

SHORT COMMUNICATION

Poplar woody root proteome during the transition dormancy-active growthD. TRUPIANO¹, M. ROCCO², G. RENZONE³, A. SCALONI³, A. MONTAGNOLI⁴, M. TERZAGHI⁴, A. DI IORIO⁴, D. CHIATANTE⁴, & G.S. SCIPPA¹

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Abstract

Woody plants living in temperate climates finely regulate their growth and development in relation to seasonal changes; their transition from vegetative to dormancy phase represents an adaptation to their environment. Events occurring in the shoot during onset/release from dormancy have been largely investigated, whereas in woody roots they remain completely unknown. In recent years, we have been interested in understanding the molecular and physiological events occurring in poplar woody root during release from dormancy. Here, we propose the results of a comparative analysis of the proteome of poplar woody root sampled at different time points: T₀ (dormancy condition), T₁ (release from dormancy), and T₂ (full vegetative condition). This study identified proteins that may be involved in the long-term survival of a dormant root or landmarking a specific time point.

Keywords: Active growth-dormancy cycle, growth seasonal changes, *Populus nigra*, root proteome, woody root

Introduction

Temperate perennial woody plants use different environmental signals, i.e. light and temperature, to coordinate their growth and development in relation to seasonal changes. The capacity to modulate the phase shift from growth to dormancy is a basic adaptation of temperate perennial plants to their environment (Jansson et al. 2010; Desotgiu et al. 2012; Vitale et al. 2012). Indeed, the transition of meristems into and out of dormant bud is crucial for plant growth and survival (Furukawa et al. 2011).

Physiological and molecular changes occurring during dormancy induction and break have been mainly investigated in the shoots of several woody plants (Arora et al. 2003, Ruttink et al. 2007; Campoya et al. 2011; Terzoli et al. 2011), whereas the effects of seasonal changes on the root functions and activities have not been well characterized yet. For example, it is well established that photoperiod and temperature are the environmental signals that trigger the transitions between rapid growth and

dormancy in the shoot of woody plants (Welling et al. 2002). Despite the recognized importance of the aboveground parts of trees, shrubs, and forests, roots have been poorly investigated (Long et al. 2011; Rewald et al. 2011; Delgado et al. 2012; Ow & Sim 2012; Verma et al. 2012), and very few studies have been interested on the variety of internal (e.g., genotype of plant species) and external (e.g., light, temperature, precipitation, and soil properties) factors that coordinate root dynamics (Abbasi et al. 2011; Palove-Balang & Mistrik 2011; Zhang et al. 2011) in relation to seasonal changes (Chiatante et al. 2005; Montagnoli et al. 2012a, 2012b; Zhang et al. 2012).

Since poplar is the first forest tree the genome of which has been determined (Tuskan et al. 2006), it has become a pivotal model for studies related to different aspects of growth and dormancy in woody plants (Durand et al. 2011; Bonadei et al. 2012). In previous studies, we investigated the effects of bending stress on the woody roots of poplar (*Populus nigra*) along a three-point time course that, based on

shoot phenological observations, was proposed to correspond to dormancy, beginning of vegetative growth, and active growth (Trupiano et al. 2012a, 2012b, 2013b). Preliminary insights were also obtained on the molecular and physiological events occurring in poplar root during the transition from dormancy to active growth (Trupiano et al. 2013b). To further investigate molecular factors regulating the root functions, which may play important roles in the long-term survival of dormant structures, we reanalyzed the proteome of poplar woody root along the three time points. In this study, we present data referring to proteins remaining unchanged during the whole time course, together with the expression trend of the most significant proteins landmarking the dormancy, the beginning of vegetative growth, and the active growth.

Materials and methods

Plant material

The seedlings of one-year-old *Populus nigra* were grown in a mixture of soil, peat, and pumice under controlled water regime, natural photoperiod, and temperature (mean annual temperature of 12–13°C) in a greenhouse. Taproots were harvested after 12 (February; T₀; dormancy), 13 (March; T₁; beginning of the vegetative growth), and 14 (April; T₂; active growth) months of plant development; sections between 10 and 25 cm were randomly sampled and used for proteomic profiling. Three biological replicates were sampled for each condition.

Protein extraction, 2D gel electrophoresis, gel scanning and analysis

Total proteins were extracted according to the phenol protocol (Mihir & Braun 2003) with minor modifications, as reported in Scippa et al. (2008), and separated by 2D gel electrophoresis (2-DE), as described by Trupiano et al. (2012a). 2-DE gels (in technical triplicate for each sample) were scanned using a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA); corresponding images were analyzed by the PDQuest software (Bio-Rad, Hercules, CA, USA). Spot detection and matching between gels were done automatically, followed by manual verification. Protein spots were annotated only if detectable in all gels. After normalization of the spot densities against the whole-gel densities, the percentage volume of each spot was averaged for three different replicates of each biological sample, and statistical Student's *t*-test analysis ($p < 0.01$) was carried out to find out significant changes in proteomic profiles between the samples. A twofold change in normalized spot densities was considered

as indicative of a differential expression. Furthermore, to identify specific time-related protein markers, analysis of variance (ANOVA – $p < 0.05$) was carried out.

In-gel digestion, mass spectrometry and protein identification

Spots were manually excised from the gels, triturated, and washed with water. Proteins were *in-gel* reduced, S-alkylated, and digested with trypsin as previously reported (Iallicco et al. 2012). Digest aliquots were removed and subjected to a desalting/concentration step on ZipTipC18 (Millipore Corp., Bedford, MA, USA) using 5% formic acid/50% acetonitrile as eluent before MALDI-TOF-MS and/or nanoLC-ESI-LIT-MS/MS analysis. Resulting MS data were used for protein identification following the procedures already described in Trupiano et al. (2012a, 2013b).

Results and discussion

In our earlier papers (Trupiano et al. 2012a, 2013b), we analyzed the effect of bending on the proteomic profile of poplar roots by performing a differential study with respect to non-bent counterparts; this study was carried out on time-course basis by analyzing the tissues from plants under a dormancy (T₀), beginning of vegetative growth (T₁), and active growth (T₂) condition, which are represented here according to their corresponding shoot phenology (Figure 1). Data related to unbent roots were simply used as a background to be subtracted to the whole proteomic data-set of the bent counterparts, with the aim to identify specific elements associable with root bending. Interpretation of the differential results for unbent roots at the three time points was achieved later; this is the topic of the short communication reported here.

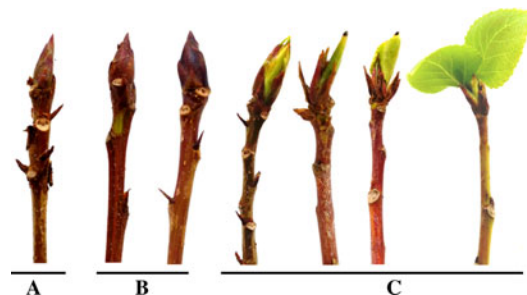


Figure 1. *Populus nigra* phenological stages assessment by bud development analysis: winter buds, stage A (February, T₀); swelling buds, stage B (March, T₁); flushing buds, stage C (April, T₂). Bud development was quantified using six levels of morphology score (0–6); minimal score (0) was given to the winter bud and maximum score (6) to buds with a growing stem.

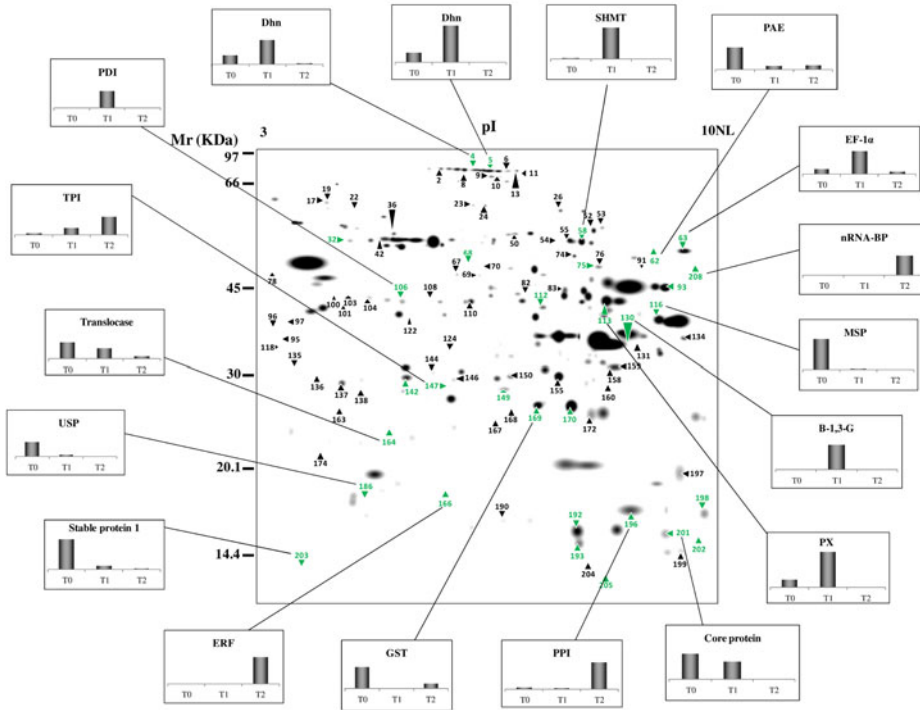


Figure 2. Two-dimensional proteome reference map of the woody poplar roots. The 31 proteins that were differentially expressed at a significant level (ANOVA – $p < 0.05$) during time course (T_0 , T_1 , and T_2) are numbered in green. The expression trends of the main identified time-related protein markers are shown by histograms. The 63 constant proteins are numbered in black. β -1,3-G, β -1,3-glucanase; Dhn, putative dehydrin; EF-1 α , elongation factor 1 α ; ERF, ethylene responsive factor; GST, glutathione-S-transferase; MSP, major storage protein; nRNA-BP, nuclear RNA-binding protein; PAE, pectin acetyltransferase; PDI, protein disulfide isomerase; PPI, peptidyl-prolyl *cis*-*trans* isomerase; PX, peroxidase; SHMT, serine hydroxymethyltransferase; TPI, triosephosphate isomerase; USP, universal stress protein.

Proteomic analysis of the unbent woody root according to a time-course basis (T_0 , T_1 , and T_2) revealed the presence of 63 constant proteins and of 31 differentially represented spots (Figure 2). As shown in Figure 3, beside proteins that regulate the main energy production pathways (glycolysis, tricarboxylic acid cycle, pentose-phosphate shunt,

gluconeogenesis, and ATP synthesis), constant components included (i) molecular species involved in lipid mobilization, beta-oxidation and synthesis, such as patatin-like protein 3 (spot 23), electron transfer flavoprotein-ubiquinone oxidoreductase (spot 124), acetyl-CoA acetyltransferase (spot 67), and two aconitase isoforms (spots 11 and 13),

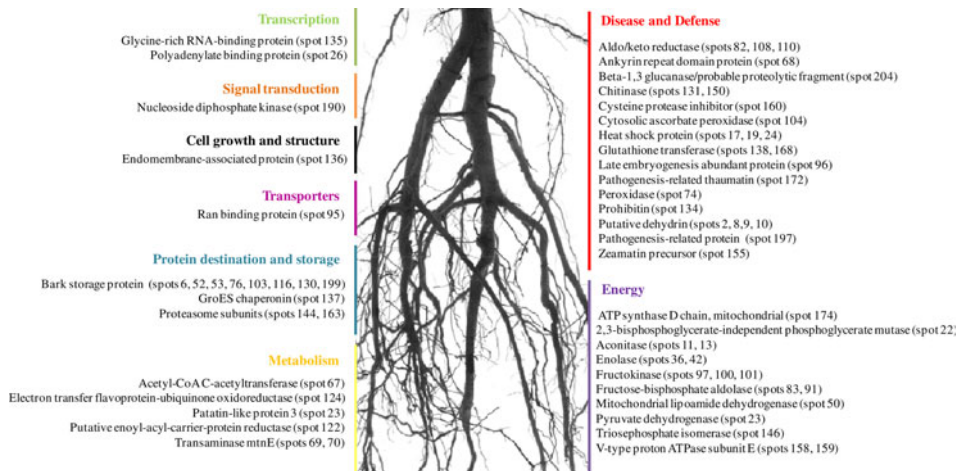


Figure 3. Constantly expressed protein spots characterizing woody taproots at T_0 , T_1 , and T_2 . Spot number and protein name of constantly expressed protein spots along the time course (T_0 , T_1 , and T_2) are listed on the basis of their functional classification provided according to Bevan et al. (1998).

already described as involved in cambial reactivation (Druart et al. 2007; Park et al. 2008); (ii) a membrane-associated putative enoyl-acyl carrier protein reductase (spot 122) belonging to the fatty acid synthase complex machinery; (iii) an endomembrane-associated protein (spot 136), which is induced in early spring to maintain plasma membrane permeability (Schrader et al. 2004; Massengo-Tiassé & Cronan 2009); (iv) mitochondrial lipoamide dehydrogenase (spot 50) and pyruvate dehydrogenase (spot 23), which are partners in the pyruvate dehydrogenase complex, thus linking cytosolic glycolytic metabolism with the tricarboxylic acid cycle. No significant changes were also observed for (i) two isoforms of transaminase mtnE (spots 69 and 70) that is involved in Met recycle thus maintaining intracellular Met and *S*-adenosylmethionine (AdoMet) levels for proper biosynthesis of ethylene and polyamines (Miyazaki & Yang 1987), which are essential plant regulators of response to abiotic stress (Gill & Tuteja 2010) and cell division in the cambial meristems (Papadakis & Roubelakis-Angelakis 2005; Love et al. 2009); (ii) a ran-binding protein (spot 95) and prohibitin (spot 134), which are both involved in the regulation of cell division and development (Dasso 2001; Ahn et al. 2006; Van Aken et al. 2007, 2010; Love et al. 2009), ethylene signaling (Christians & Larsen 2007), and oxidative stress (Ahn et al. 2006). In the latter context, ROS-scavenging enzymes, such as cytosolic ascorbate peroxidase (spot 104), aldoketoreductase (spots 82, 108, and 110), glutathione-*S*-transferase (spots 138, 168, and 104), and peroxidase (spot 74), were also observed as constantly expressed. In particular, H₂O₂ is a signaling molecule involved in triggering dormancy transition into bud break (Prassinis et al. 2011). Constant expression of H₂O₂-scavenging enzymes may be required to reduce toxicity and to allow cambium activation (Mazzitelli et al. 2007). Finally, various cold-responsive factors eventually related to the protection of reactivating cambium (Druart et al. 2007) also presented unvarying expression overtime. They included pathogenesis-related thaumatin (spot 172), zeamatin precursor (spot 155), chitinase (spots 118, 131, and 150), cysteine protease inhibitor (spot 160), late embryogenesis abundant protein (spot 96), glycine-rich RNA-binding protein (spot 135), heat shock protein (spots 17 and 19), dehydrin (spots 2, 8, 9, and 10), proteasome α and β subunits (spots 144 and 163), and GroES chaperonin (spot 137).

Conversely, 31 protein spots were found to be differentially represented in the unbent roots along the three-point time course (ANOVA – $p < 0.05$). In this study, we focus on the expression trend of these deregulated proteins, with the aim to identify important time-related landmarks. At T₀ (February), which is characterized by reduced temperature values

(–1.8 to 11.1°C) and a related water deficit, spots corresponding to factors protecting cellular structures from cold and water stress showed augmented quantitative levels. In fact, low temperatures and water deficit may determine several cellular changes in woody plants, such as perturbations in membrane structure, protein denaturation, and increased levels of toxic solutes. All these changes in turn may have induced the over-expression of (i) stress-responsive proteins, such as glutathione-*S*-transferase (spot 169), stable protein 1 (spot 203), and universal stress protein (spot 186) (Wang et al. 2002); (ii) membrane protection and cell wall remodeling factors, such as translocase (spot 164), core protein (spot 201), and pectin-acetyl esterase (spot 62) (Uemura & Steponkus 1999; Ermel et al. 2000; Follet-Gueye et al. 2000) (Figure 2). T₀ was also characterized by the over-expression of a major storage protein (spot 116) (Figure 2). Storage proteins are important for N recycling from senescing leaves to bark and back to growing leaves in the spring (Gomez & Faurobert 2002). In this context, it has been already reported that *Populus* accumulates storage proteins during autumn (Clausen & Apel 1991), which are then used in spring to provide N and C for new growth (Wetzel et al. 1989).

T₁ (March) resulted to be clearly marked by the increased levels of β -1,3-glucanase (spot 130) (Figure 2). The accumulation of this enzyme, which degrades callose (Ruonala et al. 2008; Levy & Epel 2009; Rinne et al. 2011), has been already associated with the restoration of growth-promoting substances, the transport of molecular signals between cells, and the production of cryostabilizing sugars to maintain turgor in dehydrating cells (Kaplan & Guy 2004) (Figure 2). At T₁, with temperatures comprised in the 2–15°C range, the over-representation of stress/detoxification-related proteins, such as dehydrins (spots 4 and 5), ascorbate peroxidase (spot 142) and peroxidase (spot 113), may be required for the activation of ROS signaling pathways involved in root cambium reactivation (Yakovlev et al. 2008; Hanin et al. 2011). Factors involved in protein synthesis/folding, such as serine hydroxymethyltransferase (spot 58), elongation factor 1- α (spot 63), and protein disulfide isomerase (spot 106), also marked T₁ for their increased representation; they may represent a further evidence of the molecular events preparing root to growth reactivation (Trupiano et al. 2013b) (Figure 2).

Enzymes involved in energy metabolism (triose-phosphate isomerase; spot 147) and components playing key roles in post-translational control of RNAs (nuclear RNA-binding protein; spot 208) and protein folding (peptidyl-prolyl *cis-trans* isomerase; spot 196) marked T₂ (April) for their high representation, thus indicating the occurrence of

processes related to active plant growth (Druart et al. 2007) (Figure 2). Indeed, an ethylene responsive protein factor (ERF; spot 166) also marked T₂ (Figure 2). This protein is implicated in many plant physiological functions, such as cellular proliferation and lateral roots formation (Negi et al. 2008; Neri et al. 2011; Trupiano et al. 2013a).

In conclusion, the proteomic analysis presented here provided information on the molecular species and the metabolic pathways activated in the poplar roots as a result of seasonal changes. Although preliminary information on constant and time-related protein markers was derived, our investigation was limited to a very short temporal gradient. Thus, additional studies of the entire growth cycle are required to fully understand the molecular mechanisms underlying active-dormant cycling of the tree roots. By confirming the quantitative changes described here for some proteins mentioned earlier, these studies will complete the picture resulting from investigations already done on stem cambial meristems and apical bud (Schrader et al. 2004; Druart et al. 2007; Rohde & Bhalerao 2007; Park et al. 2008; Baba et al. 2011), which emphasized the complex network of interactions existing between environmental and internal factors.

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