative 2-DE map obtained for maritime pine SE after 12 weeks of maturation. Marked spots displayed significant differences in their abundance ($P < 0.001$) between 1 week (immature SE) and 12 weeks of maturation (cotyledonary SE).

Biological interpretation

Based on the comparison of major functional classes (Fig. 2), we found that more proteins involved in metabolism are overexpressed in cotyledonary SE (12 weeks) than in immature ones (50% vs. 41% of total protein extract, respectively). Overexpressed proteins in cotyledonary SE are involved in carbon metabolism (20%), lipid synthesis (15%) and energy metabolism (15%); considering immature SE, over-expressed proteins are involved in

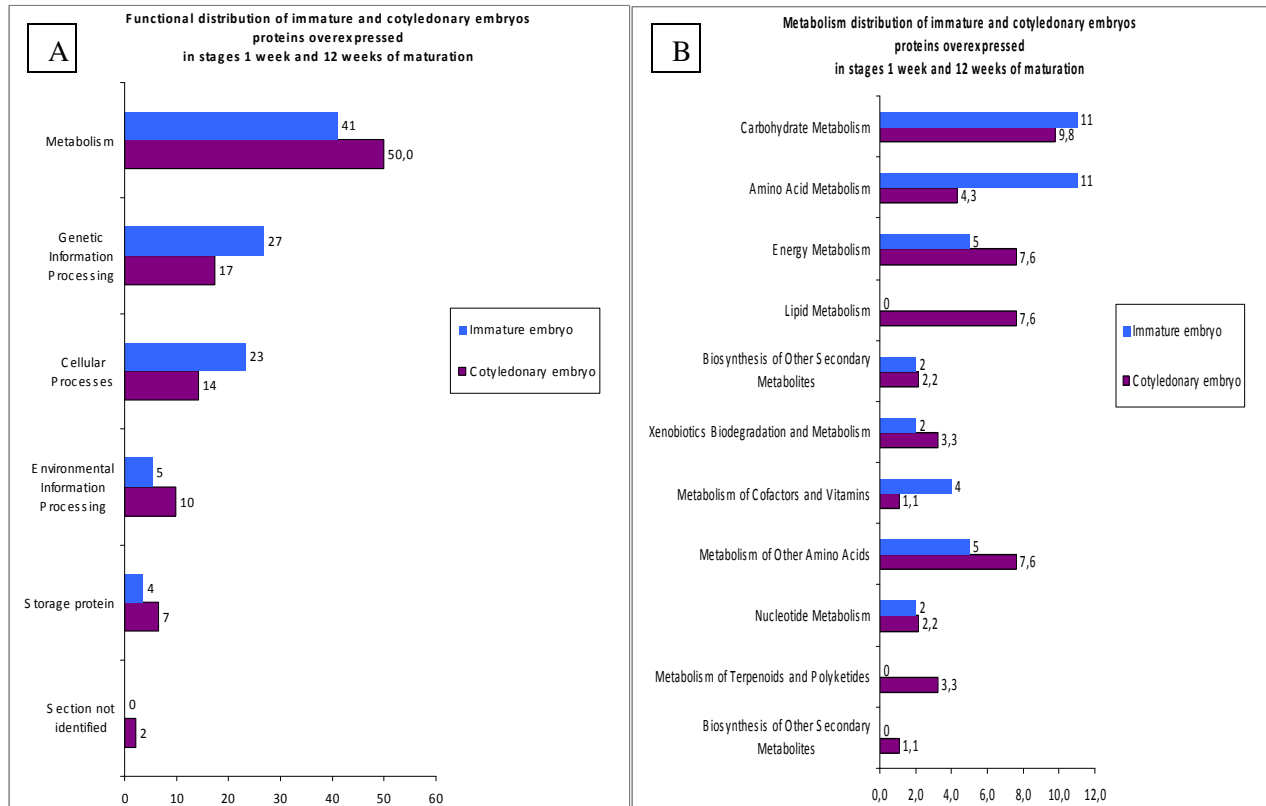


Figure 2. Distributions of the identified putative proteins among functional class (A) and metabolism class (B) according to two culture stages (immature and cotyledonary SE). Functional classification catalog Kegg: Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.jp/kegg/>. Numbers represent percentage of total proteins extracted for a stage of culture. 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.

amino acid synthesis (26%) and carbon metabolism (25%). Among the protein enzyme group, we found that ascorbate peroxidase involved in amino acid metabolism was significantly overexpressed (x 2.1) in immature SE when compared to cotyledonary SE. A similar observation was reported for Chinese fir (Shi et al., 2010) and soybean (Bailly et al., 2001) for zygotic embryos. Thus, ascorbate peroxidase could characterize the immature state of maritime pine SE. On the opposite, glutathione peroxidase, an enzyme involved in lipid metabolism, was 5.1 times more expressed in cotyledonary SE than in immature SE. An increased expression of this enzyme has been shown during SE development of *Eleutherococcus senticosus* (Shohael et al., 2007). We concluded that glutathione peroxidase is a good candidate marker of the cotyledonary state of maritime pine SE.

Conclusion and Perspectives

This preliminary work allowed us to identify putative protein markers involved in the main metabolic pathways such as energy and lipid metabolism. Metabolism of the amino acids seemed to be low in cotyledonary SE. Even if some markers have been identified, they could be related to embryogenesis and not to maturity. To answer this question it would be necessary to study the mature zygotic embryo by proteomic.

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