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INTEGRATED APPROACH TO INVESTIGATE MOLECULAR MECHANISMS IN WOODY ROOT RESPONSE TO BENDING

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GENERAL INTRODUCTION

1. Root system

Unlike mammals, plants are sessile organisms and produce organs *de novo* throughout their lifetime in order to adapt their architecture to the prevailing environmental conditions.

Plant development, in fact, is highly responsive to environmental stimuli. Such plasticity is one way in which plants overcome their inability to move toward areas of high resource availability or away from regions of adverse conditions.

Higher plants consisted of roots, which function in anchorage and absorption of water and minerals, and of stems and leaves, which provide a system well suited to the demands of life on land.

Root system has been defined as the entire below ground structure of plant (Berntson, 1994) and it has a variable and complex organization. In terrestrial plants, the root system is composed of primary, lateral and adventitious roots. The primary root is considered "embryonic" since it is laid down at the basal end of the embryo and it is the first emerging during germination. Lateral and adventitious roots are formed postembryonically in a different developmental context (López-Bucio et al., 2003). In fact while lateral roots originate from the primary roots, adventitious roots are formed from stems or hypocotyls.

A different development of primary and lateral root generates four types of root system organizations: a) the primary root greatly enlarges to become the most prominent root of the plant and is usually known as *tap root*. From the taproot many smaller branch roots may grow giving a very complex root system organization; b) the primary root is generally short lived and is replaced by numerous adventitious roots of more or less equal size. This type of root system occurs mostly in the monocotyledonous species and is known as *fibrous root* system consisting of an extensive mass of similarly sized roots; c) *heart root* system, where both large and smaller roots descend diagonally from the base; d*) plate root* system with large horizontal, lateral roots extending just below the soil surface, from which small roots branch down vertically (Cannon, 1949).

The pattern of development of a root system, in terms of number, spatial distribution and direction of growth of lateral roots represents the root system architecture and it is highly variable even among genetically identical plants (Malamy, 2005). The displacement of lateral roots at the correct positions and in appropriate numbers plays a central role in the ability of plants in establishing a root system capable to efficiently accomplish water and nutrient uptake

and to ensure a good anchorage (McCully & Canny, 1988; Varney & Canny, 1993; Bailey et al., 2002).

According to Malamy (2005) plant architecture is the result of the interaction between two pathways: (1) a "genetic" pathway that is essential for organogensis and growth, and that determine the characteristic architecture of the plant; and (2) a "response" pathway that determines how plants respond to external signals to modulate the pathway in (1). Basically, lateral root development, is guided by genetic programs but the ultimate architecture of a root system under natural conditions is largely determined by environmental factors. This high degree of variability in plant architecture provides a wide range of advantages as the development of an optimal root system is a key factor in a plant's ability to survive adverse conditions (Atkinson & Hooker, 1993).

Water availability in the soil is one of the primary elements regulating root development (Balestrini & Vartanian, 1983; Wolswinkel, 1985; Lang & Thorpe, 1986; Robinson, 1994; Chiatante et al., 2005). However, many studies are reported in the literature on the effects of the soil type, density, compaction and chemical composition, on root development (Drew, 1975; Linkor et al., 2002, Hodge, 2004) and architecture. In particular, nitrogen (Crabtree & Berntson, 1994), phosphate (Williamson et al., 2001; Glass, 2002; Ticconi et al., 2004), potassium (Lev, 2000) and sodium chloride (Waisel, 1985), together with micro-elements as cadmium (Cadiz & Davies, 1997), zinc (Cadiz & Davies, 1997) and lead (Breckle, 1997; Obroucheva et al., 1998) are the most important chemical compound in the soil affecting root system architecture. Soil temperature (McMichael & Burke, 2002), gravity, and mechanical forces deriving from wind and slope (Coutts, 1986; Watson et al, 1999; Ennos, 2000; Stokes, 2002; Chiatante et al., 2002) have been found to strongly affect root system development. Lastly, evidences have been also produced on the alterations of root systems caused by human-related environmental factors i.e fire and coppicing (Wildy & Pate, 2002; Chiatante et al., 2006).

Root has an undeniable basic role in the anchorage and stability of plant. Modifications of lateral root initiation pattern or/and number, elongation and diameters have been reported in environmental conditions affecting plant stability such as soil impedance, mechanical bending, wind, touch, and slope (Tsegaye & Mullins, 1994; Fitter & Ennos, 1989; Stokes et al., 1996; Guigo & Herbert, 1977; Chiatante et al. 2003).

The development of a root system involves mechanisms that are common to the development of all plant organs, as well as certain aspects that are unique to roots (Schiefeibein & Benfey, 1991). Despite the importance of roots the study of their biology has not received as much attention as the aerial plant organs. This has been particularly true for studies at the molecular and genetic

levels and especially for woody plants. Furthermore, with the progression of genomic and postgenomic approaches, knowledge of mechanisms regulating basic function of root biology in model plant as *Arabidopsis thaliana*, or important crop species as *Oriza sativa* and *Zea mais*, strongly increased in the last decade. However all the information available is referred to herbaceous plant and mainly to root with a primary growth, whereas almost totally unexplored remains the root biology of woody species.

The successful functioning of root system has ecological significance in terms of the competitive advantage of individual species in mixed communities but it is also economically important in the plant-based industry of agriculture, horticulture and forestry. The climatic changes, together with the increase of natural catastrophes (storms, flooding and landslides) strongly recall the attention towards the investigation on mechanisms involved in the improvement of root anchorage functions. These studies are especially important in the case of woody plants, since they may result very promising in providing knowledge for reducing landslide risk and soil erosion, on both natural and man-made slopes (Barker et al., 2004; Reubens et al., 2007; Stokes et al., 2007).

2. Root anatomical organization

The internal organization of the root, is variable but in general looks simpler and structurally also more primitive than the stem. Root axis arising from cells laid down in the seed can be divided in four different zones: the root apex, the mitosis zone, the elongation and the maturation zone (Fig.1). This longitudinal organization is also applied to any replacement root that may take over the primary root, and to any lateral and adventitious root.

The root apex is fomed by the columella and the apical meristem. The columella forms the root cap that protects the delicate apical meristem and assists in reducing friction with the rough surfaces of soil particles through which the root must grow. The root cap, in fact is made of cells secreting a mucigel that acts as a lubricant during the root elongation in the soil. The root cap plays also a very important function as sensorial organ. It is involved in the perception of a number of signals such as- soil pressure and moisture, gravity direction, and perhaps others – and modulating the growth of the main root body (reviewed in Masson, 1995). Moreover, it is generally accepted that the columella cells are the sites of gravity perception and that the amyloplasts within them are the gravisusceptors (Blancaflor et al., 1998; Chen et al., 1999). Covered by the root cap, the apical meristems represent a reservoir of undifferentiated cells producing population of proliferating cells that will further differentiate to generate the tissue systems of the primary growth (Nakajima & Benfey, 2002; Traas & Vernoux, 2002; Carles &

Fletcher, 2003). Within the apical meristem there is a cluster of cells that do not actively divide. This pad of cells is called the quiescent center (QC) and represents a reserve of cells that are recruited to replace damaged meristematic cells, or in particular conditions. Meristematic cells dividing rapidly by mitosis, characterize the so called “mitosis zone”. In this zone meristemtic initials divide generating new initials and derivates. The last ones divide again producing the “determined cells”, the fate of which is established on the base of their spatial position (Benfey et al., 1993). Above the mitosis zone there is the elongation zone in which the newly created determined cells expand in their length pushing the meristem and the root cap through the soil and contributing to root elongation. The cell elongation process involves resculpting of the wall and the cell growth by water uptake. Elongating cells begin to differentiate, acquiring specific characteristics and maturing specific cell organelles. Within the maturation zone, the differentiation process is completed giving rise to the primary growth (Figure 1). Many gymnosperms and dicotilenous undergo additional growth, that thickens the stem and root, and it is termed “secondary growth”. From ecological and evolutionary perspectives, secondary growth can be viewed as an adaptive mechanism that enables plants to secure a dominant position in ecosystems, promoting their survival and reproduction.

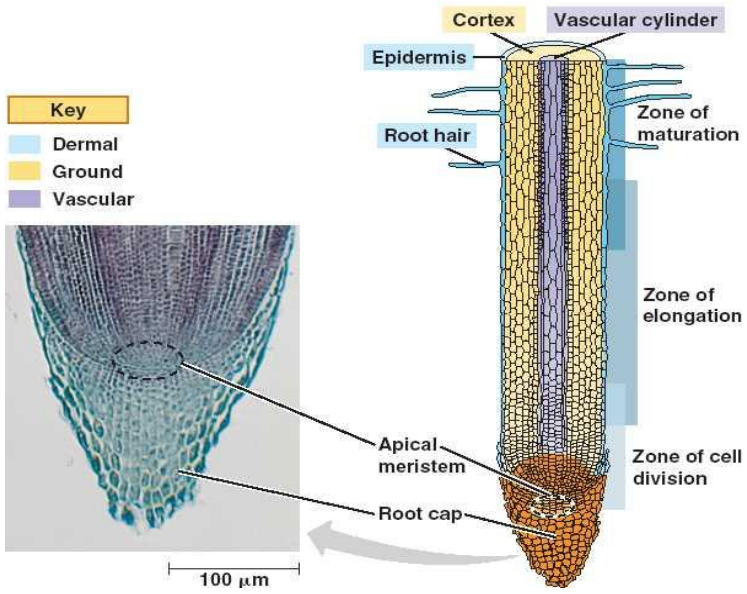

Figure 1. Longitudinal section of primary root subdivided in four main zones: root caps, the mitosis zone, elongation zone and maturation zone (http://bio1151.nicerweb.com/Locked/media/ ch35/35_12PrimaryRootGrowth. jpg).

2.1. Primary growth

The primary stage of root growth is characterized by three types of tissue systems: the dermal, the ground and the vascular system (Benfey & Scheres, 2000; Figure 2).

The dermal tissue is represented by the epidermis that covers externally and the endodermis that delimitates the vascular cylinder (Figure 2). The epidermis is a specialized protective and absorbing tissue, containing root hairs in the maturation zone, which are themselves specialized projections from modified epidermal cells known as trichoblasts (Bibikova & Gilroy, 2003). Root hairs markedly extend the absorbing surface of the root but they are often considered to be short-lived. The endodermis functions as a filter, selecting solutes in the water that is absorbed by the root hairs and that must reach the xylem vessel to be distributed in the whole plant body. In the endodermis, each endodermal cell has a Casparian strip which is a continuous band around both radial and transverse walls impregnated with suberin and lignin that form barrier to the movement of water and solutes within apoplast.

The ground tissue forming the cortex usually occupies the largest volume of roots and consists mainly of highly vacuolated parenchyma cells and commonly represents a simple, living, storage tissue.

The vascular system, forming the vascular cylinder consists of xylem and phloem surrounded by and one or more layers of non-vascular tissues, the pericycle. In the majority of dicotyledonous the vascular cylinder is formed by a core of primary xylem from which ridge-projections of xylem extend towards the pericycle (Esau, 1977). Between the ridges are strands of primary phloem. The number of the xylem ridges varies between species. Based on the ridge number roots are defined as diarch when two ridges form the vascular cylinder, triarch when there are three ridges and so on.

The pericycle is composed of parenchyma cells with primary walls but these may develop secondary walls. It gives rise to lateral roots and vascular cambium (Figure 2).

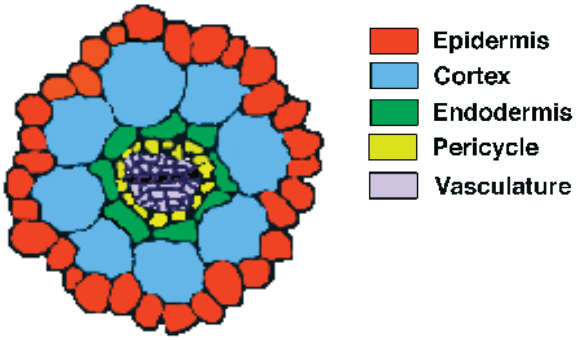

Figure 2. Anatomical organization of root in primary growth in transverse section (Benfey & Scheres, 2000)

2.2. Secondary structure

Secondary growth is characteristic of roots of gymnosperms and of most dycotiledons but is commonly absent in the monocotyledons. The secondary growth is generated by two secondary meristems (groups of cells developed from differentiated living tissues) namely the vascular cambium and cork cambium.

Vascular cambium is formed by two cell types: fusiform initials and ray initials. Fusiform initials produce axial elements of secondary vascular tissues (such as fibers, tracheids, vessel members, companion cells, etc.) while ray initials give rise to phloem and xylem rays the radial system of the secondary plant body. The secondary growth process starts with the developmet of vascular cambium. Vascular cambium develops partly out of parenchyma and partly from from the pericycle cells (Esau, 1977). Parenchyma cells that have been laid out during primary growth between the xylem rays, dedifferentiated and resume the ability to divide. The proliferarion of meristematic cells formed form the pericycle and parenchyma generate a vascular cambium that forms a continuous cylinder separating the primary xylem and floem. The activity of this vascular cambium will produce annually secondary phloem tissue on the outside and secondary xylem or wood on the inside (Figure 3).

The secondary growth is also characterized by the formation of the cork cambium. Cork cambium named phellogen initially may originate from the dedifferentiation of parenchimatic cells of the cortex, or from the epidermis or from the primary floem. This secondary meristem is annually formed and with the time will be generated from the secondary floem produced in the previous years. The differentiation of phellogen cells produces cork cells outside and cork parenchyma inside above the secondary floem. Cork cambium, cork cells and cork parenchyma together are also named periderm. Mature cork cells are dead and destined to replace the epidermis rupture during root growth; their cell walls contain suberine, a fatty substance that repels water and provides protection against desiccation and pathogens attack (Figure 3).

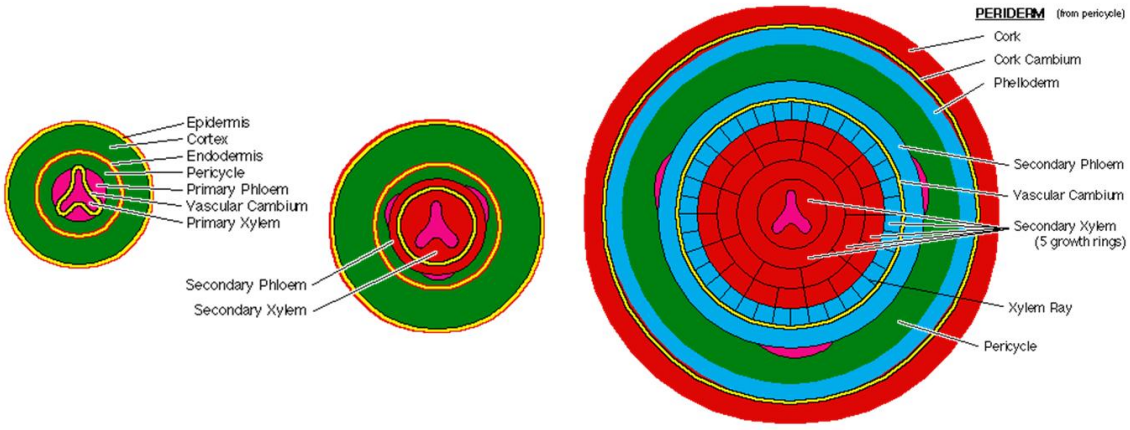

Figure 3. Development of root secondary growth from two secondary meristems namely the vascular cambium and cork cambium (http://plantphys.info/plant_biology/secondary.shtml.).

3. Development of root system: lateral root emission

In general, are defined lateral roots those that originate from pericycle of some other root (primary root, adventitious root or another lateral root). However, it must be also taken o account that lateral roots may originate from other tissues as the endodermis cells, as it occurs in ferns (Hou et al., 2004).

Lateral root (LR) initiation (LRI) and development (LRD) in higher plants are very complex processes and the knowledge about mechanisms involved are still significantly incomplete (Fukaki et al., 2007).

Reviewing all the available information, Malamy (2005) sketched basic outlines of interlinked regulatory pathways of "genetic or intrinsic" pathways that are essential for organogensis and growth, and that determine the characteristic architecture of the plant and "environmental or response" pathways that determine how plants respond to external signals modulating the genetic pathways. Most of the mechanisms involved into these pathways have been extensively studied in the plant model *Arabidopsis thaliana*; although it still remains unknown if such pathways are conserved in other plants (Hochholdinger et al., 2004). Based on investigations on several model plants (Arabidopsis, rice, mais ect) it has been observed that the development of a lateral root starts with the formation of lateral meristems (Dubrovsky et al., 2001) and continues through a process divided into eight stages as described by Peret et al., (2009). As represented in Figure 4 *Arabidopsis* lateral roots originate exclusively from pericycle founder cells located opposite xylem poles, and the first evidence of the primordia initiation is related to asymmetric anticlinal divisions of the founder cells. Division process continues with periclinal divisions producing a two-layered primordium: the outer layer (OL) and inner layer

(IL). Further periclinal division give rise to a four-layered primordium with layers OL1, OL2, IL1, IL2. At this stage the lateral root primordia has penetrated the parent endodermal layer (Stage II.-IV; Figure 4). In addition, cells in IL2 enlarge radially and divide, pushing the overlying layers up and apparently compressing the cells in IL1 and OL2. The LRP at this stage is midway through the parent cortex (Stage V; Figure 4); successively, after a periclinal division of OL2, LRP penetrated the epidermis (Stage VI; Figure 4) and begins to resemble the mature root tip, containing 3 layers that could correspond to epidermis, cortex and endodermis surrounding a potential root cap at the tip of the LRP (Stage VII; Figure 4).

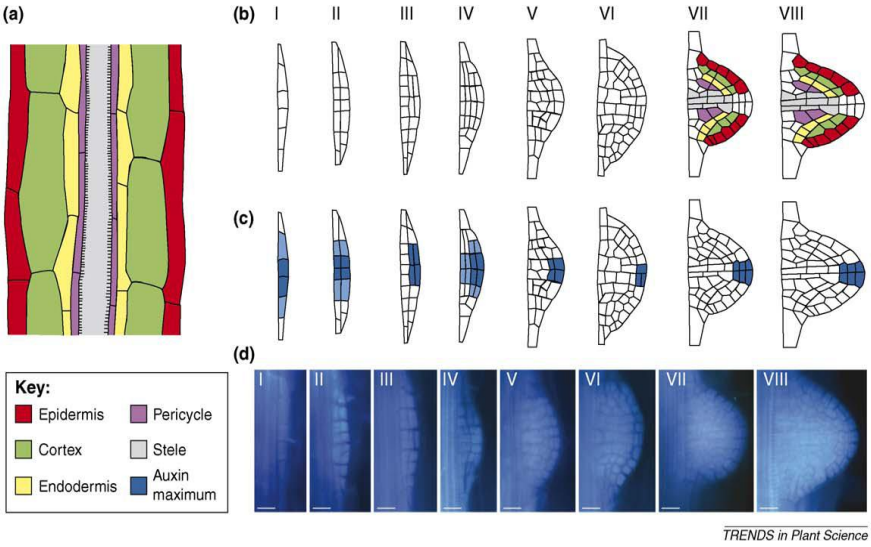

Figure 4. *Arabidopsis* Model of Lateral Root Primordium (LRP) development. Based on the information from the histological studies, color coding shows the putative derivation of each tissue from Stage I to VIII (original image in Peret et al., 2009).

The formation of a temporary root cap from tissues out of the pericycle (endodermis cells or up to a few layers of the root cortex, assists in primordium emergence (Stage VIII) through the parental root tissues (Malamy & Benfey, 1997; Dubrovsky, et al. 2001; Dubrovsky & Rost, 2003; Barlow, 2004; Peret et al., 2009). For plants not making these temporary structures, primordia was expected to break out from the parent root only by mechanical force (Charlton, 1991).

Cells in the parent root overlaying new lateral root primordia actively participate in organ emergence thanks to a transcellular auxin signalling network designed to synchronize lateral root development and emergence processes. In fact, Swarup et al. (2008) showed that IAA (indole-3-acetic acid) was detected in endodermis and is likely to influence the rate of lateral root

emergence by regulating the auxin inducible expression of cell-wall-remodelling (CWR) gene (Figure 5).

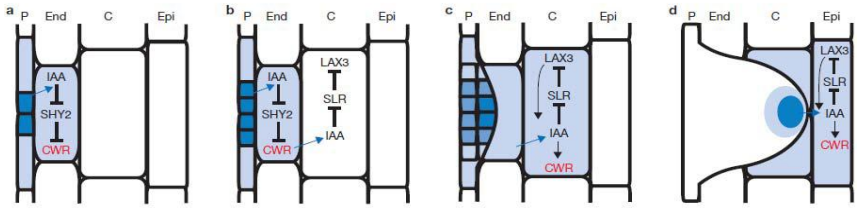

Figure 5. Auxin-dependent lateral root emergence model. Cellular auxin responses are represented as a blue color gradient. Auxin (IAA) originating from dividing pericycle (P) cells induces cell-wall-remodeling (CWR) gene expression in adjacent endodermal (End) cells and influx carrier LAX3 by targeting the degradation of the SHY2/IAA3 and SLR/IAA14 repressor proteins, respectively, facilitating the emergence of LRP (Swarup et al., 2008).

LRD ends with the differentiation of the phloem and xylem in the primordium and their subsequent connection in the parental root (Oparka et al., 1995).

Significant progress has recently been made in identifying many *Arabidopsis* genes that regulate lateral root initiation, patterning and emergence processes. A complete review of the molecular and cellular basis together with the role of the plant hormone auxin in lateral root formation has been recently published by Peret et al., (2009).

The large body of investigations on lateral root development evidences that the formation a new lateral root requires the presence, in the parental root, of specific primary tissues, the pericycle, endodermis, primary xylem and primary phloem. These tissues, in fact, are the only ones in the parental root known to be sources of lateral root meristematic cells (LRMC; Chiatante et al., 2006).

Proofs that lateral roots are formed from woody parental roots characterized by a secondary anatomical structure lacking these primary tissues are extensively reported in the literature (Chiatante et al., 2006). In fact lateral roots from wood parentals have been reported to be induced in response environmental stresses (Kozlowski, 1971; Gruber, 1992; Puhe, 2003; Paolillo, 2006), but also in physiological conditions (Paolillo & Zobel, 2002; Paolillo & Bassuk, 2005).

Few investigations carried out at anatomical level revelaed that the parenchyma in the secondary phloem, and the phellogen could be the sources for LRMC recruitment (Paolillo, 2006). However, further studies carried out by Chiatante et al. (2007) on *Fraxinus ornus* lateral root induction by environmental stresses (Figure 6) showed that the traces of new iduced laterals

were characterized by a V-shaped insertion zone similar to the one found in lateral roots emitted from woody roots of other dicots (Paolillo & Zobel, 2002).

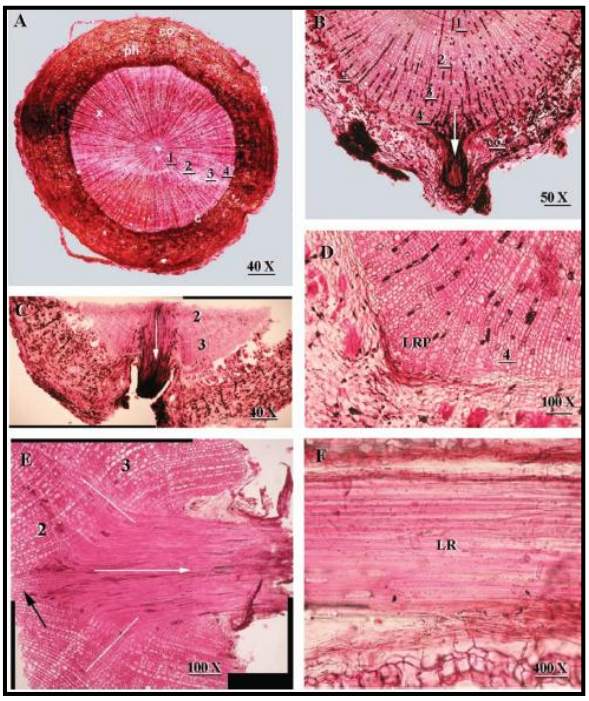

Figure 6. Transverse section of pruned and bent *Fraxinum ornus* taproot in correspondence to the zone where a new lateral root was emitted (original image in Chiatante et al., 2007).

Moreover, it has been observed that the V-shaped insertion zone ended in contact with the secondary xylem, suggesting that the formation of the new lateral probably occurs near the vascular cambium. Based on these observations it was suggested that LRMC recruitment for primordium formation involves some vascular cambium initials. This hypotheis was further supported by the finding that vascular cambium initials are not cytologically or physiologically different from the pericycle- and proto-xylemparenchymatic-cells known to be initials of lateral root primordia (Ferriera et al., 1994; Dubrowsky et al., 2000; Beckman et al., 2001; Himanen et al., 2002; Casimiro et al., 2003).

However, despite the first attempts of investigation, the mechanisms underlying lateral root development from a parental woody root have yet to be clarified, and additional work is required to confirm the the hypotheis that vascular cambium initials preserve the competence for LRMC.

4. Plant response to external factor: mechanical stresses

In nature plant frequently encounter numerous stresses, biotic (imposed by other organisms) or abiotic (imposed by physical or chemical environmental changes), that can influence their growth, development and productivity. Stress can be defined as a set of conditions that cause aberrant changes in physiological processes eventually resulting in injuries (Nilson & Orcutt, 1996). In general, any environmental condition could be a potential stress factors if its presence is extreme for a correct plant development. Plant response to environmental stress is controlled by very complex mechanism and involves alterations at different levels, depending on stress intensity, duration and severity and on plant genotype.

Plant responses to stress may be grouped in two general categories: avoidance, which prevent exposure to the stress, and tolerance, which permit the plant to withstand the stress (Levitt, 1972). Molecular factors controlling the avoidance and tolerance responses have been extensively investigated in many plants in relation to widely diffused abiotic stresses as drought, sality, heat, cold (Seki et al., 2003), and many transgenic plants have been produced with increased resistance (Wang et al., 2003; Zhang et al., 2004).

Mechanical stress is common abiotic stress in natural conditions produced by several factors, as altered gravity direction, touch, wind, soil density, soil compaction and grazing, slope ect. All these conditions have been found to induce responses involving changes at anatomical, physiological, biochemical, biophysical and molecular level (Jaffe & Forbes, 1993)

The study of the response of plants to mechanical stresses is often quite difficult, especially in the natural field, for a variety of reasons, i.e. for different intensity and continues or random exposure of plant to stress, and to overcome these difficulties, mechanical perturbations have often been reproduced in controlled conditions and have produced several data on the effects of mechanical stresses on plants (Jaffe &Forbes, 1993; Braam, 2004).

The influence of mechanical factors on plant growth and development is defined as "thigmomorphogenesis" (Boyer, 1967; Jaffe, 1973) and can be considered a physiological and morphological adaptation of plant to environmental mechanical stresses (Jaffe & Forbes, 1993). Consequently, the term, originally referred to changes in plant morphology and development, has been lately extended all the alteration induced by mechanical stress including physiological, anatomical, biomechanical and molecular changes.

Thigmomorphogenetic responses occur slowly over time and are therefore often not readily apparent or appreciated. However, these responses can be quite dramatic (Braam, 2004).

At biomechanical level, it has been observed that thigmomorphogeneis is correlated with an increase of strengthening tissue production and with an improved resistance to mechanical

damage (Jaffe et al., 1984; Biddington, 1986; Telewski and Jaffe, 1986). Moreover, it has been found that some species increase tissue rigidity, others increase tissue flexibility (Biddington, 1986; Telewski, 1995). Shoot and root morphology is profoundly altered in response to mechanical stress (Stokes et al., 1995; Crook & Ennos, 1996; Goodman & Ennos, 1996, 1997a, b). Linden (2005) investigated the response of *Populus tremuloides* shoot to bending. Results of this study revealed that to improve the rigidy stem undergo to an increase of diameter, secondary xylem (sx) production, and fibers lignifications (Figure 7A, B).

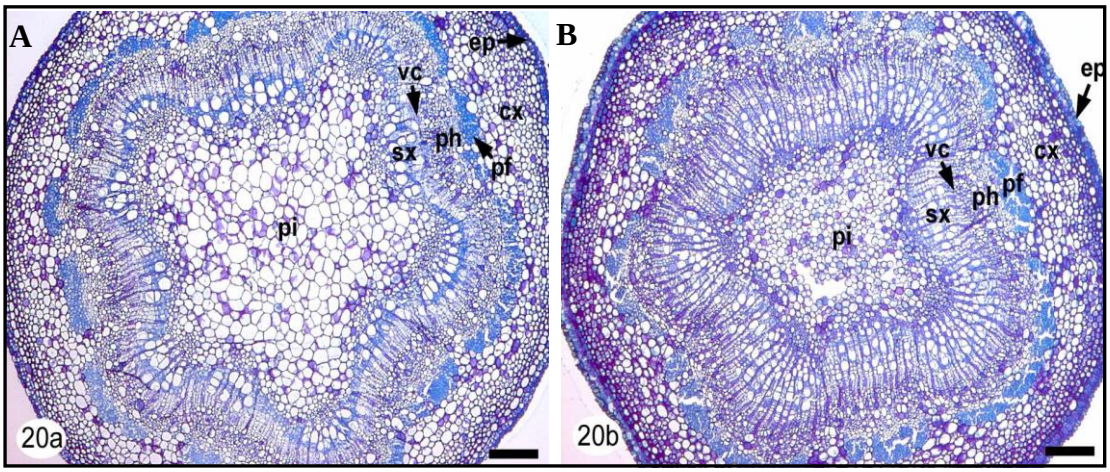

Figure 7. Light micrographs of transverse sections of control (A) and bent (B) aspen shoots stained with PAS – TBO. Shoots are relatively symmetric, with erratic piths (pi), and evidence of increased secondary xylem (sx) phloem tissue (ph) and lignification production. Note: pi, piths; sx, secondary xylem; pf, phloem fibres; ph, secondary phloem tissue; vc, vascular cambium; cx, cortex; ep, epidermis. Bar = 157 µm. (Linden, 2005).

Additional results have been obtained in several other studies (Burton & Smith, 1972; Jaffe, 1973, 1976; Phares et al., 1974; Telewski & Jaffe, 1981, 1986a; Jaffe et al., 1985; Whalley et al., 1999; Linden, 2005) showing that mechanical stress induces the decrease of stem elongation, increasing its radial growth, elastic resilience, flexural stiffness and resistance to mechanical rupture (Jaffe et al., 1984; Telewski & Jaffe, 1986b; Jaegher et al., 1985). This is usually obtained by production of so-called reaction wood characterized by the presence of fibres with a special morphology, changes in tracheid number and length and chemical composition correlated with comparable increase in lignin, as well as cell wall polymers.

Compared to the stem, the response of root systems to mechanical stresses have been less investigated. In general data obtained evidenced that common thigmomorphogenic responses of root mechanical perturbations include:

1) changes in root:shoot ratio (Stokes & Guitard, 1997);
2) alterations of root mechanical properties (Goodman & Ennos, 1998), and architecture (Nicoll & Ray, 1996; Chiatante et al., 2003);
3) increases in root cross sectional area; shift in its distribution relative to the direction of stimulation (Stokes et al., 1995; Goodman & Ennos, 1998);
4) reduction root elongation rate (Barley, 1962, 1963; Goss, 1977);
5) increase of root diameter (Barley, 1965; Atwell, 1988; Materechera et al., 1991) and biomass (Downes et al., 1994; Watson, 2000; Watson & Tombleson, 2002; Tsegaye & Mullins, 1994; Di Iorio et al., 2005; Scippa et al., 2008; Ditegou et al., 2008);
6) increase of number and thicknes of lateral roots in the direction of stimulation (Nicoll & Ray, 1996; Stokes et al. 1997a, b).

Root thigmomorphogenesis have been particularly investigated in several woody plant species, trees and shrubs growing in slope conditions (Chiatante et al., 2003; Scippa et al., 2006; Di Michele et al., 2006). Slope is a widely environmental condition that may severly affect plant stability. To improve anchorage and respond to slope, root systems develop a particular architecture with an asymmetric allocation of biomass in two preferential directions, named up-slope and down-slope (Chiatante et al., 2003 – Figure 8). Root architecture of plants growing on slope have been defined "bilateral fan-shape'' whereas symmetrical bellshape is the term used for root of plants growing in plane (Figure 8). Based on their position lateral roots are characterized by a different wood distribution, that may be related to a diverse mechanical properties and anchorage function (Figure 9).

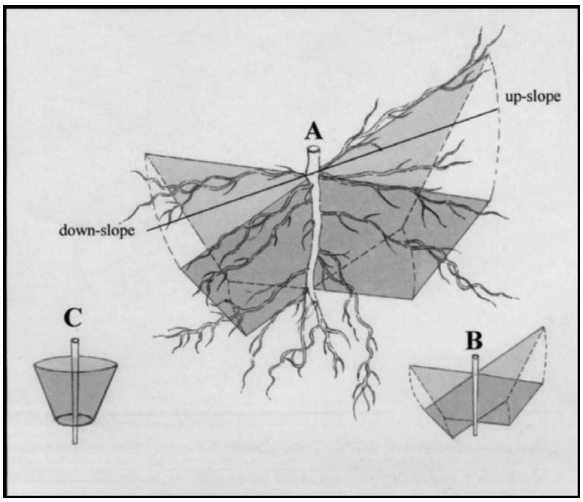

Figure 8. First-order laterals distribution of a *Fraxinus ornus* seedling within a ''bilateral fan-shape''. The bilateral-fan-shape (A) is formed by two sectors up-slope or down slope (B). When seedlings grow on a plane the first order laterals form a symmetrical bellshape (C) (Chiatante et al., 2003).

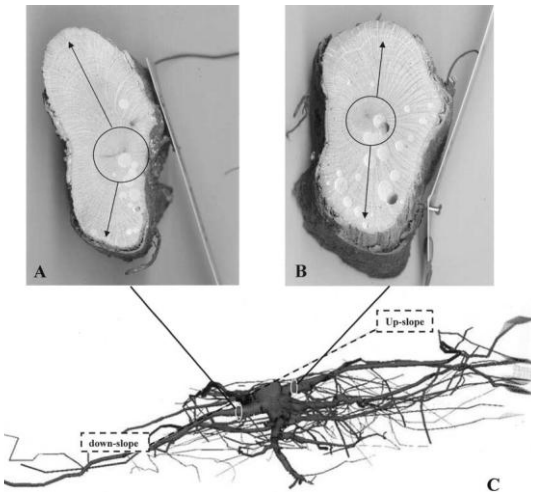

Figure 9. Woody sections of the down-slope (A) and up-slope (B) lateral roots bases (C) in *Quercus cerris*. The circles indicate the portion of wood characterized by a visible circular symmetry. The arrows indicate the directions in which wood has been added probably in response to the mechanical stimulus. The amount of wood in the down-slope lateral (A) is not equally distributed, unlike the up-slope lateral roots (B) (Chiatante et al., 2003)

Moreover it has been observed that the alterations of root system architecture and mechanical properties might partly result from a change in the mechanical function of existing laterals root and/or partly from the formation of new laterals from parental roots even if secondary growth is well developed (Chiatante et al., 2007).

Although the molecular mechanisms that regulate root thigmomorphogenesis are almost totally unknown, several studies indicated that phytohormones play a central role. It is well known that ethylene regulates auxin synthesis and transport and that auxin controls root growth orientation and lateral root development (Montiel et al., 2004; Casimiro et al., 2003; Teale et al., 2006). Based on these informations, for instance, it has been proposed that mechanical stress involve a crosstalk between the two hormones auxin and ethylene that induces lateral roots emission (Stepanova et al., 2007; Negi et al., 2008);

Several works produced very interesting resulst about the mechanisms involved in lateral roots formation and the role of auxin in response to mechanical stress. In fact Ditengou et al., (2008) and Laskowski et al., (2008) showed that manual bending of *Arabidopsis thaliana* taproot induces initiation of Lateral root primordial (LRP). In addition, Laskowski et al., (2008) proposed that curve-related LR formation reflects differential dynamics of auxin transport/uptake by cells on each side of the root driven by the differential cell geometry caused by root curvature. Alternatively, Ditengou et al., (2008) proposed that bending induction mechanisms act upstream of auxin-dependent processes. However, the exact signaling/response pathways regulating LR production to the convex side of bent roots, and whether additional components other than auxin-mediated events are involved in this process, remain to be elucidates . In the latest paper published by Richter et al., (2009) it is reported that the bending mechanical forces in *Arabidopsis* root can elicit LR emission in the convex side of the bend curve. Moreover, it has been shown that bending triggered a Ca2+ transient within the pericycle, and blocking this

change in Ca2+ also blocked recruitment of new lateral root production to the curved region of the root. Based on these results Richter et al., (2009) proposed two different a Ca2+-dependent signaling cascade operating in parallel with, an auxin-dependent pathway to initiate pericycle cell to become lateral founders (Figure 10).

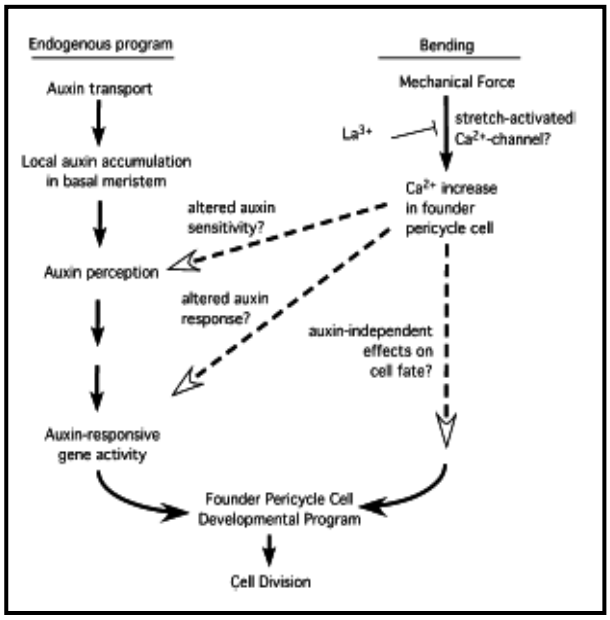

Figure 10. Model of a possible role for Ca^{2+} in stretch-induced founder pericycle cell recruitment. Ca^{2+}-dependent signaling cascade operating in parallel with, an auxin-dependent pathway to founder pericycle cell specification (Richter et al., 2009)

Although little is known about the mechanical signal transduction pathway, and it is still not clear whether signal is transmitted to the roots, it can supposed that similarly to the other environmental stress, the mechanical stress response initiates when plant recognizes the stress at cellular level.

The mechanism of mechano-signals perception has been largely investigated in the stem of herbaceous and woody plants. Resulst obtanied show that mechano-signals perception is probably based on the cytoskeleton - plasma membrane - cell wall (CPMCW) mechano-sensing network (Jaffe et al., 2002). This network, using Integrin-like protein, connects microtubule to the plasma membrane cell and then plasma membrane to the cell wall (Ingber, 2003 a, b).

Intracellular calcium (Ca^{2+}) and calcium binding proteins (calmodulins, calcineurin B-like proteins and calcium-dependent but calmodulin-independent protein kinases) together with reactive oxygen species (ROS) have long been implicated as an important second messenger in mechano-signalling and response in plant cells. In fact, coincidence of ROS and Ca^{2+} increase in many plants (Mauch et al., 1997; Gilmour et al., 1998; Arteca & Arteca, 1999; Gadea et al., 1999; Hirsinger et al., 1999; Tatsuki and Mori, 1999; Müssig et al., 2000; Oufattole et al., 2000; Lee et al., 2005) suggest that these two cellular signals may be interdependently generated and functionally linked in modulating mechanical inducible gene expression.

In the last decades many genes induced by different types of mechanical stress have been identified. In particular the literature reports the identification of a large number of touch genes (TCH) that have been found to have different functions as key factors of the signal transduction pathway, enzyme involved in the cell wall modification (Braam & Davis, 1990; Sistrunk et al., 1994; Khan et al., 1997). Several other genes have been identified to have a mechano-stimulus-inducible expression, with a wide range of functions and often related to other abiotic stresses (Ling et al., 1991; Perera & Zielinski, 1992; Gawienowski et al., 1993; Botella & Arteca, 1994; Botella et al.,1996; Mizoguchi et al., 1996; Oh et al., 1996; Royo et al., 1996; Shirsat et al., 1996; Eldick et al., 1997; Mauch et al., 1997; Gilmour et al., 1998; Arteca & Arteca, 1999; Gadea et al., 1999; Hirsinger et al., 1999; Tatsuki & Mori, 1999; Müssig et al., 2000; Oufattole et al., 2000; Lee et al., 2005).

5. Functional genomics and proteomics

With the advent of whole genome sequencing and **functional genomics**, what we know about plant biology at the genomic and phenomic level is rapidly advancing. In fact, many refer to the current epoch of genetics as the post-sequencing, or post-genomics era, due mainly to the substantial sequencing and gene function annotation efforts on model systems, moving us towards understanding root growth and development.

For example, in maize, the combination of sequencing and annotation of the its genome (Zhu et al., 2005), the available of reverse-genetics resources (Wessler, 2006) and of QTL approaches (Yu et al., 2008; Collins et al., 2008), increased the knowledge about maize primary and lateral root development defining ideal root-system architectures for different target environments stress (Dembinsky et al., 2007; Liu et al., 2008; Spollen et al., 2008).

In *Arabidopsis*, the localization of expression of more than 22,000 genes were mapped to 15 different zones of the root that correspond to cell types and tissues at progressive developmental stages (Schiefelbein & Benfey, 1991; Benfey et al., 1993; Birnbaum et al., 2003 Schmid et al., 2005); another set of studies has examined the transcriptome of different organs and developmental stages of *Arabidopsis*, including the root, in response to over 40 conditions (Montiel et al., 2004;Schmid et al., 2005; Kilian et al., 2007; Dinneny et al., 2008); moreover also the lateral root initiation and development were characterized (Malamy, 2005; Peret et al., 2009).

With the adoption of *Populus* as a model genetic tree system, genetic resources and tools developed in poplar have illustrated the potential for gene discovery and functional genomics outside of *Arabidopsis* and maize. Sequenced genome (Tuskan et al, 2006), whole-genome

microarray resources (Ralph et al, 2006, 2008; Jansson & Douglas, 2007), amenable transformation system (Han et al., 2000) and high density genetic maps allow functional genetic and genomics dissection of various traits relate to woody perennial biology.

For example, in a recent work (Prassinos et al., 2005), to better understand the genetic regulation of secondary growth in hybrid aspen (*P. tremula* × *P. alba*), a series of cDNA-amplified fragment length polymorphism (AFLP), in vertical stem segments in secondary growth, were analyzed. This approach allowed us to screen >80% of the transcriptome expressed and identify genes differentially expressed with the progress of secondary growth. In another work (Schrader et al., 2004) the transcriptomes of the six anatomically homogenous cell layers in the meristem zone were characterized.

Although functional genomic approach result an important methodology to understand plant biology it is not sufficient to answer questions concerning gene function, developmental/regulatory biology, and the biochemical kinetics of life. In fact mRNA and protein amounts might not always correlate because most often, proteins may also be regulated by post-translational modifications as glycosilation, phosphorylation, palmitoylation or sulphation (Gooley & Packer, 1977). To better comprehend the events which constitute the stress response of cells it is often necessary the use of **functional proteomics**, the global characterization of functional features of proteins. Thus proteomic analysis can nowadays be more easily used for assigning a function to gene products and for providing physiological and biological explanations for differential protein expression because provide a complete picture of the organism in a determinate condition.

Improvements in high-resolution 2-DE PAGE (Görg et al., 1999), increased content of protein and nucleotide databases, and increased capabilities for protein identification utilizing modern mass spectrometry methods (MS) as LC-MS/MS and/or MALDI-TOF-MS (Pappin et al., 1993; Yates, 1998a, 1998b; Corthals et al., 2000).

In recent years, proteomic studies have provided new insights into the regulation of e root development and response to environmental stress. These experiments either generated reference maps of the most abundant proteins of a particular development stage or treatments of many species.

For example, a recent global analysis of the *Arabidopsis* root using a proteome approach identified approximately 5159 proteins in 10-day-old roots and 4466 in 23-day-old roots (Baerenfaller et al., 2008); Di Michele et al., (2006), using an integrated transcriptomic and proteomic approach, identified response profiles of *Spartium* root subjected to slope stress; Plomion *et al.* (2006) performed amore comprehensive analysis for eight tissues/organs of adult

P. trichocarpa proteome; Du *et al.* (2006) analyzed proteins expressed in different wood regeneration stages in a system that can mimic the initiation and differentiation of cambium cells for *P. tomentosa*; many other recent works (Hochholdinger et al, 2004, 2005; Liu et al., 2006; Sauer et al., 2006; Zhu et al., 2006, 2007; Li et al., 2007, 2008; Hoecker et al., 2008) analyzed complete roots, tissues or subcellular fractions of maize roots identifying differentially expressed proteins between different genotypes or treatments.

Concluding, we are at an exciting time for root biology invetsiogations. In fact the integration of large-scale, high-resolution datasets, obtained by genomic and proteomic approach could represent the keys to deepen our knowledge about woody root biology in relation to the environment.

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OBJECTIVES AND SUMMARY

The studies presented in this PhD thesis have the general aim to contribute in understanding the complex and almost unknown biology of woody root.

In particular the work has been focused on two major issues: 1) root response to mechanical stress and 2) mechanisms involved in lateral root emission from a secondary growth.

Results of these studies are described in the chapter I and chapter II.

Chapter I: The response of *Populus nigra* woody root to mechanical stress imposed by bending.

During the first period of the PhD program, investigations have been focused on the identification of an experimental system suitable to investigate the root response to mechanical stress. The experimental system represented by bent poplar taproots has been set up, and analysed. The first results obtained by the preliminary analysis of this experimental system are reported in the paper published by Scippa et al., (2008). The data obtained showed that poplar taproot responds to mechanical stress increasing the lateral root emissions, biomass and lignin content, activating stress-responsive genes and altering the metabolic pathways. Starting from these results, the PhD project proceded in further analysing the mechanisms involved in the woody root response to bending along a temporal and spatial gradient. In particular after modeling the forces distribution along the bent taproot, the morphological and lignin changes were analyzed together with the alteration of proteins profiles. The use of 2DE coupled to the MS/MS allowed the identification of 211 well resolved proteins which represent the first woody root proteome map. In addition all the data obtained at the different level of investigation were further verified and elaborated by multivariate statistical analysis identifying important temporal and spatial protein markers. All the results obtained will be presented in the paper "The proteome of *Populus nigra* woody root: the asymmetric response to bending" in preparation to be submitted for publication.

Chapter II: Identification and characterization of an activation-tagged gene encoding an AP2/ERF protein that regulates lateral root emission.

To investigate mechanisms involved in lateral root emission from a secondary structure, a suitable experimental system is required. In details, after screening of 627 independent

activation-tagged transgenic lines in tissue culture, a transgenic hybrid poplar (*Populus tremula* X *Populus alba*), with dominant root phenotype, was identified. The cause of the observed phenotype was the hyperactivation of the gene encoding for a protein of AP2/ERF family. The mutation resulted from insertion of a strong *35S* transcriptional enhancer near the transcription start site that caused the over-expression of the poplar ERF gene (*PtaERF003*) for hyperaccumulation of its mRNA transcripts. The poplar PtaERF003 sequence was most closely related to one poorly known *At5g25190* from *Arabidopsis thaliana* . The root phenotype was increased through IAA application to the growth medium and metabolic profiling was characterized. The data obtained address the use of the transgene as model to further identify the molecular factors controlling lateral root emission from the secondary growth. In addition the possibility of producing trees with strongly lateral root phenotype could have significant economic and environmental benefits, including ensure of a good anchorage, slopes stabilization and reduced risk of landslides.

CHAPTER I

The response of *Populus nigra* woody root to mechanical stress imposed by bending.

All the studies relating with analysis of "The response of *Populus nigra* woody root to mechanical stress imposed by bending" are reported in this chapter as two papers.
The first paper, published, reports the experimental set up and preliminary results obtained from the investigation of poplar woody root response to bending treatemt.
The second paper, in preparation, details the response of woody root to bending along a temporal and spatial gradient, providing the first reference proteome map of a tree root with 211 proteins identified.

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Unravelling the response of poplar (*Populus nigra*) roots to mechanical stress imposed by bending

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Abstract
Mechanical stress is a widespread environmental condition that can be caused by several factors (i.e. gravity, touch, wind, soil density, soil compaction and grazing, slope) and that can severely affect plant stability. In response to mechanical stress and to improve their anchorage, plants have developed complex mechanisms to detect mechanical perturbation and to induce a suite of modifications at anatomical, physiological, biochemical, biophysical and molecular level. Although it is well recognized that one of the primary functions of root systems is to anchor the plant to the soil, root response to mechanical stresses have been investigated mainly at morphological and biomechanical level, whereas investigations about the molecular mechanisms underlying these important alterations are still in an initial stage. We have used an experimental system in which the taproot poplar seedlings are bent to simulate mechanical perturbation to begin investigate the mechanisms involved in root response to mechanical stress. The results reported herein show that, in response to bending, the poplar root changes its morphology by emitting new lateral roots, and its biomechanical properties by increasing the root biomass and lignin synthesis. In addition, using a proteomic approach, we found that several proteins involved in the signal transduction pathway, detoxification and metabolism are up-regulated and/or down-regulated in the bent root. These results provide new insight into the obscure field of woody root response to mechanical stress, and can serve as a basis for future investigations aimed at unravelling the complex mechanism involved in the reaction of root biology to environmental stress.

Key words: *Root system, mechanical stress, poplar,* Populus nigra, *woody root*

Introduction

Mechanical stress is a common condition caused by several factors (i.e. gravity, touch, wind, soil density, soil compaction and grazing, slope) that can severely affect plant stability. Plants have developed sensory mechanisms to detect mechanical perturbations and to induce a suite of responses (anatomical, physiological, biochemical, biophysical and molecular) collectively termed "thigmomorphogenesis" (Jaffe & Forbes 1993; Braam 2005). Thigmomorphogenesis is believed to improve plant anchorage (Mitchell et al. 1975; Jaffe & Biro 1979) and has been mainly investigated in the stem of herbaceous and woody plants. In the case of woody plants, particular attention has focused on the role of the hormones ethylene and auxin, and factors involved in reaction wood formation (Timell 1986; Sundberg et al. 1994; Little & Eklund 1999). Reaction wood represents a corrective growth that results from coordinately enhanced development of strength-contributing cells and wall metabolites (Wardrop & Davies 1964; Scurfield 1973; Barnett 1981; Timell 1986), and is one of the most critical defense systems in the long-term growth of woody species. Furthermore, in the stem of poplar mechanically stressed by bending, the formation of reaction wood is often accompanied by a stimulation of cambial cell division, whereas the cell division at the opposite site is more or less inhibited (Hellgren et al. 2004).

It is well recognized that anchorage is one of the primary functions of root systems, but although root

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thigmomorphogenesis has been interpreted as an effort of plant to improve its anchorage, root responses to mechanical stresses have been investigated, mainly at morphological and biomechanical level. In fact, it has been reported that mechanical perturbations induce changes in the mechanical properties of roots (Goodman & Ennos 1998) and in the pattern of lateral root initiation (Russell 1977; Tsegaye & Mullins 1994), and increase the number of lateral roots (Tamasi et al. 2005; Scippa et al. 2006), but investigations about the molecular mechanisms underlying these important alterations are still at an initial stage (Di Michele et al. 2006; Scippa et al. 2006). The lack of information about root response to mechanical stress may be attributed to intrinsic difficulties. For example, it is often difficult to excavate roots, especially in the case of large trees; excavation can damage the root system and thus confound the analysis of root system alterations at any level. Moreover, there are no databases of gene and protein sequences, which are indispensable tools for molecular studies.

In the present study, we used poplar seedlings as an experimental system to investigate the response of taproots to mechanical stress. In particular, the taproot was subjected to bending by forcing the root to grow on a metallic net bent to an angle of 90°. The preliminary results show that roots respond to mechanical stress by altering lateral root emission, lignin content and the expression of several stress-responsive factors. Further investigations will be carried out to investigate if different sections of the taproot (the region of the curvature, the region above and the region below) respond differently.

Material and methods

Plant material and growth conditions

One-year-old bare-root woody seedlings (seed origin) of *Populus nigra* were selected for the experiment. Root systems were gently washed to remove all traces of soil. All the lateral roots along the taproot axis were pruned by hand-scalpel to a distance of up to 3–5 cm from the lateral root–taproot junction. This short length was necessary to prevent laterals production via callogenesis directly from the taproot surface. Taproots were also pruned several centimetres from the collar to standardize the length of all samples.

Bending mechanical stress was applied by fixing the taproot of 35 lateral-free seedlings on a wide-mesh steel net (4–5 cm per mesh) bent at a right angle (Figure 1B). The taproot was bent at a distance of 18–19 cm from the base. The lower part was more flexible than the upper part. As controls, the taproots of 35 seedlings were linked to a vertical net (Figure 1A). The bent and control seedlings were scanned and then transplanted in large pots (35 cm × 45) filled with a 6:3:1 mixture of clayey loam soil : moist peat : gravel. We fixed stems horizontally to a steel wire to prevent the mechanical of the shoot from affecting the root. Bent and control seedlings were grown for 6 months in a greenhouse under controlled water

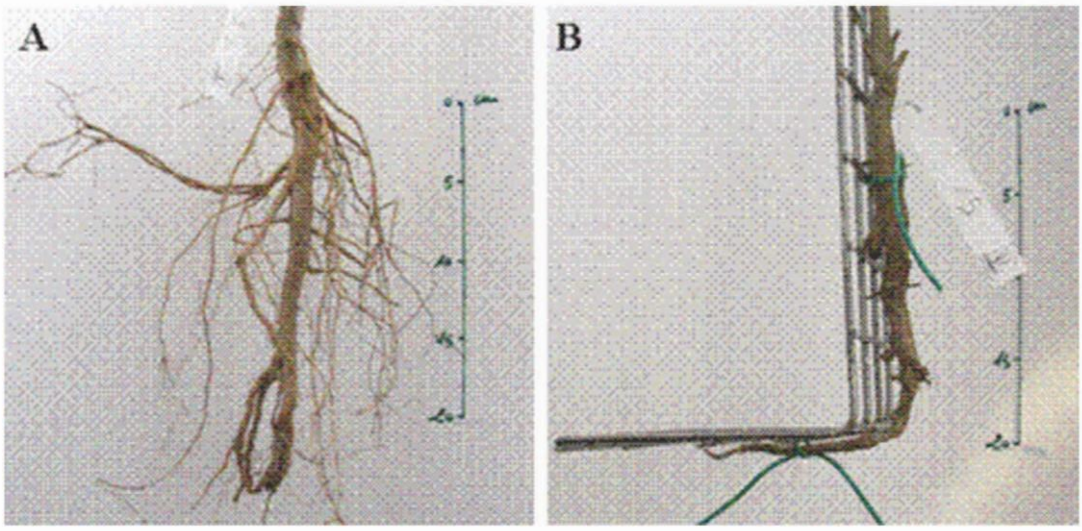

Figure 1. Taproots of 100 seedlings were freed from the first-order lateral roots. (A) For mechanical stress simulation, taproots were bent to an angle of 90° by using a metallic net. (B) Control taproots were linked to a vertical net.

and temperature conditions and randomly arranged across treatments.

Morphological analysis

The three-dimensional position coordinates (*X*, *Y*, *Z*) of the taproot and the first-order laterals (from the taproot junction up to a distance of 30 mm), together with the diameter and the topology (i.e. the branching hierarchic structure) were measured using a 3D digitizer with a Long Ranger transmitter (3 SPACE Fastrak, Polhemus Inc., Colchester, VT) (Sinoquet et al. 1997). The 3D spatial distribution of the root system had been carefully restored within a large box (50 cm square) with the vertical walls made of a transparent plastic sheet drilled at 5-cm intervals. The holes allowed the user to easily fix the orientation of a root axis by means of thin strings. All the first-order laterals preserved their orientation within the distance of 30 mm from the taproot junction, due to the rigidity of poplar root wood. All structural roots with a proximal diameter larger than 0.5 mm were digitised. Data from the digitizer and root topology were logged using Diplami software, modified for root topology as described by Danjon et al. (1999).

Because of their small size, lateral roots were assumed to be circular in cross-section. Taproots occasionally had an elliptical shape in which case we recorded the largest diameter and its orientation, as well as the diameter perpendicular at the largest diameter. The output data file was analysed using the AMAPmod software (Godin et al. 1997) which handles topological structure at several scales and provides 3D graphical reconstruction for data checking.

Root biomass and lignin content measurements

To measure root biomass, the dry weights of each whole root system were measured on 30 digitized control and stressed seedlings by weighing them before and after 24 h of desiccation in an oven at 105°C. To measure the lignin content of bent root and vertical root *Populus nigra* seedlings, about 1 g of root tissue was boiled in ethanol for 30 min, pulverized in liquid nitrogen and thawed in 10 ml of homogenization buffer (50 mM Tris–HCl, 10 g l^{-1} Triton X-100, 1 M NaCl pH 8.3). The suspension was vortexed and centrifuged at 2000 *g* for 10 min. The cell-wall pellet was washed twice with 4 ml of the homogenization buffer, 80% acetone and pure acetone, and dried in a concentrator. Each pellet was then treated with 0.4 ml thioglycolic acid and 2 ml 2 M HCl for 4 h at 95°C, centrifuged at 15,000 *g* for 10 min and washed three times with distilled water. The lignothioglycolic acid from each pellet was extracted with 2 ml 0.5 M NaOH by agitating for 16 h at 20°C. The supernatant was acidified with 0.4 ml concentrated HCl. Lignothioglycolic acid was precipitated for 3.5 h at 4°C, recovered by centrifugation at 15,000 *g* for 20 min, and dissolved in 1 ml 0.5 mol l^{-1} NaOH. The amount of lignin was calculated from the absorbance at 280 nm using a specific absorbance coefficient of 6.0 $l\,g^{-1}\,cm^{-1}$. Because this specific absorbance coefficient provides only an approximate conversion (the absorbance of lignothioglycolic acid from different sources can vary considerably; see Doster & Bostock 1988), the specimen with the highest lignin content was used as an internal standard in measurements of the percentage lignin content of the other samples. The results of three independent assays were used for statistical analysis.

Protein extraction

Total proteins were extracts from the taproot of control and stressed seedlings following a phenol protocol (Mihr & Braun 2003) with minor modifications. Independent samples (2.5 g of frozen taproot tissue) were finely powdered in liquid N_2 using a mortar, and suspended in 7.5 ml of extraction buffer (700 mM sucrose, 500 mM Tris–HCl, pH 7.5, 50 mM EDTA, 100 mM KCl, 2% w/v β-mercaptoethanol, 1 mM PMSF) by vortexing for 15 min, on ice. After addition of an equal volume of phenol-saturated 500 mM Tris–HCl, pH 7.5, the mixture was vortexed for 10 min and then centrifuged at 10,000 *g* for 15 min, at 4°C. The upper phenol phase was removed and extracted twice with the extraction buffer. Proteins were precipitated from the phenol phase by addition of five volumes of saturated ammonium acetate in methanol, overnight at −20°C. Precipitated proteins were centrifuged at 10,000 *g* for 30 min.

Two-dimensional electrophoresis

Protein pellets were washed with ice-cold methanol (once) and ice-cold acetone (three times), dried and solved in isoelectric focusing (IEF) buffer (9 M urea, 4% w/v CHAPS, 0.5% v/v Triton X-100, 20 mM DTT, 1% w/v carrier ampholytes nonlinear pH 3–10) (Bio-Rad, Hercules, CA, USA). Protein concentration was quantified using the Bio-Rad protein assay with BSA as a standard. IPG strips (18 cm pH 3–10 nonlinear, Bio-Rad ReadyStrip, Bio-Rad) were rehydrated overnight with 460 μL of IEF buffer containing 400 μg of total proteins. Proteins were focused using a Protean IEF Cell (Bio-Rad) at 12°C, applying 250 V (90 min), 500 V (90 min), 1000 V (180 min) and 8000 V for a total of 56 kVh. After focusing, proteins were reduced by incubating the

IPG strips with 1% w/v DTT for 15 min and alkylated with 2.5% w/v iodoacetamide in 10 ml of 50 mM Tris–HCl pH 8.8, 6 M urea, 30% w/v glycerol, 2% w/v SDS and a dash of bromophenol blue, for 15 min. Two-dimensional electrophoresis was carried out using a Protean apparatus (Bio-Rad) and 12% polyacrylamide gels (18 × 24 cm × 1 mm) in 25 mM Tris pH 8.3, 1.92 M glycine and 1% w/v SDS, with 120 V applied for 12 h. Each sample was run in triplicate. Protein spots were annotated only if detectable in all gels.

Image acquisition and analysis

Two-dimensional electrophoretic gels were stained with colloidal Coomassie G250 and scanned using a GS-800 calibrated densitometer (Bio-Rad). Images were analysed with the PDQuest software (Bio-Rad). Spot detection and matching between gels were performed automatically, followed by manual matching. After normalization of the spot densities against the whole-gel densities, statistical analysis (Student's t test) was performed with the PDQuest software. P values of intensity changes of each spot among three independent experiments were calculated. Protein spots with P values <0.01 were considered to show significant changes. A twofold change in normalized spot densities was considered indicative of a differentially expressed component.

Protein digestion and mass spectrophotometric analysis

Spots from two-dimensional electrophoresis were excised from the gel and digested with trypsin as previously reported (Talamo et al. 2003). Samples were desalted using μZipTipC18 tips (Millipore) before MALDI-TOF-MS analysis and/or directly analysed by μLC-ESI-IT-MS/MS. Peptide mixtures were loaded on the MALDI target together with CHCA as matrix, using the dried droplet technique. Samples were analysed with a Voyager-DE PRO spectrometer (Applera, USA). Peptide mass spectra for PMF experiments were acquired in reflectron mode; internal mass calibration was performed with peptides derived from trypsin autoproteolysis. Data were elaborated using the DataExplorer 5.1 software (Applera, USA).

Peptide mixtures were also analysed by using a LCQ Deca Xp Plus mass spectrometer (Thermo-Finnigan, USA) equipped with an electrospray source connected to a Phoenix 40 pump (Thermo-Finnigan) (Arena et al. 2006). Peptide mixtures were separated on a capillary Hypersil-Keystone Aquasil C18 Kappa column (100 × 0.32 mm, 5 μm) using a linear gradient from 10% to 60% of ACN in 0.1% formic acid, over 60 min, at flow rate of 5 $\mu l\ min^{-1}$. Spectra were acquired in the range 200–2000 m/z. Data were elaborated using the BioWorks 3.1 software provided by the manufacturer.

Protein identification

MASCOT software (Matrix Science, UK) was used to identify spots from an NCBI database containing protein or EST sequences from various *Populus* species by PMF experiments. Candidates with programme scores greater than 76 were considered identified. Eventual occurrence of protein mixtures was ascertained by sequential searches for additional protein components using unmatched peptide masses.

Results

Morphological analyses

To investigate the effect of bending on root development, the morphology of root apparatus was analysed by using the 3D digitizer (3 SPACE Fastrak, Polhemus, VT, USA; Polhemus 1993) software as described under Materials and methods. In particular, the emission of first-order new lateral roots was measured of along the pruned taproot after six months of growth. Morphological analysis showed that the number of new first-order lateral roots was significantly higher ($P<0.05$) in the right-angle bent roots compared with control roots (Table I).

Root biomass and lignin analysis

As shown in Table I, the root biomass, measured in 30 seedlings, was higher in bent root seedlings than in controls. To investigate if lignin was responsible for the different root biomass between treated and control roots, we measured content using a chemical

Table I. Root morphology and biomass measurements. Root morphology was analysed by using the 3D digitizer (3 SPACE Fastrak, Polhemus, VT, USA; Polhemus, 1993), and the emission of first-order new lateral roots was measured along 30 pruned taproot, in vertical or bent position, after 6 months of growth. For the root biomass, the dry weights of the whole root system were measured on 30 digitized control and stressed seedlings by weighing them before and after 24 h of desiccation in an oven at 105°C. Values are the mean of 30 replicates, with standard errors within brackets. Asterisk indicates significant difference (Student's t, $P<0.05$).

	Control	Right-angle curvature
Number of 1°-order laterals	41.90 (1.82)	51.47 (1.72)*
Root dry weight (g)	144.28 (7.28)	160.52 (9.64)

procedure (see Materials and methods). Indeed, lignin content was higher in the treated than in the control roots (Figure 2).

Protein maps of Populus *seedlings resolved by 2DE*

We analysed the proteome of the bent and control root by using two-dimensional electrophoresis (2DE). Figure 3 shows the proteome map of roots of poplar seedlings grown on a vertical metallic net (A, control) and on a right-angle bent metallic net (B). An average of 350–400 protein spots were reproducibly separated on Coomassie-stained gels, and the protein patterns were comparable in all samples analysed. The three replicates performed for each condition showed well-resolved spots, without streaking, and high reproducibility.

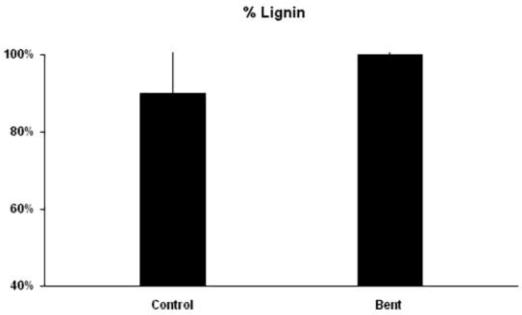

Figure 2. Lignin content in the vertical (control) and right-angle bent taproot (bent) *Populus nigra* seedlings. Bars are means of three replicates + SE. Lignin content is expressed as percentage of the lignin content in right-angle bent root, which was considered 100%.

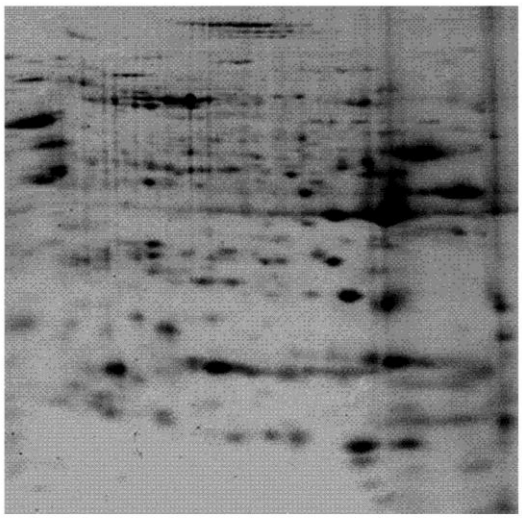

Figure 3. Two-dimensional electrophoretic gels of poplar seedling roots subjected to mechanical stress by growing in a net bent at a right angle (A) and in normal conditions (B). 2DE was performed using an equal amount (400 mg) of total protein and 18 cm Immobiline Dry Strips with nonlinear pH gradients from 3 to 10. Gels were stained with Coomassie Brillant Blue G-250 and images recorded.

To ascertain quantitative changes in proteomic maps of control and bent roots, we measured relative spot densities using software-assisted analysis. As reported in Figure 4, 15 protein spots (arrows) were differentially expressed ($P < 0.02$) between control and bent taproots. In particular, versus controls, 9 proteins were up-regulated in the bent root, with an expression change from +2.1 (spot 1, Table II, Figure 4) to +7.1 (spot 11, Table II, Figure 4), one protein spot (spot 34, Table II, Figure 4) was found only in the bent root, and five were down-regulated showing an expression change between −2.3 (spot 39, Table II and Figure 7) and −5.8 (spot 9, Table II and Figure 4). To determine their identity, the 15 abundant and/or differentially expressed spots were excised from gels, trypsin-digested and subjected to MS analysis, which generally resulted in high-quality and representative spectra. The peptide sequences were identified by database searching of uninterpreted fragment ion mass spectra with the Mascott programme, as described under Materials and Methods. Table II shows the identification of 9 out of the 15 proteins. No alignment was found for the remaining six spots, and further analysis by means of μLC-ESI-IT-MS/MS experiments are underway to identify them.

To verify identifications, we compared the theoretical and experimental molecular mass, whereas we were unable to compare the experimental pI because we used a nonlinear pI gradient for the IEF. Theoretical values were obtained from the protein identified on the SwissProt database using the Mr/pI calculator available at this website. Experimental values were deduced from protein spots on two-dimensional electrophoretic gels. In some cases, the molecular mass of protein spots differed from that of the matched proteins (Table II). Protein functions (see Table II) of the identified spots were assigned based on similarity with sequences of other plant homologs and based on data reported in the literature.

Discussion

In recent years, poplar species have emerged as model systems for woody plants (Bradshaw et al. 2000; Brunner et al. 2004). Moreover, since poplar has a small genome (5× the size of *Arabidopsis*), grows fast and is easy to propagate, both functional and structural genomics approaches can be used to investigate the response of woody taproots to

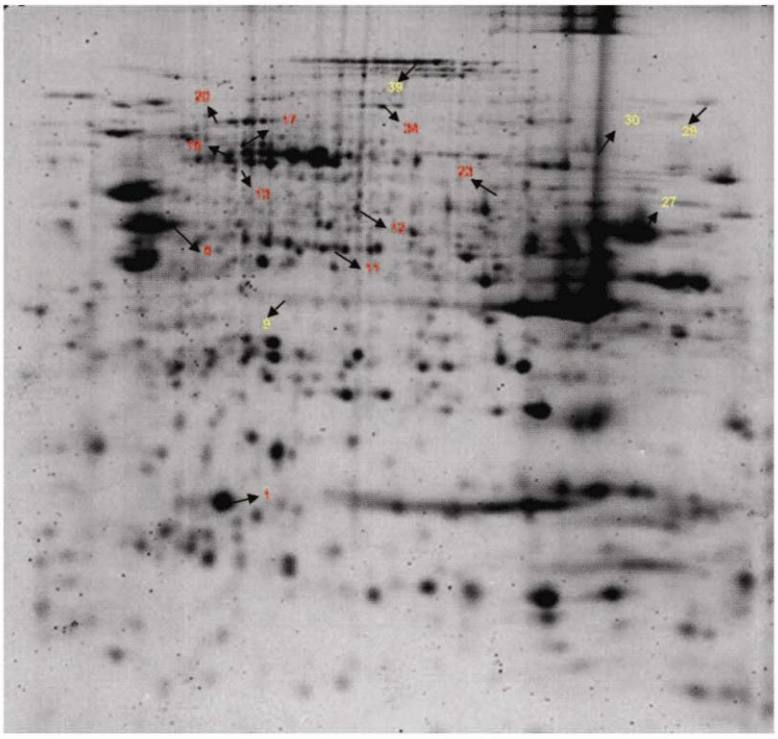

Figure 4. Two-dimensional proteome reference map of the roots of poplar seedlings (control). The 15 proteins that were differentially expressed at a significant level ($P<0.002$) and showing a change greater than twofold or detected only in one of the two conditions (vertical and right-angle bent root), with the strongest intensity on the gel (normalized volume >200), and with the greatest confidence level, are numbered. The numbers correlate with the protein identification in Table II.

environmental stress (Hertzberg et al. 2001; Wullschleger et al. 2002; Taylor et al. 2005).

In our study, we analysed the woody taproot of poplar seedlings at different levels to determine if the experimental set-up we describe can serve as an experimental system with which to probe the mechanisms involved in woody root response to mechanical stress.

It is general knowledge that mechanical stresses strongly affect plant stability, and that the function of roots in anchorage is influenced, among other factors, by: (1) the general morphology of the root system which dictates how stresses are distributed along the length and breadth of individual roots (Coutts & Lewis 1983; Coutts 1986; Blackwell et al. 1990; Ennos 1993, 1994; Nicoll et al. 1995; Crook & Ennos 1996; Stokes & Mattheck 1996; Stokes et al. 1996; Crook et al. 1997); (2) the mechanical properties of the root system (Goodman & Ennos 1998; Nicoll 2000). Studies of various plant species indicate that alterations of root morphology may be due to an increase of the number of lateral roots (Tamasi et al. 2005; Scippa et al. 2006). To investigate if bending induces alterations in the morphology of the woody taproot of poplar seedlings and if these alterations are due to the emission of new laterals, before bending, taproots were completely pruned, scanned, bent at a right angle and left to grow for 6 months in controlled conditions. After 6 months of growth the pruned control and bent seedlings emitted new laterals. However, similarly to other mechanical stresses, bending altered the morphology of roots by altering lateral root emission, as witnessed by the significantly increased number of new laterals in the bent taproots.

With regard to the mechanical properties of the root system, although we did not carry out a direct biomechanical investigation, we observed a meaningful increase of both root biomass and lignin content in response to bending. These results are in line with other reports of an association between alteration of root biomechanical properties and an increase of lignin (Patel 1971; Stokes & Guitard 1997; Jamet et al. 2000; Scippa et al. 2006), and/or the alteration the cell wall (Timell 1986; Showalter et al. 1992; Telewski 1995; Shirsat et al. 1996; Zipse et al. 1998).

Table II. Differentially expressed protein spots in poplar seedlings root. The data include: assigned spot number (see Figure 4), spot fold change where + and − indicate the spots up-regulated and down-regulated by the mechanical stress. For the spots identified: the putative assignment, the organism to which the matching protein was identified through similarity, database accession number, the identification method, the theoretical and experimental Mr, the % of coverage, the functional class according to the data reported in the literature. NI: non-identified.

Spot ID	Expression change	Protein name	Organism	Accession/ EST	ID method	Theor. Mr/ Exp. Mr	Pep./Seq. coverage (%)	Functional class
1	+2,3	Peroxiredoxin	*Populus alba* × *P. tremula* *P. trichocarpa* *P. euphratica* *P. trichocarpa* × *P. deltoides*	57894186 52401483 50067632 28609330	MF	17.4/22.0	8/58 11/52 9/58 8/56	Detoxification Antioxidant modulator of cell signaling pathways
6	+3,2	NI						
11	+7,1	Aldo/keto reductase AKR	*P. tremula* × *P. tremuloides* *Amanita muscaria*	58310994	MF	37.7/43.0	6/24	Detoxification Stress response
12	+3,4	NI						
13	+2,5	Enolase 2	*P. trichocarpa* × *P. nigra* *P. trichocarpa* *P. fremontii* × *P. angustifolia* *P. trichocarpa*	52515696 73870013 90190429 73891531	MF	47.9/56.0	8/32 9/43 8/43 6/34	Metabolism Stress response
16	+2,6	Enolase 2	*P. tremula* *P. tremuloides*	24102975 27417657	MF	47.9/58.0	6/38 6/38	Metabolism Stress response
17	+2,5	Enolase 2	*P. tremula* *P. alba* × *P. tremula*	24102975 57894302	MF	47.9/60.5	6/38 7/39	Metabolism Stress response
20	+4,9	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*P. trichocarpa* × *P. deltoides*	46843024	MF	61.2/64.0	7/31	Metabolism Stress response
23	+2,7	At2g39050/T7F6.22	*P. trichocarpa* × *P. nigra*	52516560	MF	35.6/53.0	7/27	Putative uncharacterized protein expressed protein
34	++	NI						
9	−5,8	NI						
27	−3,0	Fructose-bisphosphate aldolase	*P. tremula* × *P. tremuloides* *P. tremula* × *P. tremuloides*	18012028 (cambium) 24079330	MF	38.5/48.0	3/20 2/12	Metabolism Stress response
29	−2.8	NI						
30	−2.6	Bark storage protein B	*P. deltoids* *P. deltoids* *P. deltoides* *P. deltoides* *P. deltoides*	51874630 51875294 51875232 51875128 51875129	MF	34.2/60.5	10/28 7/25 8/29 6/45 7/30	Nitrogen storage
39	−2.3	NI						

There is a body of evidence that the hormones auxin and ethylene are involved in modulating plant response to mechanical stress and in the formation of reaction wood in the stem (Timell 1986; Sundberg et al. 1994; Little & Eklund 1999). The formation of reaction wood represents a corrective growth that is a result of coordinately enhanced development of strength-contributing cells and wall metabolites (Wardrop & Davies 1964; Scurfield 1973; Barnett 1981; Timell 1986) and is one of the most critical defence systems in the long-term growth of woody species. In addition, despite the lack of direct evidence of a correlation between the increase of lateral root number and auxin level in conditions of mechanical stress, it is well known that auxin stimulates lateral root formation (Montiel et al. 2004; Blakeley et al. 1982; Laskowski 1995; Casimiro et al. 2001) and that the number of laterals influences plant stability (Fitter & Ennos 1989; Stokes et al. 1996; Guingo & Hérbet 1997).

Our results, together with those of other, strongly support the use of the bent-root poplar seedlings model as an experimental system to investigate if and how the increase of lateral root formation is modulated by auxin. Moreover, this experimental system can serve to understand if root biomass and lignin content are related to reaction wood formation, and to investigate the role played by auxin and ethyelene.

Root alterations in response to the mechanical stress require co-ordinated growth and development control mechanisms that involve the perception of the external stimulus, the transduction of the signal, and the alteration of specific gene expression. We used comparative proteomic analysis to start investigating the gene factors involved in the response of the taproot to bending. Although preliminary, our results showed that the expression of 15 protein spots was significantly affected by bending. Nine spots were up-regulated in the bent root, one was expressed only in the bent root and five were down-regulated by bending (Figure 5). Moreover, seven of the nine up-regulated and two of the down-regulated protein spots were unambiguously identified by mass spectrometry; we are now carrying out μLC-ESI-IT-MS/MS experiments to identify the

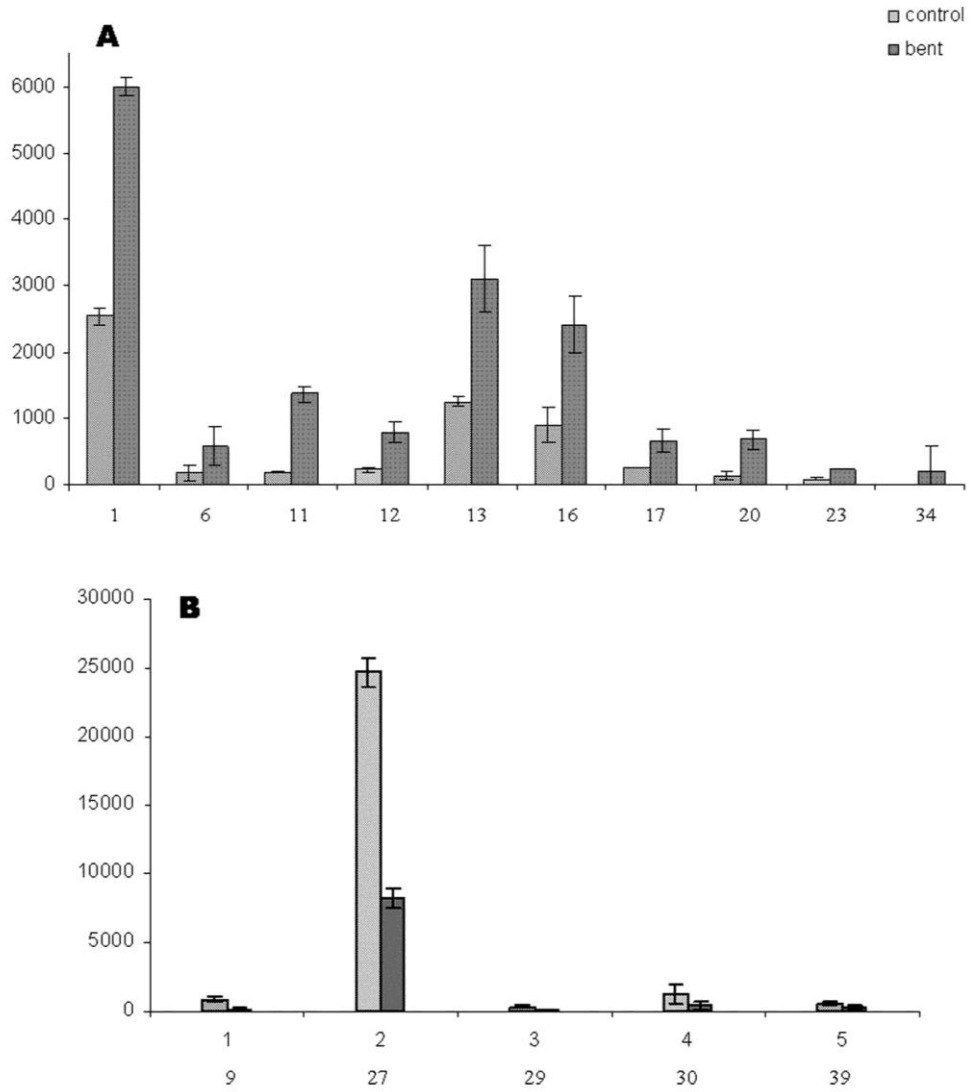

Figure 5. Differential expression of up-regulated (A) and down-regulated (B) proteins from control and bent roots. For each protein, the normalized of three gels average is reported ± SE. The numbers correlate with the protein identification in Table II.

remaining five spots. As shown in Table II, in some cases, the experimental molecular mass on the 2D gel differed slightly from the theoretical molecular mass of the matched proteins. This discrepancy can be due to the fact that the theoretical masses were calculated on the basis of the EST sequences, which may not cover the full sequences of the proteins, or alternatively to protein degradation or post-translational modifications.

Among the up-regulated protein spots, we identified a peroxiredoxin, an aldo/chetoreductase, three enolases, a biphosphoglycerate-independent phosphoglycerate mutase and an At2g39050/T7F6.22. The down-regulated spots included fructose-bisphosphate aldolase and bark storage protein. No information is available the At2g39050/T7F6.22 protein, whereas peroxiredoxins constitute the most newly identified group of H_2O_2-decomposing antioxidant enzymes. In the case of poplar (*P. trichocarpa*), nine genes have been identified, divided into four groups (Gama et al. 2007): 2-Cys Prx, Prx Q, II Prx and 1-Cys Prx.

Prx Q and 2-Cys Prx are chloroplastic peroxiredoxins and have been reported to be involved in pathogenic responses (Rouhier et al. 2004; Zhang & Riehers 2004; Kiba et al. 2005; Lamkemeyer et al. 2006) and peroxide detoxification (Broin et al. 2002; König et al. 2002; Broin & Rey 2003; Collin et al. 2003; Rey et al. 2005); type II Prx seems to be in the mitochondria. 1-Cys Prx it has been found in seeds where it seems to be involved in the germination (Haslekas et al. 2003), and in roots linked to resistance to oxidative stress generated by exogenous application of arsenate (Requejo & Tena 2005). All Prx proteins have a similar basic protein structure with a thioredoxin fold, and are resolved by dimensional gel separations; they separate with a molecular masses ranging between 17 and 22 kDa. The protein spot (Table II) we identified as peroxiredoxin has a molecular weight of 22 kDa, which is similar to the molecular weight of peroxiredoxins reported in the literature.

Prx gene expression is under the control of both developmental and environmental stimuli (Baier & Dietz 1997; Horling et al. 2002, 2003; Kandlbinder et al. 2004). Moreover, based on experimental evidence, a triple Prx function has been proposed: (i) antioxidant, (ii) modulator of cell signalling pathways, and (iii) redox sensor (Dietz et al. 2002; Rouhier and Jacquot 2002; Dietz 2003). Recent data suggest that Prx proteins modulate signalling pathways that involve reactive oxygen species and reactive nitrogen species. Reactive oxygen species are important signals in plant morphogenesis and in responses to stimuli (Mori & Schroeder 2004), and have been detected after mechanical perturbations (Legendre et al. 1993; Yahraus et al. 1995; Legue et al. 1997; Gus-Mayer et al. 1998). Based on these reports, it is conceivable that the Prx induced by bending in poplar root may be the 1-Cys type that is expressed in roots in response to oxidative stress (Requejo & Tena 2005), and that may be involved in the signalling pathways of mechanical stress.

The aldo/keto reductase was greatly increased (7.1-fold) in poplar seedlings bent roots compared with controls (Table II). Aldo/keto reductase is an NAD(P)H-dependent enzyme induced by such environmental stresses as wounding, heat, drought (Gavidia et al. 2005) and heavy metals (Bona et al. 2007). The massive increase of this protein in response to bending may be attributed to its involvement in the detoxification process in which it improves the scavenging capacity of the cell (Zhang & Riechers 2004; Zhang et al. 2005) due to the accumulation of reactive oxygen species.

Alterations of primary metabolism in response to environmental stresses has been widely studied. Here we report that metabolism is altered in mechanically stressed roots, as shown by the altered expression of three key enzymes of glycolytic metabolism. In particular, in the bent root, protein spots identified as enolases and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase were up-regulated, whereas a protein spot identified as fructose-bisphosphate aldolase was down-regulated (Table II, Figure 4). Enolase and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase are multifunctional enzymes involved in glycolysis, which have also been found to be significantly up-regulated in response to numerous plant abiotic stresses; namely, oxygen deficit, salinity or drought stress, phosphate starvation, and exposure to high or low temperatures (Forsthoefel et al. 1995; Mujer et al. 1995; Lal et al. 1998; Riccardi et al. 1998; Kollipara et al. 2002; Wang et al. 2002; Uhde-Stone et al. 2003; Yan et al. 2005). We identified three enolase spots located at different positions on the gels, with similar Mr (Figure 4, Table II), indicating that they might be isoforms of enolase or have different post-translational modifications. All three isoforms were up-regulated by bending and show a similar change in expression (Table II).

Fructose-bisphosphate aldolase is a constituent of both the glycolytic/gluconeogenic pathway and the pentose phosphate cycle, and in plants it catalyses an aldol cleavage of fructose-1,6-bisphosphate to dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate and a reversible aldol condensation (Rutter 1964). Although aldolase catalyses a readily reversible reaction, possesses no known regulatory properties, and would appear to be irrelevant for the control of metabolism and growth, small changes in its activity have marked consequences for photosynthesis, carbon partitioning and growth (Haake

et al. 1998). In fact, in plants, the fructose bisphosphate aldolase plays an important regulatory role in the flux of carbon through carbohydrate metabolism (Schaeffer et al. 1997; Pego & Smeekens 2000; Gonzali et al. 2001). Fructose-bisphosphate aldolase was found to be increased in the elongation zone of rice root in response to giberellin treatment (Konishi et al. 2004), and in *Arabidopsis* cell cultures and root apex under alternating gravity conditions (Martzivanou & Hampp 2003; Kimbrough et al. 2004). However, its expression was down-regulated in rice root treated with aluminium (Fukuda et al. 2007) and in response to low nitrogen (Lian et al. 2006).

In our study, the downregulation of fructose bisphosphate aldolase associated with the upregulation of 2,3-bisphosphoglycerate-independent phosphoglycerate mutase and enolase in bent roots may indicate the occurrence of a carbon flux shift towards a secondary metabolism as mevalonate and/or shikimate pathways. Both pathways are involved in the biosynthesis of important stress signal molecules (auxin and ABA) and lignin biosynthesis. However, although preliminary results reported in this paper, address the necessity of additional studies on other key enzymes and hormones concentration, to investigate if and how these metabolic pathways are related to the alterations of root morphology and lignin content observed in the bent poplar root.

Bark storage protein B (BSP) was downregulated in bent roots (Figure 4, Table II). This protein participates in nitrogen recycling from senescing leaves to bark and back to growing leaves in the spring (Wetzel et al. 1989; Clausen & Apel 1991; Gomez & Faurobert 2002). Major protein storage sites include shoot and root bark and xylem ray cells (Wetzel et al. 1989; Sauter and van Cleve 1990), where BSPs are stored in special vacuoles called "protein bodies" (Herman et al. 1988; Sauter et al. 1989). *Populus*, *Salix* and *Acer* accumulate BSPs during autumn (Wetzel et al. 1989; Clausen & Apel 1991), and are thought to be used during spring to provide N and C for new growth (Wetzel et al. 1989). Poplar BSPs are glycosylated and may occur as glycolytic isoforms of the base 32 kDa protein (Langheinrich & Tischner 1991; Stepien & Martin 1992) with the native form of BSP being composed of heterodimers of the different isoforms (Langheinrich & Tischner 1991). This fact may explain the significant difference we found between the theoretical and experimental weight of BSP. In fact, based on the Mr measured on our 2D SDS–PAGE gels, the Mr of the spot identified as BSP is almost double the theoretical Mr, indicating that the BSP may exist as a dimer.

Several environmental factors, among which photoperiod (Coleman et al. 1991; Langheinrich & Tishner 1991), nitrogen availability (van Cleve & Apel 1993; Coleman et al. 1994), temperature (van Cleve & Apel 1993) and wounding (Davis et al. 1993) influence BSP expression. However, the molecular mechanisms controlling BSP expression are not well characterized. We found a decrease in BSP in the root subjected to bending, where its degradation may be necessary to supply nitrogen to correct plant growth in response to the mechanical stress. In fact, our morphological and biomass analyses of bent roots show an increase of lateral root emission and root biomass, which could be related to the decrease of BSP.

In conclusion, our study clearly shows that the mechanical perturbation reproduced by bending induces, in poplar seedlings woody root, important alterations in the morphology and mechanical properties, which may improve root anchorage. In addition, using a proteomic approach, we might have identified important factors of the signal transduction pathway, detoxification and metabolism. These data provide insights into the almost unknown root responses to mechanical stress, and represent a good starting point for further dissection of the complex and coordinated mechanisms involved in woody root biology.

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The proteome of *Populus nigra* woody root: the asymmetric response to bending

(Paper in preparation)

Abstract

Mechanical stress is a widespread environmental condition caused by numerous factors (i.e. gravity, touch, wind, soil density, soil compaction and grazing, slope) and can severely affects plant stability. In response to mechanical stress and to improve their anchorage, plants have developed complex mechanisms to detect mechanical perturbation and to induce a suite of modifications collectively known as thigmo-morphogenesis. The response of woody root to mechanical stresses has been investigated mainly at morphological and biomechanical level, whereas investigations about the molecular mechanisms underlying of these important alterations are still at initial stage. *Populus* has been widely used to investigate the response of stem to different mechanical stress. Moreover since it is the first forest tree genome to be decoded, represents the model woody plant for addressing important questions including mechanisms controlling the adaptation of woody root to changing environment. In this study the woody root of *Populus nigra* seedlings subjected to bending was used to identify molecular factors controlling the response to mechanical stress. Morphologycal and biomechanical investigations revealed that the in response to bending woody root undergo to alterations that are asymmetrically distributed along three regions where the mechanical forces are differentially distributed. As initial step to the comprehensive analysis of molecular factors controlling the woody poplar taproot thigmo-response, a reference 2-D maps was produced where 213, further identified. Successively, 2-D maps of the control and three different regions of the bent taproot were compared along a time course. The integration of morphological, biomechanical and proteomic investigation, together with the results of the multivariate statistical analysis revealed important physiological temporal and spatial markers.

Keywords: *Populus, proteomics, mechanical stress, bending, mass spectrometry, roots, lignin.*

1. Introduction

Plants in natural fields are continuously exposed to a myriad of mechanical stresses that may affect their stability with important economic damages and ecological effects (Coutts, 1986; Stokes and Guitard, 1997). To cope with mechanical stresses plants have evolved complex mechanism of response involving a range of physiological, morphological, and biochemical changes. These alterations, known as thigmo-responses (Jaffe, 1973), generally occur slowly over time and are therefore often not readily apparent but can be quite dramatic. Thigmo-responses have been studied in several plant species where a reduced height, reduced leaf area, changes in root placement and structural properties, stronger and more fexible stem, and increasing ratio of branch to stem diameter have been observed (reviewed in Braam, 2005). Wind- and/or touch are he most investigated mechanical perturbation and the induced alterations have been observed in a variety of herbaceous plants e.g., Arabidopsis, maize, wheat, pea, cucumber, soybean, bean (for review see Braam, 2005), as well as in several different woody plant species as pine, fir and poplar (reviewed in Telewski, 1995; Pruyn et al., 2000; Watt et al., 2005). However, the molecular basis of plant responses to mechanical forces has been investigated in a few model systems such as *Arabidopsis* (Lee et al., 2005) and in the case of woody plant it has been mainly investigated in the stem (Azri et al., 2009). Several studies have been published showing that plant responses to environmental stress were varying among plants of different ages (Kus et al., 2002) and among different organs of the same plants (Taylor et al., 2002). A compromise between growth and defense has been suggested (Herms and Mattson, 1992; Boege and Marquis, 2005) and several hypotheses have been proposed to explain the spatial and temporal variations in plant response to environmental stress (for review see Stamp, 2003).

Plants, are sessile organisms with the majority relying on the mechanical support the complex root system provides. Anchorage, in fact, is one of the primary functions of root systems and heavily influences their architecture and morphology (Coutts, 1983, Ennos, 1991) in response to environmental stimuli.

The mechanism involved in the response of root to mechanical stress, have been extensively investigated in the model plant *Arabidopsis thaliana*, especially in relation to mechanical impedance (Okada and Shimura, 1994; Masle, 2002; Braam, 2005) and more recently, to bending forces (Ditegout et al., 2008; Richter et al., 2009). A large number of genetic factors (Braam & Davis, 1990; Sistrunk et al., 1994; Xu et al., 1995), together with the signaling

crosstalk (Fujita et al., 2006; Bostock, 2005) that evolve phytohormones such as jasmonic acid, salicylic acid, ethylene, abscisic acid and auxin have been identified. In the case of woody plants, roots response to mechanical stresses have been investigated in several trees and shrubs (Rees and Grace, 1980a, 1980b; Gartner, 1994; Goodman and Ennos, 1996, 1997a, 1997b, 1998, 2001; Stokes et al., 1995; 1997a, 1997 b; Fredericksen et al., 1994; Telewski, 1995; Lindstrom & Rune, 1999; Watson, 2000; Peltola et al., 2000; Di Iorio et al., 2005; Chiatante et al., 2006). These studies showed that common thigmomorphogenic responses to mechanical perturbations include change in root:shoot ratio (Stokes and Guitard, 1997); increase in root cross sectional area; shift in its distribution relative to the direction of stimulation (Jacobs, 1954; Stokes et al., 1995; Goodman and Ennos, 1998); and changes in the mechanical properties of the plant material (Goodman and Ennos, 1998; Di Iorio et al., 2005; Chiatante et al., 2006). However, all these reported studies are mainly focused on the analysis at morphological, biomechanical and anatomical level, while the identification of the genetic factors is still at very initial stage (Di Michele et al., 2006; Scippa et al., 2006, 2008). The absence of suitable experimental system made these investigations a very complex issue, and the amount of information collected enormously lacking (Di Michele et al., 2006, Scippa et al., 2006). Since poplar is the first forest tree genome to be decoded, it became the model woody plant for addressing important questions including mechanisms controlling the adaptation of woody root to changing environments. Furthermore, new technologies as microarrays, cDNA-AFLP and proteomics are becoming very promising, adding exciting insights for future researches.

Differential proteomic analysis has been established in the last years as an essential tool in the study of abiotic stress response in living organisms. Numerous proteomic studies have been carried out in plants facing abiotic stressing conditions, like heavy metal (Kieffer et al., 2008) and water deficit (Plomion et al., 2006) in poplar.

Here, we investigated the effects of the non-destructive, long-term mechanical stress imposed by bending, on the woody taproot of *Populs nigra*. In order to identify molecular factors controlling the response to bending a proteomic approach has been used coupled with morphological, biomechanical and biostatistical analysis. Up to our knowledge, this is the first study on mechanism involved in mechanical stress response of woody taproot and the first description of poplar woody-root complete proteome.

2. Material and Method

2.1. Plant material, growth conditions and stress treatment

One year old seedlings of *Populus nigra* were selected for the mechanical stress experiment. As reported in a previous work (Scippa et al., 2008), seedlings were cleansed of soil, freed from all laterals by cutting all the first-order lateral roots at 4-5 cm from the taproot junction and subjected to mechanical stress. At this aim the taproot 26.47 ± 0.69 cm long of 30 seedlings were bent by using a right-angle curved steel net to an angle of 90° (Fig. 2 Supporting information A) and the point of maximum bending was located at 19.60 ± 0.52 cm from collar; as control, the taproots 27.06 ± 0.67 cm long of 30 seelings were linked to a vertical widemesh steel net (4 cm per mesh) (Fig.2 Supporting information B). Stressed and control seedlings were planted in pots (35 wide x 45 high cm), with a mixture 6:3:1 of soil, peat and pumice, and grown in a green house under controlled water regime and natural photoperiod and temperature (minimum, maximum and average temperatures are reported in Figure 1 Supporting information). Roots were excavated after 12 (15-20th February 2007, T_0), 13 (15-20 th March 2007, T_1) and 14 (15-20 th April 2007, T_2) months. All the analysis were carried out along the taproot starting 15 cm downwards from the collar, where a secondary structure was well developped (Fig.2 B Supporting information). Moreover, in the case of the stressed root, the bent region was divided in three 5 cm sectors (Fig.2 A Supporting information) corresponding to: 1) sector just above the bending zone Above Bending Sector – ABS (12.09 – 17.09 cm distant from the collar); 2) sector representing the bending zone Bending Sector - BS (17.10 – 22.10 cm distant from the collar and 19.60 cm was the middle point); 3) sector just below the bending zone Below Bending Sector -BBS (22.11 – 27.11 cm distant from the collar).

2.2. Modeling bending stress along the woody taproot

A simple modeling form mechanical forces distribution in the bent root has been performed through the software Impact - Explicit Dynamic Finite Element Program. Two times have been considered, at the beginning of stress treatment (initial time -Ti) and at the end of bending stress treatment (T_2). The plant material has been considered as elasto-plastic (Fourcaud, 2007). Taproot diameters of plants at time Ti and T_2 were computed and corresponding meshes were constructed with 4 nodes elements. The bending is performed through the application of a forced displacement at the narrow end of the taproot.

2.3. Morphological analysis

Before transplanting, pruned taproots were scanned with a calibrated LA1600+ flatbed scanner (Epson America Inc., USA) coupled to a lighting system for image acquisition. The diameter and length were measured on the scanned images with the WhinRhizo software V.

2003b (Regent Instruments Inc, Quebec, Canada). Shoot and root morphological analysis measurements were carried out at the end of the whole experiment. At the end of the experiment (14 months), to make easier the measurement, first-order lateral roots of control and stressed plants were pruned again at 4-5 cm from the taproot junction. Plants were suspended and their orientation fixed by means of small strings. The three-dimensional position coordinates (X, Y, Z), diameter and topology (i.e. the branching hierarchic structure) of the taproot and the first-order laterals junction point for the root system, the stem and the first-order branches junction point for the shoot, were measured using a 3D digitizer with a Long Ranger transmitter (3 SPACE Fastrak, Polhemus Inc., Colchester, VT) (Sinoquet et al., 1997). All first-order lateral roots with a proximal diameter larger than 0.5 mm were digitized. Data from the digitizer and root topology were logged using Diplami software, modified for root topology as described by Danjon et al., (1999). Because of their small size, lateral roots were assumed to be circular in cross-section. Taproots occasionally had an elliptical shape in which case we recorded the largest diameter and its orientation, as well as the diameter perpendicular at the largest diameter. The output data file was analyzed using the AMAPmod software (Godin et al., 1997) which handles topological structure at several scales and provides 3D graphical reconstruction for data checking.

Within each growth condition and the three sectors examined, the clustering tendency of the first-order lateral emission points was evaluated using circular statistical methods, Rayleigh's Uniformity test in particular (Mardia and Jupp, 2000); calculations were performed using Oriana software v. 2.01 (Kovach Computing Services; Kovach, 1994). The null hypothesis was that data are uniformly distributed. A probability less than 0.05 was taken to indicate that data were not distributed uniformly, i.e. there was evidence of a preferred direction of lateral emission. The Z value is calculated as $Z = nm^2$, where n is the number of observations and m is the length of the mean vector. A greater mean vector length (and the resulting larger Z value) means a greater concentration of data around the mean, and thus less likelihood of the data being uniformly distributed.

2.4. Lignin content measurements

The lignin content was measured at the beginning (Ti) and at the end (T_2) of the experiment according to the Doster & Bostock (1988) protocol. For the extraction 1 g of root tissue of the control and the three sectors (ABS, BS, BBS) of the bent taproot was boiled in ethanol for 30 min, pulverized in liquid nitrogen and homogenizated in 10 mL of extraction

buffer (50 mM Tris–HCl, 10 g/LTriton X-100, 1 M NaCl pH 8.3). The suspension was vortexed and centrifuged at 10000 g for 10 min. The cell-wall pellet was washed twice with 4 mL of extraction buffer, 2 mL of 80% acetone and pure acetone and than dried in a concentrator. Each pellet was then treated with 0.4 mL thioglycolic acid and 2 mL 2 M HCl for 4 h at 95 °C, centrifuged at 15000 g for 10 min and washed three times with distilled water. The lignothioglycolic acid from each pellet was extracted with 2 mL 0.5 M NaOH by agitating for 18 h at room temperature. The supernatant was acidified with 0.4 mL concentrated HCl. Lignothioglycolic acid was precipitated for 4 h at 4 °C, recovered by centrifugation at 15000 g for 20 min, and dissolved in 1 mL 0.5 M NaOH. The amount of lignin was calculated from the absorbance at 280 nm using a specific absorbance coefficient of 6.0 L /g x cm. Because this specific absorbance coefficient provides only an approximate conversion (Doster and Bostock, 1988), the specimen with the highest lignin content was used as an internal standard in measurements of the percentage lignin content of the other samples. The results of twenty independent assays were used for statistical analysis ($P < 0,001$).

2.5. Protein extraction

Total proteins were extract from control and three regions of the bent taproots sampled in T_0, T_1, T_2 following the phenol protocol (Mihr & Braun, 2003) with minor modifications (Scippa et al., 2008).

For each sample 2g of root tissue were powdered in liquid N_2 in a mortar, and suspended in 6 mL of extraction buffer containing 700 mM sucrose, 500 mM Tris-HCl, pH 7.5, 50 mM EDTA, 100 mM KCl, 2% w/v β-mercaptoethanol, 1 mM PMSF, 1 mM Protease Inhibitor Cocktail (Sigma Aldrich). The mixture was shaken for 20 min at the room temperature, after addition of an equal volume of phenol saturated-500 mM Tris-HCl, pH 7.5, and then centrifuged at 10.000 x g for 20 min, at 4°C. Proteins were precipitated overnight at -20°C by addition of five volumes of saturated ammonium acetate in methanol. After two washing with 3 mL of saturated ammonium acetate in methanol and one washing with ice-cold acetone the proteins were centrifuged at 10000 x g, for 20 min at 4 °C, were dried and suspended in IEF buffer (9 M urea, 4% w/v CHAPS, 0.5% v/v Triton X-100, 20 mM DTT and 1% w/v carrier ampholytes pH 3–10). Two independent extraction were performed combining the root samples of three different poplar seedlings. Protein concentration was quantified by Bradford's method (1976) using the BioRad (Bio-Rad, Hercules, CA, USA) protein assay and BSA as standard.

2.6. 2DE (Two-dimensional electrophoresis)

For isoelectrofocussing, 17 cm, no linear pH 3-10 IPG strips (ReadyStrip, Bio-Rad, Hercules, CA, USA) were rehydrated overnight with 300 µl of rehydratation buffer (6 M urea, 2% w/v CHAPS, 0.5% v/v Triton X-100, 20 mM DTT and 1% w/v carrier ampholytes pH 3–10) containing 700 µg of total proteins and 5 µl di IPG buffer (isoelectrofocusing pH gradient buffer) too. Proteins were focused using a Protean IEF Cell (Bio-Rad, Hercules, CA, USA) at 12°C, applying 250 V (90 min), 500 V (90 min), 1000 V (180 min) and 8000 V for a total of 56 KVh. After focusing, proteins were reduced by incubating of the IPG strips with 1% w/v DTT in 10 mL of 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% w/v glycerol, 2% w/v SDS for 20 min and alkylated with 2.5% w/v iodoacetamide in 10 mL of 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% w/v glycerol, 2% w/v SDS for 20 min.

Electrophoresis in the second dimension was carried out on 12% polyacrylamide gels (17 x 24 cm x 1 mm) with a Protean apparatus (Bio-Rad) in 25 mM Tris-HCl, pH 8.3, 1.92 M glycine and 1% w/v SDS, with 70 V applied for 16 h, until the dye front reached the bottom of the gel. 2-DE gels were stained with colloidal Comassie G250. Samples were run in triplicate.

2.7. Scanning and PDQuest analysis

2-DE gels were scanned using a GS-800 calibrated densitometer (Bio-Rad). Image analysis was performed using the PDQuest software (Bio-Rad) to identify differentially expressed proteins. Spot detection and matching between gels were performed 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 performed to find out significant changes in protein folding between samples. A two-fold change in normalized spot densities was considered indicative of a differential expression.

2.8. In gel digestion and mass spectrometry

Spots were manually excised from gels, triturated and washed with water. Proteins were *in-gel* reduced, S-alkylated and digested with trypsin as previously reported (Vascotto et al., 2006). Digest aliquots were removed and subjected to a desalting/concentration step on µZipTipC18 (Millipore Corp., Bedford, MA, USA) using 5% formic acid/50% acetonitrile as eluent before MALDI-TOF-MS or nanoLC-ESI-LIT-MS/MS analysis.

In the first case, peptide mixtures were loaded on the MALDI target, using the dried droplet technique and α-cyano-4-hydroxycinnamic acid as matrix, and analyzed by a Voyager DE PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA), operating in positive ion reflectron mode, with an acceleration voltage of 20 kV, a nitrogen laser (337 nm) and a laser repetition rate of 4 Hz. The final mass spectra, measured over a mass range of 800-4000 Da and by averaging 50-300 laser shots, were elaborated using the DataExplorer 5.1 software (Applied Biosystems) and manually inspected to get the corresponding peak lists. Internal mass calibration was performed with peptides deriving from trypsin autoproteolysis.

In the second case, after removing acetonitrile by concentration, digests were analyzed by nanoLC-ESI-LIT-MS/MS using a LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with Proxeon nanospray source connected to an Easy-nanoLC (Proxeon, Odense, Denmark). Peptide mixtures were separated on an Easy C18 column (10 x 0.075 mm, 3 mm) (Proxeon, Odense, Denmark) using a linear gradient from 5% to 50% of acetonitrile in 0.1% formic acid, over 60 min, at a flow rate of 300 nL/min. Spectra were acquired in the range m/z 400-2000. Acquisition was controlled by a data-dependent product ion scanning procedure over the three most abundant ions, enabling dynamic exclusion (repeat count 2 and exclusion duration 1 min). The mass isolation window and collision energy were set to m/z 3 and 35%, respectively.

2.9. Protein identification

MASCOT software package (Matrix Science, UK) was used to identify spots unambiguously from an updated plant non-redundant sequence database (NCBI nr 2009/05/03) in MALDI-TOF peptide mass fingerprinting experiments by using a mass tolerance value of 40-80 ppm, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2 and Cys carbamidomethylation and Met oxidation as fixed and variable modification, respectively. Candidates with a MASCOT score > 82, corresponding to $p<0.05$ for a significant identification, were further evaluated by the comparison with their calculated mass and pI values, using the experimental values obtained from 2-DE.

MASCOT software package (Matrix Science, UK) was also used to identify spots unambiguously from the same updated plant non-redundant sequence database (NCBI nr 2009/05/03) in nanoLC-ESI-LIT-MS/MS experiments by using a mass tolerance value of 2 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2 and Cys carbamidomethylation and Met oxidation as fixed and variable modification, respectively. Candidates with more than 2 assigned peptides with an

individual MASCOT score >25, corresponding to $p<0.05$ for a significant identification, were further evaluated by the comparison with their calculated mass and pI values, using the experimental values obtained from 2-DE. Where appropriate, protein identification was checked manually to provide for a false positive rate less than 1%.

2.10. Multivariate statistical analysis

Proteins showing a differentially expressed patterns revealed by PDQuest analysis, were further subjected to uni- and multivariate analysis to identify spatial and temporal markers.

ANOVA was computed at three different levels: first it was computed for the control taproot along the temporal gradient to analyze the physiological protein patter; second it was computed between control and three stressed regions to identify proteins that changed in quantitative pattern at T_0, T_1 and T_2; third it was computed among stressed root regions, to individuate proteins with significant spatial gradient variation (ABS, BS and BBS) during time.

Cluster analysis was performed on the correlation matrix, using paired group as linkage, among proteins resulted discriminant by ANOVA ($P\leq 0.05$): for each data set, it grouped all significant proteins in relation to their quantitative relationships.

3. Result

3.1. Analysis of stresses induced by bending

Woody taproot was subjected to mechanical stress by bending around a right-angled metallic net, for a period of 14 months without any visible signs of damages. The proposed model explains the alterations in stress intensities distribution based on two main factors: diameter and wood mechanical properties variation. Modeling the mechanical forces distribution in the three different taproot sectors (ABS, BS, and BBS) at the beginning (Ti) and at end of stress treatment (T_2) revealed that the stress level is globally lower and more homogeneously distributed at initial time (Ti) than at T_2. As reported in Figure 1, the maximum value of compression stress is obtained at T_2 with 38MPa while is 31MPa at Ti. Tensile stress maximum value is obtained at T_2 with 32MPa while is 30MPa at Ti. Concerning the 3 defined spatial zones, the central one is always showing the higher stress value as in compression than in tension. The computation of the average of tensile and compression stress has been performed for all the 3 zones. Going from the ABS to the BBS, at time Ti, compression stress values vary from 12MPa to 23MPa and finally 17MPa while tensile stress vary from 11MPa to 22MPa and finally 15MPa. At time T_2, compression stress values vary from 14MPa to 27MPa and finally 21MPa while tensile stress varies from 12MPa to 24MPa and finally 15MPa (Fig.1).

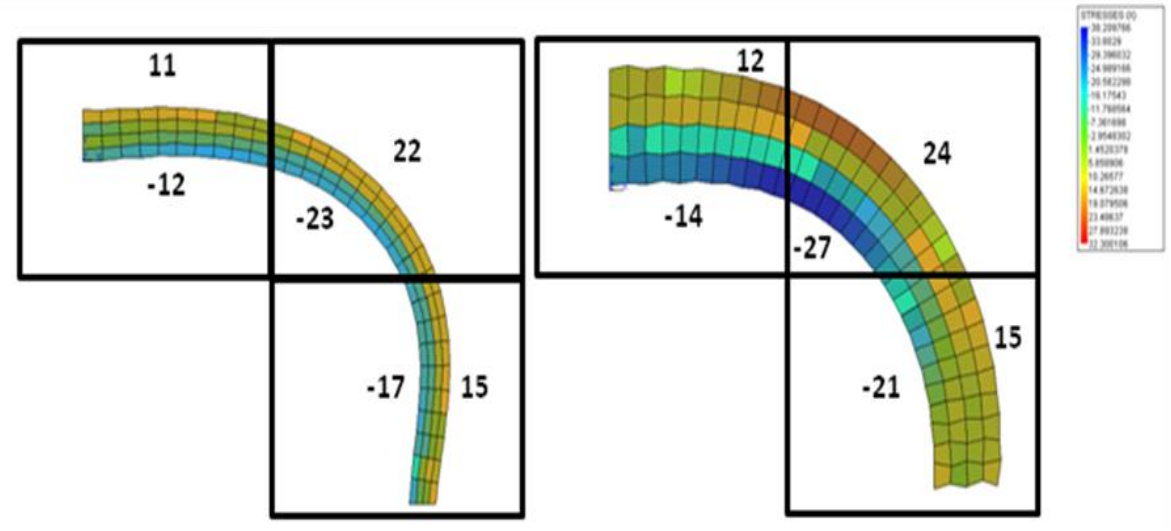


Figure 1. Model of the mechanical stress distribution. Distribution of longitudinal stresses along the taproot at time Ti (left) and time T_2 (right). Average stress for the three defined zones (ABS, BS, BBS) are indicated for both the areas in tension (positive values) and in compression (negative values).

3.2. Root and Shoot morphology

Root and shoot morphology and growth were analyzed in control and stressed plants at the end of the experiment corresponding to 14 months of growth (T_2) when the modifications are more visible. As general traits, root and shoot dry weights were slightly higher in stressed plants though not statistically significant, the same for the shoot heights (Table I). Root and shoot systems showed similar behaviors in terms of number and basal diameter of first-order laterals and branches, respectively. In stressed plants, the numbers of both first order laterals and branches were higher than control (Table I), though the branches marginally missed the 5% level (P=0.064). The basal diameter showed the opposite behavior as medians were significantly lower than control (Table I).

Traits	Control	Bending	*P*	test
Shoot				
First-order branches number (no.)	27.16 (1.57)	31.43 (1.64)	0.064	t Student
First-order branches basal diameter (mm)	5.2	4.5	**<0.001**	Median test
Shoot dry weight (g)	351.03 (22.53)	365.17 (15.10)	0.332	t Student (after log transformed)
Shoot height (cm)	284.1	282.73	0.606	Median test
Root				
First-order laterals number (no.)	44.90 (1.71)	53.47 (1.82)	**<0.001**	t Student
First-order laterals basal diameter (mm)	4.2	3.9	**<0.001**	Median test
Root dry weight (g)	156.18 (6.27)	174.62 (8.64)	0.18	t Student

Table I. Shoot and Root parameters. Values are means of 30 replicates (±SE). *P* value refers to Student's *t*-test (parametric) and median test (non parametric) depending on the fulfillment of the requirements of parametric analysis, respectively, at a significance level of $\alpha = 0.05$.

Differences emerged also in the distribution of laterals roots along the taproot. In fact, the frequency distribution of lateral emissions along the taproot was significantly different between bent and control plants (two-sample Kolmogorov-Smirnov test, P<0.001) (Figure 2). In

particular, from the detailed morphological analysis it is evident the increase of the frequency of laterals occurring in the ABS and BS sectors.

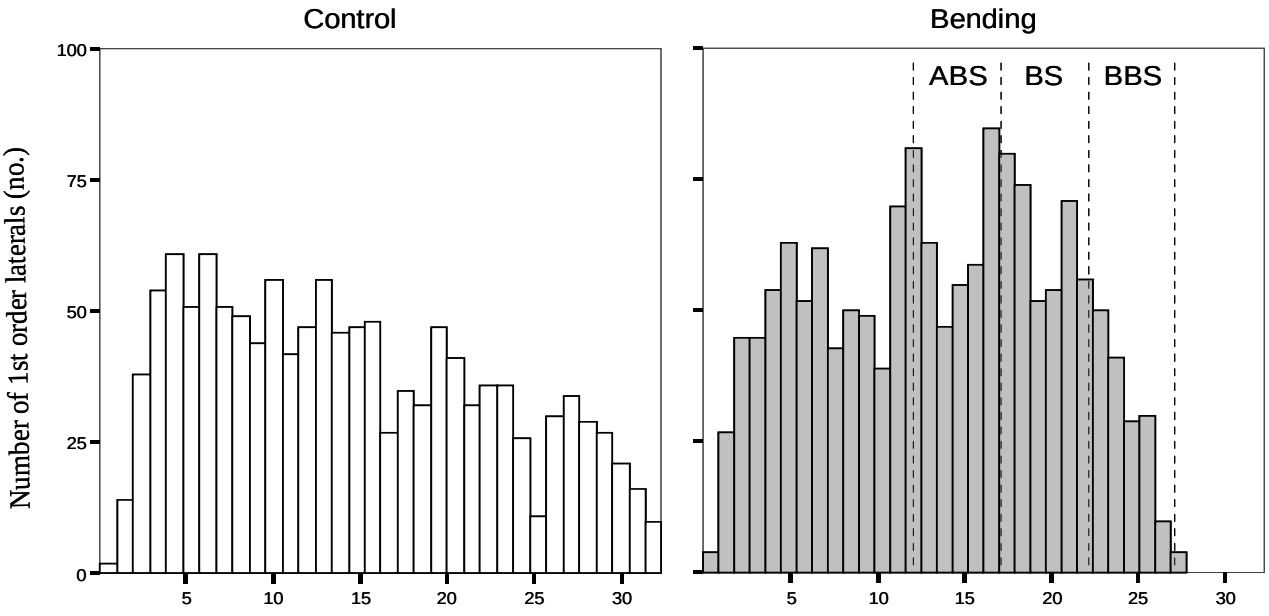


Figure 2. **Morphological analysis.** The frequency distribution of lateral emissions of first-order lateral roots was measured along pruned taproot of control and bent root after 14 months of growth (T_2). Data are referred to 30 replicates each for control and bent root. Significant differences are evidenced by Kolmogorov-Smirnov test, $P<0.001$.

The spatial distribution of the lateral root emission along the control and bent taproot was also measured. From the first-order lateral emission points analysis emerged a very evident different emission's architecture between the control and the bent taproot. In detail, the mean distribution of first-order lateral emissions in case of bent taproot pointed in different directions compared with control (Figure 3 A, B).In fact the means distributions from 10 to 25 cm taproot length pointed all towards the convex side (0° centered) for bending (Figure 3A) whereas in the case control plant the means distributions pointed in 120° roughly spaced directions (Figure 3B). At the depth within 17.1-22.1 cm, differences between control and stressed root become more significantly evident (Figure 3 C,D). In fact at this depth corresponding to the BS region in the bent root, laterals showed a signifacant clustering tendency toward the convex side for bending treated plants, (Rayleigh's test, $P<0.002$; Table II). The 95% confidence interval highlighted with red line (Figure 3 C, D) gives a clear visual evidence of this finding.

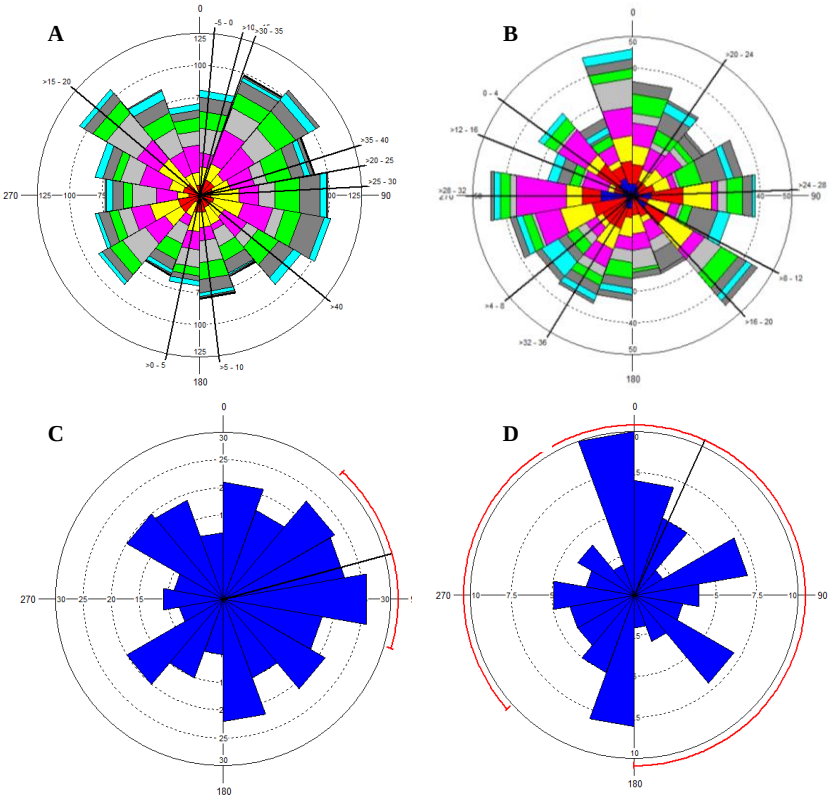


Figure 3. Analysis of emission architecture. The black lines indicate the average direction of laterals along 5 cm length taproot intervals. Average of lateral direction along the bent (A) and control (B) root at the depth intervals of 10-15, 15-20, 20-25 cm. Average of lateral direction at 17.1-22.1 cm depth corresponding to BS in the case of bent root (C). In D is represented the average lateral root direction at the same depth in the control. The solid lines from the centre indicate the mean emission direction for the different intervals. 0° coincides with convex side for bending treatment.

Table II. Parameters of the vectorial circular analysis of the first-order lateral emission points at 17.1-22.1 cm taproot length interval (BS for Bending).. The eccentricity vectors are significantly clustered at $P < 0.05$.

	Control	Bending
Mean Vector (μ)	24.308°	74.672°
Length of Mean Vector (r)	0.055	0.142
Standard Error of Mean	79.591°	16.189°
95% Confidence Interval (-/+) for μ	228.278°	42.935°
Rayleigh Test (Z)	0.259	6.2
Rayleigh Test (*P*)	*0.772*	***0.002***

3.3. Lignin content

The lignin content measured at the beginning of the experiment (Ti) was very similar in the unstressed and the three regions of bent taproot (Figure 4). However after 14 months of growth, it increased slightly in the unstressed and meaningfully in the bent taproot (Figure 4). In this last case differences were measured in the three bent taproot sectors. In particular, ABS showed similar lignin content to the control, while BS and BBS were characterized by a higher lignin values. The highest amount was measured in the BS (Figure 4).

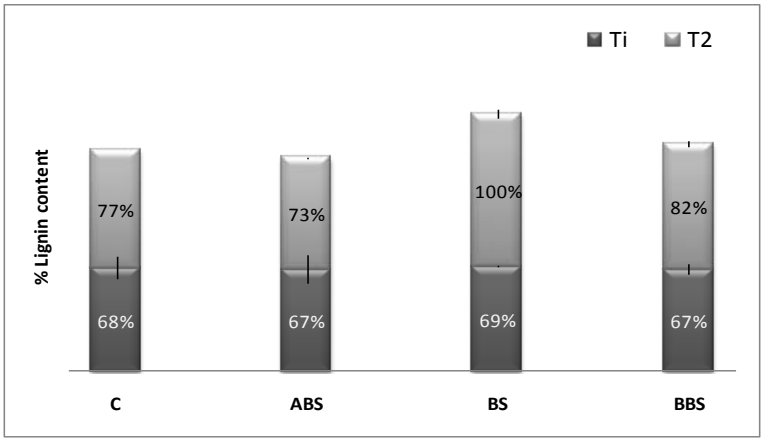


Figure 4. **Lignin content .**Lignin content of control and the three regions of stressed *Populus nigra* taproot (ABS, BS, BBS) measured at T_i , and T_2 according to the Doster and Bostock (1988) protocol. Lignin content is expressed as percentage of the lignin content in T_2 BS bent root, which was considered 100%. Bars are means of twenty biological replicates +/- SE.

3.4. The proteome of poplar woody taproot resolved by 2D electrophoresis

Due to the lack of information about the secondary root structure at molecular level, the initial step was to produce a woody root proteome map where the most abundant and well separated proteins were reported and identified. To accomplish this aim, a proteome reference maps of poplar woody root by analyzing with 2-DE whole protein extracts from C, ABS, BS, BBS in the three different times (T_0, T_1, T_2) was generated.

These 2-DE maps (twelve in number), produced in triplicate for each of two independent protein extraction experiments showed a high level of reproducibility (Figure3 Supporting Information). A master gel containing spots observed in all run replicates was firstly created for each sample; thereafter, average gels were matched by using the control sample at T_2 characterized by best qualitative and quantitative resolution, as reference (Figure 5). Average proteomic maps, on a non-linear 3-10 pH gradient, showed 350-400 well resolved spots, ranging

in a Mr value from about 97 to 11 kDa. The majority of spots detected in these maps showed analogous positions and intensities, as indicated by the degree of gel similarity between the various samples and the reference map (within 80-90% range).

A software-assisted comparison of relative spot densities between the control and the three regions of the stressed root samples revealed that one hundred forty seven protein spots resulted differentially expressed ($p<0.02$) among the four different samples in the three different times, whereas the remaining showed a constant expression .

147 variable and 66 of most abundant constant protein spots (213 in number- Figure 5) were excised from the gel and digested for further analysis by MALDI-TOF PMF and/or nLC-ESI-LIT-MS/MS.

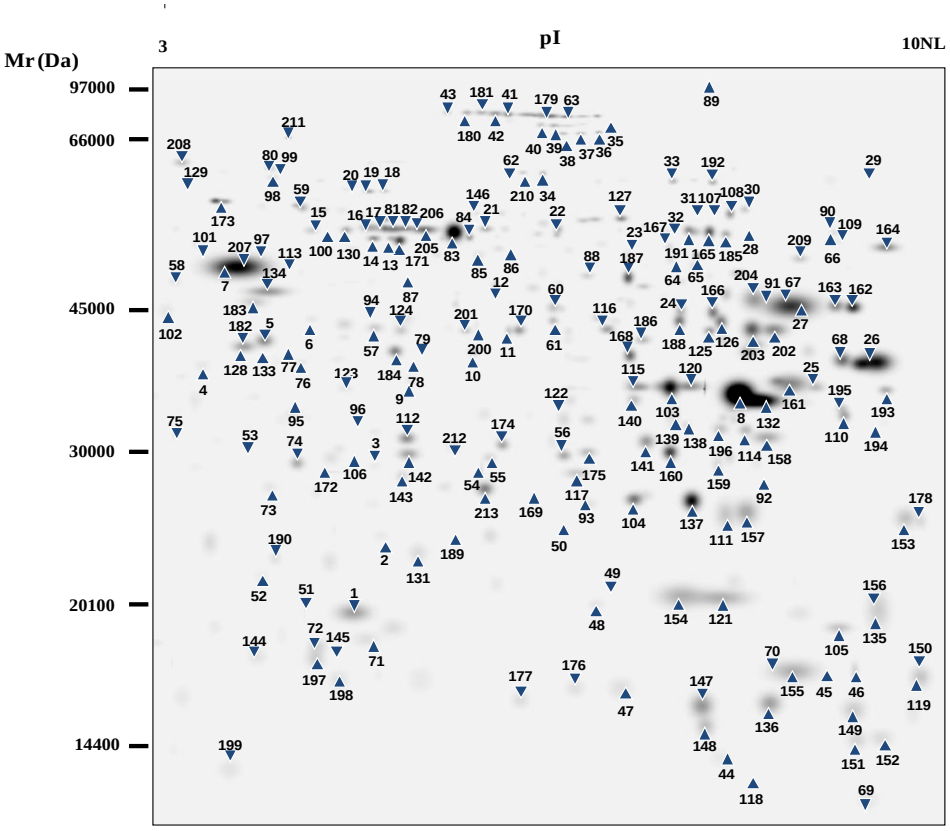


Figure 5. Poplar root master gel. Filtered image of 2DE proteome reference map of the woody root of poplar seedlings (Control at T_2) with 213 variable and constant proteins are numbered and indicated. The numbers are correlated with the protein identified reported in Table III.

211 protein spots were identified as corresponding to 273 protein accessions, 188 protein accessions for the 147 variable proteins spots and 88 protein accessions for the 66 constant proteins. In particular 147 variable spots were defined by 119 unequivocal identification accession and 26 multiple identification, while 2 protein spots remained unidentified; 66 constant

proteins were defined by 54 unequivocal identification accession and 12 multiple identifications. The list of 211 identified and 2 unidentified spots are reported in Table II (Supporting information).

Based on Bevan's et al., (1998) classification, the identified polypeptides were grouped into 13 different functional classes. As reported in Figure 6, a larger portion of this polypeptides were represented by proteins involved in Energy (28,2%) and Disease/defense (30,0%); the remaining polypeptides were represented by Protein destination and storage (15,0%), Metabolism (5,5%), Transporters (4,0%), Transcription (3,3%), Protein synthesis (2,2%), Cell structure (2,2%), Secondary metabolism (1,8%), Cell growth/division (0,4%), Signal transduction (1,1%), Intracellular traffic (0,7%), Others (5,5%).

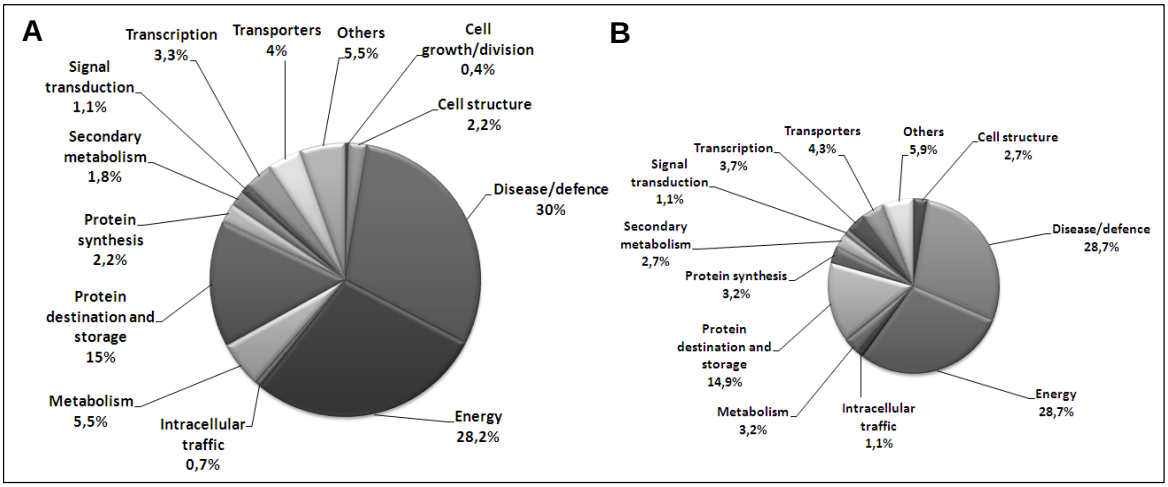


Figure 6. Functional categories. Distribution of total proteins, constant and variable, identified from woody poplar taproot. Functional classification was based upon nomenclature by Bevan et al., (1998). A) Distribution of total proteins in a number of 213; B) Distribution of 147differentially expressed proteins between control and bent, and between the three sectors (ABS;BS;BBS) of the bent root along a time gradient.

3.5. Statistical analysis

To summarize the information reported in Table III, the 147 protein spots resulting differentially expressed by the PDQuest analyses (t student test) were r subjected to Analysis of Variance (ANOVA) and further grouped according to their similar expression profile by cluster analysis.

Results of the ANOVA ($P \leq 0.05$) for the control revealed 31 significant differentially expressed protein spot during the time course. In detail as shown in Figure 7, the cluster analysis

grouped these proteins in three main clusters including 11, 13 and 7 highly expressed proteins in T_0, T_1 and T_2 respectively.

A number of 76, 78 and 57 significant proteins were found to be differentially expressed between control and the three regions of the stressed taproot at T_0, T_1 and T_2 respectively ($P \leq 0.05$).

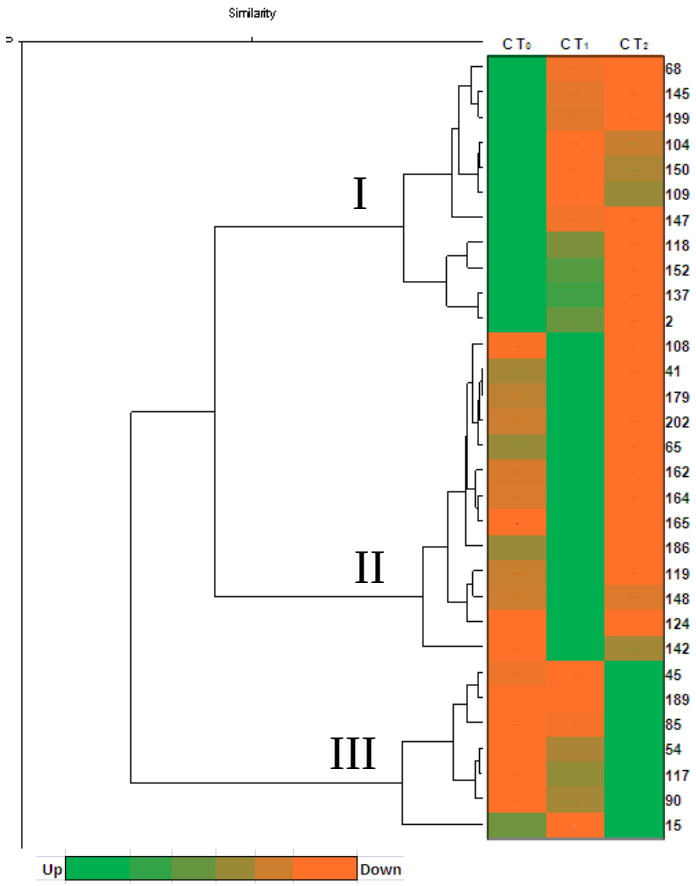
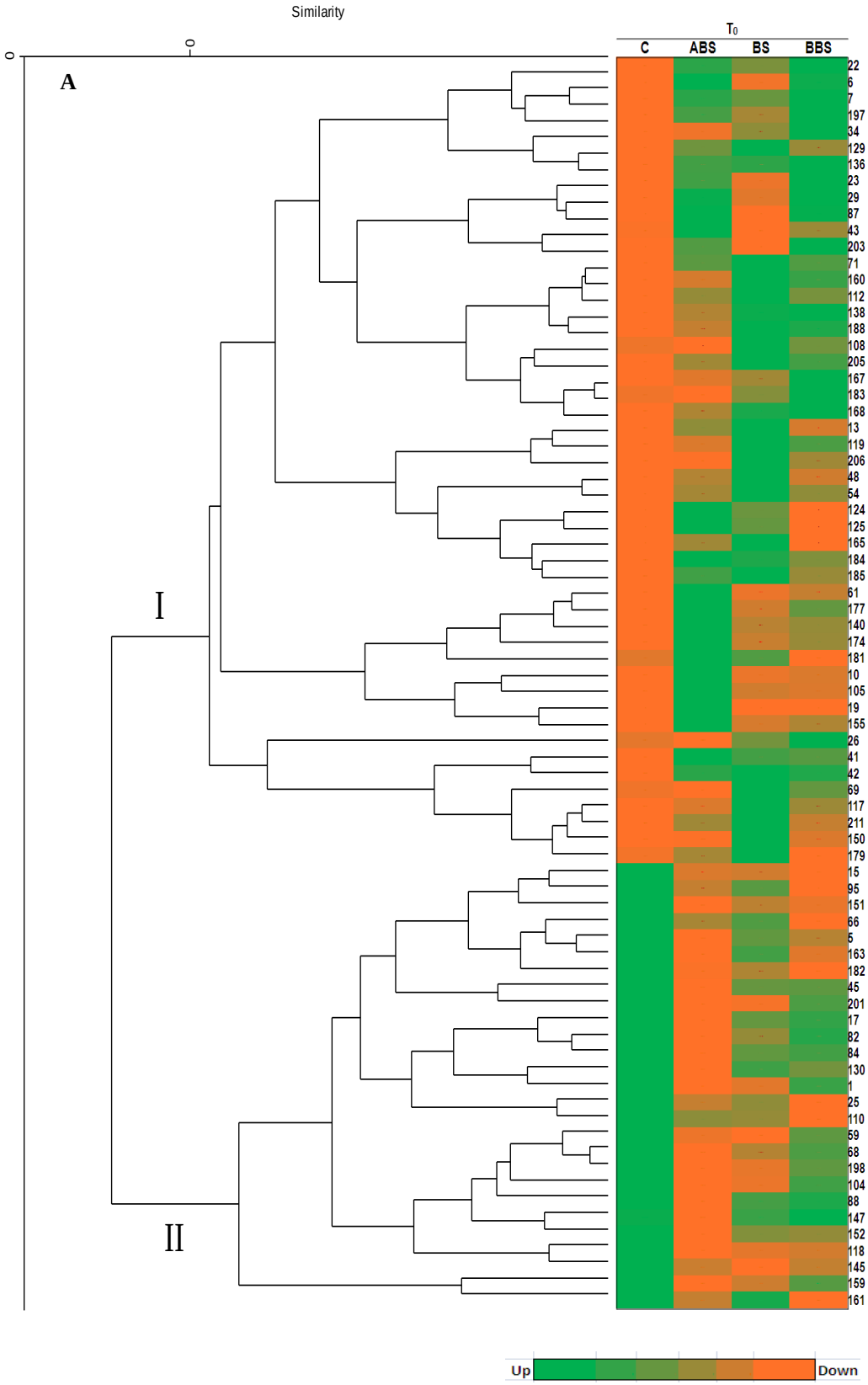


Figure 7. Cluster analysis. Hierarchical clustering of differentially expressed proteins (ANOVA, $P \leq 0.05$) in the control root at T_0, T_1, and T_2 Arabic numbers on the right side refer to proteins reported in Figure 5 and Table III.

For each time point, cluster analysis generated, for the control, two homogenous clusters grouping the significantly expressed proteins on the base of their low or high quantity (Figure 7). In particular at T_0, cluster I contained 49 proteins (spots 22-179) characterizing the control taproot for their low quantity; cluster II grouped the 27 (spots 15-161) proteins most abundantly expressed in the control (Figure 8A). Also for T_1 cluster I and cluster II contained 30 (spots 29-171) highly and 48 (spots 23-188) low expressed proteins in control respectively (Figure 8B). In T_2 clusters I and II included 31 low (spots 43-114) and 26 highly (spots 17-177) expressed proteins in the control (Figure 8C).


Similarity
0
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C
ABS
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22
6
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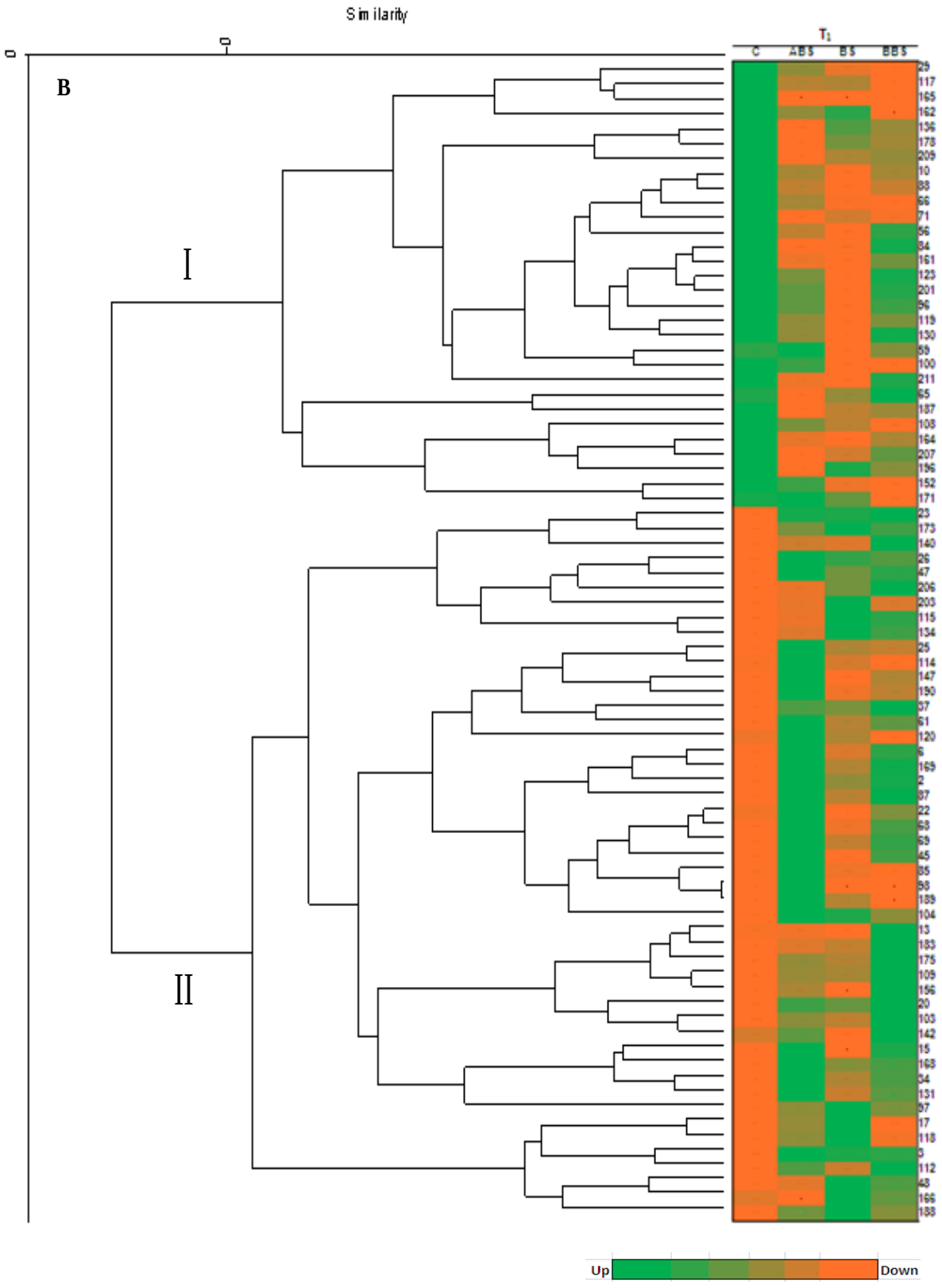
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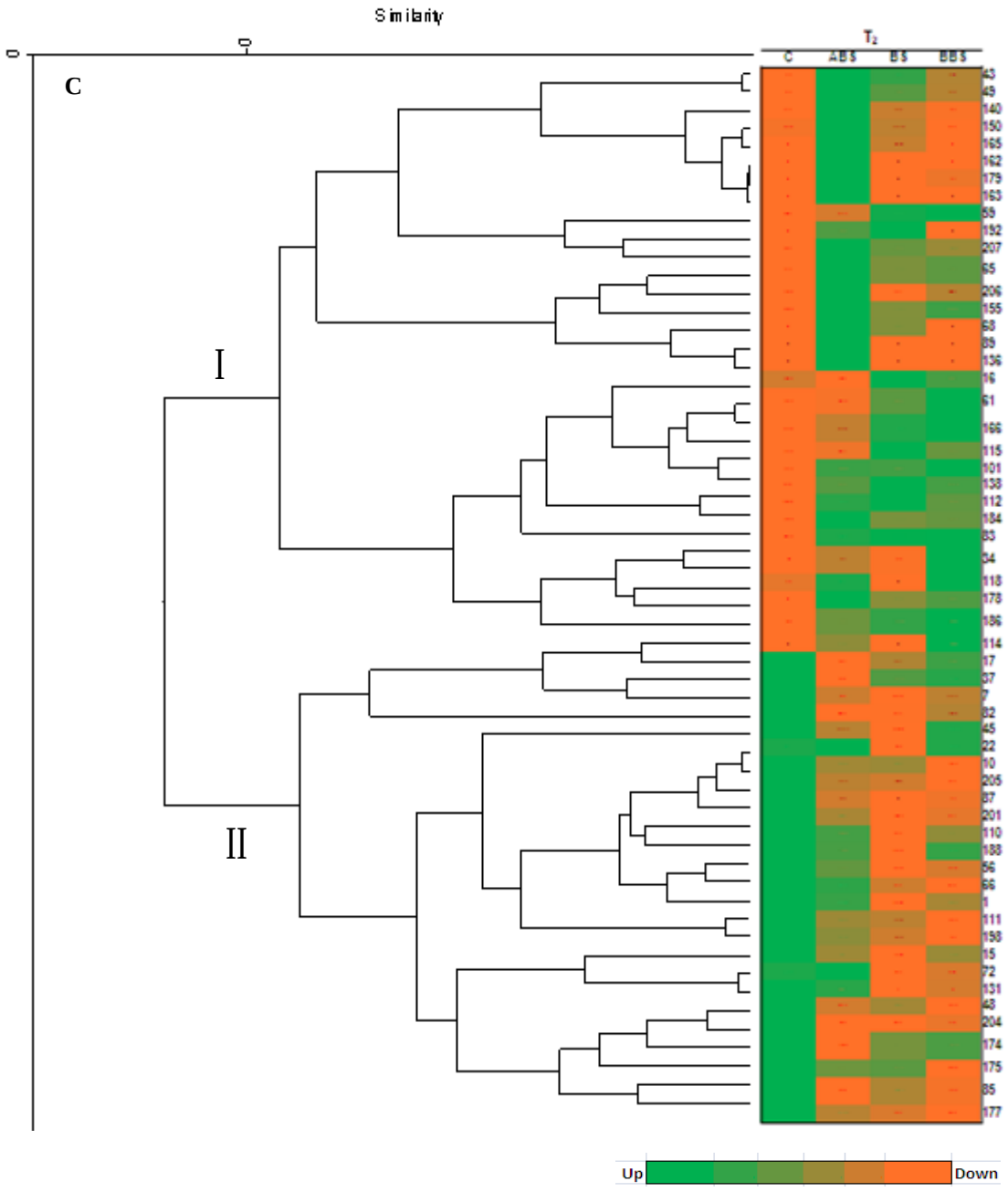

Figure 8. Cluster analysis. Hierarchical clustering of differentially expressed proteins (ANOVA, P≤ 0.05) between control and three stressed regions at T_0 (A), T_1 (B), and T_2 (C). At each time point differences in proteins abundance between control and three bent regions are well evident. Arabic numbers on the right side refer to proteins reported in Figure 5 and Table III.

Within these two main clusters generated at each time for the control, protein patterns in the three stressed regions showed a high heterogeneity raising a series of multiple different sub-

clusters (Figur e8). Moreover, different sub-clusters were differently distributed among the three stressed regions.

The ANOVA computed within the three stressed regions (ABS, BS, BBS), revealed that 9, 14 and 13 proteins resulted significant for their high expression to discriminate the stressed root regions at T_0, T_1 and T_2 respectively (Figure 9). Cluster analysis at T_0, grouped the 9 proteins in three clusters: cluster I contained 5 proteins (spots 109-164) that discriminated ABS region from the other two; cluster II and III included 2 proteins each characterizing BS (spots 51,163) and BBS (spots 88,104) (Figure 9A).

As shown in Figure 9B, at T_1, three clusters were formed: cluster I, composed of 7 proteins that principally distinguished BBS from BS and ABS (spots 13-201); cluster II, with 5 proteins distinguishing ABS from BS and BBS (spots 59-124); cluster III comprising 2 proteins that discriminated BS from ABS and BBS (spots 5, 154).

Three clusters were formed at T_2 (Figure 9C); cluster I was composed by a protein (spot 120) that characterized BBS and two more (spots 19, 22) that discriminated BSS and ABS from BS. Cluster II was characterized by 6 proteins discriminating ABS from BS and BBS (spots 70-126); cluster III included 4 proteins discriminating BS from the other two regions (spots 90-122).

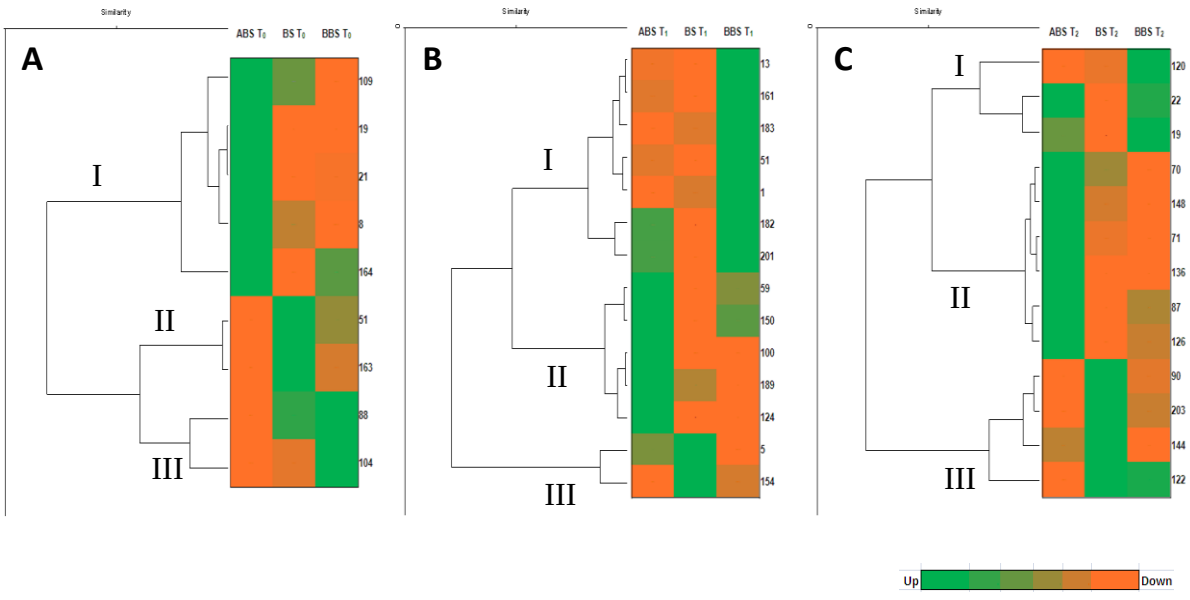

Figure 9. Cluster analysis. Hierarchical clustering of differentially expressed proteins (ANOVA, $P \leq 0.05$) between three stressed regions (ABS; BS and BBS) at T_0 (A), T_1 (B) and T_2 (C). At each time point differences in proteins abundance between three bent regions are well evident. Arabic numbers on the right side refer to proteins reported in Figure 5 and Table III. ANOVA and cluster analysis were computed among stressed root regions, to individuate proteins with significant spatial gradient variation (ABS, BS and BBS) during each time point (T_0, T_1 and T_2). Cluster analysis at T0, grouped nine significant proteins in three clusters: cluster I contained five proteins

(spots 109-164) that discriminated ABS region from the other two; cluster II and III included two proteins each characterizing BS (spots 51,163) and BBS (spots 88,104). Panel B shown that in T_1 thirteen significant proteins were grouped in three main cluster: cluster I, composed of seven proteins that principally distinguished BBS from BS and ABS (spots 13-201); cluster II, with five proteins distinguishing ABS from BS and BBS (spots 59-124); cluster III comprising two proteins that discriminated BS from ABS and BBS (spots 5, 154). Panel C shown that at T_2 cluster I was composed by a protein (spot 120) that characterized BBS and two more (spots 22, 19) that discriminated BSS and ABS from BS. Cluster II was characterized by six proteins discriminating ABS from BS and BBS (spots 70-126); cluster III included four proteins discriminating BS from the other two regions (spots 90-122).

4. Discussion

The response of woody taproot to mechanical stresses have been investigated at different levels in several plant species, and alterations in root morphology, architecture and biomechanical properties have been widely reported (Di Iorio et al., 2005; Chiatante et al., 2006). Molecular factors involved in this thigmo-responses have been little studied (Di Michele et al., 2006; Scippa et al., 2006, 2008) and the information available are enormously lacking. In a previous study to simulate mechanical stress we set up an experimental system where the woody taproots of poplar (*Populus nigra*) seedlings were bent around a 90° angled metallic net (Scippa et al., 2008). Preliminary results obtained after six months of bending, showed that poplar woody root, displays alterations in the morphology and mechanical properties, and that several important factors of the signal transduction pathway, detoxification and metabolism were identified (Scippa et al., 2008). It is well established that modifications involved in the thigmo-responses may occur slowly over time (Jaffe, 1973; Scippa et al., 2006), varying among plants of different ages (Kus et al., 2002) and among different organs of the same plants (Taylor et al., 2002). In the work here presented we used spatial and temporal investigations to add knowledge about the long term changes occurring in the woody root thigmo-responses.

To accomplish this aim the morphological and biomechanical responses of woody root to bending were analyzed after an entire vegetative growth cycle when the alterations became more evident. However, the study of the molecular factors involved, was carried out along a three points time course. Due to the lack of information about the different stages of woody root growth cycle, we based our time course on shoot phenology observations, data reported in the literature (Howe et al., 1995; Chen et al., 2002), and yearly average temperatures in the experimental site. Based on these informations, with the rationale to identify key factors controlling the root thigmo-responses at the beginning of the growing season, root samples were collected in late February (T_0), late March (T_1) and late April (T_2). We assumed, in fact, that T_0 may correspond to a dormancy time, whereas T_1, and T_2 to the beginning of vegetative growth.

4.1 Mechanical stress distribution and thigmo-response

To characterize the experimental system, a model of mechanical stress distribution is proposed (Figure 1). The model shows that stresses intensities vary along the three sectors (ABS, BS and BBS) of the bent taproot and the differences are especially evident after 14 months of growth. In fact at the beginning of the stress treatment (Ti), the forces are more homogeneously distributed showing not significant differences between the three sectors. After 14 months, a more

heterogeneous distribution of forces is observed with the BS showing higher stress value in compression than in tension. Based on this model we decided to investigate if the three sectors respond differently according to the asymmetric forces distribution. Morphological analysis after an entire vegetative growth cycle (T_2) revealed that root and shoot undergo to alterations in terms of numbers and diameters of branches and first order lateral roots in response to taproot bending. These alterations belong to a common thigmo-response observed in other plant specie roots (Di Iorio et al., 2005; Scippa et al., 2006). However, the detailed analysis of the three sectors ABS, BS and BBS, separately revealed a spatial asymmetry of the thigmo-response along the bent taproot. A first remarkable evidence of the spatial asymmetry is represented by the higher distribution of first order lateral roots in the ABS and BS sectors. Moreover, the BS sector corresponding to the curve is characterized by an additional asymmetric distribution of lateral roots that are preferentially emitted in the convex side. Mechanisms involved in the development of lateral roots in response to mechanical stress have been widely investigated in the model plant *Arabidopsis thaliana* and the role of many important gene factors and hormones have been established (revies Peret et al., 2009). Several papers have been published where lateral root induction events occur in response to the extended curves generated by a range of different mechanical/tropic mechanisms (De Smet et al., 2007; Laskowski et al., 2008; Lucas et al., 2008a). Furthermore, two very recent studies showed that artificial bending induces in *Arabidopsis thaliana* the formation of lateral roots on the convex site of the curved taproot (Ditengou et al., 2008; Richter et al., 2009). The studies also provided two pathway models, one Auxin dependent and the other Auxin independent, controlling the lateral root induction in the convex side of the bent root. If the knowledge about lateral root formation from the primary growth are highly advanced, very scarce or totally absent are the information about mechanisms involved in the first order lateral root development from a taproot in secondary growth (Chiatante et al., 2003) Data presented here, are the first, as far as our knowledge, showing an asymmetric response of taproot in secondary growth to bending (Figure 2). Furthermore, our results clearly demonstrate that as in *Arabidopsis thaliana* primary root, bending forces induce the emission of new lateral roots in convex side of the curved woody taproot (Figure 3B). Although we did not examine the mechanisms involved, the analogy of the responses observed strongly address to explore the hypothesis that common pathways are used by root in primary and secondary growth to control lateral induction in response to mechanical stimuli.

The significant increase of lignin content (Figure 4) in the taproot subjected to bending was the other important alteration confirmed by our work. In fact 14 months of stress treatment

induced a major increase of lignin that accumulated differently in the three sectors of bent taproot (Figure 4). The highest values were measured in the BS and BBS sectors. Data reported by several authors, indicated that the increase of lignin content (Patel, 1971; Stokes et al., 1997; Scippa et al., 2006), and/or the alteration of the cell wall composition (Timell, 1986; Showalter et al., 1992; Telewski, 1995; Shirsat et al., 1996; Zipse et al., 1998; Jamet et al., 2000) are important mechanisms for enhancing plant stability. In a previous work (Scippa et al., 2006) we showed that alteration in lignin content occurs when a strengthening of biomechanical properties in mechanical tissue organization and/or composition is required. Moreover the increase of lignin is reported as a important feature of the opposite wood (OW) that is produced in angiosperm trees to increase mechanical support (Scurfield, 1973; Fujii et al., 1982; Timell, 1986; Hu et al., 1999; Wu et al., 2000). In this work, we did not investigate the anatomy of the three taproot sectors, however overlapping the data of lignin measurements (Figure 4) and stress forces distribution proposed in the model (Figure 1), it might be supposed that the higher concentration of compression forces in the BS and BBS sectors can trigger the formation of OW characterized by high values of lignin.

4.2. The proteome of poplar woody taproot

The completion of the sequence of the Poplar genome, (Tuskan et al., 2006), gave rise to many proteomic studies providing a large amount of information about proteins expressed in different tissues, during different development stages and in response to environmental conditions (Plomion et al., 2006; Kieffer et al., 2008; Dafoe et al., 2009; Dafoe and Constabel, 2009; Azri et al., 2009; Bonhomme et al., 2010). Very few studies are reported in the literature on the use of proteomic approach to investigate the biology of root system of trees either in primary and secondary growth. In fact as far as we know, only the recent paper published by Plomion et al., (2006) reports the proteomic analysis of the white root (primary growth) from poplar cutting where about 1300 spots were resolved and 45 further identified.

Our work originally reports a detailed 2-DE-based proteomic analysis of poplar woody taproot, with a total of 211 protein spots identified as 271 total protein accessions (173 unequivocal and 38 multiple identification accessions, Table III). The 211 proteins identified in the two years old poplar woody taproot fell into 13 different functional categories (Figure 5A) where Energy and Disease/defense, represented the most abundant, followed by Protein destination and storage, Protein synthesis, Metabolism, Transporters, Transcription, Cell structure, Secondary metabolism, Cell growth/division, Signal transduction, Intracellular traffic and Others. In

comparison to previous proteomic analysis reported in poplar (Plomion et al., 2006) and other plant species (Méchin et al., 2004; Kim et al., 2003; Gion et al., 2005), the identification rate (99%) achieved in the present work by using MS/MS and PMF analysis, is significantly higher. Such high success of identification rate can be attributed to the recent publication of a poplar database (http://mycor.nancy.inra.fr/IMGC/PoplarGenome/index.html).

For 38 spots (17.8%) multiple identification were obtained. Multiple identification are well know and widely reported in the literature, as in recent studies in maize 12.1% (Mechin et al., 2004), and in poplar 9.3% (Plomion et al., 2006) of the spots showed multiple identification. Multiple proteins identified within a single spot have been ascribed to different explanations: a) interpretation of the same MS/MS spectrum (Plomion et al., 2006); b) contamination of the neighbouring spots (Parker et al., 1998); c) degradation product of the same proteins (Plomion et al., 2006); d) spots analysed contain multiple proteins (Giometti et al., 2002; Sun et al., 2005). Our results show that in the majority of the case, multiple proteins, with very close Mrs were identified within a single spot, indicating that spots analysed contain multiple proteins. In addition, since we found very close values of the theoretical and experimental Mr, and all the proteins manipulations have been made in presence of protease inhibitors, we can exclude the formation of degradation products. Moreover, differenced in the theoretical and experimental Mr and/or proteins resolved into multiple spots can be attributed to i) expression of multigene families encoding for different primary sequences; ii) differential proteolytic processing of expressed genes; iii) presence of dimeric and monomeric protein forms; iv) differential protein post-translational modifications.

The spatially and temporally proteome maps analysis revealed that one hundred forty-seven proteins showed changed in their expression. The residual sixty-six remained unchanged in the control and the three regions of the bent taproot during the three times analyzed.

4.2.1. Proteins with unchanged pattern during time course

The majority of proteins with unaltered expression represented different isoforms of the differentially expressed proteins that will be discussed in the next sessions. Twenty six were not isoforms and may be related to the known modification of woody plant metabolism associated with cold acclimation, including stress tolerance and detoxification, protections against osmotic stress, antifreeze activities, and fatty acid metabolism. In particular, proteins identified as aconitase (spot n. 35, 36), catalase (spot n. 106), glutathione-s-transferase omega (spot n. 94, 113), prohibitin (spot n. 193), and stress inducible protein (spot n. 210), and cysteine protease inhibitor (spot n. 213) seem to be all related with oxidative stress and detoxification metabolism

(Prasad et al., 1994; Green and Reed, 1998; Solomon et al., 1999; Kampranis et al., 2000; Loyall et al., 2000; Mueller et al., 2000; Lam et al., 2001; Agrawal et al., 2002; Di Baccio et al., 2005; Bittsánszky et al., 2005; Ahn et al. 2006; Moeder et al., 2007; Guerra et al., 2009). Osmotin (spot n. 157), zeamatin (spot n. 92) chitinase precursor (spot n. 75), class IV chitinase (spot n. 195) all included in the PR (PR3 and PR5) family may be involved in protections against osmotic stress (Zhu et al., 1995) and in the antifreeze activities (Hon et al., 1995).

Other proteins identified that may be associated to cold acclimation are two chaperonines as a Glycine-rich RNA-binding protein (spot n. 53) and GroES chaperonin (spot n. 172). Although the function of GRPs was not characterized in detail, it has been suggested that they may play a role in stress responses, (Sachetto-Martins et al., 2000; Kim et al., 2005; Kim et al., 2007) with a particular function as RNA chaperone. The GroES chaperonin (spot n. 172), has been reported to be involved in *de novo* synthesis (Bukau et al., 1998; Frydman, 2001) and stress denaturated protein folding (Wang et al., 2004).

Enzymes with different role in the metabolism as Acetyl-CoA acetyltransferase (spot n.12), Serine hydroxymethyltransferase (spot n. 30, 106, 107), Transaminase mtnE (spot n. 60, 86), Patatin-like protein (spot n.62), and Putative enoyl-acyl-carrier-protein reductase (spot n. 78), were found to be constantly expressed in control and bent taporoot, at T_0, T_1 and T_2.

Acetyl-CoA acetyltransferase and enoyl-acyl carrier protein reductases are two key enzymes of the fatty acids biosynthesis. Enoyl-acyl carrier protein reductases catalyze the last step of the elongation cycle in the synthesis of fatty acids (Massengo-Tiassé & Cronan, 2009) whereas acetyl-CoA acetyltransferase is typically involved in the mevalonate pathway where it functions in the biosynthetic direction of hormone and cholesterol synthesis (Bach et al., 1999). Patatin-like proteins, with a lipid acyl-hydrolase (LAH) activity have been found to have a large spectrum of substrates including both phospholipids and glycolipids with diverse expression patterns in plant tissues, and in response to a variety of stresses such as pathogen attack, wounding, low temperature, high salinity, abscisic acid, salicylic acid (SA), methyl jasmonate, ethylene, and iron or phosphate deficiency (Narusaka et al., 2003; Rietz et al., 2004; La Camera et al., 2005; Matos et al., 2008). Ser hydroxymethyltransferase catalyzes the reversible conversion of Ser and tetrahydrofolate (THF) to Gly and 5,10-methylene THF (Schirch, 1982) which serve as the primary sources of one carbon (C1) units of differing oxidation states, for biosynthetic reactions in amino acid, purine, pyrimidine, and lipid synthesis (Cossins, 1987). Several studies report the relation of Ser hydroxymethyltransferase with photorespiration and in protection against abiotic and biotic stresses (Navarre and Wolpert, 1995; Noctor et al., 2002; Taler et al., 2004; Wingler et al., 2000; Moreno et al. 2005). However, Clung et al., (2000)

reported a Ser hydroxymethyltransferase that accumulates specifically in roots where it has been proposed to be involved in C1 metabolism.

Transaminase mtnE (spot n. 60, 86) is involved in the last step of the methionine (Met) recycle that allows the recycling of Met from methylthioadenosine (MTA), which is formed as a by-product of polyamine biosynthesis. In plants, the Met cycle is furthermore linked to ethylene synthesis (Wang et al., 1982), which is known to be an important regulator of many important physiological and stress responses of woody plants as wood formation (Andersson-Gunnerås et al., 2003) and cell division in the cambial meristems (Love et al., 2009) and it is an important regulator of lateral root formation (Negi et al., 2008). Ran-binding proteins (spot n. 4) (RanBPs) are a group of proteins that bind to Ran (Ras-related nuclear small GTP-binding protein), and thus either control the GTP/GDPbound states of Ran or help couple the Ran GTPase cycle to a cellular process. Modulation of Ran status by a group of RanBPs regulates many cellular processes, such as nuclear protein transport in interphase cells and cell cycle progression in mitotically dividing cells (Dasso, 2001). It has been showed that that the suppression of AtRanBP1c expression in *Arabidopsis* results in a unique phenotype, which includes altered growth and development of primary roots and hypersensitivity of these roots to auxin (Kim et al., 2001). Based on these evidences reported in the literature it can be supposed that both Transaminase mtnE and Ran found in the woody root of poplar may be involved in important process as cell division of root cambium and lateral root formation.

Putative ATP synthase (spot n. 94), was also identified together with a Putative ripening regulated protein (spot n. 53), Plasma membrane polypeptide (spot n. 74), with very general function in ATP production and cell organization.

As expected the poplar woody root proteome is characterized by proteins with different functions that remained unchanged during time analyzed. *Populus nigra* as many temperate woody perennials have adapted to freezing temperatures and limited water during winter by alternating between active growth and vegetative dormancy. With higher temperatures in the spring, cold hardiness is lost and growth resumes (Mellerowicz et al., 1992). This annual growth cycle is a complex developmental process controlled by interactions between environmental and internal factors (Lang et al., 1987; Dennis, 1994). A transcriptional profile analysis during annual growth revealed that gene sets are differentially regulated during the growth cycle (Park et al., 2008).

In our study the proteome profile of poplar woody root was analyzed in a very short temporal gradient, compared to the entire annual cycle. Consequently we are unable to assess the pattern of constant proteins outside of the temporal range analyzed. In fact, the analysis of protein alterations during growth cycle in poplar woody root was beyond the scope of this study and will

be presented elsewhere. However, the identification of multiple stress-related proteins and important key enzymes of metabolism with an unchanged expression in the control and bent taproot along the entire time course may indicate that these particular proteins are likely regulated by internal factors controlling the growth cycle rather than external cues as bending.

4.2.2. Differentially expressed proteins

The proteome maps analysis, revealed that one hundred forty-seven proteins showed spatially and temporally expression changes. The statistical analysis throughout the *t-student test*, carried out by the software PDQuest, is based on the comparison of couple of proteome maps. The software assisted analysis results are rough data that often make very difficult the interpretation of their biological functions.

The particular focus of this work was the identification of important spatial and temporal protein markers of woody taproot response to mechanical stress. To accomplish this aim, the ANOVA statistical analysis followed by hierarchical clustering was carried out to elaborate the data released by the PDQuest.

The following discussion will be focus on the most prominent differences: a) within the unstressed taproot along the time course; b) between the unstressed and the three stressed region at each time point; c) within the three stressed region at each time point.

4.2.3. Differentially expressed proteins during time

In order to establish the physiological alterations occurring in the woody root proteome during the studied time course, significant (ANOVA $P \leq 0.05$) differentially expressed proteins in the control sample were clustered based on their similar patterns. Results showed that during the three times (T_0, T_1, T_2) unstressed woody root is characterized by the high expression of different isoforms (spot n. 54, 85, 137, 147) and enzymes implicated in the same (spot n.15, 65, 137) or different pathways (spot n. 186, 202) of energy and carbon primary metabolism. In addition, while T_0 and T_1 are characterized by the high abundance of proteins related with transport (spot 2, 109, 152) and protein synthesis (spot n. 118, 150, 164), T_2 distinguished for the high presence of proteins involved in folding (spot n. 45) and transcription (spot n. 90, 189). T_0 is also characterized by the high level of storage proteins (spot n. 68, 104, 145), whereas T_1 by the high quantity of proteins involved in cell structure (spot n.108, 162) and disease/defense (spot n. 41, 124, 142, 148, 179, 202). Although the analysis of factors controlling the growth cycle was not the main scope of this study, our results seem to indicate that from T_0 to T_1 and T_2 the woody taproot undergo to different physiological processes related to the transition from dormancy to

active cycle. While the information about the root annual cycle are still lacking, it has been reported that stem release from the dormant state requires prior exposure to chilling temperatures (Perry, 1971). Further, once the chilling requirement is met, warm temperatures in the spring can induce reactivation (Heide, 1993). A superinduction of several cold-responsive genes during the early stage of reactivation in the cambial cells has been reported in *Salix* (Sennerby-Forsse, 1987) and poplar stem (Druart et al., 2007). Although the exact role of this superinduction is also not clear, it has been proposed that it may reflect a need to protect the very sensitive dividing cambial cells from sudden drops in temperature during early spring (Druart et al., 2007). As reported in the stem, our data show for the first time that a similar superinduction of cold responsive factors occurs in the poplar woody root during the early stage of growth cycle reactivation. In fact, T_1 differently from the other two times is characterized by the abundance of stress/defence related proteins as Dehydrins (spot n. 41,179), β–1,3-glucanase (spot n. 148), Peroxidase (spot n. 119, 124, 202), that are well known to be involved in many abiotic stress responses including cold (Oono et al., 2006). Furthermore, we found that while T_0 was characterized by the abundance of storage proteins T_2 presented higher quantity of factors involved in folding and transcription. These data together with higher presence of different sets proteins and/or enzymes involved in the metabolism may be taken as indication that while T_0 is closer to a dormancy state, T_2 is closer to resumption of growth.

To investigate the effects of bending on the woody taproot, the ANOVA was computed between the control and the three different stressed regions at T_0, T_1, and T_2.

Data obtained show a clear asymmetry of the response of the taproot to bending along a spatial and temporal gradient. However, to simplify the discussion first will be discussed the proteins altered by the mechanical stress along the temporal gradient, without detailing their profile in the three regions of the bent taproot. Secondly, protein markers resulting from the ANOVA analysis and hierarchical clustering of each region at each time will be described to highlight the spatial asymmetric response of the bent root.

During the whole time course, compared to the control the bent taproot was characterized by the abundance of different types, numbers and protein isoforms regulating ROS detoxification and general stress response. In fact, Dehydrins (spot n. 19, 41, 42, 43, 181, 183), Peroxidases (spot n. 119, 124, 188, 203), Gluthathione-s-transferase (spot n. 103, 105, 136, 142), Heat shock proteins 70 (spot n. 34, 97, 9), Class I chitinase (spot n. 140), Aldo/ketoreductase (spot n. 15, 61, 115), Putative pathogenesis-related protein I (spot n. 45, 155), β–1,3-glucanase (spot n. 114) Universal stress proteins (spot n. 48, 144), Ankyrin (spot n. 100) are more abundantly present in the bent root compared to the control. Whereas Peroxiredoxin type II (spot n. 1), Gluthathione peroxidase

(spot n. 132) and Proteins responsive to ABA (spot n. 7, 37, 201, 207) characterized the control for their abundance. However, some of these differentially expressed proteins were found as isoforms diversely accumulated in the control and bent root. As previously discussed (Paragraph 4.3), the different isoforms may due to PTMs and then correspond to differential functional state. Furthermore, T_0 is characterized by the abundance of Dehydrins (spot n. 19, 41, 42, 43, 181, 183) and Peroxidase (spot n. 119, 124, 188, 203), T_1 by Heat shock proteins (spot n. 34, 97, 98) and Manganese superoxide dismutase-like protein (spot n. 169), whereas the low quantity of several Pathogen-related proteins (spot n. 45, 48, 72, 198) differentiates T_2.

The majority of the proteins charactering the bent root have been reported to be involved in the responses of different abiotic stresses as low temperature, drought, salinity osmotic stress, and heavy metal stresses (Plomion et al., 2006; Kieffer et al., 2008, 2009; Park et al., 2009).

Moreover, a positive correlation between the accumulation of dehydrins and ROS tolerance in plants has been demonstrated (Zhang et al., 2007). The particular dehydrins and peroxidase higher abundance detected in T_0 may result from the cold acclimation process occurring during autumn-winter. Two evidences may support this hypothesis: first as discussed above, our data seem to indicate T_0 very close to a dormancy state, second both dehydrins and peroxidase have been found to be involved in the cold acclimation of many plant species (Renaut et al., 2005). However, the higher abundance detected in the bent root compared to the control, may be required to respond to a combination of water, low temperature and mechanical stresses.

Similarly to T_0, T_1 is also characterized by the abundance of proteins involved in the stress defense and ROS detoxification. In the control, at T_1 we found an accumulation of several cold-responsive genes and according to the literature, we supposed a link with an early stage of poplar root reactivation.

Early stage of bent root reactivation may require an additional set of defense genes to respond to the mechanical bending. It is widely reported that thigmo-response requires ROS as important signaling factors (Neill et al., 2002) and that Manganese superoxide dismutase and Heat shock proteins characterizing T_1, may be key components of this signaling pathway (Timperio et al., 2008).

At T_2 the control root presented higher quantity of factors involved in folding and transcription, leading the idea that at this stage root may be closer to growth resumption. The most evident characteristic of the bent root at T_2 was the lower level of several pathogen related proteins (PRP). PR proteins originally discovered for their strong expression induced by pathogen infection, have found to be constitutively expressed in many plant organs. In fact, PR proteins have been described in xylem sap (Rep et al., 2002; Buhtz et al., 2004; Kehr et al., 2005; Alvarez

et al., 2006; Djordjevic et al., 2007; Aki et al., 2008; Dafoe et al., 2009) and beside their role in the biotic and abiotic stress response these proteins seem to be involved in degrading primary cell walls during the development of tracheary elements (Turner et al., 2007). Our data, contrarily to what reported in the literature show that bending stress induces a lower expression of several PRPs during a phase close to growth resumption. It is known that in response to bending, a modification of cell wall must occur to reinforce mechanical properties (Goodman & Ennos, 1998). Moreover, we show that at T_2, an increase of lignin occurred in the bent taproot. Based on this evidence we might suppose that at the beginning of growth resumption, a different cell wall metabolism pathway is induced by bending in order to improve stiffness characteristics.

Changes in the primary carbon metabolism were also detected along the time course. In particular the effect of the bending on root primary metabolisms is clearly evident with the alterations of several key enzymes, such as Enolase (spot n. 13, 15, 16, 17, 83, 205, 206), Fructose-bisphosphate aldolase (spot n. 25, 26, 125, 163, 185), Glyceraldehyde-3-phosphate dehydrogenase (spot n. 61, 65), Malate dehydrogenase (spot n. 10, 166, 186, 201), Alchol dehydrogenase (spot n. 88, 201), Fumarate hydratase (spot n. 185) involved in the glycolisis and the TCA cycle.

It has been reported that when buds break, respiratory metabolism changes from the pentose phosphate pathway providing reducing power to glycolysis that provides energy for growth (Rowland & Arora 1997) and the activity of several enzymes of the glycolytic and citric acid pathways increase (Wang et al. 1991, Faust & Wang 1993). The up-regulation of these enzymes have also been reported in response to numerous plant abiotic stresses (Forsthoefel et al., 1995; Mujer et al., 1995; Lal et al., 1998; Riccardi et al., 1998; Kollipara et al., 2002; Wang et al., 2002; Uhde-Stone et al., 2003; Yan et al., 2005).

Our data indicate that in response to bending an increase of energy production may be required along the entire time course. In fact it might speculate that in the bent taproot, beside the energy required for physiological activities, additional energy is required for displacement of the observed modification in root growth and morphology (i.e. increase of lateral root number, root biomass).

Additional data may support this hypothesis. During the time course, in fact, enzymes as Aspartate-semialdehyde deidrogenase (spot n. 185, 201), Dihydrodipicolinate synthase (spot n. 201), involved in the aminoacid biosythesis are present in lower quantity compared to the control.

A Tropinone reductase (spot n. 138, 160) and a Phenylacumaran benzylic ether reductase (spot n. 184), implicated in the secondary metabolism were also alerted by the bending treatment, and the

alteration was particular evident at T_0 and T_2. Interestingly, we found that all 3 portions of the bent root were characterized by a higher abundance of Phenylacumaran benzylic ether reductase (PCBER- spot n. 184) compared to the control. This is a lignin-related enzyme implicated in the reaction wood formation (Gang et al., 1999) and it might be somehow involved in the lignin accumulation found in the three regions of the bent root. Other factors involved in the cell wall structure, were altered by the bending along the time course. Two pectinesterase proteins (spot n. 25, 26) and pectin acetylesterase (spot n. 108,162) were differently accumulated in the bent root. Pectiesterases (PE) are cell wall modifying proteins responsible for methylesterification and acetylesterification of pectin and is believed to play an important role in cell wall metabolism. It is believed that many plant PE isoforms remove methyl esters in a block-wise fashion (Limberg et al., 2000). Such demethylation could allow the aggregation of pectin, via calcium interchelation, into the so-called "egg-box" structures as described by Grant et al., (1973) and, as such, could act to strengthen the wall. Alternatively, this could also make the pectin more susceptible to degradation by cell wall polygalacturonase (Pressey & Avants, 1982; Jarvis, 1984; Seymour et al., 1987; Koch & Nevins, 1989; Carpita & Gibeaut, 1993), thus acting to weaken the wall. The action of PE could serve to either strengthen or weaken the cell wall dependent on its mode of action and on the environment in which it is acting. It is therefore likely that the many PE isoforms, often found associated with plant tissues, could serve very diverse functions during development.

Different Bark storage proteins were present in the bent root in higher or lower quantity compared to the control along the time course. Bark storage proteins participate in nitrogen recycling from senescing leaves to bark and back to growing leaves in the spring (Wetzel et al., 1989; Clausen & Apel, 1991; Gomez & Faurobert, 2002). Major protein storage sites include shoot and root bark and xylem ray cells (Wetzel et al., 1989; Sauter & van Cleve 1990), where BSPs are stored in special vacuoles called ''protein bodies'' (Herman et al., 1988; Sauter et al., 1989). *Populus, Salix* and *Acer* accumulate BSPs during autumn (Wetzel et al. 1989; Clausen & Apel, 1991), and are thought to be used during spring to provide N and C for new growth (Wetzel et al., 1989).

Several environmental factors, with still unknown molecular mechanisms influence BSP expression, (Langheinrich & Tishner 1991; van Cleve & Apel, 1993; Davis et al., 1993; Coleman et al., 1994).

Bark storage proteins (spot n. 68, 71, 112, 119, 161) characterised for their high quantity the bent root at T_0. Based on the pattern reported in the literature, this higher quantity found in the bent root may be result from an increased accumulation activity induced by the stress occurring

during the vegetative growth. These increased storage accumulation activities confirm the higher energy requested during the growth resumption to face the stress condition. However, at T_0 some bark storage (spot n. 104, 182, 201) was less abundant than in the control. We can explain this lower quantity with the earlier use of these protein reserves by the bent root. Furthermore, based on our results, it seems that the storage proteins are differently required by control and bent taproot during time.

The quantity of different Cyclophilins and Protein disulfide isomerases is altered by the bending stress. Cycolphilins and protein disulfide isomerase are involved in protein folding process and their expression has been shown to be induced by both biotic and abiotic stresses (Kieffer et al., 2009; Bonhomme et al., 2010). We found that at T_0 and T_2 a cyclophilin and protein disulfide isomerase (spot n. 45, 201) were less abundant in the bent root, compared to the control. However, at T_1 two Protein disulfide isomerase (spot n. 123, 201) were present in low quantity, whereas another one (spot n. 173) together with three cyclophilin (spot n. 45, 143, 156) were found in elevated amount. As for the other stress related proteins found in this work, the different pattern of these proteins may indicate a diverse use of the defense equipment, in the bent and control root during time.

Proteolysis and protein synthesis were also altered by the bending treatment highlighting differences in the metabolic activities occurring during time. In particular, factors involved in protein synthesis (spot n. 118, 150, 178) were highly represented in the bent root along the entire time course, with highest amount at T_2. Proteins involved in the proteolysis (spot n. 3, 29, 96) were more abundant in the bent root at T_0 and T_1. Additional confirmations of different metabolic processes occurring in control and bent root at different times are represented by the diverse accumulation of several proteins (spots n. 2, 56, 59, 109,151, 152) involved in transport and metabolite exchange between the organelles and the cytosol.

Enzymes involved in transcription and signal transduction pathway showed temporal differences in their quantity on the control and bent root. Two RNA-binding proteins (spot n. 185, 197, 209), a polyadenilate binding protein (spot n. 192), a transcription factor BTF3 (spot n. 49) and an ethylene responsive protein (spot n. 189) together with a nucleoside diphosphate kinase (spot n. 177), and a phosphoglycerate kinase (spot n. 87) were present in different quantity in the bent root, along the time course. The modulated expression of these proteins is obviously linked to the regulation of the expression of a different set of genes during time that may require specific signal transduction and transcription factors. In fact, stress-general expression responses may also arise from the activities of various transcription factors, which have been viewed as a source

of interaction among otherwise distinct abiotic stress response pathways (Chen et al. 2002; Chen & Zhu, 2004).

Two transcription factors, BTF3 and ERF may be particularly interesting in the morphological response we reported in the bent woody root. BTF3 protein is an α-subunit of nascent-polypeptide-associated complex specific to plants that has been implicated in various fundamental processes as the formation of lateral roots (Malamy & Benfey, 1997; Xie et al., 2000).). Ethylene responsive protein factor (ERF) belong to a family of a large number of genes, that were found to be implicated in many diverse functions as hormonal signal transduction (Ohme-Takagi and Shinshi, 1995), response to biotic and abiotic stresses (Yamamoto et al., 1999; Stockinger et al., 1997; Liu et al., 1998; Gu et al., 2000; Dubouzet et al., 2003), regulation of metabolism (van der Fits & Memelink, 2000; Aharoni et al., 2004; Broun et al., 2004; Zhang et al., 2005), and in lateral root formation (van der Graaff et al., 2000; Banno et al., 2001; Chuck et al., 2002).

Our data indicate that in response to bending BTF3 and ERF transcription factors may play an important role in lateral root formation with different timing. BFT3 is accumulated only in the bent root late during time (T_2); ERF is present in high amount in the unstressed and bent-taproot. However its quantity was higher in the bent root at T_1 whereas in the control at T_2. Furthermore, as will be further discussed, ERF in the bent taproot showed significant quantitative difference along the spatial gradient. Lateral roots formation belongs to the physiological growth reactivation program, since they have a central role in water and mineral supply for all the initiating metabolic processes. Our data indicate that compared to the control the bent root must respond to the additional critical anchorage improvement, and then anticipate and use different lateral root induction pathways, compared to the unstressed root.

Annexin (spot n. 168) was found to be accumulated in the bent root at T_0 and T_1. Annexins are a multigene, multifunctional gene family, possessing different enzyme or other protein activities, including phosphodiesterase activity (Calvert et al., 1996; Hofman et al., 2000), peroxidase activity (Gorecka et al., 2005), F-actin binding activity and calcium channel activity (Hoshino et al., 2004) They have been found to be involved in responses to wide range of stresses including low temperature (Breton et al., 2000) and mechanical stimulation (Thonat et al., 1997). In addition annexins and have been detected in the root elongation zone of maize (Carroll et al., 1998; Bassani et al., 2004) and *Arabidopsis* where they have been found to be implicated in lateral root development (Clark et al., 2005a). In gravistimulated Arabidopsis roots, the abundance of annexins increase in roots (Kamada et al., 2005) and predominates in epidermal cells that would undergo the greatest growth rate to bend the root (Clark et al., 2005b).

Moreover, it has been proposed that annexins could operate in signaling pathways involving cytosolic free calcium and ROS, and that their expression and intracellular localization are under developmental and environmental control (review Mortimer et al., 2008). As multifunctional proteins the annexin accumulation in the bent taproot observed at T_0 and T_1, is may be linked to lateral root development, signal transduction pathway, ROS regulation, but also in the modulation of cell wall and radial growth included in the thigmo-response. Obviously although our data clearly confirm the involvement of the annexin in the thigmo-response, additional studies are required to understand the exact role played by these multifunctional proteins.

4.2.4. Differentially expressed proteins in the three stressed regions

ANOVA and hierarchical clustering computed between the three regions clearly show an asymmetric response in the bent taproot along the time course. In addition specific spatial markers were identified, that well differentiate the three different regions at each time point.

At T_0 the ABS is strongly differentiated from BS and BBS, by the higher abundance of five proteins corresponding to key enzyme of metabolism (spot n. 19, 21), transporter (spot n. 109), storage protein (spot n. 8) and elongation factor (spot n. 164). The higher quantity of 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (spot n. 19) involved in the glycolysis and d-3-phosphoglycerate dehydrogenase (spot n. 21) involved in serine biosynthesis may indicate a increased requirement, occurring in this sector, of energy produced by this pathways. In addition serine as precursor of phosphatidylcholine (phospholipid), and of strong antioxidant compoundsm has been shown to be involved in adaptive responses to abiotic stresses in plants (Youssefian et al., 2000; Tasseva et al., 2004). Thus, in the case of serine it might also be proposed roles a direct and/or indirect function in responses of root to mechanical bending although with an unclear molecular mechanisms. A Porin voltage-dependent anion-selective channel protein - VDAC, (spot n. 109), a bark-storage protein B/β -1,3 -glucanase (spot n. 8) and an Elongation factor (spot n.164) are also present in higher quantity in ABS at T_0. A Bark storage protein B (spot n.104) and the Elongation factor (spot n. 164) were also present in large amount in BBS. The high presence of bark storage proteins, at this time, as earlier discussed may represent reservoir of nitrogen of ready to be used to better respond the stress condition when growth resume. Porin voltage-dependent anion-selective channel protein has been shown to be implicated in the apoptosis, anion exchange and signal NO mediated transduction pathway (Kusano et al., 2009), whereas elongation factors may be involved in protein synthesis and/or refolding of denaturated proteins in response to abiotic stress (Shin et al., 2009).The abundance

of VDAC, β–1,3-glucanase and the elongation factor clearly indicate that they belong to the defence strategy of this region.

Fructose-bisphosphate aldolase (spot n. 163), Peroxiredoxin (spot n. 51) and Alcohol dehydrogenase (spot n. 88) characterize the BS region for their high quantity. In plants, the fructose bisphosphate aldolase plays an important regulatory role in the flux of carbon through carbohydrate metabolism (Schaeffer et al. 1997; Pego & Smeekens 2000; Gonzali et al. 2001). In addition fructose-bisphosphate aldolase was found to be increased in the elongation zone of rice root in response to giberellin treatment (Konishi et al. 2004), and in Arabidopsis cell cultures and root apex under alternating gravity conditions (Martzivanou & Hampp 2003; Kimbrough et al. 2004). In our previous study (Scippa et la., 2008), we proposed that the downregulation of fructose bisphosphate aldolase associated with the upregulation of 2,3-bisphosphoglycerate-independent phosphoglycerate mutase and enolase in bent roots, after 6 months of stress, indicated the occurrence of a carbon flux shift towards a secondary metabolism as mevalonate and/or shikimate pathways. In the present work, on the contrary we show that Fructose-bisphosphate aldolase is accumulated in most stressed region (BS) of the bent root after 12 months of stress treatment. This result may first indicate that there is a modulation of the expression of this enzyme along the time. Secondly, based on the evidences reported in the literature it might be supposed that fructose-bisphosphate aldolase in the BS region may regulate the flux of carbon to support the elongation cell growth that might occur in this region in response to altered gravity. A peroxiredoxin was found to be accumulated in the taproot after 6 months of bending (Scippa et al. 2008), and it was proposed that the Prx induced by bending in poplar root may be the 1-Cys type expressed in roots in response to oxidative stress (Requejo & Tena 2005). A similar hypothesis of the involved in the signalling pathways of mechanical stress may justify the high abundance of peroxiredoxin found in BS at T_0 and BBS at T_1, where it is present in two isoforms (spot n. 1, 51). Alcohol dehydrogenase ADH (spot n. 88) catalyses the conversion of ethanal to ethanol in anaerobic glycolysis, and it is implicated in the response to a wide range of stresses, elicitors and ABA (Matton et al., 1990; Christie et al., 1991). The abundance of ADH detected in BS, but also in BBS, is likely linked to the need of energy production to use for the modifications induced by the bending. In fact it has been shown that during late winter, genes encoding fermentation-associated proteins such alcohol dehydrogenase are up-regulated to produce energy in low oxygen condition perhaps because of ice encasement formed during freeze–thaw cycles in late winter (Bertrand et al., 2003). At T1, the most striking differences between the three sectors were shown by BS, where the majority of proteins accumulated in ABS and BBS were downregulated. In particular, both ABS and BBS were

characterised by the high abundance of several different factors involved in the stress response (spot n. 1, 51, 100, 124, 161, 182, 183, 189, 201), carbon metabolism (spot n. 13, 182, 201), protein storage (spot n. 161, 182, 201), synthesis/processing (spot n. 150, 201), and transporter (spot n. 59), indicating that in these two regions a different metabolic pathway might be active compared to BS. This last region is fact characterised by an high presence of a fructokinase, LEA protein (spot n. 5) and a cystathionine-β-synthase (CBS) domain containing protein (spot n. 154). Similarly to Fructose-bisphosphate aldolase we found accumulated in BS a T_0, the fructokinase has been reported to be upregulated in *Arabidopsis* under alternating gravity conditions (Martzivanou & Hampp, 2003; Kimbrough et al., 2004). The upregulation of fructokinase correlated with a decrease in pool sizes of fructose, suggested that in altered gravity condition the metabolism of fructose and glucose might be different. CBS domain proteins have been reported that some of these proteins of unknown function can indeed improve plants tolerance to oxidative stress (Luhua et al., 2008). Moreover they have been found to be associated with several proteins of unrelated functions, among which cystathionine-β– synthase (CBS). This particular enzyme is involved in transsulfuration pathway leading to conversion of cysteine to homocysteine that produces as final product the methionine (Kushwaha et al., 2009) that is furthermore linked to ethylene synthesis (Wang et al., 1982). It is widely known that ethylene is important regulator of many important physiological and stress responses of woody plants, including wood formation (Andersson-Gunnerås et al,. 2003), cell division in the cambial meristems (Love et al., 2009), lateral root formation (Negi et al., 2008) and many other processes included in the thigmo-respone (Telewski & Jaffe, 1986). The abundance of a CBS domain protein may be linked to the synthesis of ethylene and in turn regulates the alterations, such as reaction wood and/or lateral root formation, observed in the bent region (BS). Ethylene may be also involved in the accumulation we observed at T_1 ABS and ABS of the ethylene responsive factor, ERF (spot n. 189). As described in the previous paragraph, the ERF family members have been found to play different functions among which response to abiotic stresses and lateral root development. Here we show that a member of the ERF family is a marker of the two sectors (ABS and BS) of the bent root where we detected the highest number of lateral root emissions. This evidence may confirm the possible function of ERF in the modulation of lateral root induction in response to mechanical forces. At T_2, ABS and BBS were characterised by the high quantity of factors involved in the energy and metabolism, ATP synthase (spot n. 22), 2-3 bisphosphoglicerate-independent- phosphoglicerate mutase (spot n.19) and mitochondrial lipoamide dehydrogenase (spot n. 126). In addition these two regions showed also an abundance of bark storage proteolytic fragments (spot n. 70, 71, 120). The abundance of

factors involved in the energy production and metabolism, together with presence of several bark storage proteolytic fragments may indicate a more active metabolic activity in ABS and BBS compared to BS. Moreover, along energy and metabolism factors ABS is also characterised by the accumulation of stress/defence (spot n. 136, 148), signal transduction (spot n. 87). At this time although BS is characterised by the low presence of factors involved in the metabolism it showed the accumulation of proteins related with stress defence (spot n. 144, 203) and of electron-transfer flavoprotein:ubiquinone oxidoreductase (ETFQO) (spot n. 122), which is also accumulated in BBS. The exact function of the ETFQO is still unclear, however it has been shown that it is essential for plants to survive in sucrose-depleted conditions induced by extended growth in the dark and is involved in the Leu and fatty acid catabolic pathway (Ishizaki et al., 2005). The presence of this enzyme in the BS and BBS regions in a phase close to the growth resumption may indicate that in a condition where the supply of sugar provided by the photosynthesis did not begin yet, root to face the bending uses substrates auxiliary to those derived from sucrose, as aminoacid and fatty acid.

5. Conclusion

In this work the response of poplar woody root to mechanical stress induced by bending has been deciphered along a spatial and temporal gradient. In particular long term modifications belonging to the known thigmo-response have been observed. However, the detailed analysis of the root morphology revealed that as recently shown in *Arabidopsis thaliana*, bending induces the lateral roots formation that are preferentially emitted in the convex side of the curved region. The analogy of the response observed in primary (*Arabidopsis thaliana*) and secondary (Poplar) root growth may contribute on resolving the almost totally unknown mechanisms involved in lateral root formation from a woody structure.

This study also provides the first proteome map of a woody root, with 211 proteins identified, and 2 unidentified. The spatial and temporal analysis revealed that sixty proteins remained unchanged between control and stressed root during time, whereas one-hundred-forty-seven showed a differential expression. The data released by the software assisted analysis (PDQuest) were subjected to ANOVA and hierarchical clustering. Significant differentially expressed proteins were grouped according with their common pattern in: a) proteins differentially expressed during time in physiological conditions; b) proteins differentially expressed during time between control and bent root; c) proteins differentially expressed during time between the three regions (ASB, BS and BBS) in the bent root.

The patterns of differentially expressed proteins during time in the unstressed roots indicate that T_0, T_1 and T_2 are characterized by diverse physiological conditions. In particular T_0 may be defined ad the time closer to a dormacy, whereas T_2 to a growth resumption and T_1 as "transition" state. Proteins differentially expressed during time between control and bent root show that bending induces evident alteration in metabolism, cell structure, signal transduction pathway together with the activation of alternative stress defense machinery compared to the control. In addition all the modifications observed appeared temporally modulated.

Proteins differentially expressed during time between the three regions (ASB, BS and BBS) in the bent root confirmed the asymmetry observed in the morphological and biomechanical investigation.

The most interesting result is the induction in ABS and BS of important factors involved in the reaction wood and lateral root formation, two key processes in woody root biology and interaction with the environment.

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Table III. Variable and constant expressed protein spots in poplar root. Proteins identified in the 2-DE proteomic map of *Populus nigra* woody taproot. Spot number, accession number, protein name, experimental and theoretical pI and Mr values, method of identification, i.e. MALDI-TOF peptide mass fingerprint (PMF) or nanoLC-ESI-LIT-MS/MS (LCMS), number of peptides identified, sequence coverage, identification score, organism, and function are listed. Spot numbering refers to Figure 5. * indicates constantly expressed proteins.

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
1	Type II peroxiredoxin	A9PC26	*Populus trichocarpa*	PMF	13	75	205	5,35/18	20	Disease/defence
2	Protein translocase, putative	B9HWY0	*Populus trichocarpa*	TMS	7	54	345	6,30/19	23	Transporters
3	Proteasome subunit alpha type, putative	A9PAG0	*Populus trichocarpa*	PMF	7	32	113	5,51/26	29	Protein destination and storage
4*	Ran binding protein, putative	A9P8P1	*Populus trichocarpa*	TMS	2	14	110	4,72/25	40	Transporters
5	Fructokinase, putative	B9HH42	*Populus trichocarpa*	TMS	5	24	213	5,01/36	40	Energy
	Late embryogenesis abundant, putative	B9HXA8	*Populus trichocarpa*	TMS	8	20	369	5,16/33	41	Disease/defence
6	Pyruvate dehydrogenase, putative	A9P841	*Populus trichocarpa*	PMF	8	30	128	5,34/38	44	Energy
7	Dhn1	Q9AR85	*Populus x canadensis*	PMF	15	33	176	5,17/26	48	Disease/defence
8	Bark storage protein B	Q09117	*Populus deltoides*	PMF	11	41	148	6,90/34	38	Protein destination and storage
	Beta-1,3 glucanase	Q9M5I9	*Populus tremula x Populus alba*	PMF	12	45	140	8,78/38	38	Disease/defence
9*	Electron transfer flavoprotein-ubiquinone oxidoreductase, putative	A9PEX2	*Populus trichocarpa*	TMS	6	22	308	6,00/37	38	Metabolism
	Bark storage protein B	A9PFE2	*Populus trichocarpa*	TMS	4	15	181	6,34/36	38	Protein destination and storage
10	Malate dehydrogenase precursor	B9N6Q9	*Populus trichocarpa*	PMF	11	36	170	8,15/44	42	Energy
11	Aldo/keto reductase, putative	B9GUA6	*Populus trichocarpa*	TMS	5	14	244	5,97/38	43	Disease/defence
12*	Acetyl-CoA acetyltransferase, putative	B9IAU7	*Populus trichocarpa*	TMS	6	24	387	6,24/42	50	Metabolism
13	Enolase, putative	A9PIJ2	*Populus trichocarpa*	PMF	14	44	196	5,56/48	54	Energy
14*	Enolase, putative	A9PIJ2	*Populus trichocarpa*	PMF	18	49	233	5,56/48	54	Energy
15	Enolase, putative	A9PIJ2	*Populus trichocarpa*	PMF	13	34	133	5,56/48	55	Energy
	ATP synthase beta subunit, putative	B9HJ80	*Populus trichocarpa*	PMF	10	25	94	5,91/60	55	Energy

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
16	Enolase, putative	A9PD49	*Populus trichocarpa*	PMF	17	43	213	5,67/48	55	Energy
17	Enolase, putative	A9PD49	*Populus trichocarpa*	PMF	17	43	213	5,67/48	55	Energy
18*	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	B9MTR8	*Populus trichocarpa*	PMF	8	20	118	5,37/61	61	Energy
19	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	B9MTR8	*Populus trichocarpa*	PMF	12	26	167	5,37/61	61	Energy
20	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	B9MTR8	*Populus trichocarpa*	PMF	15	32	194	5,37/61	61	Energy
21	d-3-phosphoglycerate dehydrogenase, putative	A9PEK1	*Populus trichocarpa*	TMS	2	5	122	6,79/63	55	Energy
22	ATPase alpha subunit	Q9T756	*Asarum canadense*	PMF	8	19	108	7,15/46	55	Energy
23	Putative uncharacterized protein	A9PGH1	*Populus trichocarpa*	PMF	9	30	119	6,50/40	51	Uncharacterized
24*	Fructose-bisphosphate aldolase, putative	A9PJ53	*Populus trichocarpa x Populus deltoides*	PMF	8	29	122	7,01/39	45	Energy
25	Fructose-bisphosphate aldolase, putative	A9P9U1	*Populus trichocarpa*	TMS	14	51	787	8,63/39	41	Energy
	Pectinesterase precursor, putative	A9PE41	*Populus trichocarpa*	TMS	5	17	310	9,21/36	41	Cell structure
	Hypothetical protein	B9I618	*Populus trichocarpa*	TMS	3	9	135	9,08/20	41	Uncharacterized
26	Fructose-bisphosphate aldolase, putative	A9P9U1	*Populus trichocarpa*	TMS	14	51	787	8,63/39	43	Energy
	Pectinesterase precursor, putative	A9PE41	*Populus trichocarpa*	TMS	5	17	310	9,21/36	43	Cell structure
	Hypothetical protein	B9I618	*Populus trichocarpa*	TMS	3	9	135	9,08/20	43	Uncharacterized
27*	Fructose-bisphosphate aldolase, putative	A9P9U1	*Populus trichocarpa*	TMS	2	10	103	8,63/39	45	Energy
	Bark storage protein B	A9PGW6	*Populus trichocarpa*	TMS	5	16	242	6,21/36	45	Protein destination and storage
28*	Bark storage protein B	Q09117	*Populus deltoides*	PMF	8	31	113	6,90/34	54	Protein destination and storage
29	Xylem serine proteinase 1 precursor, putative	B9IC48	*Populus trichocarpa*	TMS	2	4	123	8,79/81	62	Protein destination and storage
30*	Serine hydroxymethyltransferase	A9PL04	*Populus tremuloides*	PMF	10	19	97	7,59/52	60	Metabolism
	Bark storage protein A	Q07469	*Populus deltoides*	PMF	10	32	113	6,84/34	60	Protein destination and storage

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
31*	Bark storage protein A	Q07469	*Populus deltoides*	PMF	10	32	113	6,84/34	58	Protein destination and storage
32*	Putative uncharacterized protein	A9PGH1	*Populus trichocarpa*	TMS	2	6	109	6,50/40	49	Uncharacterized
33*	Polyadenylate-binding protein, putative	B9GHB4	*Populus trichocarpa*	PMF	11	21	127	7,57/71	63	Transcription
34	Heat shock protein 70 (HSP70)-interacting protein, putative	B9GVR1	*Populus trichocarpa*	TMS	3	6	202	6,17/66	63	Disease/defence
35*	Aconitase, putative	B9GTX8	*Populus trichocarpa*	PMF	9	12	117	6,31/98	80	Energy
36*	Aconitase, putative	B9GTX8	*Populus trichocarpa*	PMF	10	12	130	6,31/98	80	Energy
37	Putative dehydrin	Q9FEU6	*Populus x canadensis*	PMF	12	12	122	6,12/69	70	Disease/defence
38*	Putative dehydrin	Q9FEU6	*Populus x canadensis*	PMF	12	12	122	6,12/69	70	Disease/defence
39*	Putative dehydrin	Q9FEU6	*Populus x canadensis*	PMF	12	12	122	6,12/69	70	Disease/defence
40*	Putative dehydrin	Q9FEU6	*Populus x canadensis*	TMS	7	7	209	6,12/69	70	Disease/defence
41	Putative dehydrin	Q9FEU6	*Populus x canadensis*	TMS	7	7	209	6,12/69	76	Disease/defence
42	Putative dehydrin	Q9FEU6	*Populus x canadensis*	PMF	12	12	122	6,12/69	76	Disease/defence
43	Putative dehydrin	Q9FEU6	*Populus x canadensis*	PMF	12	12	122	6,12/69	76	Disease/defence
44*	Beta-1,3 glucanase/*probable proteolytic fragment	Q9M5I9	*Populus tremula x Populus alba*	PMF	6	17	111	8,78/38	14	Disease/defence
45	Cyclophilin, putative	A9P8L4	*Populus trichocarpa*	TMS	2	12	115	8,71/18	18	Protein destination and storage
	Putative pathogenesis-related protein 1	B9GIP3	*Populus trichocarpa*	TMS	2	20	124	8,57/18	18	Disease/defence
46*	Putative pathogenesis-related protein 1	B9GIP3	*Populus trichocarpa*	TMS	2	20	132	8,57/18	18	Disease/defence
47	Putative uncharacterized protein	A9PHL8	*Populus trichocarpa*	TMS	2	36	144	8,09/9	16	Uncharacterized
48	Universal stress protein	B9GS98	*Populus trichocarpa*	TMS	2	15	156	6,43/17	20	Disease/defence
49	Transcription factor btf3, putative	A9P9Z2	*Populus trichocarpa*	TMS	2	17	116	6,74/17	20	Transcription

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
50*	Unidentified protein	-	-	-	-	-	-	-	25	Unidentified
51	Peroxiredoxin	Q8S3L0	*Populus tremula x Populus tremuloides*	TMS	2	18	116	5,56/18	19	Disease/defence
52*	ATP synthase D chain, mitochondrial, putative	A9PIU7	*Populus trichocarpa x Populus deltoides*	TMS	3	16	129	5,12/20	21	Energy
53*	Glycine-rich RNA-binding protein, putative	A9PIG6	*Populus trichocarpa*	TMS	2	10	113	4,95/25	30	Transcription
	Putative ripening regulated protein	B9H7Y9	*Populus trichocarpa*	TMS	4	38	186	5,52/13	30	Cell growth/division
54	Triosephosphate isomerase, putative	A9PE68	*Populus trichocarpa*	PMF	11	48	193	6,00/27	28	Energy
55*	Triosephosphate isomerase, putative	A9PE68	*Populus trichocarpa*	PMF	8	37	144	6,00/27	29	Energy
56	Protein yrdA, putative	A9PFJ3	*Populus trichocarpa*	TMS	4	25	207	6,18/29	29	Transporters
57	Protein disulfide isomerase, putative	A9PAI9	*Populus trichocarpa*	TMS	2	10	120	5,31/35	42	Protein destination and storage
	ABA-responsive protein-like	B9GZB1	*Populus trichocarpa*	TMS	4	15	184	5,76/31	42	Disease/defence
58*	Ankyrin repeat domain protein, putative	A9PDB7	*Populus trichocarpa*	TMS	2	7	124	4,47/39	47	Disease/defence
59	Importin alpha, putative	B9MZZ1	*Populus trichocarpa*	TMS	3	9	126	5,20/59	61	Transporters
60*	Transaminase mtnE, putative	B9HN51	*Populus trichocarpa*	PMF	9	30	122	5,75/45	45	Metabolism
61	Glyceraldehyde 3-phosphate dehydrogenase, putative	A9PA00	*Populus trichocarpa*	TMS	3	11	134	6,34/37	44	Energy
	Aldo/keto reductase, putative	B9I5E8	*Populus trichocarpa*	TMS	3	9	140	6,22/39	44	Disease/defence
62*	Patatin-like protein	B9HE67	*Populus trichocarpa*	TMS	9	29	499	6,04/42	63	Metabolism
	Pyruvate dehydrogenase E1 alpha subunit	B9HLV5	*Populus trichocarpa*	TMS	2	6	122	8,05/44	63	Energy
63*	Bark storage protein A	Q07469	*Populus deltoides*	PMF	7	17	110	6,84/34	81	Protein destination and storage
64*	Peroxidase	A9PGX5	*Populus trichocarpa*	TMS	2	7	119	5,69/39	48	Disease/defence
	Glyceraldehyde 3-phosphate dehydrogenase, putative	A9PIL9	*Populus trichocarpa x Populus deltoides*	TMS	20	60	814	7,01/37	48	Energy
	Fructose-bisphosphate aldolase, putative	A9PJ53	*Populus trichocarpa x Populus deltoides*	TMS	7	25	297	7,01/39	48	Energy

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
65	Glyceraldehyde 3-phosphate dehydrogenase, putative	A9PIL9	*Populus trichocarpa x Populus deltoides*	PMF	7	26	109	7,01/37	48	Energy
66	Putative disease resistance protein	B9MWK3	*Populus trichocarpa*	TMS	5	15	297	9,01/53	49	Disease/defence
67	Fructose-bisphosphate aldolase, putative	A9P9U1	*Populus trichocarpa*	PMF	9	36	140	8,63/39	45	Energy
68	Bark storage protein	A9PFE2	*Populus trichocarpa*	PMF	7	27	114	6,34/36	42	Protein destination and storage
69	Basic blue protein, putative	B9GEY2	*Populus trichocarpa*	TMS	2	21	88	9,61/13	11	Transporters
70	Bark storage proteinB/*probable proteolytic fragment	A9PFD1	*Populus trichocarpa*	PMF	8	21	97	6,90/36	17	Protein destination and storage
71	Bark storage protein A/*probable proteolytic fragment	Q07469	*Populus deltoides*	PMF	6	19	105	6,84/34	18	Protein destination and storage
72	Putative pathogenesis-related protein	B9N005	*Populus trichocarpa*	TMS	3	22	98	5,37/18	18	Disease/defence
73*	Proteasome subunit beta type, putative	A9PGT7	*Populus trichocarpa*	TMS	2	16	155	5,04/23	26	Protein destination and storage
74*	Plasma membrane polypeptide	B9GYR5	*Populus trichocarpa*	TMS	3	19	190	5,01/21	29	Cell structure
75*	Chitinase, precursor	B9HQY6	*Populus trichocarpa*	TMS	3	13	133	4,44/24	31	Disease/defence
76*	Fructokinase, putative	A9PEZ9	*Populus trichocarpa*	PMF	7	21	96	4,93/35	40	Energy
77*	Fructokinase, putative	B9HH42	*Populus trichocarpa*	PMF	13	43	203	5,01/36	41	Energy
78*	Putative enoyl-acyl-carrier-protein reductase	A9PBH6	*Populus trichocarpa*	PMF	8	17	120	8,70/42	39	Metabolism
79	Peroxidase	A9PGX5	*Populus trichocarpa*	TMS	3	10	178	5,69/39	42	Disease/defence
	Phenylcoumaran benzylic ether reductase 7	B9H4C7	*Populus trichocarpa*	TMS	7	24	309	5,92/34	42	Secondary metabolism
80*	Heat shock protein, putative	B9GL18	*Populus trichocarpa*	TMS	18	26	814	5,05/74	63	Disease/defence
81*	Enolase, putative	A9PD49	*Populus trichocarpa*	TMS	6	25	358	5,67/48	55	Energy
82	Enolase, putative	A9PD49	*Populus trichocarpa*	PMF	9	26	113	5,67/48	55	Energy
83	Enolase, putative	A9PD49	*Populus trichocarpa*	PMF	13	43	157	5,67/48	55	Energy
84	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Q33204	*Tetrachondra hamiltonii*	PMF	10	17	134	6,32/49	55	Energy
ID	**Protein name**	**BLAST**	**Organism**	**MS**	**matched**	**coverage**	**Mascot**	**Theor.pI/**	**Exp.**	**Functional classification**

				method	peptides	%	Score	Mr	Mr	
85	Ribulose 1,5-bisphosphate carboxylase small subunit	A9PFS6	*Populus trichocarpa*	PMF	8	47	135	8,31/21	52	Energy
86*	Transaminase mtnE, putative	B9HN51	*Populus trichocarpa*	PMF	7	20	118	5,75/45	53	Metabolism
87	Cytosolic phosphoglycerate kinase 1	O82159	*Populus nigra*	PMF	14	39	185	5,70/43	47	Signal transduction
88	Alcohol dehydrogenase	A9PG17	*Populus trichocarpa*	PMF	6	23	110	6,28/42	51	Energy
89	Bark storage protein B	A9PFE2	*Populus trichocarpa*	PMF	8	25	142	6,34/36	> 97	Protein destination and storage
90	RNA binding protein, putative	B9MV71	*Populus trichocarpa*	PMF	7	20	106	8,42/40	50	Transcription
91*	Peroxidase	A9PGX5	*Populus trichocarpa*	PMF	16	32	172	5,69/39	45	Disease/defence
92*	Zeamatin precursor, putative	A9PGI5	*Populus trichocarpa*	TMS	2	8	119	8,29/28	28	Disease/defence
93*	Glutathione-s-transferase	B9IBH2	*Populus trichocarpa*	PMF	7	38	120	5,73/24	27	Disease/defence
94*	Putative ATP synthase	B9H5L0	*Populus trichocarpa*	TMS	3	14	127	7,71/28	45	Energy
	Cytosolic ascorbate peroxidase	B9HQ83	*Populus trichocarpa*	TMS	5	27	219	5,53/27	45	Disease/defence
	Glutathione-s-transferase omega, putative	B9IIY3	*Populus trichocarpa*	TMS	2	12	87	5,52/26	45	Disease/defence
95	ATP synthase beta subunit, putative	B9HJ80	*Populus trichocarpa*	PMF	10	25	140	5,91/60	33	Energy
96	26S protease regulatory subunit 6A homolog	B9I7I1	*Populus trichocarpa*	PMF	10	35	141	4,98/48	32	Protein destination and storage
97	Heat shock protein, putative	B9GL18	*Populus trichocarpa*	TMS	10	19	474	5,05/74	52	Disease/defence
	Heat shock cognate 70 kDa protein	B9HMG8	*Populus trichocarpa*	TMS	14	27	675	5,14/71	52	Disease/defence
98	Heat shock cognate 70 kDa protein	B9HMG8	*Populus trichocarpa*	TMS	9	19	463	5,14/71	62	Disease/defence
99*	ATP synthase beta subunit, putative	B9HJ80	*Populus trichocarpa*	PMF	13	32	192	5,91/60	63	Energy
100	Ankyrin repeat domain protein, putative	A9PDB7	*Populus trichocarpa*	TMS	9	30	402	4,47/39	48	Disease/defence
101	Putative uncharacterized protein	A9P8Q0	*Populus trichocarpa*	TMS	2	10	134	4,01/19	49	Uncharacterized
	Putative uncharacterized protein	A9P9E6	*Populus trichocarpa*	TMS	2	27	110	4,08/18	49	Uncharacterized

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
102	Beta-1,3 glucanase	Q9M5I9	*Populus tremula x Populus alba*	PMF	12	42	126	8,78/38	45	Disease/defence
103	Glutathione-s-transferase	B9IBH2	*Populus trichocarpa*	PMF	8	52	113	5,73/24	38	Disease/defence
104	Bark storage protein B	A9PFD1	*Populus trichocarpa*	PMF	11	25	110	6,90/36	27	Protein destination and storage
105	Ribulose-5-phosphate-3-epimerase,putative	A9PIC8	*Populus trichocarpa*	TMS	2	17	108	5,24/24	19	Energy
	Glutathione-s-transferase	B9IBH2	*Populus trichocarpa*	TMS	3	17	105	5,73/24	19	Disease/defence
106*	Catalase	Q1XFN0	*Populus deltoides*	TMS	4	11	125	6,76/57	29	Disease/defence
	Serine hydroxymethyltransferase	A9PL04	*Populus tremuloides*	TMS	11	28	518	7,59/52	29	Metabolism
107*	Serine hydroxymethyltransferase	A9PL04	*Populus tremuloides*	PMF	8	18	111	7,59/52	58	Metabolism
108	Pectin acetylesterase, putative	A9PBM5	*Populus trichocarpa*	TMS	2	6	99	8,87/44	59	Cell structure
109	Porin/voltage-dependent anion-selective channel protein	B9H6N6	*Populus trichocarpa*	TMS	2	8	90	8,80/29	49	Transporters
110	Beta-1,3 glucanase	Q9M5I9	*Populus tremula x Populus alba*	TMS	2	8	110	8,78/38	33	Disease/defence
111	Cytosolic ascorbate peroxidase	B9HQ83	*Populus trichocarpa*	PMF	8	34	116	5,53/27	26	Disease/defence
112	Bark storage protein A	Q07469	*Populus deltoides*	TMS	5	18	209	6,84/36	31	Protein destination and storage
113*	Glutathione-s-transferase omega, putative	B9IIY3	*Populus trichocarpa*	TMS	2	12	103	5,52/26	47	Disease/defence
114	Bark storage protein B	A9PFD1	*Populus trichocarpa*	TMS	6	26	253	6,90/36	30	Protein destination and storage
	Beta-1,3 glucanase	Q9M5I9	*Populus tremula x Populus alba*	TMS	6	25	326	8,78/38	30	Disease/defence
115	Aldo/keto reductase, putative	B9IAA3	*Populus trichocarpa*	PMF	14	35	167	6,24/38	38	Disease/defence
116*	Glutathione-s-transferase theta, gst, putative	A9PHH6	*Populus trichocarpa*	PMF	7	33	123	6,32/24	43	Disease/defence
117	Undentified protein	-	-	-	-	-	-	-	29	Unidentified
118	60S ribosomal protein L22, putative	A9P9A4	*Populus trichocarpa*	TMS	2	18	122	9,54/14	13	Protein synthesis
	40S ribosomal protein S20, putative	A9PD23	*Populus trichocarpa*	TMS	3	27	154	9,65/14	13	Protein synthesis

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
119	Bark storage protein B	A9PGW6	*Populus trichocarpa*	TMS	2	9	85	6,21/36	16	Protein destination and storage
	Peroxidase	A9PGX5	*Populus trichocarpa*	TMS	2	5	126	5,69/39	16	Disease/defence
120	Bark storage proteinB/*probable proteolytic fragment	A9PFD1	*Populus trichocarpa*	TMS	11	27	529	6,90/36	38	Protein destination and storage
121	Bark storage protein A	Q07469	*Populus deltoides*	TMS	2	8	109	6,84/36	20	Protein destination and storage
122	Electron transfer flavoprotein-ubiquinone oxidoreductase, putative	A9PEX2	*Populus trichocarpa*	TMS	2	7	132	6,00/37	37	Metabolism
123	Protein disulfide isomerase, putative	A9PAI9	*Populus trichocarpa*	TMS	2	9	133	5,31/35	39	Protein destination and storage
124	Peroxidase	A9PGX5	*Populus trichocarpa*	PMF	9	24	128	5,69/39	44	Disease/defence
125	Fructose-bisphosphate aldolase, putative	A9PJ53	*Populus trichocarpa x Populus deltoides*	PMF	7	28	120	7,01/39	43	Energy
126	Mitochondrial lipoamide dehydrogenase	A9PL03	*Populus tremuloides*	PMF	10	28	101	7,25/54	44	Energy
127*	Fructokinase, putative	A9PEZ9	*Populus trichocarpa*	TMS	2	7	81	4,93/35	52	Energy
128*	Hypothetical protein	B9IK14	*Populus trichocarpa*	PMF	12	37	167	4,57/38	38	Uncharacterized
129	Mitochondrial beta subunit of F1 ATP synthase	B9HWA2	*Populus trichocarpa*	PMF	17	44	244	5,96/60	63	Energy
130	Glutathione peroxidase	B9GWH5	*Populus trichocarpa*	TMS	5	17	203	9,42/28	48	Disease/defence
131	Vacuolar ATP synthase subunit E, putative	A9P864	*Populus trichocarpa*	PMF	12	44	130	7,78/26	23	Energy
132*	Late embryogenesis abundant, putative	B9HXA8	*Populus trichocarpa*	PMF	8	24	115	5,16/33	38	Disease/defence
133*	Dhn1	Q9AR85	*Populus x canadensis*	PMF	8	28	108	5,17/26	41	Disease/defence
134	Cyclophilin, putative	A9P8L4	*Populus trichocarpa*	PMF	9	33	106	8,71/18	47	Protein destination and storage
135	Hypothetical protein	B9I618	*Populus trichocarpa*	TMS	2	9	136	9,08/20	20	Uncharacterized
136	Glutathione-s-transferase	B9IBH2	*Populus trichocarpa*	TMS	7	38	345	5,73/24	16	Disease/defence
137	Triosephosphate isomerase, putative	B9HTI4	*Populus trichocarpa*	PMF	8	37	135	6,45/27	27	Energy
138	Tropinone reductase, putative	A9PI58	*Populus trichocarpa*	TMS	2	13	120	8,98/28	32	Secondary metabolism

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
139*	Probable ATP synthase 24 kDa subunit, mitochondrial	A9PC60	*Populus trichocarpa*	TMS	5	30	216	8,50/28	32	Energy
140	Class I chitinase, putative	B9H1P7	*Populus trichocarpa*	TMS	3	17	171	8,06/35	34	Disease/defence
	Triosephosphate isomerase, putative	B9HTI4	*Populus trichocarpa*	TMS	2	11	112	6,45/27	34	Energy
141*	Cytosolic ascorbate peroxidase	A9P9X7	*Populus trichocarpa*	PMF	7	45	133	5,48/28	30	Disease/defence
142	Glutathione-s-transferase theta, gst, putative	B9GQ64	*Populus trichocarpa*	PMF	8	34	133	5,52/25	30	Disease/defence
143	RNA-binding glycine-rich protein	A9PIZ6	*Populus trichocarpa*	PMF	6	43	112	5,54/17	28	Transcription
144	Putative universal stress protein	A9PHM7	*Populus trichocarpa*	TMS	2	11	125	5,20/18	17	Disease/defence
145	Methylmalonate-semialdehyde dehydrogenase, putative	B9GH76	*Populus trichocarpa*	TMS	3	8	139	5,97/54	17	Secondary metabolism
	26S protease regulatory subunit, putative	B9IBD9	*Populus trichocarpa*	TMS	2	7	113	5,91/50	17	Protein destination and storage
	ATPase alpha subunit	Q9T749	*Calycanthus floridus*	TMS	9	26	445	6,55/46	17	Energy
146	ATP synthase subunit alpha	P24459	*Populus euphratica*	PMF	10	25	145	6,52/55	55	Energy
147	Ribulose bisphosphate carboxylase small chain, chloroplast precursor, putative	A9PI67	*Populus trichocarpa*	PMF	10	45	125	9,00/20	16	Energy
148	Beta-1,3 glucanase/*probable proteolytic fragment	Q9M5I9	*Populus tremula x Populus alba*	PMF	7	20	108	8,78/38	15	Disease/defence
149*	Putative amino acid selective channel protein	A9PFS3	*Populus trichocarpa*	TMS	4	26	225	8,99/15	16	Transporters
	Bark storage protein A/*probable proteolytic fragment	Q07469	*Populus deltoides*	TMS	10	26	522	6,84/36	16	Protein destination and storage
150	40S ribosomal protein S20, putative	A9PD23	*Populus trichocarpa*	PMF	10	54	141	9,65/14	17	Protein synthesis
151	Putative amino acid selective channel protein	A9PFS3	*Populus trichocarpa*	PMF	8	63	111	8,99/15	15	Transporters
152	Putative amino acid selective channel protein	A9PFS3	*Populus trichocarpa*	PMF	8	63	111	8,99/15	15	Transporters
153	40S ribosomal protein S5, putative	B9IIE4	*Populus trichocarpa*	PMF	12	48	159	9,75/23	25	Protein synthesis
154	CBS domain-containing protein, putative	B9I794	*Populus trichocarpa*	PMF	13	54	138	8,49/23	19	Metabolism

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
155	Putative pathogenesis-related protein 1	B9GIP3	*Populus trichocarpa*	PMF	9	65	105	8,57/18	18	Disease/defence
156	Cyclophilin, putative	A9P8L4	*Populus trichocarpa*	PMF	10	41	113	8,71/18	21	Protein destination and storage
157*	Osmotin precursor, putative	B9GKL0	*Populus trichocarpa*	PMF	12	46	153	7,85/26	25	Disease/defence
158*	Vacuolar ATP synthase subunit E, putative	A9P864	*Populus trichocarpa*	PMF	20	55	264	7,78/26	29	Energy
159	Triosephosphate isomerase, putative	B9HTI4	*Populus trichocarpa*	PMF	17	75	257	6,45/27	28	Energy
160	Tropinone reductase, putative	A9PI58	*Populus trichocarpa*	PMF	10	47	141	8,98/28	29	Secondary metabolism
161	Bark storage protein B	Q09117	*Populus deltoides*	PMF	14	41	140	6,90/34	38	Protein destination and storage
	Beta-1,3 glucanase	Q9M5I9	*Populus tremula x Populus alba*	PMF	14	48	149	8,78/38	38	Disease/defence
162	Pectin acetylesterase, putative	A9PBM5	*Populus trichocarpa*	PMF	12	43	161	8,87/44	45	Cell structure
163	Fructose-bisphosphate aldolase, putative	A9P9U1	*Populus trichocarpa*	PMF	10	44	144	8,63/39	45	Energy
164	Elongation factor	Q9FYV3	*Saccharum officinarum*	PMF	11	25	93	9,08/50	49	Protein synthesis
165	Putative uncharacterized protein	A9PGH1	*Populus trichocarpa*	PMF	11	32	134	6,50/40	54	Uncharacterized
166	Mitochondrial NAD-dependent malate dehydrogenase, putative	A9PCR0	*Populus trichocarpa*	PMF	10	39	165	8,71/36	44	Energy
167	Dihydrolipoamide dehydrogenase, putative	A9PJ55	*Populus trichocarpa x Populus deltoides*	PMF	12	34	153	6,95/54	50	Metabolism
168	Annexin, putative	A9PA39	*Populus trichocarpa*	PMF	24	60	324	6,16/36	41	Cell structure
169	Manganese superoxide dismutase-like protein	B9I887	*Populus trichocarpa*	PMF	14	69	164	6,80/25	28	Disease/defence
170*	Aldo/keto reductase, putative	B9GUA6	*Populus trichocarpa*	PMF	11	29	145	5,97/38	43	Disease/defence
171	Enolase, putative	A9PIJ2	*Populus trichocarpa*	PMF	15	49	182	5,56/48	54	Energy
172*	Groes chaperonin, putative	B9INC6	*Populus trichocarpa*	PMF	9	54	126	7,77/27	28	Protein destination and storage
173	Protein disulfide isomerase-like protein	B9GU26	*Populus trichocarpa*	PMF	21	48	261	4,76/56	61	Protein destination and storage

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
174	Probable ATP synthase 24 kDa subunit, mitochondrial	A9PC60	*Populus trichocarpa*	PMF	15	49	179	8,50/28	31	Energy
175	Triosephosphate isomerase, putative	B9HTI4	*Populus trichocarpa*	PMF	14	61	213	6,45/27	30	Energy
176*	Nucleoside diphosphate kinase, putative	B9GP74	*Populus trichocarpa*	PMF	10	64	192	6,31/16	17	Signal transduction
177	Nucleoside diphosphate kinase, putative	A9PAF2	*Populus trichocarpa*	PMF	7	47	115	6,09/16	16	Signal transduction
178	40S ribosomal protein S7, putative	A9PCT6	*Populus trichocarpa*	PMF	18	70	277	9,73/22	25	Protein synthesis
179	Putative dehydrin	B9GQ42	*Populus trichocarpa*	TMS	11	22	432	8,98/51	81	Disease/defence
180*	Bark storage protein B	A9PFD1	*Populus trichocarpa*	TMS	4	18	217	6,90/36	81	Protein destination and storage
	Putative dehydrin	Q9FEU6	*Populus x canadensis*	TMS	15	16	535	6,12/69	81	Disease/defence
181	Putative dehydrin	Q9FEU6	*Populus x canadensis*	TMS	16	14	564	6,12/69	81	Disease/defence
182	Bark storage protein B	A9PFD1	*Populus trichocarpa*	TMS	7	25	390	6,90/36	43	Protein destination and storage
	Putative peroxidase	B9H4R4	*Populus trichocarpa*	TMS	8	23	406	4,92/37	43	Disease/defence
	Fructokinase, putative	B9HH42	*Populus trichocarpa*	TMS	6	21	282	5,01/36	43	Energy
	Late embryogenesis abundant, putative	B9HXA8	*Populus trichocarpa*	TMS	5	13	252	5,16/33	43	Desease/defence
183	Dhn1	Q9AR85	*Populus x canadensis*	PMF	16	44	230	5,17/26	44	Desease/defence
184	Phenylcoumaran benzylic ether reductase	O65882	*Populus trichocarpa*	PMF	14	39	183	5,51/34	39	Secondary metabolism
185	Fructose-bisphosphate aldolase, putative	A9P9U1	*Populus trichocarpa*	TMS	7	32	461	8,63/39	48	Energy
	RNA binding protein, putative	A9PCV9	*Populus trichocarpa*	TMS	7	14	223	6,45/38	48	Transcription
	Fumarate hydratase, putative	B9GSD7	*Populus trichocarpa*	TMS	11	22	402	8,40/53	48	Energy
	Putative alanine aminotransferase	B9HTU4	*Populus trichocarpa*	TMS	3	8	179	6,92/54	48	Metabolism
186	Mitochondrial NAD-dependent malate dehydrogenase, putative	A9PCR0	*Populus trichocarpa*	PMF	9	27	124	8,71/36	41	Energy

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
187	Aldo/keto reductase, putative	B9IAA3	*Populus trichocarpa*	PMF	19	45	260	6,24/38	51	Disease/defence
188	Peroxidase	A9PGX5	*Populus trichocarpa*	PMF	22	54	252	5,69/39	36	Disease/defence
189	Putative ethylene-responsive protein	B9IJM2	*Populus trichocarpa*	TMS	6	28	232	5,97/20	25	Disease/defence
190	ATP synthase D chain, mitochondrial, putative	B9HU25	*Populus trichocarpa*	PMF	10	41	117	5,20/20	24	Energy
191*	Putative uncharacterized protein	A9PGH1	*Populus trichocarpa*	PMF	12	30	155	6,50/40	54	Uncharacterized
192	Polyadenylate-binding protein, putative	B9GHB4	*Populus trichocarpa*	PMF	10	18	114	7,57/71	63	Transcription
193*	Prohibitin, putative	A9PD98	*Populus trichocarpa*	PMF	10	55	167	9,26/32	38	Disease/defence
194	Porin/voltage-dependent anion-selective channel protein	B9H6N6	*Populus trichocarpa*	PMF	10	40	134	8,80/29	36	Transporters
195*	Porin/voltage-dependent anion-selective channel protein	A9PCP8	*Populus trichocarpa*	TMS	12	43	647	8,46/29	37	Transporters
	Class IV chitinase	A9PH35	*Populus trichocarpa*	TMS	3	13	169	8,32/30	37	Disease/defence
196	Vacuolar ATP synthase subunit E, putative	A9P864	*Populus trichocarpa*	TMS	4	19	204	7,78/26	32	Energy
	Proteasome subunit alpha type, putative	A9PJR2	*Populus trichocarpa*	TMS	19	60	1035	6,86/27	32	Protein destination and storage
197	RNA-binding glycine-rich protein	A9PIZ6	*Populus trichocarpa*	PMF	9	44	116	5,54/17	18	Transcription
198	Putative universal stress protein	A9PHM7	*Populus trichocarpa*	PMF	12	67	132	5,20/18	17	Disease/defence
199	Putative uncharacterized protein	A9PIZ5	*Populus trichocarpa*	TMS	4	45	129	5,02/12	14	Uncharacterized
200*	Alcohol dehydrogenase, putative	A9P8L8	*Populus trichocarpa*	TMS	12	45	839	5,78/34	42	Energy
	Malate dehydrogenase, putative	A9P8R3	*Populus trichocarpa*	TMS	6	22	315	6,11/36	42	Energy
	Protein disulfide isomerase, putative	A9PAI9	*Populus trichocarpa*	TMS	7	26	319	5,31/35	42	Protein destination and storage
	Aldo/keto reductase, putative	B9GNC4	*Populus trichocarpa*	TMS	13	33	611	5,86/38	42	Disease/defence

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
201	Alcohol dehydrogenase, putative	A9P8L8	*Populus trichocarpa*	TMS	9	39	631	5,78/34	44	Energy
	Malate dehydrogenase, putative	A9P8R3	*Populus trichocarpa*	TMS	4	15	248	6,11/36	44	Energy
	Glyceraldehyde 3-phosphate dehydrogenase, putative	A9PA00	*Populus trichocarpa*	TMS	4	17	139	6,34/37	44	Energy
	Bark storage protein B	A9PFD1	*Populus trichocarpa*	TMS	6	22	395	6,90/36	44	Protein destination and storage
	Aldo/keto reductase, putative	B9GNC4	*Populus trichocarpa*	TMS	11	29	601	5,86/38	44	Disease/defence
	ABA-responsive protein-like	B9GZB1	*Populus trichocarpa*	TMS	5	16	220	5,76/31	44	Disease/defence
	Aspartate-semialdehyde dehydrogenase, putative	B9HJH4	*Populus trichocarpa*	TMS	5	20	212	6,72/41	44	Metabolism
	Protein disulfide isomerase, putative	B9I9L2	*Populus trichocarpa*	TMS	11	37	610	5,63/40	44	Protein destination and storage
	Dihydrodipicolinate synthase	Q43038	*Populus trichocarpa x Populus deltoides*	TMS	5	15	150	6,97/42	44	Metabolism
202	Mitochondrial NAD-dependent malate dehydrogenase, putative	A9PCR0	*Populus trichocarpa*	PMF	9	33	123	8,71/36	43	Energy
	Peroxidase	A9PGX5	*Populus trichocarpa*	PMF	17	28	174	5,69/39	43	Disease/defence
203	Peroxidase	A9PGX5	*Populus trichocarpa*	PMF	16	42	199	5,69/39	43	Disease/defence
204	Fructose-bisphosphate aldolase, putative	A9P9U1	*Populus trichocarpa*	PMF	10	44	119	8,63/39	45	Energy
205	Enolase, putative	A9PD49	*Populus trichocarpa*	PMF	27	67	309	5,67/48	55	Energy
206	Enolase, putative	A9PD49	*Populus trichocarpa*	PMF	27	67	325	5,67/48	56	Energy
207	Dhn1	Q9AR85	*Populus x canadensis*	PMF	18	44	244	5,17/26	48	Disease/defence
208	Bark storage protein B	A9PFE2	*Populus trichocarpa*	TMS	4	13	224	6,34/36	64	Protein destination and storage
	Ara4-interacting protein, putative	B9GKN0	*Populus trichocarpa*	TMS	2	4	91	4,80/65	64	Intracellular traffic
	Hypothetical protein	B9IK14	*Populus trichocarpa*	TMS	2	8	114	4,57/38	64	Uncharacterized

ID	Protein name	BLAST	Organism	MS method	matched peptides	coverage %	Mascot Score	Theor.pI/ Mr	Exp. Mr	Functional classification
209	Fructose-bisphosphate aldolase, putative	A9P9U1	*Populus trichocarpa*	TMS	8	28	532	8,63/39	48	Energy
	RNA binding protein, putative	B9MV71	*Populus trichocarpa*	TMS	8	18	341	8,42/40	48	Transcription
	Bark storage protein A	Q07469	*Populus deltoides*	TMS	5	17	213	6,84/36	48	Protein destination and storage
210*	Stress-inducible protein, putative	A9PEQ3	*Populus trichocarpa*	TMS	14	29	610	7,58/58	63	Disease/defence
211	Transitional endoplasmic reticulum ATPase, putative	B9I3G9	*Populus trichocarpa*	PMF	14	26	177	5,16/90	70	Intracellular traffic
212*	Probable ATP synthase 24 kDa subunit, mitochondrial	A9PC60	*Populus trichocarpa*	TMS	16	50	736	8,50/28	30	Energy
	Proteasome subunit alpha type, putative	A9PCM5	*Populus trichocarpa*	TMS	13	66	708	5,96/28	30	Protein destination and storage
	Triosephosphate isomerase, putative	A9PE68	*Populus trichocarpa*	TMS	6	33	403	6,00/27	30	Energy
	Proteasome subunit alpha type, putative	B9HDL1	*Populus trichocarpa*	TMS	7	25	311	5,74/27	30	Protein destination and storage
213*	Cysteine protease inhibitor, putative	A9P9T5	*Populus trichocarpa*	PMF	8	44	128	5,76/22	28	Disease/defence

SUPPORTING INFORMATION

Figure 1. Seasonal climatic change. Monthly minimum (T min), maximum (T max) and natural average temperatures (T average) during the year July 2006-June 2007 at *Fondazione Minoprio* thermopluviometric station (Vertemate – ITALY), "cutnitt" locality where *Populus nigra* seedlings were grown in control and stressed condition.

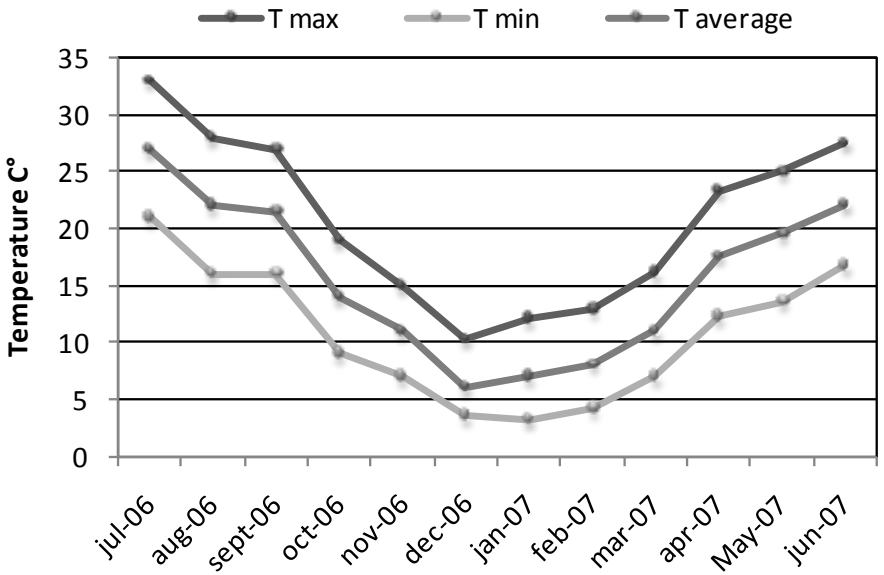


Figure 2. Experimental system. Taproots of 100 seedlings were freed from the first-order lateral roots. (A) For mechanical stress simulation, taproots were bent to an angle of 90°, by using a metallic net, and were analyzed 3 portion of taproots: ABS= above bending stress, BS= bending stress and BBS= below bending stress. (B) Control taproots were linked to a vertical net and was analyzed the central portion of taproots. Lines are equivalent to 5 cm.

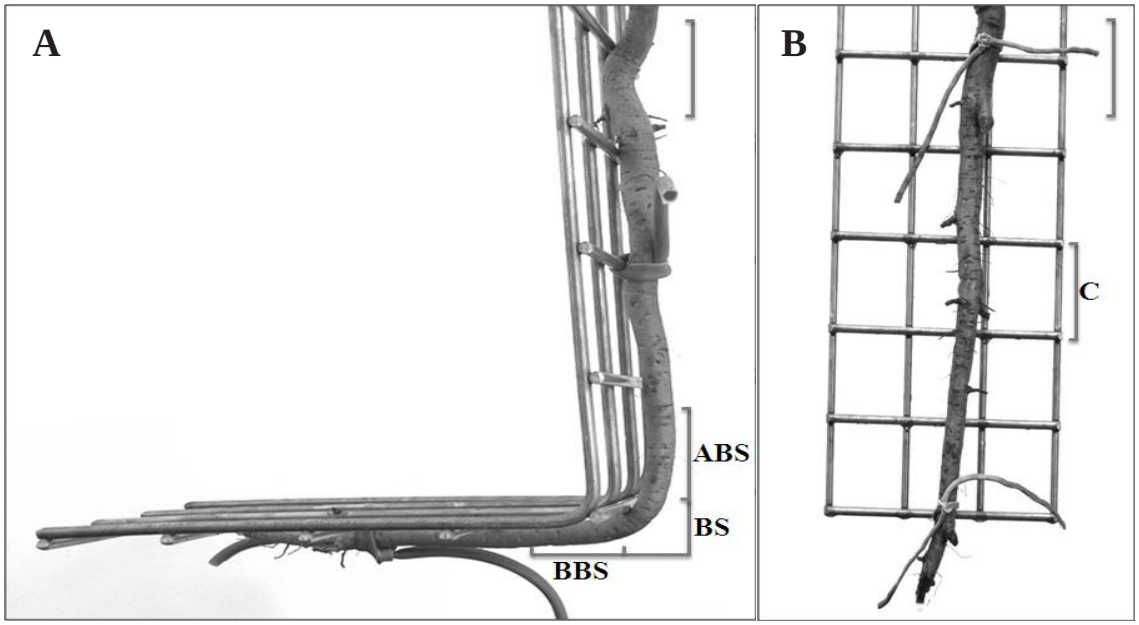
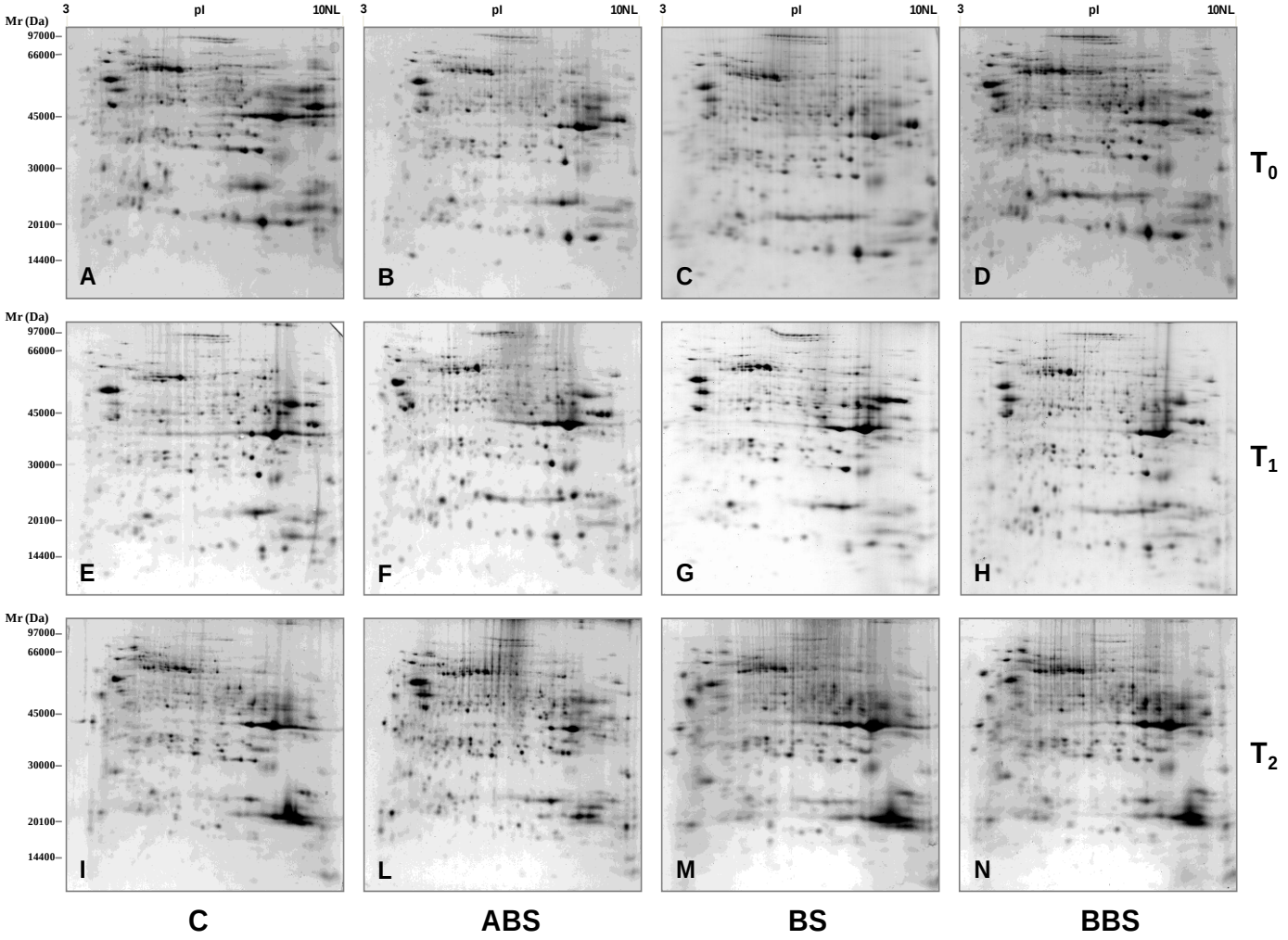


Figure 3. 2-D analysis of the total proteins from *Populus nigra* woody root: Control at T_0 (A), T_1 (E) and T_2 (I); Three sectors of the bent root. ABS at T_0 (B), T_1 (F) and T_2 (L); BS at T_0 (C), T_1 (G) and T_2 (M);. BBS at T_0 (D), T_1 (H) and T_2 (N). Gels were stained with colloidal CBB R-250. An equal amount (400 mg) of total protein extracts was loaded in each gel.



CHAPTER II

Identification and characterization of an activation-tagged gene encoding an AP2/ERF protein that regulates lateral root emission.

Thi chapter reports the study concerning the "Identification and characterization of an activation-tagged gene encoding an AP2/ERF protein that regulates lateral root emission". The researches activities summarised in a paper (*in preparation*), have been focused on the use of a functional genomic approach, for the identification and characterization of a gene encoding an ethylene responsive factor (ERF). Results obatined highlighted the importance of ERF factor in regulating many important processes among which lateral root formation. Based on these results, in the future, this gene could represent an essential resource for functional characterization of complex networks that regulate lateral roots formation from woody taproot.

1. Introduction

Poplar is the established model taxon for dissection of woody perennial growth (see for review Boerjan 2005). Sequenced genome, whole-genome microarray resources, amenable transformation system and high density genetic maps allow functional genetic and genomics dissection of various traits relate to woody perennial biology.

One of the major limitations for gene function analysis of tree species is the lack of mutagenized populations. Because most loss-of-function are recessive and require generations of selfing, these approaches are impractically long with trees (e.g., one generation cycle can last decades). Activation tagging is a dominant approach to produce mutations. Because the dominant nature of the lesions, they can be identified in the primary transformats and thus overcome the limitations imposed by then long generation cycles in trees. The method was originally developed by Walden and colleagues (Hayashi et al., 1992) and further tested by Weigel et al. (2000). A T-DNA vector with four copies of an enhancer element from the constitutively active promoter of the cauliflower mosaic virus (CaMV) 35S gene is randomly inserted into the genome via transformation. Insertion of the vectors can cause transcriptional activation of proximal genes, and, because activated genes. Association of the mutant phenotype with the T-DNA insertion has led for this method to become known as activation tagging. Because the dominant nature of the gene activation can be screened rapidly within the primary transformants without the need for backcrossing/selfing, it represents the best-suited mutagenic strategy currently available for trees.

Activation tagging is particularly suited for dissection of large gene families where functional redundancy makes dissection of individual gene effects with loss-of-function lesions even more difficult. The AP2/ERF family of plant transcriptional regulators is one of the largest, with 147 predicted members in *Arabidopsis thaliana* (Nakano et al., 2006). It is characterized by a DNA-binding domain first identified in APETALA2 (AP2; Jofuku et al., 1994) and the Ethylene-Response Factors (ERF; Ohme-Takagi & Shinshi, 1995). Members of this family have important regulatory functions ranging from growth and development to the response to environmental stimuli (Nakano et al., 2006). However, the exact function for many is unknown.

Zhang et al. (2008) identified 200 AP2/ERF genes in *Populus* which could be classified into four subfamilies: AP2 (Apetala 2), DREB (Dehydration Responsive Element Binding), ERF (Ethylene Responsive Factor) and RAV (Related to ABI3/VP1).

Here, we report the discovery via activation tagging and characterization of a gene encoding putative AP2/ERF transcription factor with unknown function in both Populus and Arabidopsis. We show transgenic, expression and metabolic data suggesting function of this gene in likely auxin-mediated lateral and adventitious root formation.

2. Material and method

2.1. Transformation of poplar with activation tagging vector

We used standard *Agrobacterium*-mediated transformation protocols (Tzfira et al. 1997; Han et al. 2000), INRA717-1B (*Populus tremula* x *alba*) clone and pSKI074 activation tagging vector (Fig. 1; Weigel et al. 2000).

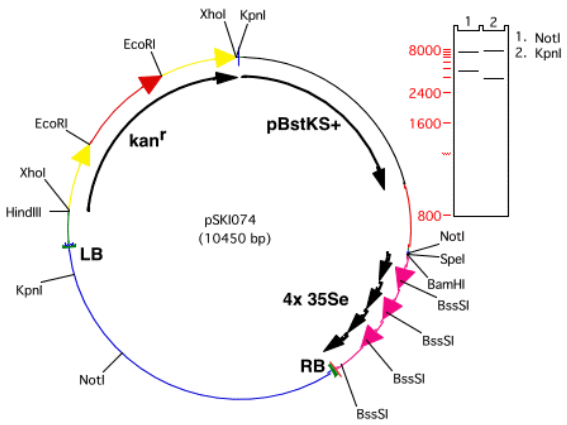


Figure 1. pSKI074 vector used to produce activation tagging lines. Original image from Weigel et al. (2000)

In vitro P. tremula x *P. alba* plants (clone 717-1B4) were sterilely propagated on ½ MS media: 1/2 MS; ½ Murashige and Skoog, (1962) salts and Gamborg's vitamins (Sigma), 1.28 mM 4-morpholinoethanesulfonic acid (MES) pH 5.7, 3 % sucrose, 1 mg/L L-cysteine, 200 mg/L L-glutamine and 0.8 % Phytagar (Invitrogen) in Magenta vessels. Plants were grown in a growth chamber at 16 hr light/8 hr dark photoperiod and 24°C. Prior to co-cultivation with *Agrobacterium*, 0.8 – 1.2 cm leaf and stem explants were placed on Induction Media (IM; MS media salts and Gamborgs vitamins, pH 5.7, 3 % sucrose, 1 mg/L L-cysteine, 200 mg/L L-glutamine, 0.5 % Phytagar, 0.2% Phyatgel (Sigma), 10 µM 1-naphthaleneacetic acid (NAA), 5 µM 6-(γ,γ-dimethylallylamino) purine (2iP) and incubated in the dark at 24°C for 3d. The *Agrobacterium* strain GV3101, containing the plasmids pMP90RK and pSKI074, was grown from -80°C-stored glycerol stocks. A 10 mL overnight culture in Luria-Bertani selection broth (LB; 10 g/L of tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH to 7, 100 mg/L rifampicin, 100 mg/L ampicilin, 50 mg/L gentamycin, and 50 mg/L kanamycin) with 150 rpm shaking at 28 °C

until an O.D. of approximately 0.6 was reached. Bacteria were centrifuged at 1600 x g for 15 min at 4°C and the pellet was resuspended in Liquid IM with 100 μM acetosyringone to an O.D. between 0.3 and 0.4. The explants were removed from the IM plates and incubated in the *Agrobacterium* broth for 60 min, blot dried on sterile filter paper and placed onto Co-culture Media (CM; IM and 100 μM acetosyringone) plates and incubated at 24°C in the dark for 3 d. The explants were then washed at least 5 times in sterile water containing 300 mg/L cefotaxime and placed on Selection Media (SM; IM and 300 mg/L cefotaxime and 50 mg/L kanamycin) and incubated in the dark at 24 °C for 2-3 weeks. The explants were moved to fresh SM, and incubated with a 16 hr/8 hr photoperiod at 24°C and transferred to new SM every two weeks. Calli of 1-2 cm size (2-6 weeks) were transferred to Organogenesis Media (OM; MS Media, 300 mg/L cefotaxime, 50 mg/L kanamycin) containing 0.1 μM thidiazuron (TDZ) for 2 weeks. All subsequent transfers on OM contained 0.01 μM TDZ. Shoots approximately 2-4 cm in height were harvested and placed on Rooting Media (RM: 1/2 MS media with 2% sucrose and 50 mg/L kanamycin for approximately 1 month. These plants were then multiplied as described above and incubated on ½ MS media until the roots and stems appeared healthy enough to transfer to soil. To ensure that each line in the population represented independent transformation events, only one shoot was selected from each explant. Once plants had been rooting for 4-5 weeks they were removed from the Magenta box, and the roots were washed of media under warm water.

2.2. Positioning of the tag in the *Populus* genome

Genomic DNA was isolated from greenhouse grown poplar using Qiagen Plant DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The location of the T-DNA was determined by TAIL-PCR as described by Liu et al. (1995). The following PCR primers were use to generate the four probes for four regions of the pSKI074 activation tagging vector:

pre LB 5'-TGTAGATGTCCGCAGCGTTA-3', 5'-ATCTAAGCCCCCATTTGGAC-3';

post RB 5'-CTCGGGAGTGCTTGGCATT-3', 5'-ATCATCCTGTGACGGAACTTTGG-3';

Kanr5'-GCGTGGCTTTATCTGTCTTTGTATTG-3',5'GGCCTACTTTAATTGCTTCCAC-TGTTA-3';

35S 5'-CGACACTCTCGTCTACTCCAA-3', 5'-TATCACATCAATCCACTTGCTTT-3'.

Three arbitrary degenerate primers were used: A1 [NGTCGA(G/C)(A/T)GANA (A/T)GAA], A2 [GTNCGA(G/C)(A/T)CANA(A/T)GTT], and A3 [(A/T)GTGNAG(A/T)A NCANAGA]. TAIL-PCR products of tertiary reactions were analyzed by electrophoresis on an 0.8% agarose gel. Following the electrophoresis, the specific bands were cut from the gels and purified using Quiaquick kit gel extraction (Qiagen, Valencia, CA) according to the

manufacturer's protocol and sequenced. Recovered sequences flanking the T-DNA were BLASTn against the *Populus* genome sequence and position of the closest gene determined using the *Populus* Genome Browser (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Poptr1_1&advanced=1).

2.3. Sequence analysis and homology

Sequencing was performed using capillary 3100 Genetic Analyzers (Applied Biosystems, Foster City, CA) and ABI Prism BigDye Terminator Cycle Sequencing v2.0 Ready Reaction with AmpliTaq DNA Polymerase (Applied Biosystems Foster City, CA). The poplar *PtaERF003* cDNA was sequenced in both directions.

Sequence homology searches and sequence analyses were performed using the National Center for Biotechnology Information BLAST server. A multiple alignment analysis was performed with ClustalW (http://bioinformatics.ubc.ca/resources/tools/clustalx; Chenna et al., 2003). Phylogenetic trees were constructed using the neighbor-joining (NJ) method and MEGA4 (http://www.megasoftware.net/mega.html; Tamura et al., 2007).

2.4. Vector construction and poplar transformation with the candidate gene

The full open reading frame of the gene was inserted into pK2GW7 binary vector using the GATEWAY (Invitrogen) system and subsequently sequence-verified. The binary vectors were transformed into *Agrobacterium* strain C58 using the freeze and thaw method. *Agrobacterium*-mediated transformation and the same clone INRA717-1BA was used as previously described (Han et al. 2000). Multiple (20+) independent events were produced as described above. Three independent events (named *35S:PtaERF003-1*, *35S:PtaERF003-2* and *35S:PtaERF003-10*) were used to recapitulate the phenotype of the original activation tagging mutant.

2.5. Measurement of adventitious and lateral root length and diameter

Plants were grown on propagation medium (0.25 g l^{-1} MES, 0.1 g l^{-1} myo-inositol, 2.15 g l^{-1} MS, 0.2 g l^{-1} L-glutamine, 20 g l^{-1} sucrose, 1 mg l^{-1} nicotinic acid, 1 mg l^{-1} hydrochloride, 1 mg l^{-1} calcium pantothenate, 1 mg l^{-1} thiamine hydrochloride, 1 mg l^{-1} L-cysteine, 0.5 mg l^{-1} biotin, pH 5.8) containing 5mM indole-3- butyric acid (IBA -Sigma, St. Louis, MO) and no IBA.

Digital images of roots were captured after 2 month of growth by Nikon Coolpix. Changes in adventitious and lateral root number, length and diameter were quantified using the Image J program from NIH. For each genotype and conditions we measured roots from 10 clonally-propagated seedlings.

2.6. RNA Extraction

RNA was extracted from approximately 0.2 g of expanding stems, roots, leaves and apices tissues using a modified Qiagen RNeasy Mini kit protocol (Qiagen, Valencia, CA). Tissues were ground with a mortar and pestle to a fine powder in liquid nitrogen, lysis buffer was added, and the slurry was homogenized using a polytron. A 0.4 volume of 5 m K-acetate was added to the homogenate and incubated on ice for 15 min. The extracts were spun for 15 min at 4°C at top speed in a tabletop centrifuge. A one-half volume of 100% (w/v) ethanol was added to the supernatant, and the mix was applied to the RNeasy mini column. We followed the remaining kit procedures precisely. RNA concentrations were measured using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), and RNA integrity was checked on 1.5% (w/v) agarose Tris-acetate EDTA ethidium bromide gels.

2.7. cDNA synthesis and RT-PCR

cDNA was synthesized using 3.0 µg of total RNA, poly(A) oligonucleotide primer and Superscript III reverse transcriptase (Invitrogen Co., Carlsbad, CA, USA). We used standard PCR conditions and gene specific primers (5'-GGCCAGAC CGCAACAGCGA-3'; 5'-CATT TGCTCAATGTGGTGATC-3'). The amplified fragment was gel purified using the QIAquick Spin kit (Qiagen, Valencia, CA) and sequenced as described above. Ubiquitin (UBQ) cDNA fragment was used as a loading control using the same RT reaction and the following primers: 5'-CTCAAAGTGAAAGGCCAGGATG-3'; 5'-ACTGTCAAAGCTCTTGGTGAG-3'. All PCR reactions were performed in 25 µl volumes using Taq recombinant polymerase (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA prepared from 5 ng of total RNA was used as template in all reactions.

Reaction conditions for thermal cycling were 95 °C for 5 min, 35 cycles of 95 °C for 35 s, 55°C for 35 s, 72 °C for 50 s and 72 °C for 7 min. Two independent biological replicates were run for each sample, each with two technical replications. Results were analyzed using Image J 1.41o software (Wayne Rasbanb National Institute of Health, USA; http//rsb.info.nih.gov/ij). To account for small differences in RNA loadings data was normalized to ubiquitin gene expression.

2.8. Indole-3-acetic acid (IAA) treatments

To deplete endogenous sucrose and auxin, leaves from greenhouse-grown INRA 717-IB4 plants were washed and incubated in 1/2MS liquid media for 24 h under 140 rpm agitation and 22°C.

To measure changes in gene expression as a function of time of auxin treatment leaves were transferred to 1/2MS liquid media supplemented with IAA (indole-3-acetic acid) 1 mM and IAA 10 mM. Samples were taken 0, 1, 4, 8, 20 h pre-treatment (control) and 0, 1, 4, 8, 16, 24 and 48 h post treatment. Following the treatment, leaves were flash frozen in liquid nitrogen and stored at -80^{o}C until processed. Three independent biological replicates were used for all treatment conditions.

2.9. Metabolic profiling

Leaf and fine root samples were fast-frozen on dry ice and stored at -80 ºC until analyzed. After grinding to a fine powder in liquid nitrogen, approximately 40-90 mg of plant tissue (fresh weight) were twice extracted with 5 mL 80% ethanol overnight and then combined prior to drying a 0.5-ml aliquot in a helium stream. Sorbitol (200 µl of a 1 mg/mL aqueous solution) was added before extraction as an internal standard to correct for differences in extraction efficiency, subsequent differences in derivatization efficiency and changes in sample volume during heating. Dried extracts were dissolved in 500 µL of silylation–grade acetonitrile followed by the addition of 500 µL N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce Chemical Co., Rockford, IL), and samples then heated for 1 h at 70 °C to generate trimethylsilyl (TMS) derivatives. After 4 days, 1-µL aliquots was injected into an Agilent Technologies Inc. (Santa Clara, CA) 5975C inert XL gas chromatograph-mass spectrometer, fitted with an Rtx-5MS with Integra-guard (5% diphenyl/95% dimethyl polysiloxane) 30 m x 250 µm x 0.25 µm film thickness capillary column. The standard quadrupole GC-MS is operated in the electron impact (70 eV) ionization mode, with 6 full-spectrum (50-650 Da) scans per second. Gas (helium) flow is set at 1.37 mL per minute with the injection port configured in the splitless mode. The injection port, MS Source, and MS Quad temperatures are set to 250 ºC, 230 ºC, and 150 ºC, respectively. The initial oven temperature is held at 50 ºC for 2 min and is programmed to increase at 20 ºC per min to 325 ºC and held for another 11 min, before cycling back to the initial conditions. All peaks above a set minimum threshold were integrated, whether or not their identity was known. A large user-created database (>1200 spectra) of mass spectral EI fragmentation patterns of TMS-derivatized compounds were used to identify the metabolites of interest to be quantified (i.e., those that approached statistically significant thresholds, relative to the control plants) were reintegrated and reanalyzed using a key selected ion, characteristic m/z fragment, rather than the total ion chromatogram, to minimize integrating co-eluting metabolites. Peaks were quantified by area integration and the concentrations were normalized to the quantity of the internal

standard (sorbitol) recovered, amount of sample extracted, derivitized, and injected. The response of the each transgenic line was divided by the response of the appropriate control line to determine a relative transgenic response. The average relative response of seven transgenic lines was then presented, and contrasted with the average relative response of four phenotypically high expressing transgenic lines.

3. Results

3.1. Isolation and characterization of a poplar mutant with increased adventitious/lateral root formation

We identified an activation tagging event with increased lateral and adventitious root formation (Fig.2; Table I).

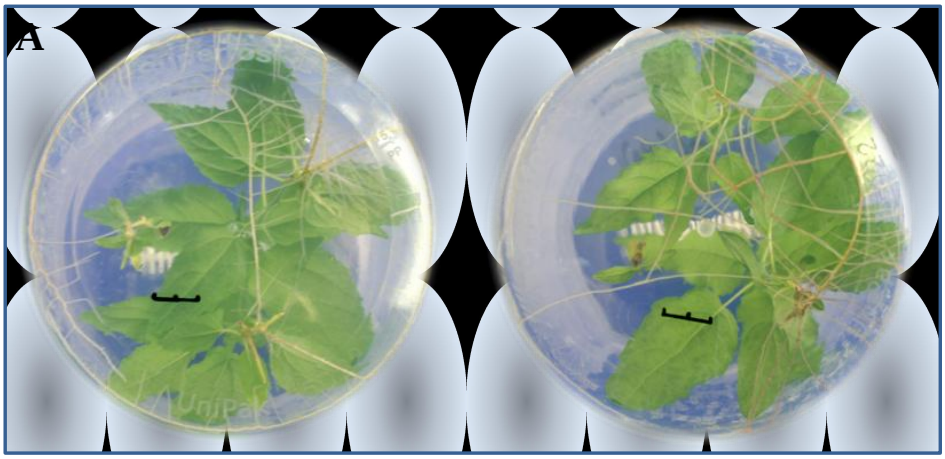


Figure 2. Phenotypic effects of *PtaERF003* up-regulation in poplar seedlings. A) WT and B) original mutant pictures of representative plants that were grown for 2 months in MS media without IBA as described in the Materials and methods.

In order to identify the gene that conditions the mutant phenotype we first amplified and sequenced a genome fragment flanking the left border of the tag. Recovered sequence flanking the T-DNA was positioned in the *Populus* genome on LG_XVIII: 6025980-6026040 bp (Fig.3).

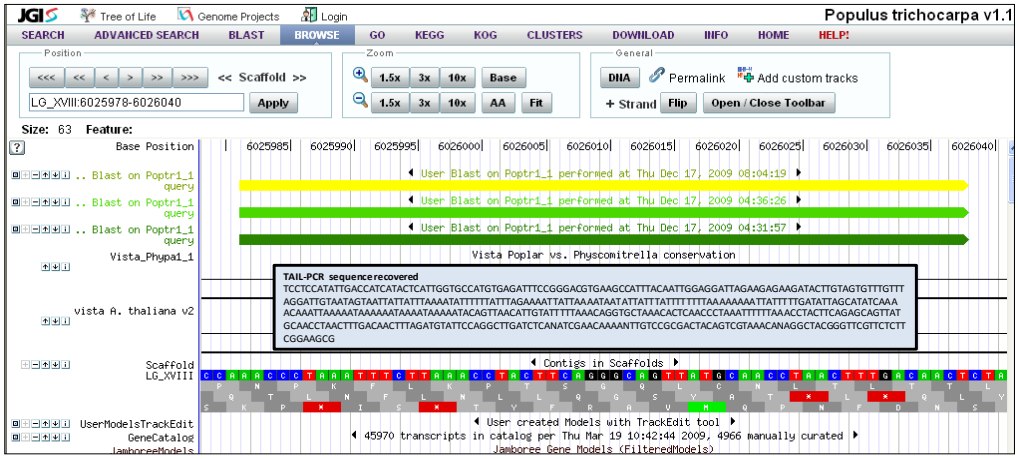


Figure 3. Position of the sequence identified by TAIL-PCR in the poplar genome. Recovered sequence flanking the T-DNA (reported in the black squared) was positioned in the *Populus* genome (http://genome.jgi-psf.org/cgi-bin/runAlignment? db=Poptr1_ 1&advanced=1) on LG_XVIII: 6025980-6026040 bp.

Using the poplar genome browser (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Poptr1_1&advanced=1), we searched for predicted gene models within region upstream and downstream from the insertion sites. One putative candidate genes corresponding to model **extfgenesh4 _Kg_0016** was found in this region on LG_XVIII: 6019203-6020290 bp (Fig. 4).

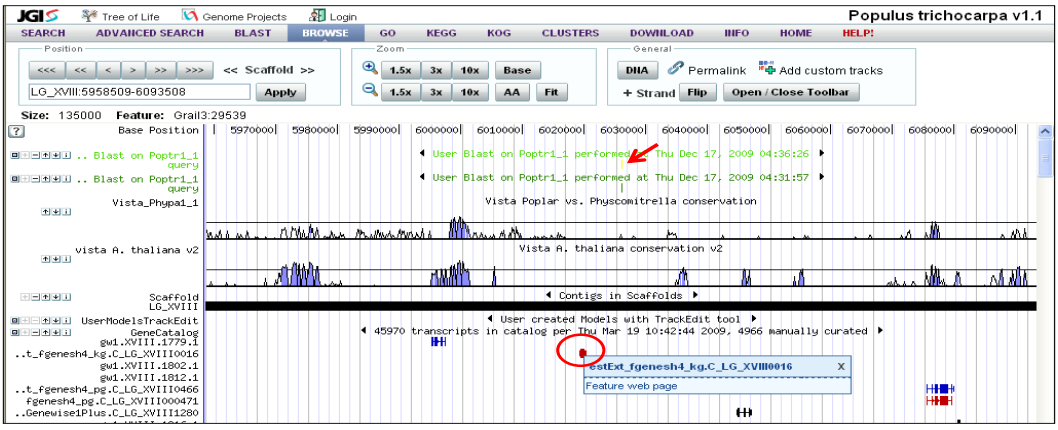


Figure 4. Position of the sequence identified by TAIL-PCR (reported with red arrow) in the poplar genome sequence (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Poptr1_1&advanced=1). Candidate genes (red circles) within about 5000 bp upstream region of the activation tagging inserts.

According to JGI annotation, the gene encodes a pathogenesis-related, ethylene-responsive transcriptional factor of the AP2/ERF superfamily (Fig. 5).

The AP2/ERF superfamily is plants-specific group of transcription factors defined by the AP2/ERF domain, which consists of about 60 to 70 amino acids and is involved in DNA binding. Based on the number of AP2/ERF domains and the function of the genes, AP2/ERF members were classified into four subfamilies - AP2, DREB, ERF and RAV. The AP2 family proteins contain two repeated AP2/ERF domains, the ERF family proteins contain a single AP2/ERF domain, and the RAV family proteins contain a B3 domain, which is a DNA-binding domain conserved in other plant-specific transcription factors, including VP1/ABI3, in addition to the single AP2/ERF domain (Riechmann et al., 2000).

It has been demonstrated that the AP2/ERF proteins have important functions in the transcriptional regulation of a variety of biological processes related to growth and development, as well as various responses to environmental stimuli.

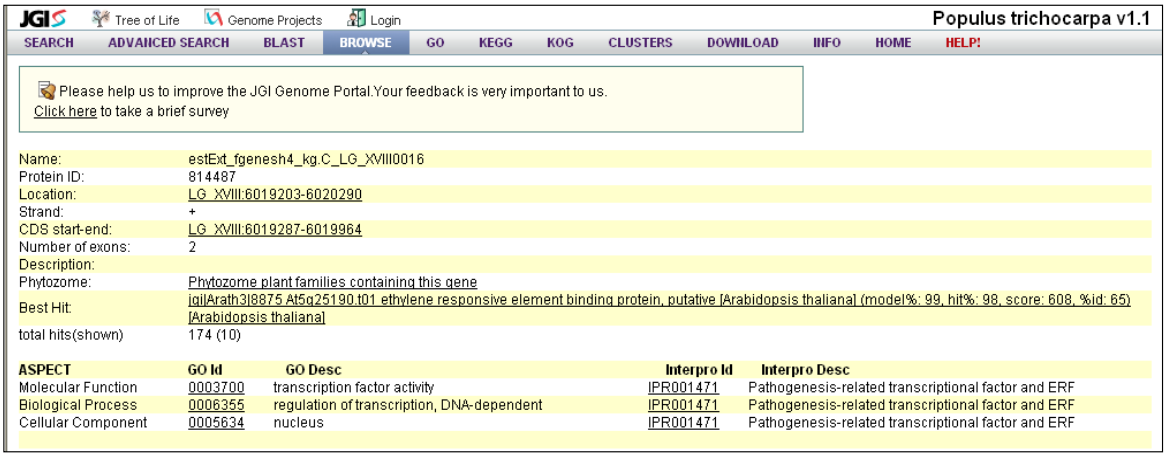


Figure 5. Annotation of the candidate tagged gene represented by extfgenesh4 _Kg_0016 model.

Following the initial identification of the tobacco ERFs (Ohme-Takagi & Shinshi, 1995), other proteins of the ERF family in various plant species were characterized and implicated in many diverse processes including hormonal signal transduction (Ohme-Takagi & Shinshi, 1995), response to biotic (Yamamoto et al., 1999; Gu et al., 2000) and abiotic stresses (Stockinger et al., 1997; Liu et al., 1998; Dubouzet et al., 2003), regulation of metabolism (van der Fits & Memelink, 2000; Aharoni et al., 2004; Zhang et al., 2005), and root/shoot development (van der Graaff et al., 2000; Banno et al., 2001; Chuck et al., 2002). Using the availability of the Arabidopsis genome sequence Nakamo et al. (2006) attempted a full characterization of the family. Out of 147 AP2/ERF genes, 122 genes belong to the ERF family. Phylogenetic analysis identified 10 major groups (I-X) within the superfamily. The poplar candidate gene, which we discovered in this study shows highest homology to AtERF003 (At5g25190) in *Arabidopsis*

from group V. For consistency with the *Arabidopsis* annotation we named the candidate gene PtaERF003 (*Populus tremula* x *alba*).

Arabidopsis group V consists of two subgroups, Va and Vb (Fig. 6). The four genes in subgroup Va are closely related to each other, sharing two motifs, CMV-1 and CMV-2, in the C-terminal regions. AtERF#003 contains CMV-2 and part of CMV-1. Only a single gene, AtERF#002, which does not contain these motifs, was assigned to subgroup Vb. Two motifs, CMV-3 and CMV-4, were identified in AtERF#002 through comparison with the rice ERF genes in subgroup Vb. Recently, two independent reports demonstrated that the overexpression of WIN1/SHN1 (AtERF#001) results in the enhanced accumulation of epidermal wax (Aharoni et al., 2004; Broun et al., 2004). SHN2 (AtERF#004) and SHN3 (AtERF#005) shared a similar function with WIN1/SHN1 (AtERF#001; Aharoni et al., 2004; Broun et al., 2004). Aharoni et al. (2004) also predicted that these three ERF proteins would have two conserved motifs corresponding to motifs CMV-1 and CMV-2, respectively. Their preliminary results showed that the over-expression of AtERF#003 (At5g25190) did not result in the typical morphological *shn* phenotype (Aharoni et al., 2004) suggesting that this gene plays distinct function. There is no information regarding the function of AtERF#002. None of the proteins in this group in Arabidopsis was found to be associated with root formation.

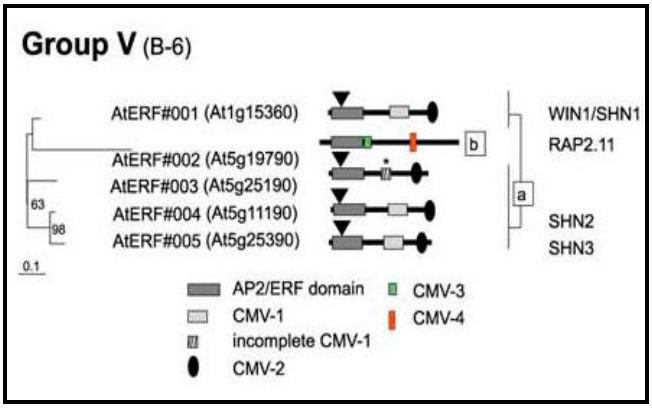


Figure 6. Phylogenetic relationships among the group V of *Arabidopsis* ERF genes (Original image Nakuma et al., 2006).

3.2. The candidate gene is up-regulated in the mutant *larD1* plant

In order to determine if the gene neighboring the T-DNA insertion was activated by the presence of the 35S enhancers in the activation tagging, we studied the expression of the gene in the mutant *larD1* and WT-717 plants. Primers were designed based on candidate gene EST

sequences from *P. tremula* as well as predicted gene model sequences in *P. trichocarpa*. We also wanted to study if the activation tagging resulted in ubiquitous over-expression of the gene or merely up-regulated that native expression pattern. We therefore extracted RNA from WT and mutant *larD1* leaves, stems, roots and apices. A housekeeping gene, ubiquitin (UBQ), was used in every reaction as a normalization control of mRNA concentrations and stability (Brunner et al, 2004). PtaERF003 transcript was detected in all four organs in WT 717-1BA plants (Fig.7). Consistent with the observed phenotype in the *larD1* mutant, highest expression was found in roots. We found a significant up-regulation of PtaERF003 in all studied tissues, suggesting that the presence of the tag indeed led to transcription activation of the gene (Fig 7). Despite the activation of the gene in *larD1*, the expression pattern across the different organs was similar. The gene was most abundant in roots followed by leaves and showed lower and approximately similar expression levels in stems and apices. Therefore the conclusion of this experiment is that the candidate gene was up-regulated but the native expression was preserved. Because of the position of the tag and activation of the gene we hypothesized that PtaERF003 up-regulation is conditioning the mutant *larD1* phenotype.

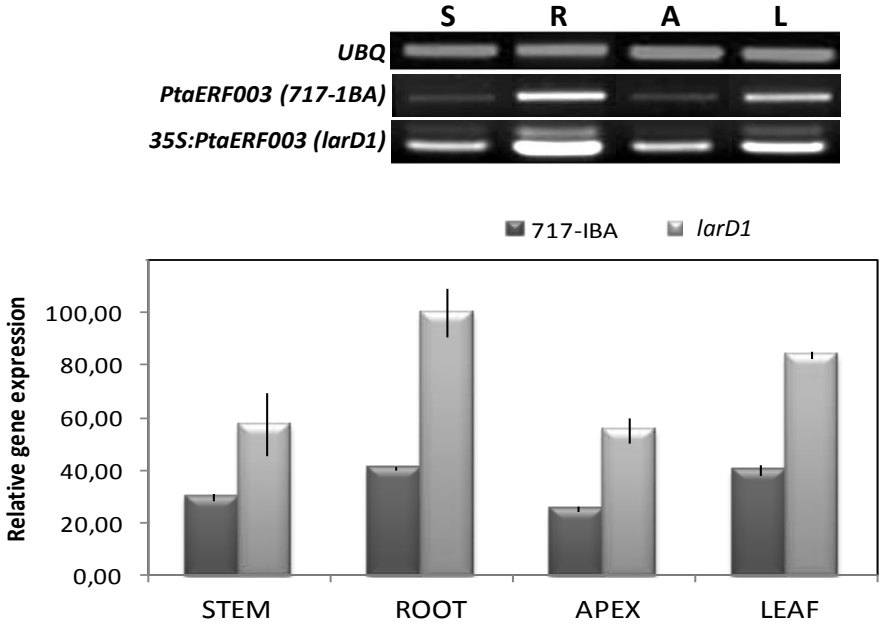


Figure 7. *PtaERF003* expression in three different organs of WT-717 and *larD1* plants. Stem (S), root (R), apex (A) and leaf (L) tissues were harvested from 717-1BA (dark grey) and *larD1* (light grey) mature plants (eight-weeks-old). Expression was studied by RT-PCR as described in Materials and methods. Expression levels of PtaERF003 were normalized to ubiquitin (*UBQ*) expression level, using the gel images and ImageJ software. Bars represent means and standard errors of at least two independent biological replications.

3.3. PtaERF003 belongs to a small subfamily

Using the poplar genome sequence we identified 10 close members of PtaERF003. Therefore the size of the subfamily relative to *Arabidopsis* (5 members) has doubled. *Populus* has experienced recent genome-wide duplication that has occurred post divergence with *Arabidopsis* lineage. Therefore many gene families are double in size while others have lost the duplicate copies. It has been speculated that the retention of the duplicates is associated with neofunctionalization needed to accommodate unique woody perennial developmental and adaptive processes. Phylogenetic analysis of poplar, *Arabidopsis* and one functionally-characterized *Medicago* protein indicate three likely functionally divergent distinct lineages in the subfamily (Fig.8). Proteins from group one are involved in regulation of suberin/cutin biosynthesis. Group three contains only two proteins with no poplar member both involved in processes related to root development. Group2 predominantly consists of poplar proteins with only one *Arabidopsis* protein AtERF003 whose function is yet unknown.

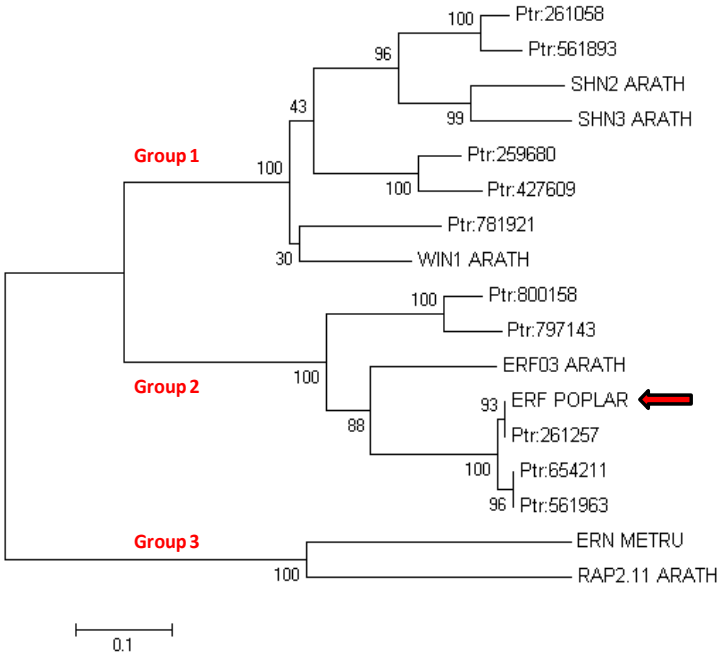


Figure 8. Phylogenetic analysis of poplar, *Arabidopsis* and *Medicago* close ERF protein orthologs. The tree was generated using MEGA4 software (http://www.megasoftware.net/). Sites containing alignment gaps were excluded from further analysis, and the distance between sequences represent the proportion of amino acid sites at which the two sequences compared are different. The tree was constructed using the neighbor-joining method. The bootstrap percentage indicated at each joint point was created from 1,000 iterations. Nodes with less than 50% bootstrap confidence were collapsed. The tagged poplar gene is indicated with an arrow.

3.4. Recapitulation of the mutant phenotype

To recapitulate mutant phenotype we fused PtaER0003 cDNA to strong *CaMV35S* promoter and transformed into the same 717-1BA background. We recovered multiple independent transgenic events which were PCR-verified for the presence of the transgene. We also validated the overexpression of *PtaERF003* in three independent events (35S:PtaERF003-1, 35S:PtaERF003-2, 35S:PtaERF003-10) that were selected for further analysis (Figure 9). *PtaERF003* transcript was highly overexpressed in all three events. In two events (35S:PtaERF003-2, 35S:PtaERF003-10) the transgene expression levels exceeded these found in the original *larD1* mutant.

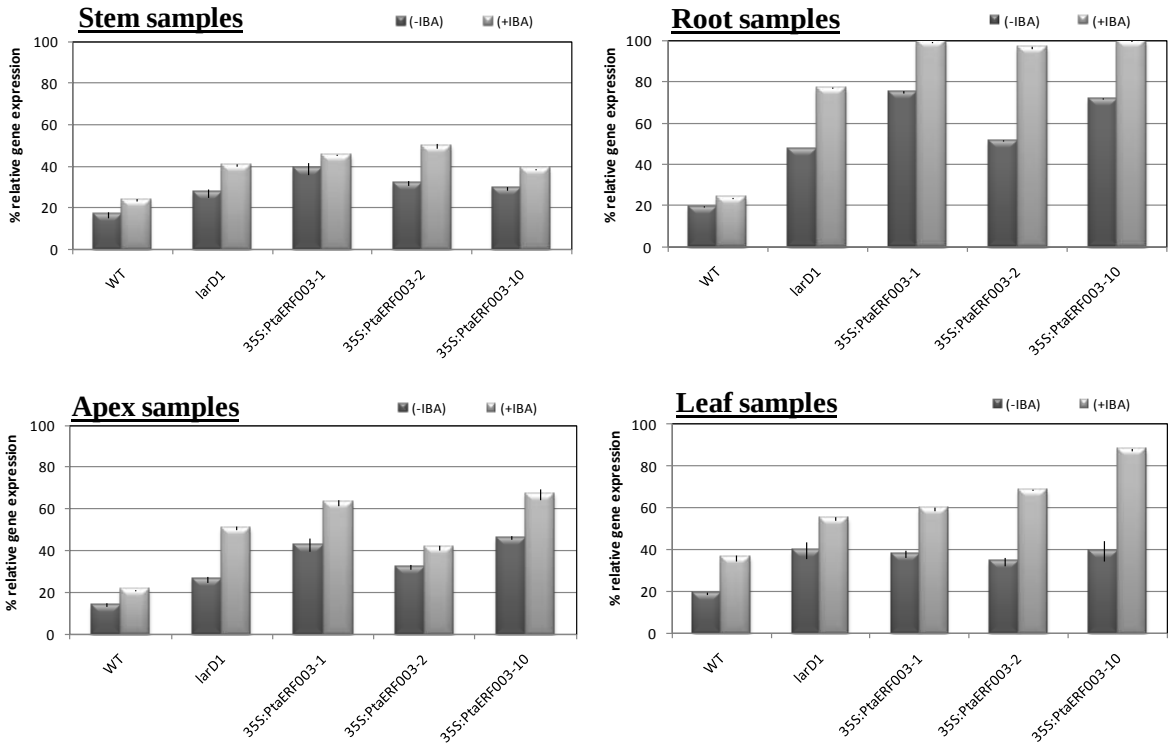


Figure 9. Expression of PtaERF003 in WT (717-1BA), larD1 and three independent recapitulation events transformed with 35S:PtaERF003 (#1,2 and 10). Total RNA was extracted from roots of eight weeks-old mature plants grown on 5 mM IBA (light grey) and without IBA (dark grey). RT-PCR analyses were performed as described in the Material and method. The graphs represent relative expression levels normalized to expression of an ubiquitin-like gene. Bars and standard errors represent means of at least two biological replications

We studied root development in the three selected events and compared it to WT-717 and *larD1* (Fig.10, Table I). We studied a number of root characteristics including length and diameter of the adventitious roots, and number, length and diameter of lateral roots of the first orders (Table I).

Samples	Adventitious lenght (cm)		n°. Lateral roots		Lateral roots lenght (cm)		Lateral roots diameter(cm)		Fold change n°. Lateral roots		Fold change n°. Lateral roots
	(-) IBA	(+) IBA	(-) IBA	(+) IBA	(-) IBA	(+) IBA	(-) IBA	(+) IBA	(-) IBA	(+) IBA	(+)IBA/(-)IBA
WT	11,765	8,053	23,5	24,5	1,481	1,158	0,053	0,048	-	-	1,12
larD1	9,231	5,872	55,0	68,0	2,467	1,128	0,063	0,090	2,34**	2,77*	1,42**
35S:PtaERF003-1	13,081	9,060	64,5	72,0	1,896	1,531	0,052	0,068	2,74**	2,93**	1,29
35S:PtaERF003-2	6,303	12,109	74,5	79,5	1,012	1,222	0,072	0,095	3,17**	3,24**	1,32
35S:PtaERF003-10	12,140	14,921	62,5	75,0	1,856	1,496	0,066	0,086	2,65**	3,06**	1,31

Table I. Roots development of 717-1BA (WT), original mutant (*larD1*) and 3 independent events (35S: PtaERF003-1, 35S:PtaERF003-2 and 35S:PtaERF003-10). Plants were grown for 2 months in MS +/- IBA 5 mM . Values represent mean of 10 seedlings and fold change (-) IBA and (+) IBA represent the increase or decrease of new lateral root formation numbers in CaM35S mutants compared to WT, grown in absence and in presence of IBA, respectively; fold change (-)IBA/(+)IBA represents the increase or decrease of new lateral root numbers rate in samples of the same typology, growing in absence and presence of IBA. * and ** indicate a significant difference at $P < 0.01$ and $P<0.05$, respectively, and – indicates the data absence.

Consistent with the *larD1* root phenotype, all three events over-expressing PtaERF003 showed significant ($P<0.01$ or $P<0.05$) increases in number of lateral roots (Table I). In fact the higher over-expression in the recapitulation events led to even greater number of lateral roots than in the original mutant. Although some decrease in primary (adventitious) root length was detectable in original mutant and one of the recapitulation events (#2), the differences were not of the magnitude of LR increases and two of the recapitulation events (#1, 10) showed primary root lengths larger than WT. Therefore, increased LR proliferation cannot be explained with decrease in primary root growth. Although some other root parameters did show changes, they were not of the magnitude and consistency of LR proliferation. Interestingly, presence of auxin in the media even further increased the differences between LR development in WT and the *larD1*/PtaERF003 over-expressing transgenic plants.

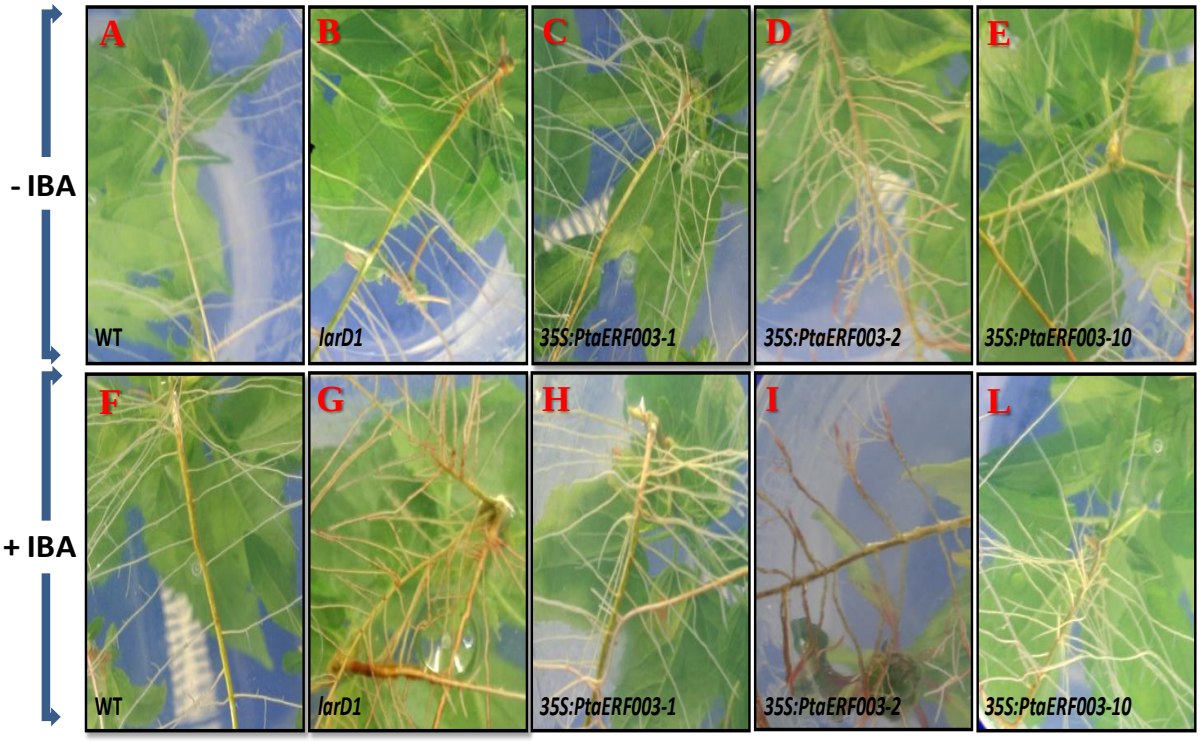


Figure 10. Phenotypic effects of *PtaERF003* up-regulation in the original mutant and recapitulation events. Pictures were taken of representative plants that were grown for 2 months in MS media with or without IBA as described in the Materials and methods.

3.5. PtaERF003 is early auxin-responsive gene

Because of the leading role of auxin in lateral root initiation and emergence and the enhancement of LR phenotypes in presence of auxin (Table I) we decided to test if *PtaERF003* is regulated by auxin. Accumulation of *PtaERF003* mRNA was quantified in poplar WT leaves before and after IAA treatment (Fig. 11). Leaves were first depleted of auxin by floating in nutrient media. During this period we monitored the expression of the gene and applied auxin when the expression of *PtaERF003* leveled off (~20 hr) suggesting of complete auxin depletion. We then applied auxin in two levels (1mM and 10mM) and followed the response up to 48 hr after the treatment. *PtaERF003* transcript abundance peaked early at 1 hr post IAA treatment then gradually decreased as early as 4 h post treatment but stayed at higher than pretreatment levels for the whole monitored period (Fig. 11). The expression levels were higher in the 10mM IAA treatment suggesting that the observed responses at the relatively high 1mM IAA concentrations are not saturated.

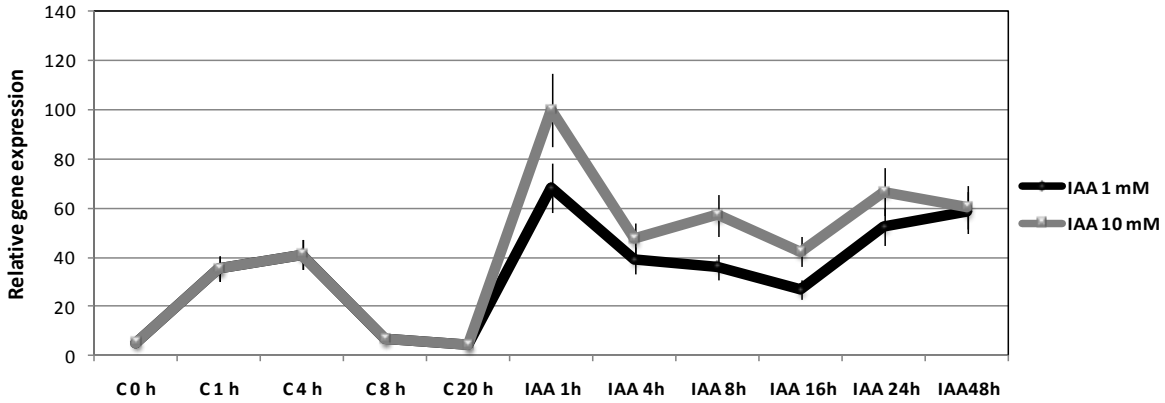


Figure 11. *PtaERF003* auxin responsiveness. Leaves segments of 717-1BA clone were incubated in ½ MS nutrient medium to deplete of endogenous auxin. After 20 h, two IAA treatments were performed: the first (reported in black) using 1mM IAA and the second (reported in grey) with 10 mM IAA. Expression of PtaERF003 gene was analyzed by using RT-PCR and normalized to expression of ubiquitin gene. Three biological replicates were used in each time point.

3.6. Metabolic profiling

While traditional approaches to quantifying small molecules have targeted single metabolites, metabolic profiling provides a snapshot of the levels of many small molecules within a tissue and how the levels change under various circumstances, including different genetic modifications.

We therefore next sought to determine whether using metabolic profiling we can detect compositional changes correlated with *larD1* root phenotype. Only metabolites that were increased by >1.5 or decreased to <0.6 are shown. Amino acids, sugars and secondary metabolites intermediates were detected to be significantly changed in the mutant and overexpression transgenics compared to control transgenics plants expressing reporter GUS gene.

L-(U-'4C)-5-oxoproline (pyrollidone carboxylic acid or pyroglutamic acid) increased in both *larD1* and recapitulation events while asparagine and ornithine increased only in original *larD1*mutant while decreased in recapitulation events (the asparagine was almost unchanged in 35S:ERF003-2).

TCA cycle intermediate, malic acid, were less abundant in 35S:ERF003-1. Unknown carbohydrate and sucrose-6-phospate showed similar changes.

Many of the nonstructural carbohydrates, fructose, galactose, glucoside and glucose were enriched in the *larD1* and the three recapitulation events (Table II). Moreover, the myoinositol,

the initial precursor for the formation of uronic acids in plants, was also slightly increased in larD1 and the three PtaERF003 overexpressing plants (Table II).

The (+) catechine, a phytoalexin important in plant defense, was increased in all PtaERF003 upregulated plants with a maximum of abundance in original mutant. Finally the salicortin, salicylates synthesized from phenylalanin via the shikimate pathway (Zenk, 1967), were also more abundant in *larD1* and 35S:ERF003-10 but less abundant in the other two independent events (Table II).

Roots samples	*larD1*	35S:PtaERF003-1	35S:PtaERF003-2	35S:PtaERF003-10
oxo-proline	1,82	1,71	7,80	2,51
asparagine	6,76	**0,73**	1,02	**0,75**
ornithine	14,61	**0,30**	**0,58**	**0,45**
fructose	1,44	1,07	6,31	3,29
galactose	1,57	1,26	6,23	2,57
glucose	1,78	1,24	5,19	2,47
carbohydrate	2,17	**0,90**	3,87	1,51
glucoside	7,77	1,24	3,46	2,95
malic acid	5,03	**0,95**	2,21	1,14
sucrose-6-phosphate	1,92	**0,87**	1,96	1,19
myoinositol	1,07	1,02	2,17	1,29
(+) catechin	13,33	1,75	1,85	2,04
salicortin	37,75	**0,45**	**0,22**	2,04

Table II. Metabolite concentrations were determined by GC/MS from roots of 717-1BA, *larD1*, 35S:ERF003-1, 35S:ERF003-2 and 35S:ERF003-10. Concentrations were compared with the levels of 717-1BA. Values reported in bold character represent the 35S mutant metabolites with negative fold change compared with 717-1BA.

4. Discussion

Gene discovery techniques based on functional genomics tools like for example microarrays require equally matched throughput resources for functional genomics characterization. Expression and sequence analysis are rapid and valuable tools for initial identification and characterization of gene sequences. However functional analyses through mutagenesis or transgenics remain the gold standard for functional gene characterization (Meinke et al, 1998). Loss-of-function or knock-down approaches using RNAi or artificial micro RNAs (miRNAs) provide the ultimate proof of gene(s)' roles but suffer significant limitations. For example, characterization of genes within families is difficult because gene redundancy precludes functional analyses by masking the knock-out of close family member (Nakazawa et

al, 2003). Furthermore, loss-of-function lesions are typically recessive and require rounds of selfing to expose the lesion's effect. Such approaches are therefore impractical for species that can not tolerate selfing and/or have long generation cycles like most woody perennials. Thus gain-of-function dominant approaches like activation tagging may be better suited for gene discovery in species like *Populus* (Busov et al., 2003; Harrison et al, 2007).

Using activation tagging, we have identified and characterized a novel gene from *Populus*. To our knowledge this is the first forward discovery of a gene that affects root morphology in any tree species. In addition to its novelty as 'tree' gene, the most obvious ortholog in *Arabidopsis* was similarly not characterized. Therefore we demonstrate that using these techniques in *Populus* we can start provide functional analysis of genes that are of completely unknown function in any plant species and therefore enable insights in plant development in a broader sense.

Leveraging the power of the poplar genome and availability of the tag as a molecular beacon we could rapidly and efficiently link the phenotype with the putative affected gene. Therefore this technique as a means of forward gene discovery approaches are only practical in a genome is available. Using expression analyses and retransformation experiments we show that the increased lateral roots phenotype in the *larD1* mutant result from and activation of a gene on LG XVIII corresponding to extfgenesh4 _Kg_0016 model and encodes putative transcription factor of the AP2/ERF superfamily. Our molecular characterization experiments showed that the tag does not constitutively activate the gene but rather up-regulates its native expression pattern. In WT the gene was predominantly expressed in roots. Similar pattern was observed *larD1* but at a much higher expression level. This finding suggests that the mutant phenotype is not result of ectopic expression but rather a result of enhanced native expression and function. The fact that the gene is predominantly expressed in roots and the activation causes root-related phenotype is also in support of a native role of this gene in root formation process.

The poplar candidate gene encodes protein that showed highest sequence homology to AtERF003 (At5g25190) from *Arabidopsis* (Nakamo et al., 2006). For consistency the gene was named PtaERF003. AP2/ERF proteins constitute one of the largest gene families in plants with at least 126 members in *Arabidopsis* and 147 in *Populus*. As mentioned above activation tagging is particularly valuable in identification of genes from gene families where loss-of-function phenotypes are masked by significant functional redundancy within the family. Our work validates the power of activation tagging for identification of genes form large gene families.

Similar to the original mutation, the retransformation experiments under constitutive 35S promoter showed phenotypes largely linked to root development and particularly lateral root formation. The increased lateral root density in the mutant was even further enhanced in the recapitulation events. This is likely as we show due to the higher expression of the gene in these events. Our experiments show that the differences with respect to lateral root density were further accentuated when plants were grown on auxin containing media. We further show that the gene was rapidly (1 hr) and highly responsive to auxin and that responsiveness increased at very high (10mM) concentrations suggesting of high saturation level of the transcription response. Although it is premature to speculate about the mechanism of this gene's action, our experiments suggest that gene is likely playing role through the auxin signal transduction pathway. This is not surprising as auxin plays major role in lateral root formation at both the initiation and growth phases (Zimmerman & Hitchcock, 1942; Estelle & Somerville, 1987; Hobbie & Estelle, 1995; Monroe-Augustus et al., 2003).

To better understand the molecular mechanisms that influence the observed phenotypic alterations we used metabolic profiling. Results of metabolic profiling show that PtaERF003 is a key transcription factors whose up-regulation leads to major changes in aminoacids, carbohydrates and hormones pathways.

In particular, mutants showed an increase, in the soluble sugar content, polysaccharide concentration, salicortin, (+) catechin, myo-inositol, malic acid and sucrose-6 phophate. The concentrations of oxo-proline, asparagines and ornithine aminoacids was increased in *larD1*original mutant compared to WT roots. Oxo-proline is synthesized from either glutamic acid (or glutamine) or ornithine (Mestichelli et al., 1979). Higher than normal utilization rates for oxo-proline was noted in mutant roots leading to increased free amino acid production and interconversion. These findings suggest that protein catabolism prevails over protein synthesis in the mutant plant which may be interpreted as a mechanism to sustain the increased growth of lateral roots in the *larD1* plants.

Salicortin is a salicylic acid intermediates is not generally involved in plant growth and development and is mainly involved in pathogen defense (Close & McArthur, 2002). Gutierrez et al. (1998) shown that application of the test levels of salicylic acid to soybean plants positively influenced their growth pattern (shoot and root length, fresh and dry weights of shoots and roots and leaf area) increasing pigments content as well as the rate of photosynthesis (Zhao et al., 1995; Sinha et al., 1993). Furthermore ERF proteins are often found to mediate stress responses including pathogen attack. Therefore it is logical that PtaERF003 may stimulate production of defense compounds. It may be speculated that the increased lateral root production regulated by

PtaERF003 may be part of a concerted broader mechanism to counteract stress via various mechanisms including production of defense or stress-related chemicals.

Myo-inositol is commonly used in the plants culture medium and it is considered as a promoting or even an essential medium component in many types of plants tissue cultures (Loewus & Dickinson, 1982) because involved in: signal transduction, the transport of indole acetic acid (IAA), the control of IAA-induced growth and cell elongation (Loewus & Murthy, 2000) and the transport of cytokinins (Gur et al., 1987). Therefore increased concentrations of myo-inositol may indicate increased demands for these hormones during LR formation in the mutant plant.

Catechins have been implicated to have a possible protective role against insect predators, injuries, and nitrogen detoxification (Roepenack-Lahaye et al., 2003; Kanazawa & Sakakibara, 2000; Hennion et al., 2000; Swiedrych et al., 2004). They have been shown to promote plant tissue growth (Protacio et al., 1992); somatic embryogenesis from in vitro cultures (Kuklin & Conger, 1995) and flowering (Khurana et al., 1987). Catechins inhibit indole-3-acetic acid oxidation and enhance ethylene biosynthesis (Elstneret al., 1976; Vrchotová et al., 2004).

In conclusion, the activation tagged population described here could be a valuable gene in production tissue-specific marker lines and in applications requiring lateral root gene expression or suppression and could be represent a tremendous resource for functional characterization of all participants at the complex network that regulate the woody lateral root formation. The novelty and significance of the mutant and gene identification warrants further investigation into the broader mechanisms of this function through knock-down, promoter analysis and microarray experiments.

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