GENETIC ANALYSIS OF EARLY ADVENTITIOUS ROOT DEVELOPMENT IN A PSEUDO-BACKCROSS POPULATION OF *Populus*

By

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To my Mom, Dad, and sisters

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The ability of stem cuttings to form adventitious roots (AR) is critical for propagation of trees species that are cultivated for bioenergy, pulp and paper, and timber production. However, genes and molecular mechanisms that control AR formation are largely unknown. To identify quantitative trait loci (QTLs) that regulate AR development in poplar – a tree model species – we measured rooting response in a pseudo-backcross population derived from a cross between a hybrid female *Populus trichocarpa* × *Populus deltoides*, and an unrelated male parent *P. deltoides*. Apical cuttings were collected from 234 individuals and grown in a hydroponic system for 18 days. The number of ARs was recorded daily. After 18 days, roots were harvested and scanned to measure length, volume, and surface area of total, primary (first order) roots, and root branches, as well as dry biomass and average diameter. QTLs for number of roots and root architectural traits were identified using composite interval mapping performed on the progeny's mother map. Significant QTLs for most of these traits mapped consistently in two regions of linkage groups II and XIV.

Next, each genotype was categorized depending on the allele carried in each of the two main QTL regions identified previously. Genotypes carrying *P. trichocarpa*

alleles in both QTL regions generally had higher number of roots than genotypes carrying the *P. deltoides* alleles. Three genotypes were selected within each QTL category for gene expression analysis using whole-transcriptome microarrays, at multiple time points.

This study enabled the detection of significant differences in transcript abundance across time points and between extreme genotypes, and identification of candidate genes that control number of ARs. Of particular importance among these was the gene *superroot2* (SUR2), which was more highly expressed in poor rooters. Mutants for SUR2 have been reported to cause overproduction of auxin and higher number of adventitious roots in Arabidopsis. This study also allowed insights into the genes expressed during different phases of AR formation in *Populus*.

CHAPTER 1
LITERATURE REVIEW

Introduction

Species of the genus *Populus* comprise hardwood trees that belong to the Salicaceae family and include poplars, cottonwoods and aspens (Eckenwalder, 1996). Generally referred to as poplars (Heilman, 1999), the genus *Populus* consist of 29 species grouped into six sections: *Abaso, Turanga, Leucoides, Aigeiros, Tacamahaca* and *Populus* (Eckenwalder, 1996). Although there is no doubt about the existence of the genus *Populus,* the definition of species within the genus is still not well established, as well as their classification into each section. Problems in defining species and delineating sections are due to the great morphological diversity and extensive interspecific hybridization (Eckenwalder, 1996, Zsuffa, 1975). Despite these difficulties, poplar species have been traditionally placed into sections according to morphological and reproductive characteristics, as well as by their hybridization potential (Zsuffa, 1975). Species within a section can freely intercross, but crosses of species of different sections are more difficult to occur.

One of the remarkable characteristics of *Populus* species is their adaptability to a variety of ecological habitats. Poplars are widely distributed in the Northern Hemisphere, from far north to the tropics (Dickmann, 2001, Dickmann and Kuzovkina, 2008). They are found naturally in riparian sites, but also in well drained uplands, with few species doing well in both types of sites (Farmer Jr, 1996). Except for section *Turanga*, all sections have at least one species native to North America (Eckenwalder, 1996). The *Tacamahaca* section contains nine species, three of them native to North America (*P. balsamifera, P. trichocarpa,* and *P. angustifolia*), while the *Aigeiros* section

consists of three species, of which two occur naturally in North America (*P. deltoides* and *P. fremontii*) (Dickmann, 2001). In 2007, indigenous *Populus* species occupied more than 17 million hectares in the United States, and nearly 70 million hectares in the world (FAO, 2008). The United States is the third country with the greatest reported area of indigenous poplars, after Canada (approximately 28 million hectares) and the Russian federation, with around 21 million hectares (Ball et al., 2005). Most of the world's explored poplars grow in natural forests and are used for wood production (Heilman, 1999). Significant annual removals are reported in the Russian Federation and Canada, which remove around 100 and 16 million cubic meters, respectively (Ball et al., 2005).

All species in the genus *Populus*, except for *P. lasiocarpa* Oliv., are dioecious, thus obligate outcrossers (Eckenwalder 1996). The vast natural geographic range of poplars can be explained by their pollination and seed dispersal mechanisms: *Populus* species are wind-pollinated, producing very small and light seeds (300 to 16000 seeds per gram) that contain many cottony hair-like structures that are easily dispersed (Dickmann and Kuzovkina, 2008). These structures allow them to be carried for more than ten kilometers by the wind, or even more distant on the surface of moving water (Dickmann and Kuzovkina, 2008).

Poplars colonizing ability is also facilitated by their remarkable growth rates, which have attracted attention to the commercial use of these species. In forest plantations, rotations of 6-7 years are used for harvesting pulp, and 10 years or longer for solid wood products (Heilman, 1999). In addition, poplars are easy to propagate vegetatively, a critical characteristic for deployment of superior genotypes in forestry plantations.

Commercially, the wood of poplars has numerous uses: paper, veneer, plywood, and engineered wood products like oriented strand board (Heilman, 1999). The country with the largest area of poplar plantations is China (4.9 million hectares, representing more than 70 % of the global poplar plantation area), where poplars are used for wood production and environmental purposes (Ball et al., 2005).

In addition to their economic relevance, poplars play an important role in environmental restoration. Most poplar species inhabit riparian sites (Eckenwalder, 1996), where they provide shelter to wildlife and protect the margins of the rivers against soil erosion (Braatne et al., 1996) and runoff of agricultural chemicals (Ball et al., 2005). Moreover, poplars are used in reforestation of degraded areas due to their fast growth rates and short lifespan (Braatne et al., 1996). In several countries, including the United States, poplars have been used for phytoremediation purposes: decreasing pollutant concentrations in contaminated soils (Ball et al., 2005). Poplars are also used in carbon sequestration strategies due to their rapid growth.

More recently, poplars have become important in scientific research as model organisms for tree biology studies. *Populus trichocarpa* Torr. &Gray was the first woody perennial plant to have its genome sequenced (Tuskan et al., 2006), and only the second dicotyledon, after *Arabidopsis thaliana*. The *Populus* genome size is estimated to be approximately 485 MB, with 45,555 putative genes (Tuskan et al., 2006).

Another important characteristic of poplars is their relatedness with the model plant *Arabidopsis* (Bradshaw et al., 2000). Divergence of *Arabidopsis* and *Populus* is recent (100 to 120 million years ago [Mya], Tuskan et al., 2006) when compared to the divergence of angiosperm and gymnosperms (~ 300 Mya, Bowe et al 2000). The

availability of both genome sequences facilitates comparative and evolutionary genomic studies, as well as the application of high-throughput genomic technology (Yang et al., 2009). Developmental processes such as wood formation, seasonality of growth and adaptability of perennial plants cannot be studied in *Arabidopsis*, but are well suited for analysis in *Populus* (Bradshaw et al., 2000). A remarkable similarity between *Arabidopsis* and poplar is described in the study of Bohlenius et al. (Bohlenius et al., 2006), where it was shown that the CONSTANS/FLOWERING LOCUS T (*CO/FT*) regulatory module, which controls flowering time in response to the day length in *Arabidopsis*, also controls flowering time in *Populus*. However, this module also induces growth cessation and regulates bud set in the late season in *Populus,* a process absent in *Arabidopsis*. In addition, as it has been pointed out by Wullschleger et al., ecological questions can also be answered using *Populus* as a model, because of the wide distribution of this genus and its adaptability a variety of sites (Wullschleger et al., 2002).

Interspecific Hybrids in *Populus*

Interspecific hybridization among poplars occurs in nature between species of the same section and, eventually, between some species of different sections, as long as their range overlaps. The only exception occurs with species of the section *Populus*, which are reproductively isolated from species of other sections (Eckenwalder, 1996). For example, intersectional hybrids between species in the *Aigeiros* and *Tacamahaca* sections occur readily in nature (Dickmann and Kuzovkina, 2008). Most hybrids are fertile because all species of this genus are diploid, with the same number of chromosome (2n=38) (Rae et al., 2007). Therefore, interspecific hybridization has been

widely used to combine desirable traits of different species in selected genotypes (Stettler et al., 1996).

Also, F1 hybrids have shown greater performance than the parents, a phenomenon known as heterosis or hybrid vigor (Heilman and Stettler 1985; Stettler et al 1988). In addition, interspecific hybridization has an important function in the stability of phenotypic variance of the hybrids in a variety of ecological habitats since hybrids combine traits of trees from different habitats (US EPA 1999).

There are eight native species of *Populus* in North America, where several regions of natural hybridization occur (Eckenwalder, 1996). These zones are usually relatively narrow (10-15 km), containing F_1 individuals and subsequent interspecific generations, such as F_2 and backcrosses (US EPA, 1999). The most common interspecific hybrids originate from crosses between members of the *Aigeiros* and *Tacamahaca* sections of *Populus*. Selective crosses between Asian and North American species are known as Asian-american hybrids, between Asian and European species as 'Eurasian hybrids', between European and North America species as 'Euroamerican', and between North America species as 'Intra-american' hybrids. The Euramerican hybrid between *P. deltoides* × *P. nigra* is the most extensively used cross in forest plantations of poplars in North America. Hybrids between *P. trichocarpa* × *P. deltoides* have also become important, mostly in commercial plantations of northwestern North America (Dickmann 2008).

Quantitative Genetics of Growth and Development

Assuming genetic variability among parental species, interspecific hybrid crosses can provide insight into the genetic basis of species differences (Stettler et al., 1996) based on the analysis of trait segregation in F_2 progeny and backcrosses. These

multigeneration pedigrees can be used in genetic mapping experiments, to identify genes that regulate phenotypes (Rae et al., 2007). However, most traits of interest in forest trees, like growth and root ability, do not follow simple Mendelian monogenic inheritance, being controlled by several genes with high influence of the environment. These traits are often referred to as quantitative traits, due to continuous distribution of values measured in segregating populations. Thus, the loci associated to these traits are known as quantitative trait loci (QTL) (McClean, 1998).

QTL analysis is used to study the genetic basis of quantitative phenotypic variation, determining the position of a locus causing variation in the genome, and estimating the effect of the alleles and mode of action (Mackay et al., 2009). The goal of QTL mapping is to identify markers in close genetic distance to the causal loci (Mackay, 2001) that, consequently segregate together due to lower probability of recombination. Therefore, this probability tends to increase with the physical distance (Mackay et al., 2009).

Several traits have been evaluated through QTL analysis in *Populus* hybrids, including important adaptive traits that enhance survival in forest trees, like bud set and bud flush (Frewen et al., 2000), commercially important traits, like stem growth and form (Bradshaw and Stettler, 1995), and wood composition and biomass traits (Novaes et al., 2009).

***P. trichocarpa* × *P. deltoides* Hybrids**

Hybrids between *P. trichocarpa* and *P. deltoides* are commonly used in commercial plantations because of their superior growth performance (Heilman and Stettler, 1985; Stettler et al., 1988). The commonly called black cottonwood *P. trichocarpa* is the largest hardwood tree found in western North America and is the

largest of the American poplars (DeBell, 1990). *P. deltoides*, or eastern cottonwood, is one of the fastest-growing commercial species in North America and one of the largest eastern hardwoods, found mostly in the entire eastern region to the arid west of the United States (US EPA, 1999). Under favorable conditions, growth of these hybrids is superior to any other temperate tree species (Eckenwalder 2001). The superior properties of these hybrids relative to the pure species may be due to their accelerated radial growth (Heilman and Stettler, 1985). Among the intersectional crosses between *Tacahamaca* and *Aigeiros,* the cross between *P. trichocarpa* and *P. deltoides* subsp. *deltoides* (southern cottonwood) is the most vigorous (Eckenwalder, 2001).

The success of *Populus* hybrids is not only due to their remarkable growth rate and good performance, but also to the ease of their vegetative propagation which facilitates breeding and operational deployment of superior individuals (White et al., 2007). Vegetative propagation enables the replication of superior genotypes, therefore capturing the additive and non-additive variance that contributes to the higher phenotypic value (Bradshaw 2000). *Populus* individuals have been vegetatively propagated as hardwood cuttings for centuries (Ritchie, 1994). The existence of root primordia in the inner bark allows the rapid root development. However, only species and hybrids from sections *Aigeiros* and *Tacahamaca* have root primordia. Species form other sections do not typically develop adventitious roots and root poorly (Dickmann 2008). All species of the section *Tacamahaca* are easily propagated by hardwood cuttings. Most species of the section *Aigeiros* also root well, with the exception of *P. deltoides*, which is not easily propagated by hardwood cuttings (Rae et al., 2007). This limitation is significantly improved in hybrids of *P. deltoides* and *P. trichocarpa*.

Adventitious Root Formation

Adventitious roots are formed in parts of the plant other than the embryonic root produced during embryogenesis (Barlow, 1986). Adventitious roots can develop from a variety of tissues. North American aspens (*Populus tremuloides* and *P. gradidentata*), for example, regenerate largely by vegetative suckering from the residual root systems (Pregitzer and Friend, 1996). Commercial poplar plantations are based mostly on the propagation through adventitious rooting of hardwood cuttings (Ritchie, 1994).

Adventitious root formation (ARF) is an organized developmental process that is generally divided into phases according to physiological, histological and biochemical observations (Davies and Hartmann, 1988). Generally, ARF is divided into three phases: the dedifferentiation phase, before any histological event; the induction, where cells start to divide to form an internal root meristem; and elongation or differentiation phase, where the root-primordia grows and emerges from the stem (De Klerk et al., 1999).

Lateral and adventitious root formation follow a similar organogenesis process, except that the later roots need to acquire competence for cell proliferation in most of the species. This involves the dedifferentiation of cells that were committed to a different developmental process (Srivastava, 2002). But both processes share the second ‘induction phase’, and third ‘elongation phase’ (Ozawa et al., 1998). Although the identification of each phase may not be straightforward in all species, studies have shown that each phase has its own requirements (De Klerk et al., 1999).

Some species have pre-defined sites for the formation of root primordia (Lovell and White, 1986). Usually, adventitious root primordia arise close to the phloem and cambium, at the ray cells, or in bud or leaf gaps. They might also arise in the pericycle,

which is the tissue located between endodermis and phloem in roots (Lovell and White, 1986).

ARF may have important function in supporting and anchoring plants, as can be observed in vine plants and fig trees with the banyan habit (Barlow, 1986). ARF contributes to water-use efficiency and extraction of nutrients from the soil. In these cases, adventitious roots are formed with no severance. However, in the case of stem cuttings, there is an added dimension of wound response (Srivastava, 2002). The breakage of the connection with the root system in a cutting exposes stem tissue, interrupts transport of substances to roots and from them, and activate a series of repair responses and systemic signaling cycles (Lovell and White, 1986).

The timing of each phase of adventitious root formation varies among species and depends on external stimuli. Ahkami et al (Ahkami et al., 2009) studied anatomical changes during ARF in *Petunia hybrid* and found that the root initiation phase may occur in the first 72 hours after severance of the cutting. At 72 hours after excision, signaling for meristematic cells marks the transition from initiation phase to induction phase. It was also observed that the first root meristem was visible after 96 hours, but the root primordium was only completely formed after six days of excision. Roots were formed after eight days. This timing is very similar to that described in previous studies of De Klerk and colleagues (De Klerk et al., 1999, De Klerk, 2002), which identified three phases of adventitious root formation in apple microcuttings.

Factors Regulating ARF

Many environmental and endogenous factors regulate rooting. Critical endogenous factors in adventitious root formation are phytohormones. They can act directly on cell division and growth, or indirectly, interacting with other molecules or

phytohormones (Correa and Fett-Neto, 2004). Auxin is the principal phytohormone that initiates rooting (Srivastava, 2002). A major advance in vegetative propagation was the discovery of the effect of auxin on the production of adventitious roots from cuttings in the 1930s (Thimann and Went, 1934). Although auxins are required for rooting during the first and second phases of the adventitious rooting process (the dedifferentiation and induction stages), they inhibit the process during the elongation phase (De Klerk, 2002). Indole-3-acetic acid (IAA) is the main endogenous auxin. However, Indole-3-butyric acid (IBA), another endogenous auxin, is more stable than IAA (Epstein and Lavee, 1984). Therefore, IBA is most commonly used for rooting in commercial operations (De Klerk et al., 1999).

There is significant evidence that ethylene interacts with auxin to control adventitious rooting in stems or stem cuttings (Srivastava, 2002). Ethylene is a small, readily diffusible hormone that has an important role integrating developmental events with external stimuli (Klee, 2004). It influences developmental processes such as seed germination, fruit ripening, abscission, and senescence (Abeles et al., 1992). It is also an important stress hormone. Adverse biotic or abiotic stimuli usually lead to ethylene synthesis. Ethylene slows down plant growth until the stress is removed. At the level of gene expression, ethylene induces transcription of many genes in response to a multitude of environmental and developmental stimuli (Klee, 2004). It seems that ethylene has a promotive effect only in the presence of an auxin source. Some studies suggest that auxin promotes dedifferentiation through stimulation of ethylene synthesis (Sun and Bassuk, 1993) . Although ethylene is promotive during the dedifferentiation phase I, it is inhibitory during the induction phase (De Klerk, 2002). Application of

inhibitors of ethylene biosynthesis or ethylene action also reduces the number of adventitious root formed.

Another phytohormone class that plays a role in ARF are the cytokinins. The effect of cytokinins on rooting, however, varies with concentration as well as duration of treatment. Higher concentrations and longer treatments are inhibitory, whereas lower concentrations and shorter treatment times may have an enhancement effect (Srivastava, 2002). It is also well known that exogenous as well as endogenous balances between auxins and cytokinins can favor a developmental pattern or orient an organogenic program (Gaspar et al., 2003). Ramirez-Carvajal et al. (Ramirez-Carvajal et al., 2009) have shown the effect of cytokinin on ARF through the alteration of the type-B response regulator PtRR13 in *Populus* plants. Their results suggest that cytokinin acts through PtRR13 to repress adventitious root development in intact *Populus* plants. Although cytokines have a negative effect on ARF, being inhibitory during the induction phase, they are required at low levels to promote dedifferentiation.

Jasmonic acid (JA) and methyl ester methyl jasmonate (MeJA), usually referred to as jasmonates, are also plant growth regulators whose role on adventitious root has been reported recently (Fattorini et al., 2009). It has been shown that MeJA, combined with root-inductive hormones, enhances adventitious rooting in tobacco. Jasmonates are important in plant defense against mechanical wounding (Srivastava, 2002). As a wounding response in vegetative propagation through stem cuttings, the JA and MeJA have been suggested to affect the dedifferentiation phase, responsible to make cells prompt to respond to auxin (De Klerk, 2002).

Genes Related to ARF

Few genes have been described to be specifically involved in ARF. Konishi and Sugiyama (Konishi and Sugiyama, 2006) have analyzed the phenotype of the ROOT PRIMORDIUM DEFECTIVE 1 gene (*rpd1*) in *Arabidopsis thaliana* mutants. Seeds of the Landsberg *erecta* strain of *Arabidopsis* were mutagenized by treatment with ethyl methanesulfonate (EMS). The mutation in the *rpd1* gene prevents the development of root primordia in adventitious root formation in hypocotyl segments. It was observed that *rpd1* mutants form the initial root primordia, but development is retarded beyond two- to four-cell-layer stages.

Sorin and colleagues (Sorin et al., 2005) used two classes of *Arabidopsis* mutants to study adventitious rooting, *superroot* (SUR1 and SUR2) and *argonaute1* (AGO1) mutants described previously (Boerjan et al., 1995, Delarue et al., 1998, Bohmert et al., 1998). SUR1 and SUR2 are auxin overproducers that develop excess adventitious and lateral roots. Both genes were isolated from an EMS-mutagenized *Arabidopsis thaliana* seed stock, described before by Boerjan and colleagues (Boerjan et al., 1992). Therefore, *superrroot* genes are probably negative regulators of adventitious root formation. On the other hand, AGO1 mutants are defective in adventitious root formation. Using an allelic series of AGO alleles and AGO SUR double mutants, alteration of auxin homeostasis and a hypersensitivity to light were observed. Their results show mRNA accumulation of the *Auxin Response Factor17* (ARF17) in hypocotyls of AGO1. This suggest that ARF17 might be the principal negative regulator of adventitious root formation, by regulating genes involved in auxin and light signaling during adventitious root development. In a follow-up study, Gutierrez et al. (Gutierrez et al., 2009) showed that *Auxin Response Factor6* and *8* are positive regulators of

adventitious root formation. These three genes are regulated by light and act in a complex regulatory network.

With the aim to identify gene markers regulating ARF in Arabidopsis, Sorin et al. (Sorin et al., 2006) used proteomic analysis of Arabidopsis mutants defective in ARF to identified 11 proteins that might influence endogenous auxin content, number of adventitious root primordia developed, and/or number of adventitious roots formed. Analyzing molecular and biochemical processes in ARF of *Petunia hybrida*, Ahkami et al. (Ahkami et al., 2009) showed RNA accumulation of cyclin B1 gene after 48 hours of excision that is not expressed in roots suggesting that it can serve as a marker for root initiation phase of ARF.

The gene LRP1 (*lateral root primordium-1*) has been shown to be specifically expressed in lateral and adventitious root primordia of *Arabidopsis thaliana* (Smith and Fedoroff, 1995). Also, gene HRGPnt3 (*hydroxyproline-rich glyprotein*) is expressed in adventitious and lateral roots of tobacco (Vera et al., 1994). It is expressed after the first cell division of lateral root primordium formation.

Adventitious rooting ability of woody perennial species has been suggested to be under strong genetic control (Borralho and Wilson, 1994, Ronnberg-Wastljung et al., 2005). However, large variation in broad-sense heritability of adventitious root-related traits has been reported in poplar (Table 1-1). In general, ARF is under moderate to strong genetic control in poplar. Variation can be due to high diversity among different species of poplar and because of genotype × environment interactions. Table 1-1 suggests that the presence of a *P. trichocarpa* background is associated with lower

heritability of adventitious root-related traits in *Populus*, but no conclusions can be made since growth conditions and type of cuttings used differ significantly among studies.

Genetic Control of Gene Expression

Functional genomics tools like DNA microarrays are useful for studies of global gene expression. Through microarray technology, the expression levels of essentially all genes can be quantified simultaneously based on hybridization of labeled transcripts to glass slides (Yang et al., 2009). The basic principle of DNA microarrays is to bind an unknown sample to an ordered array of known DNA molecules fixed in a precise location of a two-dimensional surface (Gershon, 2002). There are two main microarray platforms commonly used: cDNA microarrays, in which amplified cDNA clones are printed directly onto slides. Alternatively, in oligonucleotide microarrays, shorter probe sequences developed based on gene sequence information is synthesized in situ or printed on a microarray (Murphy, 2002).

DNA microarrays have broad application. Microarrays enable the identification of genes expressed in a certain time and under specific treatment. Furthermore, microarrays can be used to assess differentially expressed genes of a given tissue under different conditions (Wang et al., 2007b), as well as changes in gene expression at different stages of a developmental process (Hertzberg et al., 2001, Brinker et al., 2004).

Applications of microarrays also include genotyping individuals for genetic differences, such as single-nucleotide polymorphisms (SNPs) associated with a specific phenotype (Cutler et al., 2001). Microarrays are also being used to discovery information about a gene's function through comparison of patterns of gene expression among known genes (Alberts et al., 2002).

The completion of the genome sequence of *Populus* has allowed the development of whole-genome oligonucleotide microarrays for these species, which was first designed by Oak Ridge National Laboratory in collaboration with NimbleGen (Madison, Wi, USA) (Yang et al., 2009). Quesada and colleagues (Quesada et al., 2008) have used this array to compare transcribed genes in vegetative organs of *P. trichocarpa* genotype Nisqually-1 to *A. thaliana* orthologs. The greatest variety of expressed genes occurred in woody stem, where was also observed the highest proportion of unknown transcripts. *Populus* whole-genome oligonucleotide microarrays were also used by Ramirez-Carvajal and colleagues (Ramirez-Carvajal et al., 2008) to investigate expression pattern of the cytokinin response regulator gene family in *P. trichocarpa* genotype Nisqually-1, and Bocock et al. (Bocock et al., 2008), to identify genes encoding invertase, an enzyme important in carbon utilization because it catalyzes the hydrolysis of sucrose into glucose and fructose.

Studies have shown that variation in transcript levels is genetically controlled and heritable. Because gene expression can assume continuous values, it can be studied as a quantitative trait. As the poplar genome sequence and DNA microarrays are available, these two techniques can now be combined with the QTL mapping information in an approach known as Genetical Genomics (Jansen and Nap, 2001). The principle of this strategy is based on the genetic variation between related individuals in a segregating population. Therefore, the expression profile of all individuals for each gene is a phenotypic trait, hence quantitative trait that is combined with a genetic map, enabling the assessment of the genetic map positions of gene expression QTLs, known as *eQTLs* (Kirst and Yu, 2007). If the eQTL co-localizes with the physical position of

genes that affect their own expression, they are called *cis*-acting eQTL. Otherwise, this gene expression QTL is trans-regulated, in other words, regulated by factors that are not the genes themselves (Jansen and Nap, 2001).

Table 1-1. Broad-sense heritabilities of adventitious root-related traits of different types of stem cuttings of *Populus* species under different conditions of growth and type of material.

Population studied	Growth conditions	Type of cutting	Number of adventitious roots	Root dry weight	Total length of primary roots	Reference
P. deltoides	Sharkey clay and 1:1:2 sand:peat:loam substrates	Apical Hardwood	0.44-0.56	0.36-0.58	0.33-0.52	Wilcox and Farmer (1968)
P. deltoides	Sand	Dormant hardwood	0.85-0.91			Ying and Bagley (1977)
F_2 of BC (Family 331)	*in vitro*	Greenwood stems discs	0.32		0.29	Han *et al*. (1994)
P. trichocarpa	Sand	Hardwood	0.15-0.18	0.20-0.33	0.23-0.28	Riemenschneider and Bauer (1997)
BC*, D*, DM*, DN* and NM*	Field in three geographic locations	Dormant hardwood		0.09-0.11		Zalesny Jr. *et al*. (2005)
P. deltoides × *P.* ×*canadensis* syn. *euramericana*	Hydroponic	Greenwood apical	0.80-0.85		0.58-0.71	Zhang *et al*. (2009)
pBC (Family 52-124)	Fafard 4MIX soil	Greenwood apical		0.28-0.33		Novaes *et al*. (2009)

*BC: Backcross (*P. trichocarpa* × *P. deltoides*) × *P. deltoides*; D: *P. deltoides*; DM: *P. deltoides* × *P. maximowiczii*; DN: *P. deltoides* × *P. nigra*; NM: *P. nigra* × *P. maximowiczii*; pBC: pseudo-backcross (*P. trichocarpa* × *P. deltoides*) × unrelated *P. deltoides.*

CHAPTER 2
GENETIC ANALYSIS OF EARLY ADVENTITIOUS ROOT DEVELOPMENT IN A PSEUDO-BACKCROSS POPULATION OF POPULUS

Introduction

Adventitious roots are post-embryonic roots formed on plant shoots. Although adventitious and lateral roots have common developmental properties, adventitious root formation involves additional dedifferentiation of already committed cells (Srivastava, 2002). Generally, the formation of adventitious roots occurs in three phases that may partially overlap. The first phase is of dedifferentiation, when cells are prepared to respond to rhizogenic stimuli from auxin, and further are going to originate 'root primordia' (De Klerk et al., 1999). In woody plants, the onset of adventitious roots may occur in several tissues. Although adventitious roots form more frequently from young secondary phloem cells, they also originate from vascular rays, cambium or pith cells (Davies and Hartmann, 1988). The second stage in the formation of adventitious roots is the 'induction', when cells 'activated' in the first phase become committed to form root primordia, which elongates in the third phase (De Klerk et al., 1999).

Adventitious root formation from cuttings is used extensively for propagating superior genotypes vegetatively. Clonal propagation through rooted cuttings is used particularly in *Eucalyptus* and in species of the Salicaceae family, which includes poplars (*Populus*) and willows (*Salix*). The ability of poplars to readily form adventitious roots from hardwood cuttings is one of the reasons for their wide use in commercial plantations (Zsuffa et al., 1996). In 1994, more than 50 percent of all poplar plantings worldwide were represented by fewer than 10 clones (Ritchie, 1994).

Considerable variation in adventitious root production is found between species, cultivars, different ages and organs (Lovell and White, 1986). There are very few tree

taxa that are able to produce adventitious roots promptly on hardwood cuttings, which are segments of dormant stem wood. Species and hybrids in the *Aigeiros* and *Tacamahaca* sections of the genus *Populus* are among them. But even in these sections, there is considerable variation in the degree and vigor of adventitious rooting (Dickmann and Hendrick, 1994). In poplar, most of the adventitious roots originate from preformed root primordia in the periderm of the stem (Dickmann and Hendrick, 1994). Even though rooting ability is a complex trait with high phenotypic plasticity, it has been shown to be under strong genetic control in species of *Eucalyptus* (Borralho and Wilson, 1994), *Salix* (Ronnberg-Wastljung et al., 2005) and *Populus* (Wilcox and Farmer, 1968, Zhang et al., 2009, Rae et al., 2007).

Poplar is a suitable model for studying the genetic control of root development because there is extensive variation for the trait and well-established genetic and genomic resources (Bradshaw et al., 2000, Taylor, 2002, Wullschleger et al., 2002, Brunner et al., 2004). Available genomic resources include the genome sequence of the *P. trichocarpa* genotype Nisqually-1 (Tuskan et al. 2006) and whole-transcriptome microarrays (Jansson and Douglas, 2007), enabling the integration of genomic information from quantitative trait loci (QTL) analysis and gene expression data (Jansen and Nap, 2001). QTL mapping allows the identification of genomic regions that regulate trait variation and, in combination with gene expression data, can be used to identify genes that underlie quantitative trait variation (Schadt et al., 2003a). This integrative strategy, also called genetical genomics, was initially proposed by Jansen and Nap (Jansen and Nap, 2001) and is being extensively utilized in medical research (De Haan

et al., 2003, Schadt et al., 2003b). In forest species, only a few studies utilized genetical genomics in *Eucalyptus* (Kirst et al., 2004) and in *Populus* (Street et al., 2006).

The objective of this study was to dissect the quantitative genetic control of adventitious root formation in *Populus*, and the transcriptional program that differentiates individuals that carry alternative alleles for root development in the main genetic loci that control the trait. This analysis allowed the identification of two QTL involved in the regulation of root formation, and several candidate genes co-located in these QTL intervals. A transcriptome analysis identified genes that are differentially regulated between genotypes with distinct rooting ability. Finally, the combination of QTL and transcriptome information was used to define genes possibly involved in initial adventitious root development in the hybrid population.

Material and Methods

Plant Materials

The family (52-124) used in this study is a pseudo-backcross between the hybrid female parent 52-225 [*Populus trichocarpa* (clone 93-968) × *P. deltoides* (clone ILL-101)] and the unrelated male parent D124 (*Populus deltoides*), established by the Natural Resources Research Institute of the University of Minnesota. The parent D124 is from northern Minnesota. The *P. trichocarpa* parent of the hybrid came from western Washington, whereas the *P. deltoides* parent material originated in Illinois.

Phenotypic Measurements

Twelve centimeter long apical cuttings were collected from 234 individuals of family 52-124, as well as the parental genotypes. Cuttings were placed in 23×16.2×6 inch-containers, with up to 59 cuttings, which were maintained in hydroponic culture (H_2O buffered at pH 5.7, with 0.5 g L^{-1} of MES) for the duration of the experiment. The

experimental design was an incomplete block design with four blocks and three replications, for a total of 708 cuttings. Root emergence was recorded daily at the same time (10 am), until the 18th day of culture. After 18 days, roots from each cutting were excised and scanned using a Canon LiDE 600F scanner (in grayscale at 600 dpi). Images were analyzed with WinRHIZO^{TM} (version 2007d, Regents Instruments Inc., Quebec, Canada) to measure root architectural traits: length, volume, and surface area of total, primary (first order roots), and root branches (second and third order roots), as well as average diameter. After being scanned, the roots were dried in a 65^{o}C drying room. Dried biomass was weighed in a high-precision scale after samples were placed for 72 hours at room temperature and humidity in the laboratory. Table 2-1 describes all traits measured, as well as maximum and minimum phenotypic values, compared to parental values.

Statistical Analysis

Covariance parameters were estimated for all traits using PROC MIXED (SAS Institute Inc. 9.2® 2004, Cary, NC, USA), considering all variables random in the following model:

$$y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_{k(j)} + e_{ijkl}$$

where y_{ijkl} is the phenotypic value of the *i*th clone in the *j*th block within the *k*th replication, μ is the overall mean, α_i is the random effect of the clone; β_j is the random effect of replication, $\gamma_{k(j)}$ is the random effect of incomplete block (within replication) and e_{ijkl} is the residual error.

Clonal repeatability was calculated using the covariance parameter estimates in the following formula:

$$H^2 = \frac{\sigma_c^2}{\sigma_c^2 + \sigma_e^2}$$

where σ_c^2 and σ_e^2 are the variance components corresponding to clone and residual effect across the three replications, respectively.

A log transformation was applied to all traits, except for number of roots. Least-square means used in the QTL analysis were calculated by including clone as a fixed effect in the model, using PROC MIXED.

QTL Analysis

QTLs for root-related traits were identified based on a linkage map previously described (Novaes et al., 2009). The linkage map consists of 181 markers distributed homogenously in the hybrid female parent, with an average density of one marker every 16 cM. QTLs were identified using composite interval mapping (Zeng, 1993) in Windows QTL Cartographer v.2.5 (Wang et al., 2007a) using standard model 6 with walk speed of 2 cM. A genome-wide significance level of $P<0.05$ was established based on 1000 permutations (Churchill and Doerge, 1994).

Selection of Genotypes with Alternative Alleles for Genomic Regions that Control Adventitious Root Formation

QTL for the trait number of roots were consistently mapped on LG II and XIV (see Results). We classified each genotype depending on the allele (*P. trichocarpa* or *P. deltoides*) that was observed in both QTL regions. Four categories were defined: (1) genotypes carrying *P. deltoides* or (2) *P. trichocarpa* alleles at both QTL, and (3) genotypes carrying *P. trichocarpa* alleles at the QTL in LGII and *P. deltoides* alleles in

QTL on LG XIV, and (4) vice-versa. Genotypes with recombination between markers flanking in each of the two QTL were not grouped into any of the categories. As expected, genotypes carrying *P. trichocarpa* alleles in both QTL regions generally had more roots than genotypes carrying the *P. deltoides* alleles in those intervals. These are referred hereafters as the *Pt*QTL and *Pd*QTL categories, respectively. Within each one of these two categories, we identified three genotypes with extreme phenotypes for number of roots. For these six genotypes we collected 12 centimeter-long cuttings and established them in the same hydroponic conditions used previously in the QTL detection experiment. The number of new roots formed in these genotypes was recorded daily for 12 days, and samples were collected for transcriptome analysis.

Tissue sampling

To measure gene expression during adventitious root formation in the three selected genotypes from each QTL category (*Pt*QTL and *Pd*QTL), a section of 1 centimeter, measured from the base of each cutting, was collected at 0, 1, 2, 4 and 8 days after placing them in the hydroponic solution. Samples were flash-frozen in liquid nitrogen for posterior RNA extraction. Four biological replicates were collected from each genotype, at each time point. In addition, five biological replicates of each genotype were maintained in hydroponic growth conditions until day 12 to verify that the root development was consistent with the phenotype observed in the QTL detection experiment.

RNA extraction, cDNA synthesis and labeling

Total RNA was extracted (Chang et al., 1993) from the bottom 1 cm stem section collected from each sample. The sample included xylem, phloem and bark. RNA was purified using RNeasy Mini Kit columns (Qiagen), and DNase treated with RNase-Free

DNase set (Qiagen). RNA quality was evaluated in 1% w/v agarose gels. RNA was amplified and cRNA synthesized and labeled using Two Dyes Agilent Low Input Quick Amp Labeling Kit. The microarray platform used consisted of single 60-mer probes designed for each of 43,803 annotated gene models from the sequenced genome of *P. trichocarpa*. These probes were previously selected for being adequate for analysis of gene expression in this mapping population (Drost et al., 2009)

Microarray experimental design and data analysis

A total of 60 microarrays were used in the transcriptome analysis. Gene expression of each of six genotypes was analyzed in five time points (0, 1, 2, 4 and 8 days), with four biological replicates per genotype and time point, following a "bird cage" design (Figure 2-1). Median values of signal intensities were quantile normalized (Bolstad et al., 2003) and $\log_2$ transformed. Normalized signals were analyzed in SAS 9.2 (SAS Institute Inc. 9.2® 2004, Cary, NC, USA) using a mixed-model ANOVA with genotype and genotype × time interactions as fixed effects, and microarray as random effect.

Differences in expression between the group of genotypes from the *Pt*QTL and *Pd*QTL categories were estimated at each time point, and the significance was determined based on a false discovery rate (FDR) of 5% (Storey and Tibshirani, 2003). Genes showing a similar pattern of expression differences between genotypes from the *Pt*QTL and *Pd*QTL categories, at all time points, were clustered using a Modulated Modularity Clustering graph-based technique using Spearman correlation (Stone and Ayroles, 2009).

Annotation

Populus gene model transcript sequences were annotated by searching for sequence similarities using BLASTx against *Populus* (JGI v.1.1) and The *Arabidopsis* Information Resource (TAIR v8.0) gene models.

Results

Analysis of Parental Genotypes

Adventitious roots developed almost simultaneously in both parental genotypes. However, the number of roots developed was significantly different (P<0.01) until the 17th day in hydroponic culture, with the female parent (*Populus trichocarpa* ×*P. deltoides* hybrid) developing more roots than the pure *P. deltoides* male parent (Figure 2-2). After the 17th day, the number of roots developed was no longer significantly different between the two parents. Therefore, both parents appear to have a similar capacity to form roots, but development of adventitious roots appears to be delayed in *P. deltoides* relative to the hybrid.

Phenotypic Variation

Three classes of traits were measured in the progeny of Family 52-124: (1) number of roots, measured from day 9 to day 18, (2) root architecture and (3) total root biomass, measured in day 18 (Table 2-1).

Number of roots. A Poisson distribution was observed for the trait number of roots, which displayed transgressive segregation in the hybrid population (Figure 2-3). The hybrid parent had higher number of roots in all days measured, when compared to the pure *P. deltoides* parent. Considerable variation in the day of root emergence was also observed in the progeny. After nine days in the hydroponic solution half of the genotypes had produced roots (Figure 2-4).

Root architectural traits and biomass. After 18 days in hydroponic solution, roots were harvested to measure architectural traits, including total length, surface area, total volume of first order roots and root branches, and average diameter. Root architectural traits and total dry-weight also showed transgressive segregation in the progeny (Figure 2-5). The hybrid *P. trichocarpa* × *P. deltoides* parent presented higher values for most of the traits, with the exception of VOL, DIAM, PRISA, PRIV and DRYWT (Figure 2-5).

Genetic Control of Early Root-Related Traits

Clonal repeatability (*H*) was estimated for all traits, and was generally low, ranging from 0.115 for L1 to 0.342 for day16 (Table 2-1). The trait number of roots showed higher *H*, ranging from 0.274 for day12, to 0.342 for day16, than root architectural traits

Phenotypic Correlations

Phenotypic correlations were calculated between all pairs of traits (Table 2-2). In general, root architectural traits and number of roots were highly correlated, except for DIAM, which had lower correlations (ranging from 0.17 to 0.52) relative to the root architectural traits. Also, phenotypic correlations among number of roots and root architectural traits were moderate to high, ranging from 0.35 to 0.68 (excluding DIAM). Phenotypic correlations were positive for all traits.

QTL Analysis of Number of Root

QTL analysis was performed for number of roots, measured after 9 days in hydroponic culture. Eleven QTLs for number of roots were identified on the mother map, using the 95th percentile of 1000 permutations as a threshold (P<0.05) (Table 2-3, Figure 2-6). An average of 1.83 QTLs was identified, each day that the number of roots was measured. QTLs for number of roots mapped consistently in LG II and LG XIV. The

effects of the QTLs mapped in these two linkage groups tended to decrease over time (Table 2-3). Each QTL explained between 6.66 to 10.66% of the phenotypic variation, depending on the trait (Table 2-3). The total variance explained for each trait averaged 9%.

QTL Analysis of Root Architectural Traits

QTLs were detected for eight of the ten root architectural traits measured. In total, 15 significant QTLs were mapped, seven on LG XII and six on LG XIV (Table 2-4, Figure 2-7). The phenotypic variation explained by each QTL ranged from 5.8 to 10.87%, similar to the range explained by QTL for the number of root traits. All QTL alleles positively affecting the traits were derived from *P. deltoides,* grand-parent of the pedigree.

QTL Analysis of Root Biomass

A QTL for root dry weight was detected on LG XVII, the only trait to have a QTL detected in this interval (Table 2-4, Figure 2-7). In total, 7% of the variance in this trait was explained by this QTL. Alleles positively affecting this trait come from *P. trichocarpa*.

Genotypes with Alternative Rooting Alleles

Genotypes carrying alternative alleles at both QTLs on LG II and XIV were selected for gene expression analysis (Figure 2-8). Genotypes in the *Pt*QTL category (UF352, UF498 and UF926) developed higher number of roots (P<0.1) than those in the *Pd*QTL category (UF717, UF209 and UF912). The rooting properties of these genotypes were confirmed in a separate experiment using the same hydroponic growth conditions as those in the QTL detection experiment (Figure 2-9).

Transcriptome Analysis of Adventitious Root Development

The transcriptome response of cuttings in hydroponic solution was analyzed to identify genes differentially expressed in different experimental conditions. Based on the analysis of variance, genes differentially regulated across time points, between genotypes in the *Pt*QTL and *Pd*QTL categories across the entire experiment and in individual time points were identified.

Gene expression analysis of time effect

A total of 26,121 putative genes were identified as significantly differently expressed (FDR<0.001) between at least two time points in the experiment. To identify the time point at which the most significant changes in gene expression occurred, a contrast of the expression between consecutive time points (i.e. time points 0 and 1, 1 and 2, 2 and 4, 4 and 8, and 8 and 0) was performed. Most differences in transcript levels occurred during the first 24 hours of the experiment (i.e. time points 0 and 1, Figure 2-10). On the other hand, only ten genes were differentially regulated between 4 and 8 days in hydroponic culture. This suggests that these two time points are within the same rooting phase.

Among genes previously described to be involved in root development in model plants, the gene eugene3.153750001 (putative homolog of *superroot2*) was detected as being significantly more highly expressed in time point 0 compared to time point 1. A mutant of SUR2 has been described as causing auxin overproduction and abnormally high number of adventitious roots (Delarue et al., 1998). The expression of SUR2 also increases as a response to wounding (Barlier et al., 2000). Within the same contrast, but more highly expressed in time point 1 instead, is the gene CPC902 (Condensin complex components subunit C, eugene3.00051316) which is a homolog of the

Arabidopsis gene SMC1 (*structural maintenance of chromosomes* 1). SMC1 encodes for one of the proteins of the cohesion complex family (Schubert et al., 2009), necessary for correct chromosome segregation during nuclear divisions, possibly indicating the initiation of cell divisions necessary for root meristem organization. The contrast of time points 1 and 2 showed higher expression of gene grail3.0049011101, homolog of *Arabidopsis* gene TOR1 (*tortifolia*1) in time point 1. TOR1 encodes a protein associated with microtubules, which regulates the direction of organ growth (Furutani et al., 2000). In time point 2, the gene estExt_Genewise1_v1.C_440031, a putative homolog of the *Arabidopsis* gene TPL (*topless*), was highly expressed. TPL is involved in transcriptional repression of root-promoting genes during the transition stage of embryogenesis (Osmont and Hardtke, 2008), Among genes more highly expressed in time point 4, fgenesh4_pm.C_LG_VIII000556, homolog of *Arabidopsis* gene PIN3, was detected. PIN3 is an auxin efflux regulator involved in root development and elongation (Blilou et al., 2005).

Gene expression analysis of genotype effect

A total of 1929 genes were identified as differentially expressed between genotypes in the *Pt*QTL and *Pd*QTL categories (FDR<0.05), in at least one time point (Figure 2-11a). Only 45 of these genes were differentially expressed across all time points. These included the genes estExt_fgenesh4_pg.C_LG_II0581 and gw1.XIV.1942.1, homologs of the *Arabidosis* genes RUB1 (*related to ubiquitin* 1, AT1G31340) and OMT1 (*caffeic acid O-methyltransferase 1*), respectively, which were expressed primarily in the *Pt*QTL category. RUB1 was found to be involved in response to auxin stimulus and regulation of ethylene production (Bostick et al., 2004). Gene OMT1 is involved in lignin biosynthesis (Goujon et al., 2003). In the *Pd*QTL category,

genes estExt_fgenesh4_pm.C_LG_IV0339 1 (putative homolog of the gene COL2) and gw1.XV.655.1 (putative DEAD-box protein) were more highly expressed, respectively. The gene COL2 is related to flowering time in *Arabidopsis* but known to control growth cessation in *Populus* (Bohlenius et al., 2006).

Gene expression comparison between extreme genotypes within time point

In order to identify genes regulating rooting ability, we analyzed genes differentially regulated between genotypes in the *Pt*QTL and *Pd*QTL categories at each time point. An interesting gene found to be significantly more highly expressed in the *Pd*QTL category in time point 0 was gene gw1.XI.1499.1, homolog of STM *Arabidopsis* gene (*shoot meristemless*). Yanai et al (Yanai et al., 2005) demonstrated that the activation of STM proteins increase cytokinin levels dramatically. Low levels of cytokinins are necessary to promote dedifferentiation of cells in the first phase of adventitious root formation. Also in time point 0, but more highly expressed in the genotypes of the *Pt*QTL category, is gene eugene3.00190357, a homolog of *Arabidospsis* gene APY2 (*apyrase 2*). APY2 has been reported to be expressed in fast growing tissues, including meristematic region of root tips (Wu et al., 2007). In time point 1, the gene estExt_fgenesh4_pg.C_LG_IV1532, which in *Arabidopsis* encodes a basic chitinase (CHIB) was identified. CHIB is involved in the signaling pathway mediated by ethylene and jasmonic acid during pathogen response (Zhou et al., 2005). We found the gene grail3.1392000301, homolog of *Arabidopsis* gene SUR1 (*superroot1*) to be expressed only in time point 2. This gene is highly expressed in the genotypes of the *Pt*QTL category in our experiment. SUR1 was first isolated by Boerjan and collegues (Boerjan et al., 1995), and is also an auxin overproducer that causes development of an excess of adventitious roots. Also in time point 2, but more highly expressed in the genotypes of

the *Pt*QTL category, is gene eugene3.00012559, homolog of *Arabidospsis* gene AIR9 (auxin-induced in root cultures 9). AIR9 doesn't have homology to any known proteins and was first isolated from cDNA library of auxin-treated root culture. In time point 4, gene gw1.VIII.2487.1, P5CS1 (*delta1-pyrroline-5-carboxylate synthase 1*), involved in root elongation response (Szekely et al., 2008) is expressed in poor rooters. In time point 8, the gene fgenesh4_pg.C_LG_II000237, SUR2 (*superroot2*) is expressed in the *Pd*QTL category.

Several auxin-responsive genes were detected as differentially regulated in this study. Most of them were differentially expressed between genotypes in the *Pt*QTL and *Pd*QTL categories. Among genes involved in auxin signaling, we detected gene estExt_fgenesh4_pm.C_LG_VIII0154, homolog of PGP1 *Arabidopsis* gene, differentially expressed in genotypes of the *Pd*QTL category in time point 1. Also gene grail3.0003074001, a homolog of *Arabidopsis* gene AUX1, expressed in the *Pd*QTL category in time point 8, estExt_fgenesh4_pg.C_LG_II0422, TSA1 in poor rooters in times 2 and 8.

Cluster analysis

A total of 1929 genes were differentially regulated between genotypes in the *Pt*QTL and *Pd*QTL categories at any time point. For these genes, the difference in transcript abundance between the set of genotypes in each of the two QTL categories were estimated. The estimates were used to cluster genes with common transcriptional response differences between genotypes in the *Pt*QTL and *Pd*QTL categories, across all time point. Sixty clusters were identified, varying from 2 to 148 transcripts in size, with eight unclustered genes (Figure 2-12). Further analysis showed one cluster with a noticeable pattern of expression, with a linear increase in the difference in transcript

abundance between genotypes in the *Pt*QTL and *Pd*QTL categories across each time point (Figure 2-13). Of the 91 genes in this cluster, 36 showed an increase in expression over time in genotypes of the *Pd*QTL category. A set of 55 genes showed the opposite pattern, with higher expression in the *Pt*QTL category. For example, the gene estExt_fgenesh4_pg.C_1630059, homolog of *Arabidopsis* gene ARF4 (*auxin response factor* 4) was significantly more highly expressed in genotypes in the *Pd*QTL category. ARF4 is a member of the ARF gene family of transcription factors that mediates auxin responses. In Arabidopsis, ARF4 seems to have the same function of ARF3 in specifying abaxial cell identity (Pekker et al., 2005). In the group of genes more highly expressed among genotypes in the *Pd*QTL category, the gene fgenesh4_pg.C_LG_III001778, homolog of SGS3 *Arabidopsis* gene (*suppressor of gene silencing* 3), was observed. A previous study (Peragine et al 2004) hypothesized that SGS3 promotes maintenance of the juvenile phase by repressing the expression of adult-promoting genes. In the same study, the accumulation of ARF4 transcripts was detected in shoot apices of SGS3 mutants, indicating a possible function of this gene in ARF4 regulation.

Among genotypes in the *Pt*QTL category, a couple of genes related to cell wall, cellulose biosynthesis and lignin were identified as more highly expressed. For instance, gene grail3.0594000101, CESA3 encodes cellulose synthase isomer (Burn et al., 2002). Also, gene eugene3.00110748, TBL38 (trichome birefringence-like), which encodes a member of the TBL gene family containing a plant-specific DUF231 domain of unknown function. Two other genes of the same family have been shown to be involved in the synthesis and deposition of secondary wall cellulose (Bischoff et al., 2010). Also more

highly expressed in genotypes of the *Pt*QTL category, COB-like (gw1.155.39.1) is a key regulator of the orientation of cell expansion in roots (Schindelman et al., 2001). The gene fgenesh4_pg.C_scaffold_246000012, homolog of *Arabidopsis* gene RHD3 (root hair defective 3), encodes a putative GTP-binding protein that might be involved in cell wall biosynthesis and actin organization (Wang et al., 1997).

Genetical genomic analysis of QTLs for root number

A set of 81 genes differentially expressed between genotypes in the *Pd*QTL and *Pt*QTL categories co-localize with the QTL interval for number of roots in LG II and XIV. Of particular interest are two genes (estExt_fgenesh4_pg.C_LG_II0422 and fgenesh4_pg.C_LG_II000237) that are part of the tryptophan biosynthesis pathway. Tryptophan is a precursor of auxin, a phytohormone well known to affect production of adventitious rooting in cuttings (De Klerk et al., 1995). fgenesh4_pg.C_LG_II000237 is the homolog of *Arabidopsis* gene SUR2 (*superroot2*), also found to be significantly expressed in the contrast of time points 0 and 1, as previously described. The gene estExt_fgenesh4_pg.C_LG_II0422 is the homolog of TSA1 (*tryptophan synthase alpha chain*), which catalyzes the conversion of indole-3-glycerolphosphate to indole, the penultimate reaction in the biosynthesis of tryptophan.

Several transcription factors located in the QTL intervals were also identified as being differentially regulated. Genes eugene3.00020393 and fgenesh4_pg.C_LG_III000900, are highly expressed in genotypes of the *Pd*QTL and *Pt*QTL categories, respectively. The gene eugene3.00020393 is a homolog of *Arabidopsis* gene MYB4R1, a putative MYB protein containing four R1R2-like repeats, which is unusual for MYB proteins and whose biological function has not been explored to date (Stracke et al., 2001). Fgenesh4_pg.C_LG_III000900 is a homolog of

Arabidopsis gene WRKY21, which encodes WRKY DNA-binding protein 21. It is significantly expressed in time point 2. The detailed function of WRKY proteins is still largely unknown, but several WRKY proteins have been associated to the response to pathogen infection and other stresses (Eulgem et al., 2000). Both genes are located in the QTL interval in Linkage Group II.

Also with higher expression in genotypes of the *Pd*QTL category, but in time points 4 and 8, gene estExt_Genewise1_v1.C_LG_II1725, homolog of *Arabidopsis* gene GASA1, is involved in response to gibberellins stimulus, brassinosteroid, abscisic acid stimulus and unidimensional cell growth (Bouquin et al., 2001). Expressed in genotypes of the *Pt*QTL category in time points 2 and 4, a homolog of *Arabidopsis* gene EOL1, estExt_Genewise1_v1.C_LG_II2213, encodes a paralog of ETO1, which is a negative regulator of ACS5, a key enzyme in ethylene biosynthesis pathway (Christians et al., 2009).

Discussion

This study investigated the genetic control of adventitious root formation and early root-related traits of a pseudo-backcross population (family 52-124) originated from a cross between the hybrid *Populus trichocarpa* × *P. deltoides*, and an unrelated *P. deltoides* parent. Some previous quantitative genetics studies have shown that adventitious root formation is under strong genetic control in *Populus* species (Ying and Bagley, 1977, Zhang et al., 2009, Wilcox and Farmer, 1968). However, we found weak to moderate genetic effect in rooting (H^2 = 0.12 - 0.34), in line with a previous study in the same family (H^2 = 0.28 - 0.33) (Novaes et al., 2009), and a few studies in other *Populus* species (Zalesny et al., 2005, Riemenschneider and Bauer, 1997). Differences

might be attributed to the use of different growth conditions, the type of cuttings that were used or differences in the genetic control of the trait among species of *Populus*.

QTL Identification

QTLs were discovered for a broad range of early root-related traits. Few QTL mapping studies have been carried out to identify genes related to adventitious root formation, and most of them were carried out in crop species such as rice (Zheng et al., 2003), maize (Mano et al., 2005) and common bean (Ochoa et al., 2006). Only two QTL studies on adventitious rooting of *Populus* had been previously reported (Han et al., 1994, Zhang et al., 2009). Han et al. (1994) studied the quantitative genetic aspect of *in vitro* adventitious root formation and shoot regeneration in F_1 hybrids of *P. trichocarpa* and *P. deltoides,* and segregating populations (F_2 and BC_1). That study mapped two QTLs involved in control of organogenesis *in vitro.* However, no QTL was detected for root number or length. Zhang et al. (2009) used functional mapping to detect QTLs for number of roots and maximum root length measured at five time points. The population used was an interspecific hybrids between *P. deltoides* and *P. euramericana*, from which cuttings were also grown in hydroponics. Three QTLs affecting maximum root length and total root number were detected, but no common QTL affected both traits. This result might be due to the fewer number of individuals used (93 genotypes, relative to 234 genotypes used in our experiment) and the mapping approach used. In that previous study, the proportion of the phenotypic variance explained by QTLs detected for root number and root length ranged from 0.01 to 0.20 and 0.01 to 0.14, respectively.

Adventitious Rooting Analysis

Adventitious root development on the bottom of apical cuttings has been suggested to be due to higher concentrations of auxin translocated to that region,

probably in response to wounding (Zalesny Jr et al., 2003). The work of Zalesny Jr. and Wiese (Zalesny Jr and Wiese, 2006) has shown that time of the year and ancestry, and their interaction, are highly significant factors in determining the number of root and root dry weight of *Populus* hardwood cuttings. Also, it has been observed that superior rooting of apical cuttings occurs for species from the section *Tacamahaca* compared to those from *Aigeros*. On the other hand, species from section *Aigeiros*, such as *P. deltoides* and *P. nigra*, rooted better when using middle and basal cuttings. Zalesny Jr. et al (Zalesny Jr et al., 2003) have also shown that stem position on the stool plant accounted for approximately 6% of the variation in rooting of different species of poplar, including *P. deltoides*.

Previous studies in adventitious rooting ability, that included several species of *Populus* (Zalesny et al., 2005), concluded that dormant hardwood cuttings of *P. deltoides* don't root as well as other *Populus* species, possibly because fewer number of preformed root primordia are present. Our study suggests that a slower development of roots in *P. deltoides* may be at the source of this difference. Depending on the growth condition, this effect might be critical in the survival of cuttings. Studies linking number of roots and existence of preformed root primordia are still lacking.

Successive Phases in Adventitious Root Formation

The phases of adventitious root formation possibly follow similar pattern in *Populus* as in apple microcuttings, where the process has been studies in much detail. Roots were observed in the present study after six days in culture. In apple microcuttings, roots are generally observed after five days (De Klerk et al., 1999). Wu (Wu, 2004) observed root outgrowth from the stem after six days of culture, in the hybrid *Populus tremula* × *P. alba*. Therefore, these woody species appear to follow

approximately the same timing of adventitious root formation. The transcriptome information also indicates that in the first 24 hours, described previously as the dedifferentiation phase (De Klerk et al., 1999), the largest number of genes is differentially regulated (Figure 2-10). This might be due to several internal changes such as hormonal changes and gene regulation. Also, probably most of the wound response takes place during this phase. Cells start to become less specialized and de-differentiate to a meristematic state that is capable of cell division.

Among the most interesting genes observed within each putative adventitious root formation phase, we found gene CPC902 (eugene3.00051316) involved in chromosome segregation during nuclear divisions in time point 1. Probably due to meristematic root organization during the induction phase. Following induction phase, intense rhizogenesis activity in stem root primordia causes the mobilization of lipids reserves probably for energy source (Ciamporova, 1983). Interestingly, we found a number of lipid-related genes differentially expressed between genotypes in the *Pt*QTL and *Pd*QTL categories (fgenesh4_pg.C_LG_VII001302, eugene3.00640195, gw1.VII.1867.1, eugene3.01850030, fgenesh4_pg.C_LG_X000784, estExt_fgenesh4_pg.C_LG_XVIII0095, estExt_Genewise1_v1.C_LG_X3279, gw1.III.811.1, gw1.147.138.1, eugene3.00640241, fgenesh4_pg.C_scaffold_7085000001). Also genes P5CS1 and PIN3 (differentially expressed in time point 4), and gene LAC15, (differentially expressed in time point 8), are known to influence root elongation, which is in agreement with the respective rooting phase. Significantly expressed in genotypes of the PtQTL category during time

point 8, gene gw1.155.39.1, homolog of *Arabidopsis* COB-like gene, is a key regulator of the orientation of cell expansion in roots.

The most promising gene, however, is the gene fgenesh4_pg.C_LG_II000237 *superroot2* (SUR2). EMS *Arabidopsis* mutants of SUR2 result in overproduction of auxin hormone and abundant adventitious root development. Therefore, this gene is a negative regulator of adventitious root. SUR2 is significantly overexpressed in poor rooters when compared to good rooters in our experiment suggesting that this gene is also controlling adventitious root formation in *Populus.* This gene might act by negatively affect auxin biosynthesis, auxin conjugate hydrolysis, sensitivity to auxins, or by increasing cytokinin levels. Cloning of gene SUR2 in *Populus* might be a valuable tool to study how this gene alters number of adventitious root in this species.

Conclusions

QTL mapping and microarray data was used to dissect adventitious root formation in poplar stem cuttings. Parental genotypes presented differential rooting response to early number of adventitious roots. The pseudo-backcross progeny showed segregation for all phenotypic traits measured. This allowed the detection of major QTLs related to early number of adventitious roots. Evaluation of genotypes showing extreme phenotypes through whole-transcriptome analysis made possible the identification of candidate genes of particular importance to rooting that co-locates with major QTLs interval detected, as well as insights into root development processes in *Populus* species.

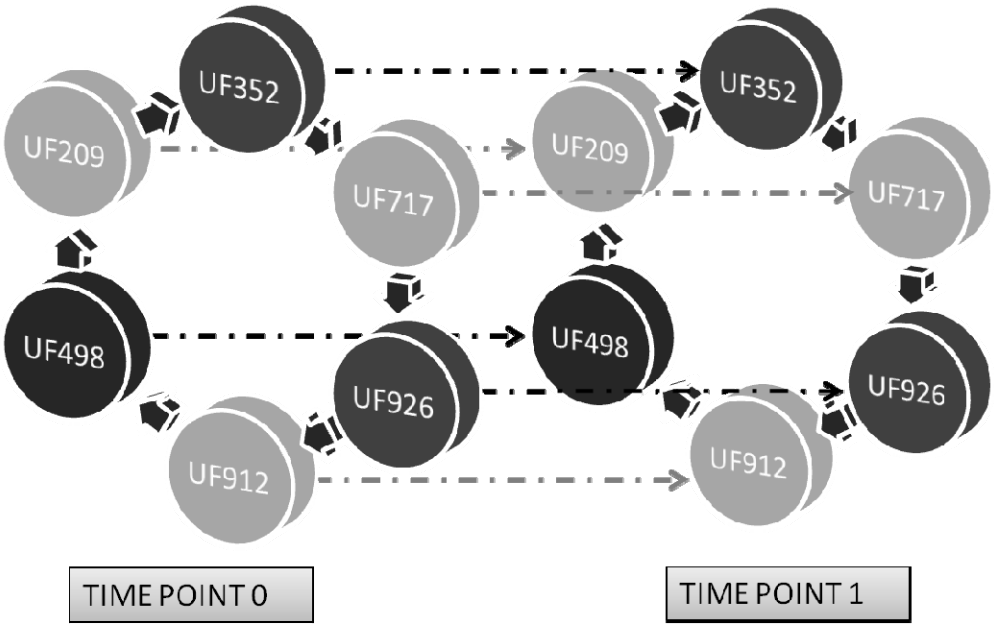

Figure 2-1. Subset of the microarray experimental design showing only two time points. Each arrow is a microarray chip. Arrows indicate dye orientations: The sample at the tail of the arrow is labeled with red (Cy5) dye, and sample at the head of the arrow is labeled with green (Cy3) dye. Dark gray color circles represent genotypes in *Pt*QTL category, while light gray represents genotypes in *Pd*QTL category. Comparisons were made for all consecutive time points as indicated in the figure.

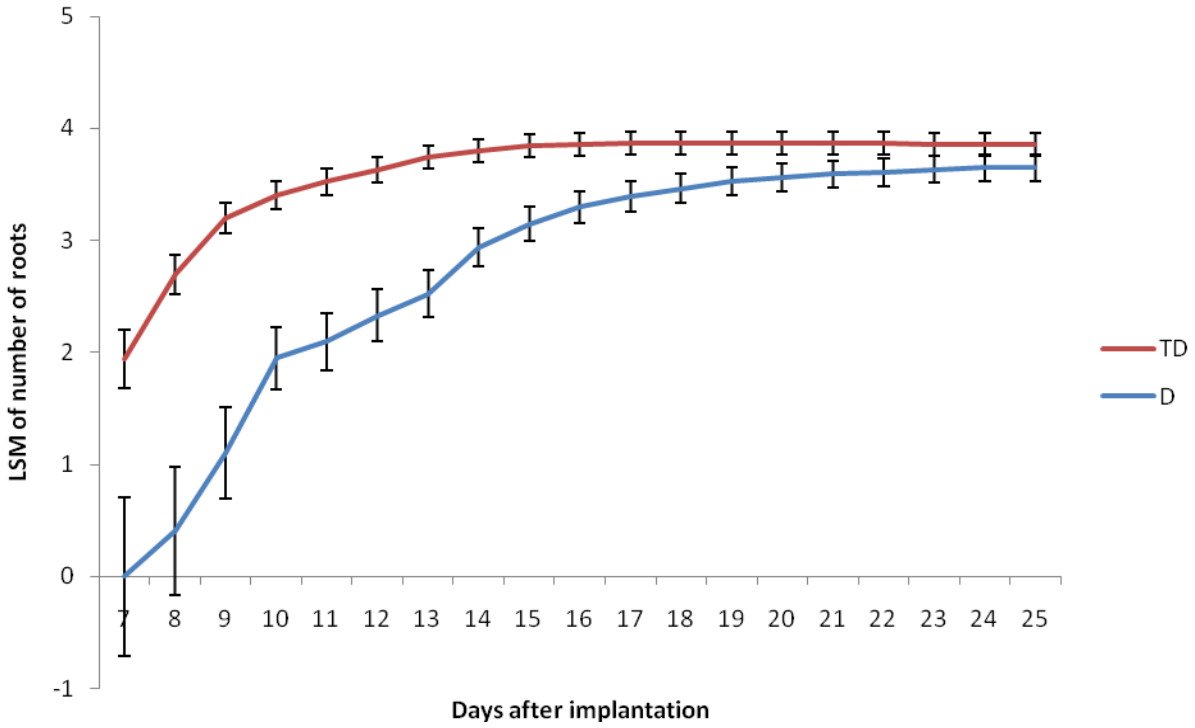

Figure 2-2. LSM of number of adventitious roots developed on the female hybrid parent *Populus trichocarpa* × *P. deltoides* 52-225 (**TD**), and the unrelated male parent *P. deltoides* D124 (**D**), maintained in hydroponic solution for 25 days. Error bars show standard error. Parents means are significantly diferent ($p<0.05$) from day 7 to day 19.

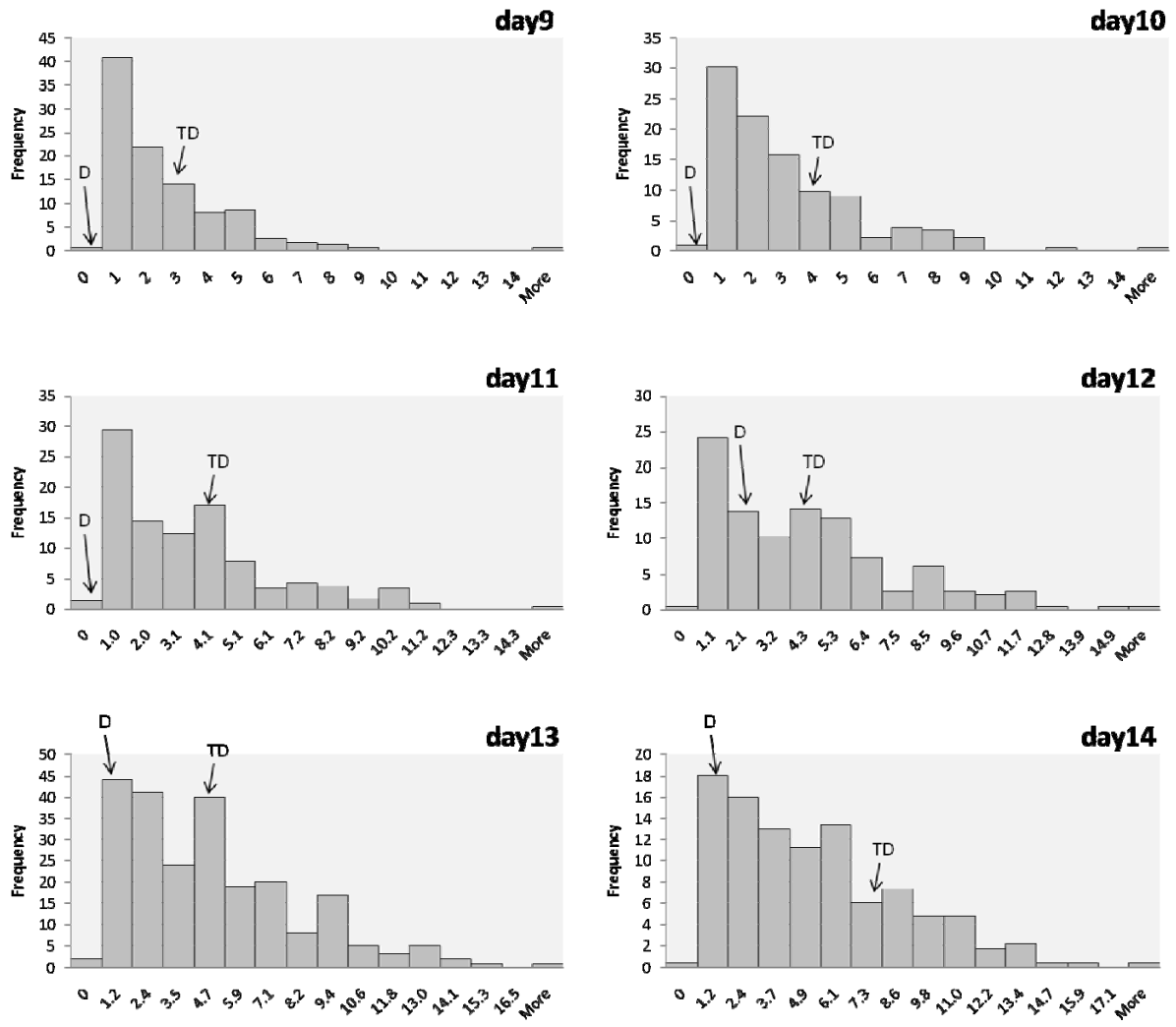

Figure 2-3. Distribution of Least Square Mean (LSM) estimates for number of root traits of 236 individuals of family 52-124 and their parents ‘P. deltoides’ (D) and P. trichocarpa × P.deltoides) × P.deltoides (TD).

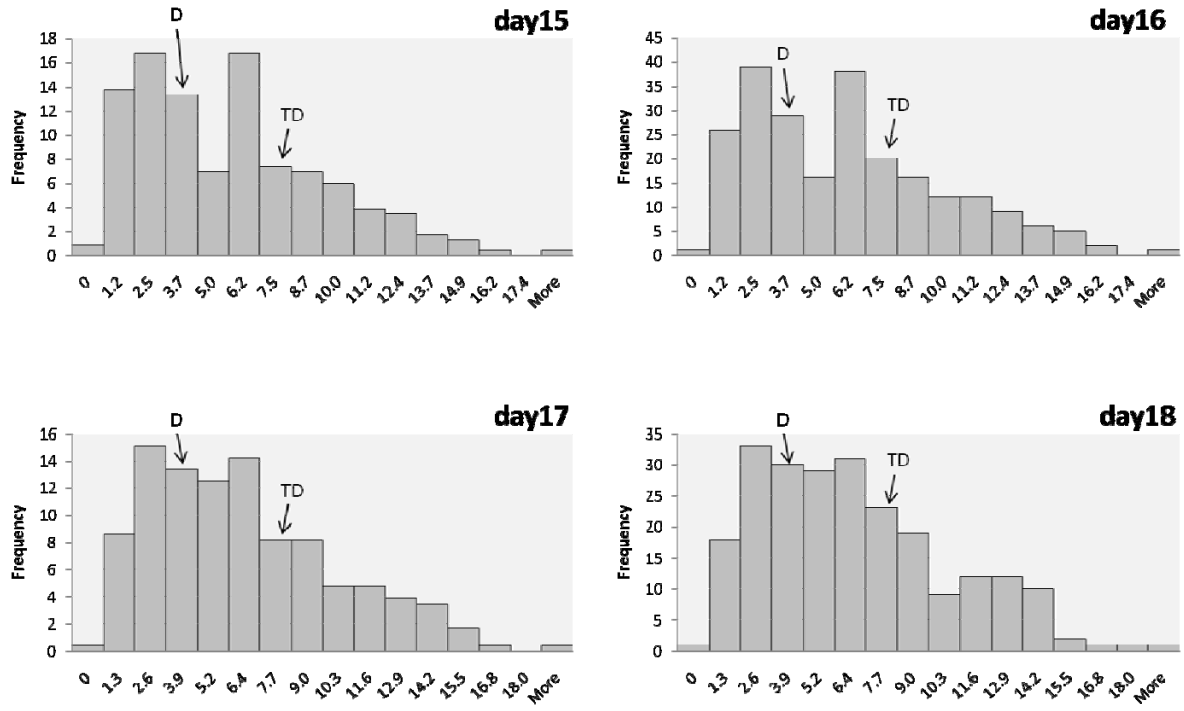

Figure 2-3. Continued.

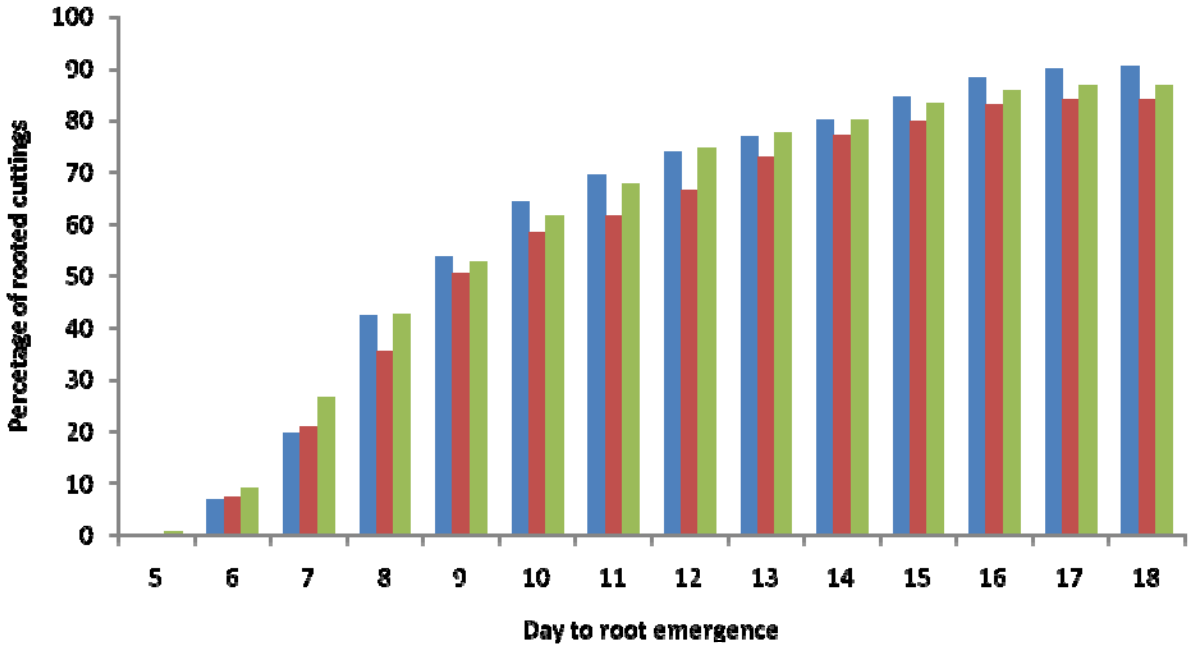

Figure 2-4. Cumulative percentage of genotypes rooted during the experiment in each replication. Each color bar represents a replication.

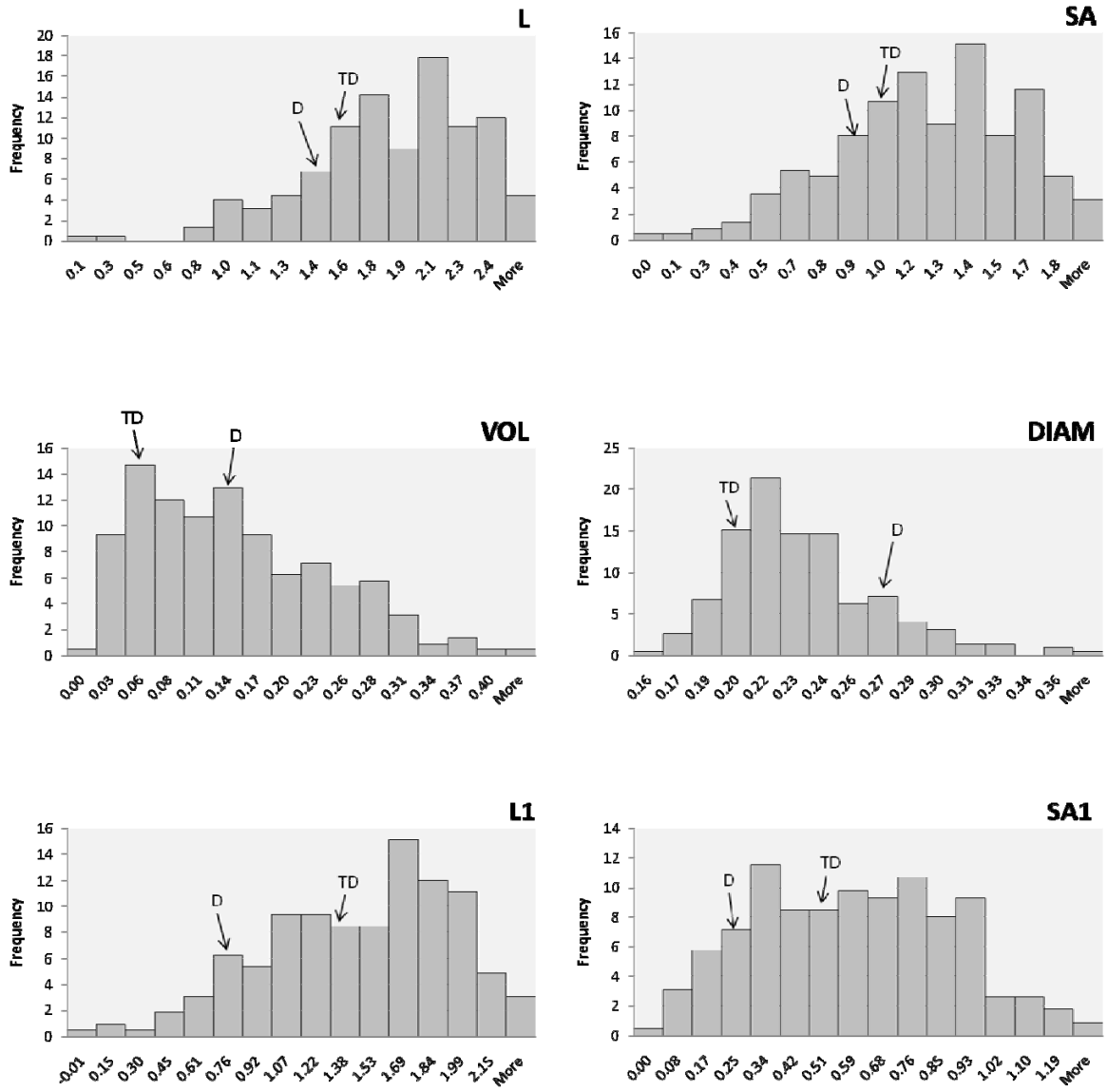

Figure 2-5. Distribution of Least Square Mean (LSM) values for root architectural and biomass traits of 225 individuals of family 52-124 and their parents '*P. deltoides'* (D) and (*P. trichocarpa* × *P.deltoides*) × *P.deltoides* (TD), measured after 18 days in hydroponic solution.

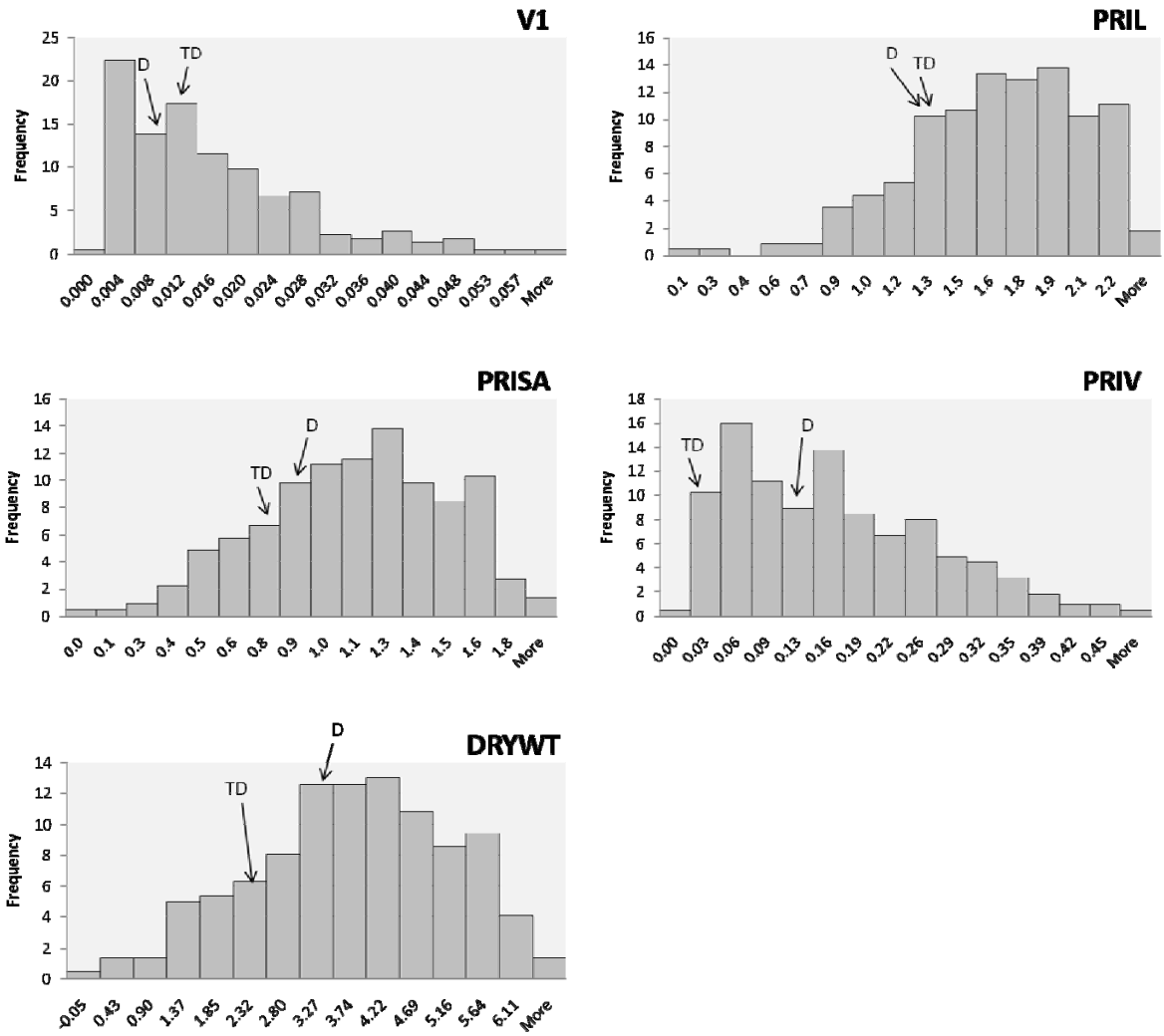

Figure 2-5. Continued.

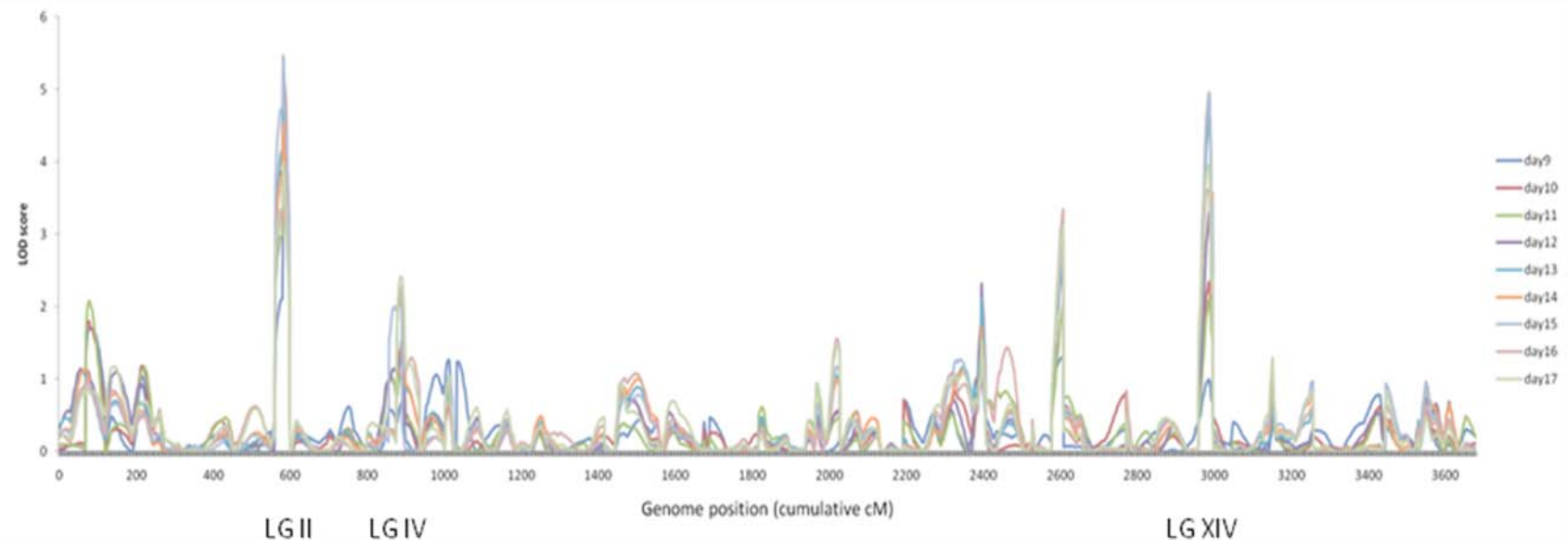

Figure 2-6. Localization of 11 quantitative trait loci (QTLs) detected on the mother map for number of roots. QTLs were identified as in Table 2-3.

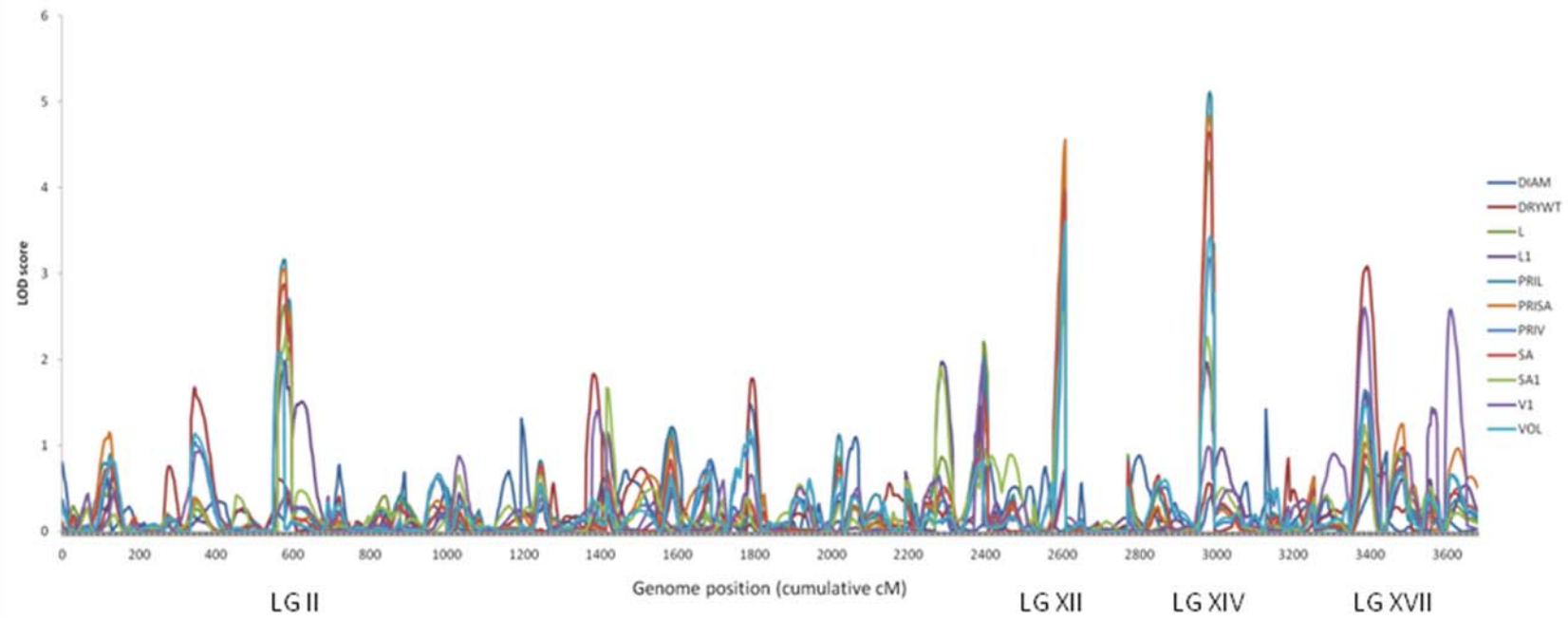

Figure 2-7. Localization of 15 quantitative trait loci (QTLs) detected on the mother map for root architectural traits and root biomass. QTLs were identified as in Table 2-4.

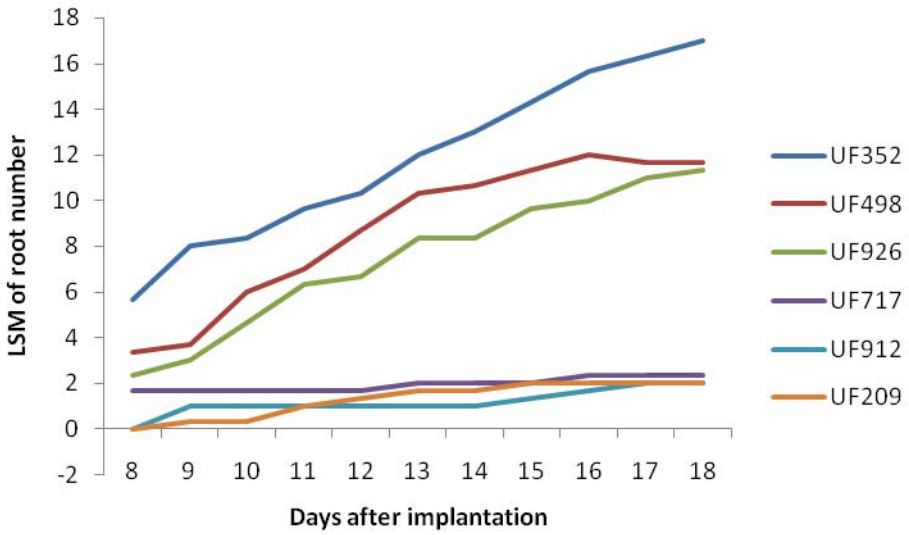

Figure 2-8. Least square means (LSM) of number of adventitious roots developed on selected genotypes for each day measured. Genotypes UF352, UF498 and UF926 carry *P. trichocarpa* allele, whereas genotypes UF717, UF912 and UF209 carry *P. deltoides* allele. Number of roots is significantly different between extreme genotypes for all days.

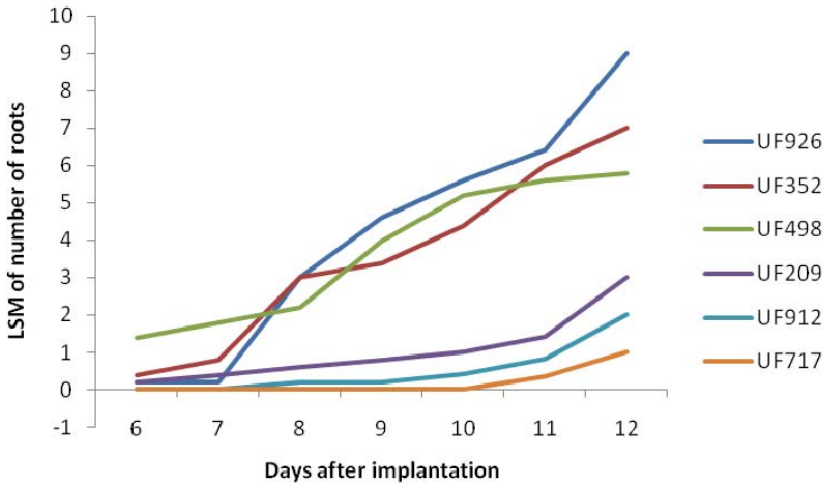

Figure 2-9. Least Square Means (LSM) of number of adventitious roots developed on extreme genotypes selected for expression analysis. Genotypes UF352, UF498 and UF926 carry *P. trichocarpa* allele, whereas genotypes UF717, UF912 and UF209 carry *P. deltoides* allele.

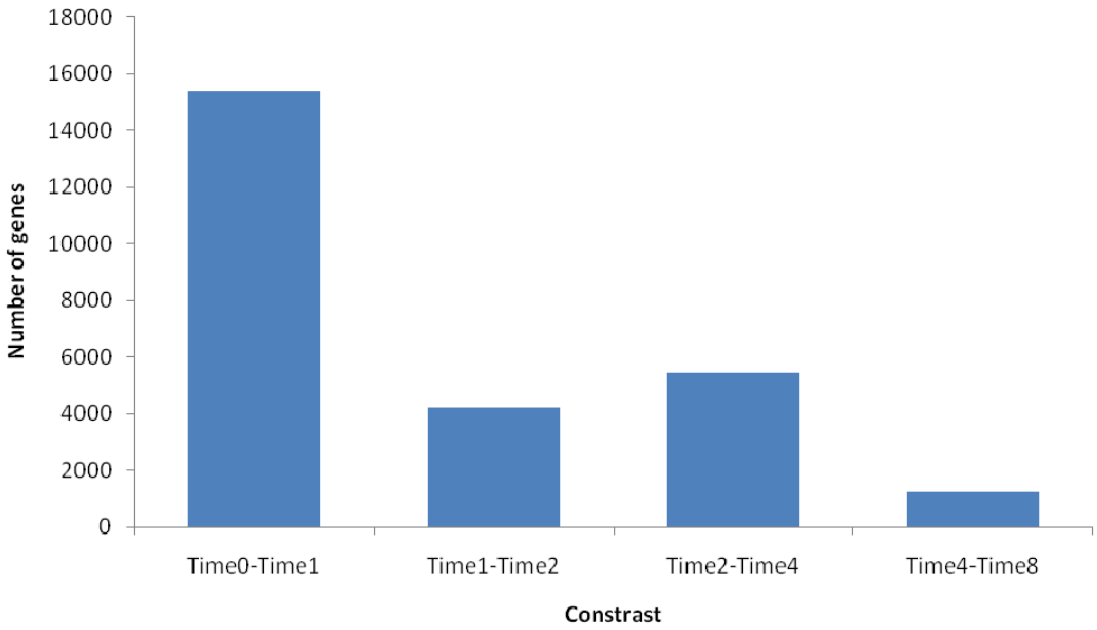

Figure 2-10. Number of genes differentially expressed when contrasting consecutive time points.

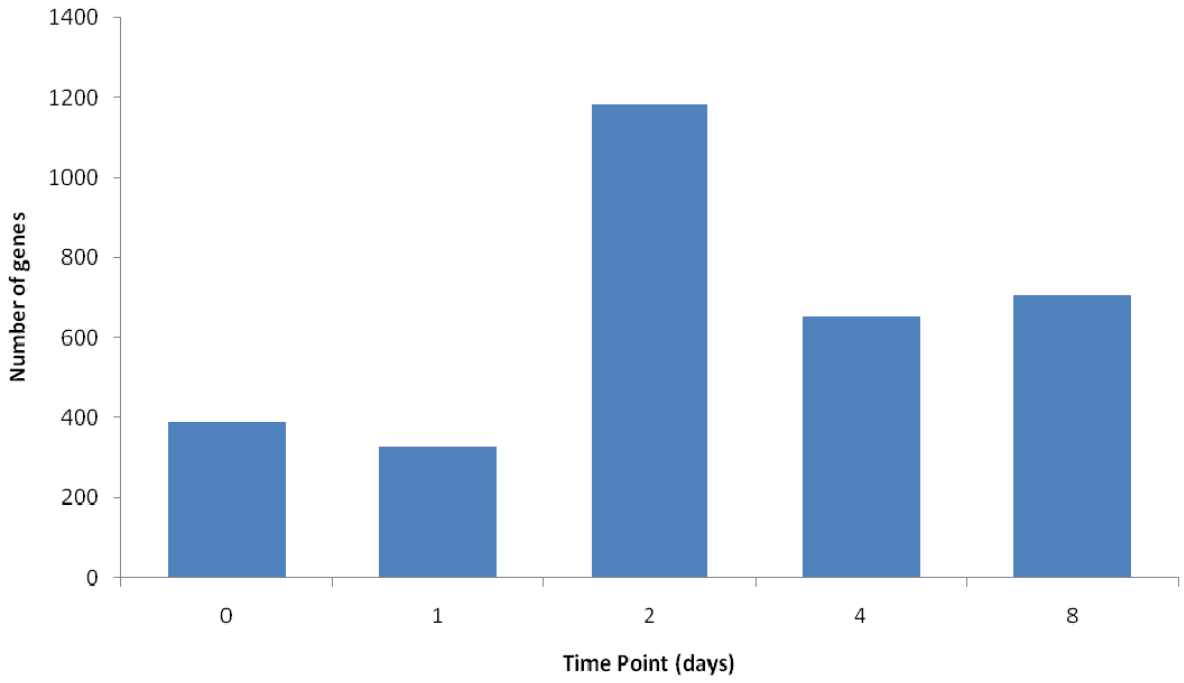

Figure 2-11. Total number of genes differentially expressed between genotypes in the *Pt*QTL and *Pd*QTL categories within each time point.

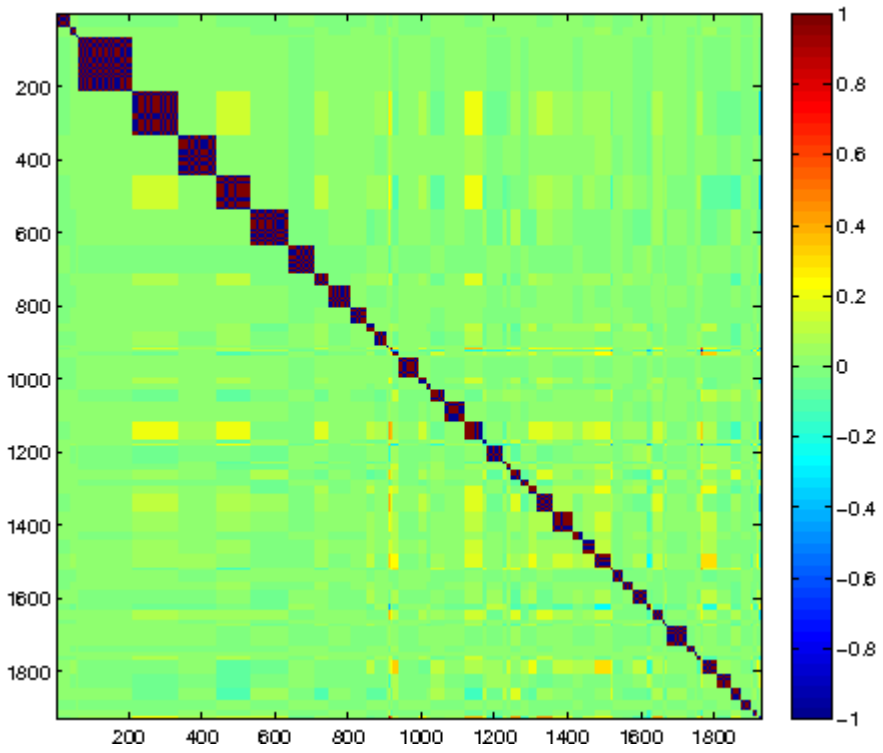

Figure 2-12. Correlation matrix of the signal difference between genotypes in the *Pt*QTL and *Pd*QTL categories estimated for 1929 genes identified as differentially expressed (FDR<0.05) between the two categories in at least one time point.

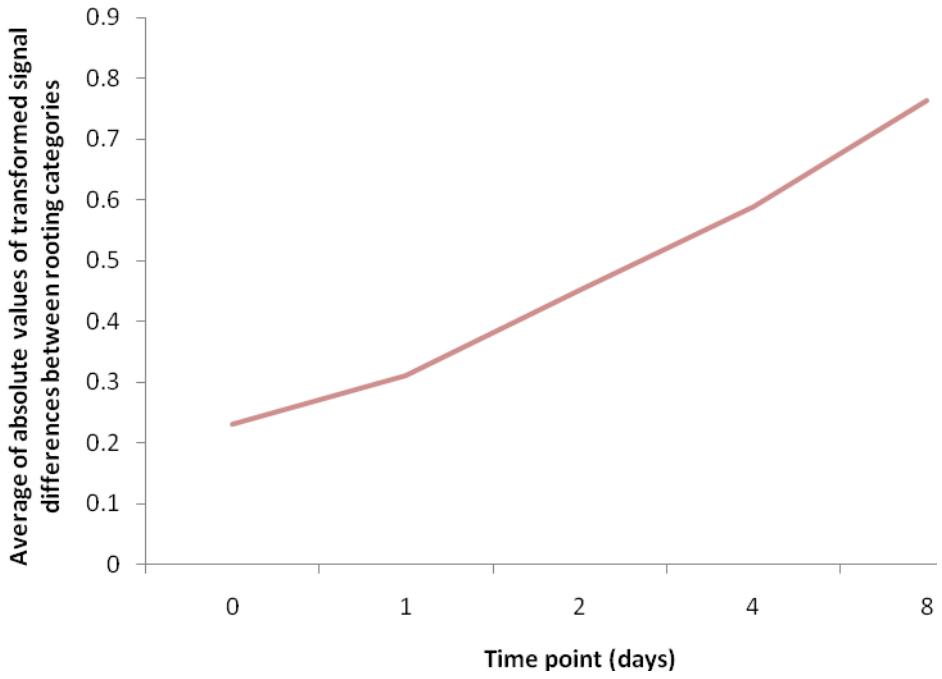

Figure 2-13. Average of absolute values of signal differences between genotypes in the *Pt*QTL and *Pd*QTL categories across each time point for the only cluster with a linear increase in the difference in transcript abundance between the two categories.

Table 2-1. Clonal repeatability estimates for all adventitious root-related phenotypes measured.

Trait	Acronym	Phenotypic values Mean ± SD *P. trichocarpa* × *P. deltoides*	Phenotypic values Mean ± SD *P. deltoides*	Progeny Variation	Clonal repeatability H^2 ± SD
Root architectural traits					
Total root length (cm)	L	56.5	86.4	123.1 ± 94	0.153 ± 0.051
Total root surface area (cm^2)	SA	10.0	23.4	25.1 ± 19	0.169 ± 0.052
Total root volume (cm^3)	VOL	0.14	0.51	0.42 ± 0.3	0.185 ± 0.053
Average diameter (mm)	DIAM	0.56	0.93	0.70 ± 0.2	0.236 ± 0.055
Length of root branches (cm)	L1	25.6	28.5	55.7 ± 49	0.115 ± 0.049
Surface area of root branches (cm^2)	SA1	2.90	2.40	4.33 ± 3.8	0.117 ± 0.049
Volume of root branches (cm^3)	V1	0.28	0.20	0.034 ± 0.03	0.120 ± 0.049
Total length of primary roots (cm)	PRIL	30.8	57.6	67.1 ± 50	0.193 ± 0.053
Surface area of primary roots (cm^2)	PRISA	6.90	20.1	19.4 ± 15	0.187 ± 0.053
Volume of primary roots (cm^3)	PRIV	0.19	0.61	0.49 ± 0.42	0.177 ± 0.053
Number of root traits					
Count of roots on day 9	day9	3.79	0.46	1.76 ± 1.97	0.294 ± 0.052
Count of roots on day 10	day10	4.64	1.08	2.36 ± 2.35	0.297 ± 0.052
Count of roots on day 11	day11	5.21	1.25	3.05 ± 2.72	0.281 ± 0.052
Count of roots on day 12	day12	5.79	1.58	3.69 ± 3.09	0.274 ± 0.052
Count of roots on day 13	day13	6.50	1.92	4.24 ± 3.35	0.289 ± 0.053
Count of roots on day 14	day14	6.86	2.92	4.62 ± 3.55	0.304 ± 0.054
Count of roots on day 15	day15	7.21	3.58	5.05 ± 3.66	0.311 ± 0.054
Count of roots on day 16	day16	7.59	4.16	0.14 ± 0.47	0.342 ± 0.056
Count of roots on day 17	day17	7.36	4.58	5.71 ± 3.88	0.336 ± 0.056
Count of roots on day 18	day18	7.36	4.92	5.92 ± 3.95	0.341 ± 0.056
Root biomass (mg)	DRYWT	4.73	27.5	19.3 ± 19.7	0.144 ± 0.051

Table 2-2. Pair-wise estimates of phenotypic correlations between all traits.

Trait	L	SA	DIAM	VOL	L1	SA1	V1	PRIL	PRISA	PRIV	DRYWT	day9	day10	day11	day12	day13	day14	day15	day16	day17	day18
L	X	0.98	0.34	0.92	0.95	0.94	0.92	0.95	0.94	0.92	0.91	0.46	0.49	0.52	0.55	0.55	0.58	0.58	0.58	0.57	0.56
SA		X	0.44	0.98	0.88	0.87	0.85	0.98	0.99	0.98	0.96	0.45	0.47	0.51	0.53	0.53	0.57	0.57	0.58	0.57	0.57
DIAM			X	0.51	0.21	0.19	0.17	0.44	0.49	0.51	0.48	0.17	0.15	0.12	0.13	0.12	0.17	0.17	0.18	0.18	0.18
VOL				X	0.78	0.77	0.76	0.97	0.99	0.99	0.96	0.42	0.45	0.47	0.49	0.50	0.54	0.55	0.56	0.55	0.55
L1					X	0.99	0.97	0.80	0.80	0.80	0.81	0.38	0.38	0.41	0.43	0.42	0.44	0.43	0.42	0.42	0.40
SA1						X	0.99	0.79	0.79	0.79	0.80	0.37	0.37	0.40	0.42	0.41	0.42	0.42	0.41	0.40	0.39
V1							X	0.77	0.77	0.77	0.78	0.38	0.37	0.40	0.41	0.41	0.42	0.41	0.41	0.40	0.39
PRIL								X	0.99	0.96	0.91	0.50	0.54	0.58	0.62	0.63	0.66	0.67	0.68	0.67	0.67
PRISA									X	0.99	0.95	0.45	0.48	0.52	0.54	0.55	0.59	0.59	0.60	0.60	0.59
PRIV										X	0.97	0.39	0.42	0.44	0.46	0.47	0.50	0.51	0.52	0.52	0.51
DRYWT											X	0.35	0.37	0.40	0.41	0.41	0.45	0.45	0.46	0.45	0.44
day9												X	0.92	0.87	0.80	0.76	0.75	0.72	0.70	0.69	0.67
day10													X	0.93	0.88	0.84	0.82	0.79	0.77	0.75	0.73
day11														X	0.96	0.93	0.91	0.88	0.85	0.83	0.81
day12															X	0.98	0.96	0.93	0.90	0.88	0.86
day13																X	0.98	0.96	0.93	0.92	0.89
day14																	X	0.99	0.96	0.95	0.93
day15																		X	0.99	0.97	0.96
day16																			X	0.99	0.98
day17																				X	0.99
day18																					X

Table 2-3. Phenotypic variance explained by each QTL interval identified for number of root traits and the respective linkage group (LG), flanking markers location, LOD peak and origin of positive allele.

			Flanking Markers				
QTL	Trait acronym	LG	Marker 1	Marker 2	LOD peak	Origin of positive allele	Phenotypic variance explained (%)
1	day9	II	G734	rG876	5.34	*P. deltoides*	10.12
2	day9	IV	O349	G961	4.22	*P. trichocarpa*	7.94
3	day11	II	S96	rG876	5.48	*P. deltoides*	10.34
4	day14	II	S96	rG876	5.60	*P. deltoides*	10.66
5	day14	XIV	rO386a	G674	4.99	*P. deltoides*	9.67
6	day15	II	S96	rG876	5.00	*P. deltoides*	9.33
7	day15	XIV	rO386a	G674	5.03	*P. deltoides*	8.93
8	day16	II	S96	O461	3.72	*P. deltoides*	7.20
9	day16	XIV	rO386a	P2515	3.87	*P. deltoides*	7.86
10	day17	II	S96	rG876	4.75	*P. deltoides*	9.19
11	day17	XIV	rO386a	P2515	3.63	*P. deltoides*	6.66

Table 2-4. Phenotypic variance explained by each QTL interval detected for root architecture traits and root biomass with the respective linkage group (LG), flanking marker location, LOD score and origin of positive allele.

			Flanking markers				
QTL	Trait acronym	LG	Marker 1	Marker 2	LOD peak	Origin of positive allele	Phenotypic variance explained (%)
1	L1	XII	G2643	G2673	3.80	*P. deltoides*	7.64
2	L	XII	G2643	G2673	3.86	*P. deltoides*	7.18
3	L	XIV	rO386a	P2515	4.30	*P. deltoides*	10.0
4	PRIL	II	S96	O461	3.16	*P. deltoides*	5.8
5	PRIL	XII	G2643	G674	4.45	*P. deltoides*	8.12
6	PRIL	XIV	rO386a	G674	5.12	*P. deltoides*	10.87
7	PRISA	XII	G2643	G2673	4.56	*P. deltoides*	8.54
8	PRISA	XIV	rO386a	G674	4.84	*P. deltoides*	10.84
9	PRIV	XII	G2643	G2673	3.17	*P. deltoides*	6.12
10	PRIV	XIV	rO386a	P2515	3.18	*P. deltoides*	6.76
11	DRYWT	XVII	rG880	P648	3.08	*P. trichocarpa*	7.04
12	SA	XII	G2643	G2673	3.95	*P. deltoides*	7.40
13	SA	XIV	rO386a	G674	4.64	*P. deltoides*	10.03
14	VOL	XII	G2643	G2673	3.61	*P. deltoides*	6.92
15	VOL	XIV	rO386a	P2515	3.43	*P. deltoides*	7.22

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BIOGRAPHICAL SKETCH

Cynthia M. Silva was born in Santo André (São Paulo state, Brazil) in 1985. At the age of 17, she entered into University of São Paulo in Piracicaba (ESALQ-USP, Brazil) to pursue her Bachelor of Science in Agronomic Engineering. As an undergrad, she joined the Ph.D. student Marines Karasawa, supervised by Professor Dr. Elizabeth Ann Veasey, to study the genetic diversity of the rice species *Oryza glumaepatula* using microsatellites markers. When she concluded her project in Dr. Veasey's lab, she started a new project with the Ph.D. student Juliana Teixeira on the development of molecular markers for disease resistance in *Eucalyptus*, under the supervision of Dr. Luis Camargo. During her last year as an undergrad, Dr. Camargo introduced her to Dr. Matias Kirst, who accepted her as an intern in his Forest Genomics lab at the University of Florida. During her three-month internship in the U.S., she first learned about the tree genus *Populus*, and was introduced to the world of QTL mapping. She came back to Brazil to obtain her Bachelor of Science in January of 2008. She returned to the United States in August of 2008, to pursue her Master of Science degree in the School of Forest Resources and Conservation at the University of Florida, as a member of Dr. Kirst's Forest Genomics research group.