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Mechanisms of cadmium tolerance in Populus

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MECHANISMS OF CADMIUM TOLERANCE IN POPULUS

Brahma Reddy Induri

**Dissertation submitted to the
Eberly College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy
in
Biology**

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Department of Biology

**Morgantown, West Virginia
2013**

**Keywords: Cadmium, candidate genes, genotype, heritability, homeostasis, phenotype,
phytoremediation, *Populus*, quantitative trait loci
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ABSTRACT

Mechanisms of Cadmium Tolerance in *Populus*

Brahma Reddy Induri

Cadmium (Cd), a known carcinogen and one of the leading heavy metal pollutants on a global scale, is released to the atmosphere through various industrial processes such as smelting and mining and other applications including manufacture of batteries and phosphate fertilizers. It causes many deleterious effects in plants, and is harmful to humans through its entry into the food chain. Traditional methods of remediating Cd-contaminated soils, such as excavation and land-filling, are expensive and disturb the natural topography. The emerging method of phytoremediation in which plants are employed to remediate contaminated soils has become increasingly popular because of its cost effectiveness and environmentally friendly characteristics. *Populus* trees have been extensively studied for their effectiveness in phytoremediation due to their size, productivity, and status as a model organism. My dissertation had the following objectives with the ultimate focus on developing transgenic *Populus* trees for phytoremediation of Cd contaminated soils: 1.) Map QTL (Quantitative Trait Loci) for Cd tolerance and identify candidate genes for Cd tolerance from QTL intervals, microarray studies and comparative genomics in *Populus*; 2.) Map QTL for growth, biomass and phenology traits in *Populus* under greenhouse and field conditions; 3.) Study the influence of Fe on Cd toxicity effects on growth and biomass traits of Cd tolerant and Cd susceptible *Populus* genotypes. In Chapter 2, I conducted a greenhouse hydroponic study in which 252 genotypes of a pseudo-backcross *Populus* progeny of family 52-124 were grown and treated with Cd. The phenotypic data recorded on these genotypes were used to identify tolerant and susceptible genotypes, and also for identifying QTL for Cd tolerance. The individuals used in the hydroponic experiment were scored for SNP (Single Nucleotide Polymorphism) markers, and statistical associations were detected between the phenotypic and genotypic data, indicating the presence of QTL. Candidate genes were identified from these QTL intervals using microarray studies and comparative genomic approaches. The same pedigree was grown in a field trial at West Virginia University Agronomy Farm and extensive phenotypic data was recorded on growth, biomass and phenology traits during 2008 and 2009 seasons. In Chapter 3, I compared biomass and phenology trait QTLs across the above hydroponic experiment and from the field study. Extensive phenotypic variation was observed among the growth and biomass traits in both the studies and the change in the QTL positions across experiments and across years suggested changing genetic control on the traits. For Chapter 4, a greenhouse hydroponic experiment was carried out in which a Cd tolerant and a Cd susceptible *Populus* genotype were studied. This experiment was a dosage study with two levels (0 μ M and 25 μ M) of Cd and two (0 and 100 μ M) levels of Fe. All of the treated plants were examined for Cd toxicity effects under high Fe and Fe deficiency conditions on growth and biomass traits. This experiment demonstrated that high Fe conditions mitigated the toxic effects of Cd, and that this effect was more pronounced in the Cd susceptible genotype than in the Cd tolerant genotype. In the future, the candidate genes identified from these studies will be functionally characterized using transgenic approaches and tested for their production and phytoremediation capabilities under greenhouse and field trials. My research has therefore laid the groundwork for the improvement of *Populus* as a crop for biofuels production and phytoremediation.

DEDICATED TO

My Beloved Parents - Yellamanda Reddy and Indira

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CHAPTER I

GENERAL INTRODUCTION

Cd contamination

Cadmium (Cd) is one of the most widespread pollutants of soil and water in industrialized nations and is one of the major pollution problems on a global scale (Zacchini *et al.*, 2009). Cd was ranked seventh in the top ten priority hazardous substances list of the American Agency for Toxic Substance and Disease Registry (Kamnev and Lelie, 2000). Cd is used in the manufacture of batteries, pigments, plastics, electroplating, and fertilizers and is also released into the atmosphere from mining, smelting and smoke from factories and recycling plants. Cd is being released from coal burning, zinc smelting, application of mineral phosphate fertilizers, vehicular traffic, metal industries, volcanoes, and continental dust released by weathering of bedrock (Pinot *et al.*, 2000; Schützendübel *et al.*, 2002; Zacchini *et al.*, 2009). Application of sewage sludge, Cd rich phosphate fertilizers and mining are adding Cd to the soils (McLaughlin *et al.*, 1999, Pinto *et al.*, 2004, Rodriguez-Serrano *et al.*, 2009) which, over time, accumulates in soil and crops risking human health and crop production (Hall, 2002). The cleaning of these contaminated lands by traditional methods such as excavation followed by land filling, soil washing and chemical treatment is ineffective, expensive and not eco-friendly. Because of this, there is a huge demand for eco-friendly and cost effective methods of remediation.

Phytoremediation is an emerging cost-effective and environment friendly technology that utilizes plants to remove, transform, or stabilize contaminants including organic pollutants located in water, sediments, or soils (Cherian and Oliveira, 2005). Phytoextraction, i.e., removal of heavy metals from the soil and storing them in the harvestable plant parts (Padmavathiamma and Li, 2007), is an important component of phytoremediation. The contaminant is removed from the site permanently by harvesting and subsequent incineration of these plants (Cherian and Oliveira, 2005).

Cd toxicity on humans

Cd is the leading heavy metal pollutant and its worldwide pollution in food production systems and increased movement in soil-plant systems is posing serious threat to human health (Shao *et al.*, 2008). Cd causes many disorders and diseases in humans, and is reported to affect the endocrine, renal, and reproductive systems and is also reported to cause kidney dysfunction, leukemia, and cancer of the lung and prostate (Henson *et al.*, 2004).

Cd effects on plants

Cd causes many harmful effects on plants and was reported to be the most toxic heavy metal to plants by causing 50% reduction in the dry weight in some plants (Almeida *et al.*, 2007). Apart from affecting protein structure and membrane lipid composition (Vanassche *et al.* 1990; Ouariti *et al.*, 1997), Cd was found to be toxic even at minute concentrations by developing symptoms such as leaf roll, chlorosis and root and shoot growth reduction (Milone *et al.*, 2003). Cd decreases the photosynthetic activity of plants by disturbing chloroplast ultrastructure (Sandalio *et al.*, 2001) and altering stomatal closure. Apart from affecting the ion and water balance of plants, Cd has been shown to affect various physiological processes such as photosynthesis, respiration, nitrate assimilation and metabolite accumulation (Solti *et al.*, 2008), transpiration and various cellular processes such as enzyme activity (Smeets *et al.*, 2005). Cd also interferes with the uptake and distribution of other nutrients (Shao *et al.*, 2007; Rodriguez-Serrano *et al.*, 2009). Cd disturbs iron (Fe) metabolism (Siedlecka and Krupa, 1999; Fodor *et al.*, 2005; Solti *et al.*, 2008). Cd has also been shown to affect the electron transport system, interact with antioxidative defense systems and induce lipid peroxidation (Smeets *et al.*, 2008; Rodriguez-Serrano *et al.*, 2009).

Developing plants that can tolerate and accumulate toxic levels of Cd is of utmost importance, considering the harmful effects Cd has on humans and plants. Some herbaceous plants accumulate higher levels of metals in their cells naturally, but their slow growth rate, annual habit, small stature, low biomass and/or narrow geographic adaptability make them unsuitable for phytoremediation on an operational scale (Eapen and D'souza, 2005). Higher growth rate, easy propagation by cuttings, suitability for replicated trials, wide range of adaptability, high bulk root volume, very high transpiration rate and large stature make *Populus* an ideal choice for phytoremediation (Rockwood *et al.*, 2004).

Populus is regarded as a model forest tree (Rae *et al.*, 2007; Taylor, 2002; Wullschlegel *et al.*, 2002) because of the availability of many genetic tools including marker saturated genetic linkage maps and whole genome microarrays along with the availability of multiple generation pedigrees (Yin *et al.*, 2004). Along with these genetic tools that are beneficial for genetic studies, most importantly, a whole genome sequence is available for *Populus* (Tuskan *et al.*, 2006), thereby facilitating the identification and isolation of genes that may be important for Cd tolerance. Cd tolerance is also reported to segregate in the genus *Populus* (Robinson *et al.*, 2000). Therefore, *Populus* provides an excellent system both for exploring the basic mechanisms of Cd tolerance, as well as for developing a viable system for phytoremediation. Apart from having ideal phytoremediation characteristics, *Populus* is currently being employed to remediate soils polluted by atrazine (Burken and Schnoor, 1998), trichloroethylene (Newman *et al.*, 1997), chloroacetanilide herbicides (Gullner *et al.*, 2001), Cd (Robinson *et al.*, 2000), Se (Pilon-Smits *et al.*, 1998) and zinc (Di Baccio *et al.*, 2003). Phytoremediation is increasingly becoming popular because of the low cost and limited funds available for remediating environmental pollution (Pilon-Smits, 2005). Salt *et al.*, (1995) and Robinson *et al.*, (2000) reported a phytoremediation

cost of \$60,000 to \$100,000 per hectare, substantially lower than the cost of traditional methods. Apart from the low costs, the solution with phytoremediation is permanent, has low risk of pollution by leaching and the soil will be fertile (Robinson *et al.*, 2000).

Mechanisms of Cd tolerance and metabolism in plants

Plants have many mechanisms to cope with the heavy metal stress. Heavy metal tolerance is defined as the ability to survive soil metal concentrations that are toxic to other plants (Hall, 2002). In general, heavy metal tolerance in plants is by either reduced uptake or elevated plant internal sequestration (Yang *et al.*, 2005b; Macnair *et al.*, 2000; Hall, 2002). Plants developed various mechanisms of heavy metal tolerance including chelation of metals in the cytosol by various ligands, such as phytochelatins, metallothioneins, metal-binding proteins, organic acids and amino acids (Yang *et al.*, 2005b; Hall, 2002), by molecular chaperones and metal trafficking, increased expression of heat-shock-proteins (Yang *et al.*, 2005b), storage of heavy metals in vacuoles, epidermal cells and trichomes and enhanced expression of their enzymatic and nonenzymatic antioxidant defense responses (Milone *et al.*, 2003; Zhao *et al.*, 2005), thereby maintaining homeostasis in plant cells.

Different protein families involved in conferring tolerance to heavy metals including Cd are metal transporters such as heavy metal (or CPx-type) ATPases, natural resistance associated proteins (Nramp), cation diffusion facilitator (CDF) proteins, zinc-Fe permease (ZIP) proteins, other yet to be identified metal transporters (Yang *et al.*, 2005b), increased expression of ABC-type (ATP-Binding Cassette) transporters, vacuolar transporters like *CAX1* and *CAX2* (Clemens, 2001), ZRT (zinc transporters)- and IRT (Fe transporters) like proteins and cation antiporters (Cherian and Oliveira, 2005). Membrane transporters play an important role in metal uptake and homeostasis as demonstrated in yeast and *Arabidopsis* (Clemens, 2001). Some plants called

hyperaccumulators naturally accumulate very high concentrations of heavy metals in their above ground portions (Robinson *et al.*, 2000). Plants having high heavy metal tolerance and very high accumulation rates are of great use in phytoremediation. In fact, the concept of phytoremediation was inspired by them (Chaney *et al.*, 1997).

Mechanisms of Cd tolerance in Populus

Populus tolerates Cd either by sequestration of Cd into vacuoles (Zacchini *et al.*, 2009) or by reduced translocation from roots to shoots to protect the photosynthetic machinery of the plants (Dos Santos Utmazian *et al.*, 2007). Overexpression of phytochelatins, metallothioneins and antioxidative enzymes conferred Cd tolerance to *Populus* as shown below. The biochemical and molecular mechanisms of Cd accumulation and translocation are unknown and are still being investigated by many researchers (Zacchini *et al.*, 2009).

Biotechnological approaches to enhancing Cd tolerance

There have been many biotechnological efforts to enhance Cd tolerance levels, including several studies in *Populus*. Transferring unique genes from plant hyperaccumulators, bacteria, yeast and animals to fast growing and high biomass producing species has been practiced to improve the phytoremediation capabilities by employing genetic manipulation and plant transformation technologies including overexpressions of chelators like phytochelatins, metallothioneins, glutathione enzymes, and various antioxidative enzymes involved in oxidative stress and different metal transporters to improve the phytoremediation potential of many plants (Cherian and Oliveira, 2005).

Populus has been used in remediating various soils contaminated with atrazine, trichloroethylene and selenium. Transgenic *Populus*, overexpressing γ -glutamyl-cysteine synthetase (γ -ECS), with increased Cd uptake capacity and increased GSH levels, was used for

remediating heavy metals and herbicide polluted soils (Gullner *et al.*, 2001). Bacterial gene, *gsh1*, when transferred to *Populus*, enhanced the tolerance to (Arisi *et al.*, 1997, 2000) and accumulation of Cd in roots (Rennenberg and Will, 2000). Simultaneous transfer of many genes into appropriate candidate phytoremediation plants will solve the problem of mixed contamination (organic and inorganic pollutants) of soils (Cherian and Oliveira, 2005).

Importance of QTL mapping

Some phenotypic characters are governed by single genes (qualitative) and some characters are controlled by many genes with cumulative effects and are called quantitative or polygenic characters. These characters are also called complex traits as many genes are involved and the traits show a continuous range of variation and are influenced more by the environment. This is in contrast to qualitative characters, which show discontinuous variation of the phenotype and are influenced more by the genotype compared to the environment (Kearsey, 1998). The discovery of molecular markers is a milestone development in the quest for unraveling the genetic basis/ dissection of complex traits with which we can develop the linkage maps of the pedigree we study, thus providing the basis for QTL analysis (Doerge, 2002). QTLs are the regions in the genome controlling a phenotypic trait of interest.

QTL mapping is a very powerful tool for elucidating the genetic architecture of complex traits (Rae *et al.*, 2008). Information regarding the number, genomic location and individual effects of genes controlling complex traits can be used to unravel their genetic architecture, and thus help in the breeding programs of forest trees (Zhang *et al.*, 2006). By growing pedigrees segregating for a trait of interest one can test for correlations between the phenotypes and the molecular markers (genotypes) to gain an understanding of the molecular basis of complex traits (Wheeler *et al.*, 2005). Identification of the genes underlying the QTL intervals leads to efficient

selective breeding, and opens up the possibility of using transgenic technology to alter polygenic traits, facilitating discovery of alleles responsible for the process of evolution (Falconer and Mackay, 1996).

Estimation of heritability is one of the first aims in the genetic dissection of a quantitative character (Falconer and Mackay, 1996). Heritability estimates separate the contributions of genetic and environmental variances to the total proportion of variation. Heritability in general can be defined as the proportion of the phenotypic variation in a population that is explained by the differences in the genotypic values of individuals in that population. Broad-sense heritability determines the degree to which an individual's phenotype is controlled by the genotypes whereas narrow-sense heritability determines the degree to which the individuals' phenotype is determined by the actual genes transmitted from their parents (Falconer and Mackay, 1996). Heritability is the reason for degree of resemblance between relatives and it is the proportion of the total variance due to the variation in breeding values (Falconer and Mackay, 1996) and the prediction of genotypic value by the phenotypic value as the most important function of heritability. Heritability will give the strength of correspondence between these two values, thus helping the breeders in estimating the future performance of the population. Broad-sense heritability is the ratio of genotypic variance, $\text{Var}(G)$ to the phenotypic variance, $\text{Var}(P)$. The trait that is being mapped should be under at least moderate genetic control without which the trait cannot be mapped.

QTL mapping of responses to a wide variety of environmental cues enabled researchers to better understand plant responses to the environment (Rae *et al.*, 2007) including salinity (Koyama *et al.*, 2001), drought (Lanaceras *et al.*, 2004), nitrogen supply (Loudet *et al.*, 2003) and soil aluminum concentration (Hoekenga *et al.*, 2003). QTL methodology will give us some

of the first data on the genomic response and on the underlying genes responsible for Cd tolerance in *Populus*. Here, QTL mapping of Cd tolerance will be carried out using an interspecific pedigree.

QTL mapping in forest trees

QTL analyses in forest trees are very important in that they help in dissecting the genetic architecture of quantitative traits (Wu and Lin, 2006). The linked markers with the QTLs also provide the basis for designing marker assisted selection methods. QTL mapping in forest trees is difficult because a standard QTL methodology will involve a number of experimental crosses, which is time consuming and often impossible in trees. Another important prerequisite of QTL mapping is the presence of sufficient phenotypic variance and marker polymorphism in the population being mapped (Karp *et al.*, 1997; Lynch and Walsh, 1998; Lexer *et al.*, 2004). QTL analysis in unstructured intra-specific populations of trees is not possible because of the rapid decay in linkage disequilibrium between molecular markers and traits in large outbreeding populations (Lexer *et al.*, 2004; Hedrick, 2000). QTL analysis is generally carried out by employing three-generation families such as F₂ and backcross from inbred stocks, but lack of inbred lines in forest trees, the long generation times (Hayashi and Awata, 2004; Tulsieram *et al.*, 1992) and inbreeding depression (Verhaegen *et al.*, 1997) of forest trees makes it virtually impossible to create F₂ and backcross pedigrees. QTL analysis is, therefore, difficult in forest trees due to lack of multi-generational pedigrees (Tulsieram *et al.*, 1992).

Because of the higher heterozygosity of forest trees than crop plants due to outcrossing, intra- or inter-specific crossing between the individuals leads to substantial segregation due to gene recombination, thus making QTL analysis possible in the full-sib or half-sib (two-generation) populations of forest trees (Hayashi and Awata, 2004). The pseudo-test cross

strategy (backcross model) is generally used for construction of linkage maps in forest trees (Hayashi and Awata, 2004). Although most trees do not have multigeneration pedigrees, they do have parents and full sibs or maternal half sibs. Because of this, in the present study, the pedigree is derived from a pseudo-back cross (Grattapaglia and Sederoff, 1994) between heterozygote female parent and homozygous null male parent. This approach has been employed in many mapping studies successfully, e.g., eucalyptus (Grattapaglia and Sederoff, 1994) and also in potato (Gebhardt *et al.*, 1989).

Populus can undergo interspecific hybridization and first generation intercross (F_2) and first generation backcross (BC_1) progeny of interspecific hybrids segregate for a wide range of complex traits (Bradshaw and Stettler, 1995; Taylor, 2002). Separate linkage maps are generally constructed for each of the parents by employing two-way pseudo-backcross configuration of markers heterozygous in one parent and homozygous in the other parent (Hayashi and Awata, 2004) and these two linkage maps are combined with the codominant markers segregating in both the parents (Hayashi and Awata, 2004; Wu *et al.*, 2000).

QTL mapping in Populus

Although there is considerable difficulty in working with forest trees, the *Populus* genus is a more convenient system than other trees, owing to its widespread distribution in contrasting environments (Rae *et al.*, 2007) and considerable genetic variation (Wullschleger *et al.*, 2005). There have been many QTL studies in *Populus* for various characters, including leaf morphology and variation (Wu *et al.*, 1997), bud set and bud flush (Frewen *et al.*, 2000), leaf stomatal and epidermal cell development in response under elevated CO_2 (Ferris *et al.*, 2002), dormancy related traits (Chen *et al.*, 2002), leaf level productivity traits (Bunn *et al.*, 2004), growth and biomass distribution (Wullschleger *et al.*, 2005), leaf morphology and spring bud flush (Zhang *et*

al., 2005), flavonoid biosynthesis (Morreel *et al.*, 2006), wood chemical properties (Zhang *et al.*, 2006), osmotic potential (Tschaplinski *et al.*, 2006), drought resistance (Street *et al.*, 2006), leaf growth and delayed senescence under elevated CO₂ (Rae *et al.*, 2006), biomass under elevated CO₂ (Rae *et al.*, 2007), and yield in bioenergy *Populus* (Rae *et al.*, 2008). The present study of QTL mapping for Cd tolerance will provide some of the first data on the genomic regions responsible for Cd tolerance in *Populus*.

Microarray analysis

Microarrays are powerful genomics tools developed to unravel differential gene expression within cells. Most importantly, they can facilitate a global view of a gene expression across an entire genome (Jaluria *et al.*, 2007). Based on this expression pattern of the genes from microarrays, potential defense functions or mechanisms can be identified (Miranda *et al.* 2007). A microarray is defined as a collection of microscopic spots arranged in an array or grid-like format and attached to a solid surface or membrane (Jaluria *et al.*, 2007). Thus, a DNA microarray consists of arrayed series of thousands of microscopic spots called features, each containing picomoles of a specific DNA sequence from a specific section of a gene or other DNA fragment. These spots are called features or probes and are designed in such a way that each probe binds to a specific nucleic acid sequence in the target gene by a process called hybridization (Li *et al.*, 2002). The target sequence, which is bound to the probe, is generally labeled with some signal emitting molecule or dye such as a fluorophore (Butte, 2002). These probes are used to hybridize to fluorescent or chemiluminiscent target cDNA samples under stringent conditions. The resulting intensity of the fluorescence or labeling dye signal from the probe-target hybridization, after scanning, will provide relative abundance of the expression of the specific gene containing the target sequence (Jaluria *et al.*, 2007; Li *et al.*, 2002). Function of

unknown genes can be identified from the large collection of the reference data that is generated from different mutants, tissues and treatments (Rensink and Buell, 2005). Accessibility, cost efficiency and amenable protocol standardization have made microarray a preferred choice although there are many modern and comprehensive wide genome gene expression technologies (Jares, 2006).

Spotted cDNA arrays and oligonucleotide arrays are the two major classes of arrays and these two arrays basically differ in the arrayed material with spotted cDNA array probes being oligonucleotides, complementary DNA (cDNA), fragments of Polymerase Chain Reaction (PCR) products, expressed sequence tag clones, or long genome cloned fragments using vector or gene specific primers (Jares, 2006) and the probes in oligonucleotide arrays coming from oligonucleotides.

Microarrays in Populus

Release of the *Populus trichocarpa* ‘Nisqually-1’ genome sequence has led to development of many novel genomic tools, including whole genome microarrays to analyze the transcriptome of *Populus*, which helps in unraveling molecular mechanisms of variation underlying plant growth, development, environmental response, and eventually in understanding adaptation and evolution (Quesada, Li *et al.*, 2008). Several whole-genome oligonucleotide microarrays have been developed for *Populus* with Oak Ridge National Laboratory and NimbleGen developing the first whole genome oligonucleotide microarray (Yang *et al.*, 2009 unpublished and Tsai *et al.*, 2009). *Populus* presently has different whole-genome microarray platforms, including NimbleGen (first and second generation), Affymetrix and Agilent microarrays.

There have been many gene expression profiling studies (Yang *et al.*, 2009, unpublished) using the first-generation *Populus* DNA microarray, 13K POP1 array developed by Andersson *et al.*, (2004) to identify potential regulator genes of cambial stem cell identity across the wood forming meristem (Schrader *et al.*, 2004), to identify the genes involved in change in the carbon flow into various cell wall components during wood formation (Andersson-Gunneras *et al.*, 2006), to identify drought response (Street *et al.*, 2006), to better understand environmental and hormonal regulation during dormancy (Druart *et al.*, 2007) and to study auxin-regulated wood formation process (Nilsson *et al.*, 2008). By employing the second-generation *Populus* DNA microarray, 25K POP2 array, developed by Moreau *et al.*, (2005), transcript profiling has been accomplished to monitor nocturnal gene expression changes in leaf growth of *Populus* leaf tissue (Matsubara *et al.*, 2005), to drought response in *Populus* genotypes of mapping population Family 331 showing extreme sensitivity and insensitivity (Street *et al.*, 2006) and in apical bud formation and dormancy induction (Ruttink *et al.*, 2007). Microarray studies were also carried out to study the gene expression changes in young *Populus* plants that were exposed to gradually increased water deficit for 4 weeks in a greenhouse (Bogeat-Triboulot *et al.*, 2007) and in *Populus* hybrid plants infected by leaf rust (Miranda *et al.*, 2007). A whole genome microarray analysis was carried out in the present study to understand the molecular mechanisms of Cd tolerance in two tolerant and two susceptible genotypes of *Populus*.

Candidate gene identification

A candidate gene is defined as any gene that has been identified in one organism that is predicted to influence a similar phenotype in another organism (Fitzpatrick *et al.*, 2005; Piertney and Webster, 2008). There are different ways to identify candidate genes including forward genetics approaches in which we create mutant phenotypes by using insertional mutagenesis or

by disrupting a gene function and then studying the phenotypic variation (Grattapaglia *et al.*, 2009). Reverse genetics approaches, such as QTL analysis and association mapping, will help in identifying functionally important loci. Another refined reverse genetics approach is integration of gene expression data (metabolic and peptide data to a particular stress response) with QTL mapping information – called the genetical genomics approach, thereby facilitating the identification of candidate genes. Consolidated QTL positions and the availability of a genome sequence facilitates the identification of candidate genes and this approach has been successful in many plant species for complex traits (Grattapaglia *et al.*, 2009). QTL positional information will be crucial to identify candidate genes in the context of availability of large mapping pedigrees and high throughput genotyping technologies (Grattapaglia *et al.*, 2009). Microarray, i.e., parallel profiling of all genes in a genome, facilitates identification of expression patterns associated with traits of interest (Street *et al.*, 2008). Generally, researchers use these microarray data sets just to shortlist candidate genes. Thus, QTL positional data along with association mapping studies, genome-wide insertional mutagenesis studies, and whole genome microarray studies is an invaluable tool for identification of candidate genes for various phenotypes.

Cd and interaction with other elements

Cd has a similar ionic radius to that of some essential macronutrients and micronutrients, including potassium, calcium, magnesium, iron, manganese, copper, nickel and zinc, and is reported to inhibit their uptake and distribution because of competition for binding sites of common transmembrane transporters (Clemens, 2001; Shukla *et al.*, 2008; Rodriguez-Serrano *et al.*, 2009). In fact, mineral nutrient homeostasis disturbance is one of the most important Cd toxicity effects in plants (Shao *et al.*, 2007; Liu *et al.*, 2003). One of the primary interactions occurs with Fe. Cd disrupts Fe metabolism (Wallace *et al.* 1992; Siedlecka and Krupa 1999;

Fodor *et al.*, 2005; Solti *et al.*, 2008) and decreases the shoot Fe content by affecting its translocation from root to shoot in *Populus* (Fodor *et al.*, 2005; Solti *et al.*, 2008). Siedlecka and Krupa (1999) reported that Cd affecting Fe translocation from roots to leaves as the prominent aspect of Cd-Fe interaction.

Solti *et al.* (2008) reported that Cd disturbs the Fe homeostasis in *Populus*. Fe is essential for photosynthesis and chlorophyll biosynthesis. Furthermore, because of its capacity to donate and accept electrons, it functions as a cofactor for many enzymes with important roles in respiration, nitrogen metabolism and DNA biosynthesis (Jeong and Connolly, 2009). A cell-membrane bound transporter transports both Fe and Cd (Shao *et al.*, 2007, Lombi *et al.*, 2002). Cd treated and Fe deficient plants look similar in their effects on thylakoid structure changes, suggesting an interaction between Cd and Fe in *Populus* (Fodor *et al.*, 2005). High Cd concentration results in chlorosis in plants and is associated with decreased translocation of Fe to leaves, thus inducing Fe deficiency (Sarvari *et al.*, 2001; Shao *et al.*, 2007; Solti *et al.*, 2008). Furthermore, competition between Fe and Cd ions for uptake by a membrane bound Fe transporter caused decreased Fe levels in leaves at increased concentrations of Cd (Shao *et al.*, 2007).

Cd toxic effects were recovered by high Fe concentrations (Siedlecka *et al.*, 1997; Fodor *et al.*, 2005; Meda *et al.*, 2006; Shao *et al.*, 2007), suggesting an interaction between the two elements. Thus, Cd sensitivity depends on the concentration of Fe in the medium (Solti *et al.*, 2008). Cd and Fe interaction may vary among the Cd tolerant and Cd susceptible genotypes of *Populus*. The level of induction of iron transport systems, based on the external Fe concentration, determines Cd uptake in higher plants (Shao *et al.*, 2008). Under Fe deficiency conditions, induction of Cd uptake is stronger, resulting in increased Cd levels in shoots (Shao *et al.*, 2008).

In the Cd microarray study conducted by DiFazio and Ellis (personal communication), the expression of Fe transporters including Yellow Stripe-Like 5 and Yellow Stripe-Like 8 were significantly overexpressed in the susceptible genotypes, but not the tolerant genotypes suggesting the Cd-Fe interaction plays a significant role in contributing to Cd uptake and sensitivity in *Populus*. In the same study, the genes that were significantly expressed in Cd susceptible genotypes compared to the tolerant genotypes are NRAMP3, YSL5, YSL8, HMA6 (PAA1), YSL7, YSL5, FRD3, YSL8, and MTP3.

Main Objectives

- 1). Map QTL (Quantitative Trait Loci) for Cd tolerance and identify candidate genes for Cd tolerance from QTL intervals, microarray studies and comparative genomics in *Populus*.
- 2). Map QTL for growth, biomass and phenology traits in *Populus* under greenhouse and field conditions.
- 3). Study the influence of Fe on Cd toxicity effects on growth and biomass traits of Cd tolerant and Cd susceptible *Populus* genotypes.

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CHAPTER II

IDENTIFICATION OF QUANTITATIVE TRAIT LOCI (QTL) AND CANDIDATE GENES FOR CADMIUM TOLERANCE IN POPULUS

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Authors' contribution

Brahma Reddy Induri wrote the manuscript and performed the greenhouse experiments and Cd assays. Tongming Yin and XinYe Zhang performed the marker analyses, developed the genetic map, performed QTL mapping and provided text describing those methods. Wellington Muchero and Gerald Tuskan contributed toward QTL mapping. Danielle Ellis performed the microarray experiment, analyzed the microarray data, and contributed text describing this work. Gancho Slavov contributed to the statistical analysis. Stephen DiFazio directed the project and performed microarray analysis and contributed toward the writing.

Abstract

Understanding genetic variation for the response of *Populus* to heavy metals like cadmium (Cd) is an important step in elucidating the underlying mechanisms of tolerance. In this study, a pseudo-backcross pedigree of *Populus trichocarpa* Torr. & Gray and *P. deltoides* Bart. was characterized for growth and performance traits after Cd exposure. A total of 16 Quantitative Trait Loci (QTL) at logarithm of odds (LOD) ratio ≥ 2.5 , were detected for total dry weight, its components, and root volume. Major QTL for Cd responses were mapped to two different linkage groups and the relative allelic effects were in opposing directions on the two chromosomes, suggesting differential mechanisms at these two loci. The phenotypic variance explained by Cd QTL ranged from 5.9% to 11.6% and averaged 8.2% across all QTL. Leaf Cd contents also showed significant variation in the pedigree independent of variation in Cd tolerance, suggesting that there is potential for improving the phytoextraction potential of *Populus* genotypes. A whole-genome microarray study led to the identification of nine Cd-responsive genes from these QTL. Promising candidates for Cd tolerance include an NHL repeat membrane-spanning protein, a metal transporter, and a putative transcription factor. Additional candidates in the QTL intervals include a putative homolog of a glutamate cysteine ligase, and a glutathione-s-transferase. Functional characterization of these candidate genes should enhance our understanding of Cd metabolism and transport and phytoremediation capabilities of *Populus*.

Introduction

One of the challenges posed by phytoremediation of cadmium (Cd) contamination is the development of plants that are tolerant of the deleterious effects of this toxin, which has no known nutritional value in plants. Symptoms of Cd toxicity include leaf roll, chlorosis and reductions in root and shoot growth, even at minute concentrations (Almeida *et al.*, 2007; Milone *et al.*, 2003). Cadmium compromises the structure and function of many proteins by acting on sulphhydryl groups (Vanassche and Clijsters, 1990) and affects the permeability of membranes by altering lipid composition (Ouariti *et al.*, 1997).

In addition to disrupting the ion and water balance of plants, Cd affects various physiological processes such as photosynthesis, respiration, nitrate assimilation, metabolite accumulation, and enzyme activity (Smeets *et al.*, 2005; Solti *et al.*, 2008). Cd decreases the photosynthetic activity of plants by changing chloroplast ultrastructure (Sandalio *et al.*, 2001) and altering stomatal closure. Cd also interferes with the uptake and distribution of other nutrients (Rodriguez-Serrano *et al.*, 2009; Shao *et al.*, 2008), most notably iron (Fe) (Fodor *et al.*, 2005; Siedlecka and Krupa, 1999; Solti *et al.*, 2008). Cd has also been shown to affect the electron transport system, interact with antioxidative defense systems and induce lipid peroxidation (Rodriguez-Serrano *et al.*, 2009; Smeets *et al.*, 2005).

Most phytoremediation research thus far has been carried out on herbaceous plants that accumulate high levels of metals in their cells naturally. However, their slow growth rates, annual habit, small stature, low biomass and/or narrow geographic adaptability make them unsuitable for phytoremediation on an operational scale (Eapen and D'Souza, 2005). *Populus*, with its rapid growth, makes an ideal plant species for phytoremediation of soils polluted by heavy metals (Cunningham and Ow, 1996). Furthermore, *Populus* is readily-propagated from

vegetative cuttings, and has a wide range of adaptability, high bulk root volume, high transpiration rate and large stature, all of which should enhance the efficacy of phytoremediation (Rockwood *et al.*, 2004). Previous studies have assessed the potential of using *Populus* to remediate soils polluted by atrazine (Burken and Schnoor, 1997), trichloroethylene (Newman *et al.*, 1997), Cd (Robinson *et al.*, 2000), selenium (Pilon-Smits *et al.*, 1998), and zinc (Di Baccio *et al.*, 2009). In addition, some hybrid *Populus* clones have shown elevated tolerance of Cd in field plantings relative to other tree species (Migeon *et al.*, 2009), and there is considerable natural variation within the genus that could be exploited to enhance Cd tolerance further using biotechnological approaches.

To further characterize Cd tolerance in *Populus*, we 1) quantified phenotypic and genetic variation for Cd tolerance and Cd accumulation in a pseudo-backcross multiple generation *Populus* pedigree that was grown under greenhouse conditions 2) detected QTL for traits related to Cd tolerance including dry weight components and root volume and 3) identified candidate genes in the QTL intervals based on transcriptional data from a microarray study.

Material and Methods

Plant materials

An interspecific mapping population was developed at the University of Minnesota Natural Resources Research Institute by crossing *P. trichocarpa* clone 93-968 from western Washington to *P. deltoides* clone ILL-101 from southern Illinois. The resulting F₁ hybrid, 52-225, was in turn backcrossed to *P. deltoides* clone D-124 from Minnesota. Two-hundred and fifty-two full-sib progeny from the ‘pseudobackcross’ pedigree (Family 52-124) were used for the current study. Dormant cuttings were collected from a stoolbed in early spring prior to bud break and were stored at 4°C for five weeks to meet chilling requirements. This facilitated uniform rooting of the cuttings, and enabled selection of plants with approximately uniform sizes at the initiation of our experiment. Two ramets per clone were used for both control and Cd treatments. A total of 1008 vegetative cuttings (252 genotypes x 2 treatments x 2 cuttings per genotype) were examined in this experiment.

Cuttings with approximate lengths of 15 cm and diameters of 1 cm were dipped in 1:10 dilution of the commercial liquid rooting hormone solution (Dip N Grow 1% IBA, 0.5% NAA) for 15 seconds, surrounded by foam stoppers and plugged into 3 cm holes at 8 cm spacing in the lids of polyethylene containers. Approximately 5 cm of the base of the cuttings was submerged beneath a dilute, continuously aerated nutrient solution 0.1X Johnson’s solution; (Siddique *et al.*, 1990). The nutrient solution was composed of 400 µM NH₄NO₃, 400 µM KNO₃, 200 µM Ca(NO₃)₂·4H₂O, 100 µM MgSO₄·7H₂O, 50 µM K₂HPO₄, 20 µM KCl, 25 µM H₃BO₃, 0.5 µM Na₂MoO₄·2H₂O, 2 µM MnSO₄·4H₂O, 0.5 µM CuSO₄·5H₂O, 2 µM ZnSO₄·7H₂O, 0.5 µM CoCl₂ and 20 µM Fe-Na EDTA. The pH was approximately 5.4. One primary shoot was maintained per cutting.

Once the cuttings developed roots and shoots, they were transplanted into 126 polyethylene containers (24 cm x 12 cm x 38 cm) following a partially balanced incomplete block design, with two treatments (Cd and control), 126 containers as incomplete blocks, and two replicates of each genotype in each treatment. Each container had one ramet from each of eight different genotypes at 9 cm spacing and contained 7L of the 0.1X Johnson's solution described above. The solutions were changed every three days throughout the experiment (from 05/19/07 to 07/10/07) to maintain nutrient and oxygen levels and to avoid algal growth.

The experiment was carried out with a target day temperature of 25°C and a night temperature of 21°C and a photoperiod of 18 h. Artificial lighting was provided by high-density mixed halide lights and photosynthetically active radiation (PAR) was recorded for every container used in the study, at plant height (Average PAR = 224.05 $\mu\text{mol}/\text{m}^2/\text{s}$ and standard deviation = 55.08). Temperature was maintained by an evaporative cooling system.

Cuttings were grown for 40 days and one half of the containers were randomly selected to receive a 25 μM CdCl_2 treatment for an additional 20 days. The remaining half of the containers were maintained as described above for two weeks and used as controls. The Cd concentration was selected based on pilot experiments that revealed substantial phenotypic variation in this family without excessive mortality. Fresh CdCl_2 was added each time solutions were changed. Before and after 20 days of treatment, measurements were taken on shoot length, cutting diameter, shoot diameter, photosynthetically active radiation (PAR), root collar diameter (the diameter recorded right above the origin point of the uppermost root on a cutting), and rooted length for each cutting (portion of the cutting occupied by roots). Furthermore, root volume was estimated using water displacement in graduated cylinders. After two weeks, roots and shoots were harvested, separated into paper bags and dried in an oven at 60°C for 6 days.

Roots were washed with deionized water and blotted on paper towels before being stored in the bags. Dry weights were recorded for leaves, roots, and shoots separately, and total dry weights were calculated by summing all components.

Cd analysis

The dried leaf samples with the petioles of all genotypes used in the QTL hydroponic study, were ground to pass through a 20-mesh sieve. Approximately, 0.1 g of ground leaf tissue per genotype was placed into 75 ml microwave digestion tubes. These samples were acid digested with 5 ml of 70% nitric acid (NF, technical grade) and 2 ml of 30% hydrogen peroxide by a closed vessel MARSXpress microwave digester, version 5.0. (CEM Corporation, Matthews, NC, USA). Digestions were completed at 200°C for 2 h (Jones and Case, 1990).

The volumes of the digests were brought to 75 ml with deionized water and all digests were filtered through Whatman No. 44 filter paper to remove any particulate matter. Quality procedures included running blank and control samples in each microwave digestion as well as including a reference genotype in all microwave digestions. Cd contents of the filtrates were determined by using a Varian 220FS flame atomic absorption spectrophotometer, FAAS, (Varian, Inc., Mulgrave, Vic., Australia) and results were verified for a subset of 50 samples using an ICP-OES (Inductively Coupled Plasma – Optical Emission Spectra).

Statistical analysis of phenotypic data

Phenotypic data were tested for normality using the Shapiro-Wilk test (Shapiro and Wilk, 1965) and logarithmic transformations were performed to mitigate heteroscedasticity of variance versus predicted values. Data were analyzed with mixed linear models using SAS JMP, version 8.0 (SAS Institute Inc, Cary, NC). The general form of the statistical model was:

$$Y_{ijk} = \mu + T_i + B_j + G_k + \epsilon_{ijk},$$

where Y_{ijk} was the measurement of response variable Y (total dry weight, root dry weight, leaf dry weight, stem dry weight, or root volume) for genotype k in container j under treatment i , μ was the population mean, T_i was the effect of treatment i , B_j was the effect of incomplete block (container) j , G_k was the effect of genotype k , and ε_{ijk} was the experimental error. When significant ($P \leq 0.05$), the rooted length and dry weight of each cutting and PAR for each container were also included as covariates. Genotype and all interactions with Genotype were treated as random effects in the model, whereas all remaining variables were treated as fixed effects.

Broad-sense heritabilities (H^2) were calculated as $H^2 = V_G/(V_G + V_\varepsilon)$ based on the variance components for Genotype (V_G) and Error (V_ε) estimated using restricted maximum likelihood (REML) in mixed linear models that were fitted separately for control and Cd treatments. This was done to assess differences in variances and heritabilities under these two conditions; where, V_g is the genotypic variance component and V_p is the total phenotypic variance, each of which was estimated using estimated using the restricted maximum likelihood (REML) method.

The effect of Cd on each genotype (hereafter referred to as “Cd effects”) was estimated by subtracting its Best Linear Unbiased Predictor (BLUP) under Cd conditions from that under control conditions (Coles *et al.*, 2010) for all biomass traits described above. The genotypes with the largest BLUP differences were identified as Cd sensitive and those with the smallest BLUP differences were identified as Cd tolerant. Genetic correlations among traits were calculated using BLUPs and the REML method in SAS JMP, version 8.0.

Linkage map construction

To construct a genetic map for Family 52-124, 188 progeny were genotyped using 590

amplified fragment length polymorphism (AFLP) markers. In addition, 418 progeny were genotyped using 287 simple sequence repeat (SSR) markers (Yin *et al.*, 2009) that were chosen based on their physical locations along chromosomes to achieve an even distribution of markers on each linkage group. Four aneuploid trees were identified by genotyping with fully informative SSR markers and these individuals were later removed from the mapping data set. Marker generation, genotyping and nomenclature were performed as described previously (Tuskan *et al.*, 2004; Yin *et al.*, 2004). Map construction was conducted using JoinMap 3.0 under the CP cross type (Van Ooijen, 2001).

QTL analysis

QTL were identified using MapQTL version 5.0 (Van Ooijen, 2004). Interval mapping was performed initially to identify markers associated with putative QTL. Markers with significant effects in this analysis were then used as cofactors in restricted Multiple QTL Model (MQM) mapping (Van Ooijen, 2004). LOD scores were calculated at 1.0 cM intervals. Only QTL with LOD score > 2.5 are reported here.

Microarray study

A whole-genome microarray study was conducted using two genotypes that demonstrated differential Cd effects in the above QTL hydroponic study: a Cd tolerant genotype (1-183), and a Cd susceptible genotype (182). The experiment consisted of 6 polyethylene containers as described above but with only 4 plants per container. Each genotype was represented by two ramets per container with randomly assigned locations. Three containers were treated with 25 μM CdCl_2 and the remaining three containers were under control conditions. Three ramets from each genotype x treatment combination were harvested at 24 h, and three at 72 h after Cd

treatment. Roots from each plant were thoroughly washed in deionized water and blotted dry and all material was quick frozen in liquid nitrogen and stored at -80°C.

Plant tissue was ground in liquid nitrogen, RNA was extracted using the Qiagen RNA Mini kit (Qiagen, Germantown, MD), and double stranded DNA was synthesized using the Invitrogen SuperScriptTM double-stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, California). Labeling, hybridization, and scanning were performed by NimbleGen (Roche NimbleGen, Madison, WI). The raw data were normalized using the robust multichip averaging (RMA) procedure, which performs a convolution background correction, quantile normalization, and summarization based on a multi-array model fit robustly using the median polish algorithm (Bolstad *et al.*, 2005). Differentially expressed genes (Cd treated vs control) were determined using rank product analysis (Breitling *et al.*, 2004) using 1000 permutations and a percentage of false positives cutoff of 0.05.

Results

Cadmium effects

The effect of Cd was evident for all measured traits (Table 2.1). Cd symptoms included necrotic spots on leaves and browning of roots (Figure 2.1). The genotypes under Cd conditions showed poor growth compared to those under control conditions (Table 2.1). The effects of genotype, treatment and their interaction were highly significant ($P < 0.001$) for total dry weight, root dry weight, leaf dry weight, stem dry weight and root volume. The covariate effects of cutting rooted length, cutting dry weight, and PAR were also significant ($P < 0.001$) for all traits except PAR for root volume (Table 2.2).

There was extensive variation in Cd responses among genotypes (Figures 2.2, 2.3.A). Broad-sense heritabilities of total dry weight and its components were relatively low and comparable between control (range $H^2 = 0.19 - 0.39$) and cadmium conditions (range $H^2 = 0.11 - 0.34$, Table 2.1). Root volume showed the lowest heritabilities under either control ($H^2 = 0.19$) or Cd ($H^2 = 0.11$) conditions. In contrast, total dry weight had heritabilities of 0.36 and 0.34 in control and Cd conditions respectively (Table 2.1).

Leaf cadmium content

There was excellent correspondence between the Cd concentrations estimated by FAAS and those estimated by ICP-OES ($R^2 = 0.998$; Supplemental Figure 2.1), indicating the reliability of our analysis method. Genotypes showed significant variation for leaf Cd content as depicted in the histograms (Figures 2.3.B and 2.3.C). However, the broad-sense heritability estimate for leaf Cd content was only 0.12. There was a poor correlation between leaf Cd contents of different ramets for the same genotype ($r=0.014$) (Figure 2.4.A). However, there was a high correlation between Cd measurements of two independent digestions and analyses from the same ramet,

($r=0.948$) (Figure 2.4.B). Furthermore, there was little variation among Cd measurements from subsamples of leaves collected from the same plants of two different genotypes (Coefficient of Variation = 0.018 and 0.019, respectively, for the two genotypes). Therefore, the variation observed between ramets from the 40 replicates appears to reflect true sample-to-sample variation among the replicates rather than lack of precision in the Cd measurements themselves. The total dry weight and Cd contents of leaves were uncorrelated ($r=0.012$; Figure 2.4.C.), suggesting that these traits varied independently in this experiment.

QTL identification

QTL were mapped for 16 phenotypes with a minimum LOD score of 2.5. Four of these QTL were identified in control conditions and 12 under Cd conditions. QTL were located on LG III and XVI (Figure 2.5; Table 2.3). The phenotypic variation explained by QTL ranged from 5.9% to 11.6% and averaged 8.2% across all traits. Excluding co-localizing QTL (± 1 LOD) and accounting for correlations among phenotypic traits (Table 2.4; Figure S.2.2), these 16 QTL correspond to four independent positions within the genome (Table 2.3), and ranged in size from approximately 15 to 30 cM (Figure 2.5).

Three QTL for dry weight and its components under control conditions were co-located on LG III along with three QTL for Cd effects on dry weight, collectively referred to as locus cd1 (Table 2.3). Two clusters of QTL were mapped to LG XVI at two discrete positions, the first of which (cd3) contained QTL for leaf dry weight under control conditions and Cd effects on total dry weight and its components (Table 2.3). For both cd1 and cd3, the allelic effects for dry weight measures under control conditions were in the same direction as those for QTL for Cd effects. However, the relative effect of the allele originating from *P. trichocarpa* was in opposite directions for these two loci decreasing Cd tolerance at cd1 and enhancing Cd tolerance at cd3.

The second cluster (cd2) on LG XVI contained QTL for total dry weight and its components under Cd conditions, as well as three QTL for Cd effects. In contrast, to the other two loci, the allelic effects were opposite for dry weight measures and Cd effects. In this case, the allele from *P. trichocarpa* conferred reduced Cd tolerance on average. Finally, an apparently independent locus on LG XVI (cd4) explained 11% of the variation in leaf Cd concentration under Cd conditions (Table 2.3).

Candidate gene identification

The Cd response QTL intervals encompassed 1571 predicted genes from v2.0 of the *Populus trichocarpa* genome annotation. In addition, across all genotypes and treatments there were 1748 different genes that showed up-regulation, and 672 genes that showed down-regulation in response to Cd treatment (Figures S.2.4, S.2.5). Nine of these differentially expressed genes were also present in the Cd response QTL intervals (Table 2.6). Candidate genes that had no detected level of expression in the microarray study but present in the QTL intervals were also identified (Table 2.7).

Table 2.1. Summary statistics and heritability estimates for total dry weight and other measured traits along with their variance components.

<u>CONTROL</u>	Mean (g)	SD ^a	Range	SE ^b	V _G ^c	V _p ^d	H ² _{BS} ^e
Total dry weight C	6.35	4.11	24.98	0.19	0.170	0.471	0.361
Leaf dry weight C	3.57	2.28	13.05	0.10	0.160	0.499	0.321
Root dry weight C	1.14	0.88	7.34	0.04	0.201	0.581	0.346
Stem dry weight C	1.63	1.16	7.39	0.05	0.233	0.597	0.390
Root volume C	1.62	0.94	6.00	0.04	0.045	0.235	0.191
<u>CADMIUM</u>							
Total dry weight Cd	2.52	1.63	8.24	0.07	0.153	0.450	0.340
Leaf dry weight Cd	1.46	1.01	5.23	0.04	0.147	0.533	0.276
Root dry weight Cd	0.45	0.33	2.51	0.01	0.179	0.558	0.322
Stem dry weight Cd	0.60	0.43	2.70	0.02	0.153	0.529	0.288
Root volume Cd	0.97	0.64	9.00	0.02	0.024	0.218	0.110
Cd mg/g	0.34	0.27	1.51	0.01	0.108	0.859	0.126

^astandard deviation,

^bstandard error,

^cgenetic variance,

^dphenotypic variance (V_G + V_e),

^ebroad-sense heritability

Table 2.2. Results of ANOVA for traits measured in the greenhouse hydroponic study that showed significant differences between Cd and control conditions.

Trait	Total dry weight		Leaf dry weight		Root dry weight		Stem dry weight		Root volume	
	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>
Genotype	1.97	<.0001	1.86	<.0001	1.76	<.0001	1.97	<.0001	1.61	<.0001
Treatment	589.94	<.0001	525.75	<.0001	335.03	<.0001	555.28	<.0001	215.37	<.0001
Genotype x Treatment	1.49	0.0002	1.41	0.0009	1.28	0.0124	1.53	<.0001	1.34	0.0044
Rooted length of cutting	10.08	0.0016	5.99	0.0147	16.76	<.0001	7.71	0.0057	13.92	0.0002
Cutting dry weight	139.78	<.0001	130.92	<.0001	56.23	<.0001	146.18	<.0001	51.94	<.0001
PAR	6.87	0.0091	4.85	0.0281	4.30	0.0388	9.18	0.0026	0.70	0.4027

Table 2.3. Linkage group (LG), QTL positions, Logarithm of Odds ratio (LOD) scores, Percent Variation Explained (PVE), and direction of effect of each QTL are given under the control (C), Cadmium treated (Cd) conditions.

Locus	Trait	Linkage Group	Position	LOD	PVE	Left marker	Right marker	T Effects ^a	D Effects ^b
cd1	Total dry weight C-Cd ^c	III	109.267	3.33	8.9	*CCCGA_8	*CACAC_218	-0.047	0.043
cd1	Root volume C-Cd	III	110.267	3.04	9	*CCCGA_8	*CACAC_218	-0.025	0.022
cd1	Leaf dry weight C-Cd	III	111.267	3.06	7.7	*CCCGA_8	*CACAC_218	-0.046	0.039
cd1	Total dry weight C	III	111.676	2.51	6.1	*CCCGA_8	*CACAC_218	-0.025	0.022
cd1	Root volume C	III	114.726	2.5	6.8	*CCCGA_1	*CCCAC_22	-0.014	0.013
cd1	Root dry weight C	III	110.267	2.66	6.6	*CCCGA_8	*CACAC_218	-0.021	0.022
cd2	Root dry weight C-Cd	XVI	22.784	2.99	7.4	G_3141	*CCCCT_202R	0.045	-0.030
cd2	Total dry weight C-Cd	XVI	26.609	2.81	6.8	*CCCCT_202R	*TCCGT_8R	0.047	-0.033
cd2	Leaf dry weight C-Cd	XVI	26.609	4.26	10	*CCCCT_202R	*TCCGT_8R	0.056	-0.042
cd2	Total dry weight Cd	XVI	26.609	3.14	7.8	*CCCCT_202R	*TCCGT_8R	-0.029	0.021
cd2	Root dry weight Cd	XVI	26.609	2.99	7.3	*CCCCT_202R	*TCCGT_8R	-0.027	0.017
cd2	Leaf dry weight Cd	XVI	26.609	4.6	11.6	*CCCCT_202R	*TCCGT_8R	-0.037	0.028
cd3	Leaf dry weight C	XVI	56.812	2.9	5.9	*CCCCT_246R	*CTCAG_356R	0.033	-0.016
cd3	Total dry weight C-Cd	XVI	56.812	3.61	7.4	*CCCCT_246R	*CTCAG_356R	0.059	-0.028
cd3	Leaf dry weight C-Cd	XVI	56.812	5.41	11	*CCCCT_246R	*CTCAG_356R	0.072	-0.036
cd4	Leaf dry weight Cd	XVI	68.558	4.61	11.3	*CGCCA_308R	*CTCTC_100R	-0.040	0.025

^a The relative effect of the allele originating from *P. trichocarpa* ; ^b The relative effect of the allele originating from *P. deltoides*; ^c Difference in BLUP between control and Cd conditions (Cd effect).

Table 2.4. Genetic (above diagonal) and phenotypic correlations among the variables measured.

Traits all correlations	Total dry weight C	Root dry weight C	Leaf dry weight C	Stem dry weight C	Root volume C	Total dry weight Cd	Root dry weight Cd	Leaf dry weight Cd	Stem dry weight Cd	Root volume Cd	Total dry weight C-Cd	Root dry weight C-Cd	Leaf dry weight C-Cd	Stem dry weight C-Cd	Root volume C-Cd
Total dry weight C	1	0.8838	0.9765	0.9463	0.7703	0.268	0.2562	0.2242	0.2822	0.1859	0.9197	0.7918	0.8783	0.8825	0.5893
Root dry weight C	0.9022	1	0.796	0.8016	0.7862	0.2106	0.2084	0.1769	0.2149	0.1401	0.8235	0.9271	0.7186	0.757	0.6323
Leaf dry weight C	0.973	0.8317	1	0.8841	0.7102	0.2648	0.2556	0.2259	0.2669	0.1816	0.8968	0.7036	0.901	0.8232	0.537
Stem dry weight C	0.9431	0.8152	0.8895	1	0.7313	0.2674	0.2454	0.2148	0.3103	0.1941	0.8646	0.7131	0.7899	0.9278	0.5485
Root volume C	0.6294	0.626	0.6059	0.5817	1	0.2165	0.205	0.1793	0.2338	0.1961	0.7043	0.7131	0.6315	0.6758	0.7931
Total dry weight Cd	-0.3557	-0.3091	-0.2795	-0.2959	-0.0793	1	0.8005	0.961	0.8843	0.5168	-0.1319	-0.0947	-0.1627	-0.0663	-0.1229
Root dry weight Cd	-0.2765	-0.3656	-0.1976	-0.2234	-0.0563	0.8157	1	0.655	0.6965	0.4847	-0.0628	-0.1733	-0.0356	-0.0157	-0.1135
Leaf dry weight Cd	-0.3188	-0.2572	-0.3007	-0.2286	-0.0624	0.9129	0.6886	1	0.7598	0.4666	-0.161	-0.0729	-0.219	-0.0727	-0.1258
Stem dry weight Cd	-0.3146	-0.2638	-0.2208	-0.356	-0.0569	0.8559	0.721	0.689	1	0.4719	-0.0701	-0.0505	-0.071	-0.0667	-0.0792
Root volume Cd	-0.079	-0.1164	-0.0457	-0.0305	-0.1963	0.4798	0.4724	0.4145	0.46	1	-0.0194	-0.0446	-0.0259	0.0186	-0.4417
Total dry weight C-Cd	0.8291	0.7409	0.7669	0.7582	0.4363	-0.8175	-0.6584	-0.7426	-0.706	-0.3353	1	0.8533	0.9699	0.935	0.6565
Root dry weight C-Cd	0.7198	0.8329	0.6294	0.6347	0.4196	-0.6752	-0.8196	-0.5678	-0.591	-0.3515	0.8475	1	0.7372	0.7683	0.6802
Leaf dry	0.7977	0.6723	0.803	0.69	0.412	-0.7424	-0.5519	-0.8099	-0.5664	-0.2869	0.9358	0.742	1	0.8569	0.5939

weight C-Cd															
Stem dry weight C-Cd	0.7824	0.6716	0.6941	0.8424	0.405	-0.682	-0.558	-0.5429	-0.8034	-0.2839	0.8902	0.7452	0.7662	1	0.6068
Root volume C-Cd	0.474	0.4948	0.4376	0.4119	0.796	-0.3495	-0.3295	-0.2978	-0.3221	-0.7498	0.5016	0.5003	0.4553	0.4488	1

Table 2.5 Paired correlations and their ‘p’ values estimated by the REML method.

Variable 1	Variable 2	Correlation	‘p’ value
Total dry weight	Cd mg/g	0.111	0.0877
Rooted length	Cd mg/g	0.094	0.1490
Rooted length	Total dry weight	0.137	0.0342*
Cutting dry weight	Cd mg/g	-0.199	0.0021*
Cutting dry weight	Total dry weight	0.518	<.0001*
Cutting dry weight	Rooted length	-0.016	0.8006
PAR ^a	Cd mg/g	0.226	0.0005*
PAR	Total dry weight	0.043	0.5098
PAR	Rooted length	0.112	0.0853
PAR	Cutting dry weight	-0.050	0.4414

^a PAR = Photosynthetically Active Radiation. * Denotes significant effects.

Table 2.6. Cd tolerance candidate genes in QTL intervals that also showed altered expression levels in the microarray experiment. Corresponding Arabidopsis genes were identified based on best reciprocal blast hits and the putative functions were derived from the TAIR 9 annotation. Fold Change is the ratio of normalized expression levels under Cd versus control conditions. ns, Not significant with false discovery rate of 0.05.

<i>Populus</i> Gene	Arabidopsis Gene	QTL LG	Gene Symbol	Fold Change	Pfp	Clone	Time (h)	Putative Function
gw1.XVI.2928.1	AT1G70280.2	XVI	None	12.3	3E-04	Both	24	NHL repeat-containing membrane protein
fgenes4_pg.C_LG_III001134	AT3G59140.1	III	<i>MRP14</i>	4.7	0.003	183	72	Multidrug Resistance-Associated Protein; ATP:-type transporter
gw1.XVI.2910.1	AT2G28305.1	XVI	<i>LOG1</i>	3.3	0.013	182	72	Unknown function, expressed during 4 leaf senescence stage
eugene3.00031003	AT4G11450.1	III	<i>F25E4.70</i>	3.1	0.021	183	24,72	Unknown function, expressed during leaf senescence
estExt_fgenes4_pg.C_LG_III1085	AT4G23496.1	III	<i>SPIL5</i>	0.32	0.016	182	72	Unknown function; Potentially involved in anisotropic cell expansion
estExt_fgenes4_pg.C_LG_III1187	AT4G24015.1	III	None	0.27	0.041	182	24	Zinc Finger/Unknown function
fgenes4_pg.C_LG_III001060	No Hits	III	None	0.27	0.028	182	24	None
gw1.XVI.2786.1	AT5G03150.1	XVI	<i>JKD</i>	0.24	0.017	182	24,72	Zinc Finger Transcription Factor; root development, regulation of cell division, cell differentiation, meristem growth
fgenes4_pm.C_LG_XVI000330	AT2G37220.1	XVI	<i>F3G5.1</i>	0.13	0.001	182	24	Chloroplast RNA binding protein, response to ABA stimulus, response to cold

Table 2.7. Candidate genes identified from the QTL intervals with no detected change in expression in response to Cd treatment.

<i>Populus</i> Gene	Arabidopsis Gene	QTL LG	Gene symbol	Annotation
estExt_fgenes4_pm.C_LG_III0405	AT4G23100.1	III	GSH1, CAD2	Glutamate-cysteine ligase
estExt_Genewise1_v1.C_LG_III2138	AT1G63440.1	III	HMA5	Heavy metal ATPase
fgenes4_pm.C_LG_III000450	AT5G41610.1	III	CHX18	Monovalent cation:proton antiporter
estExt_fgenes4_pg.C_LG_III1026	AT5G41210.1	III	GST10	Glutathione S-transferase
fgenes4_kg.C_LG_III000037	AT4G11600.1	III	GPX	Glutathione peroxidase

Figure 2.1. Morphological effects of Cd treatment in a hydroponic experiment. A) Control roots B) Cd treated roots C) Control leaf D) Cd treated leaf.

Figure 2.2. A) Genotypic variation in visible effects of Cd treatment. A) Differences between control (right two plants) and Cd (left two plants) treatments; B) Variation among genotypes under Cd conditions. Blue bar is 30.5 cm.

Figure 2.3. A) Distributions of the difference in BLUPs of total dry weight between control (C) and Cadmium (Cd) treatments. Genotypes from the left portion of the curve are relatively tolerant of Cd treatment B). Distributions of BLUPs of Cd contents among 240 genotypes and C). BLUPs of Cd contents among 40 genotypes with replication.

Figure 2.4 A). Dipiction of variation within the genotypes B). Correlation between independent digestions for the same samples and C) Correlation between Cd contents and total dry weights among the pedigree.

Figure 2.5. LOD scores from the QTL analysis. A) Total dry weight Control: Linkage Group III, B) Total dry weight Cd: Linkage Group XVI, C) Total dry weight Control-Cd: Linkage group III, and D) Total dry weight Control-Cd: Linkage group XVI.

Supplementary Figure S.2.1. Correlation between ICP-OES and FAAS analyses of leaf Cd.

Supplementary Figure S.2.2. Matrix of dot plots showing pairwise relationships among all measured traits.

Supplementary Figure S.2.3. Matrix of dot plots showing pairwise relationships among all measured traits with PAR.

Supplementary Figure S.2.4. Venn diagram of number of up-regulated genes from microarray experiment for two genotypes after 24 and 72 hours. Tol, Cd-tolerant genotype (clone 1-183); Susc, Cd-susceptible genotype (clone 182).

Supplementary Figure S.2.5. Venn diagram of number of down-regulated genes from microarray experiment for two genotypes after 24 and 72 hours. Codes are as in Fig. S2.

Discussion

We observed substantial phenotypic variation in Cd effects on plant biomass and leaf Cd accumulation in Family 52-124, a pseudo-backcross pedigree derived from *P. trichocarpa* x *deltoides* hybrids. The effects of genotype, Cd treatment and their interactions were significant for all response variables measured in this study, including total dry weight, root dry weight, leaf dry weight, stem dry weight, and root volume. Substantial variation in Cd tolerance is commonly observed within and among species. For example, Pietrini *et al.*, (2010) used 50 μM of Cd (i.e., twice as high as in our experiment) to compare responses of multiple hybrid *Populus* clones involving six species. They observed significant variation among clones in the effects of Cd on biomass accumulation, photosynthetic responses, Cd content of plant parts, and concentration of photosynthetic pigments. Interestingly, the variation in Cd responses observed among full siblings in our inter-specific cross is of similar magnitude to the variation observed among highly diverse clones that were exposed to a higher concentration of Cd. This illustrates the tremendous phenotypic variation captured by our crossing design and experimental conditions. The Cd-tolerant and Cd-susceptible genotypes identified in this study are a useful resource for future experiments targeted at physiological and molecular dissection of Cd tolerance.

Heritability estimates

Our broad-sense heritability estimate for total dry weight was somewhat lower than those for growth and yield traits measured for *Populus* in recent field studies (Dillen *et al.*, 2009; Marron *et al.*, 2006; Marron *et al.*, 2010; Rae *et al.*, 2008), though certainly within the range of estimates in earlier studies (Riemenschneider *et al.*, 1996). The broad-sense heritabilities calculated for total dry weight and its components under control conditions were not substantially different from the heritabilities calculated for these traits under Cd exposure. Root

volume had the lowest heritabilities under both conditions, whereas root dry weight had heritabilities that were comparable to those for other dry weight components. This discrepancy may reflect difficulties in accurately measuring root volume through water displacement.

Leaf Cd analysis

Significant variation was observed for leaf Cd contents among the 240 genotypes analyzed. Heritability of this trait was low. Significant biological variation was observed between the replicates of a genotype for Cd content. PAR had significant positive correlation with Cd content (Table 2.5). It was hypothesized that PAR was one of the reasons for significant biological variation observed in Cd contents and also for the significant phenotypic variation observed among 240 genotypes. When the correlations of Cd contents were analyzed with different covariates by REML, PAR had the highest, significant positive coefficient with Cd content (0.22) (Table 2.5 and Supplemental Figure S.2.3). Higher PAR might have resulted in higher transpiration rates in some ramets, thereby increasing uptake of nutrient-Cd solution, which eventually resulted in higher Cd contents. In the study by (Pietrini *et al.*, 2010) significant variation was observed for leaf as well as root Cd contents among the *Populus* clones subjected to 50 M CdSO₄ exposure in hydroponics for three weeks. Variation in the leaf Cd content was observed when different *Populus* clones were grown on a metal contaminated site (Migeon *et al.*, 2009). In another study by Laureysens *et al.*, (2004) significant variation was observed for Cd accumulation among the *Populus* clones grown on a waste disposal site. In a study conducted in pots with different levels of metals, by using *Populus* clones, significant variation was observed for leaf Cd contents (Dos Santos Utmazian and Wenzel, 2007). In another hydroponic study by (Pilipovic *et al.*, 2005) variation in the leaf Cd contents was observed when *Populus* clones were treated with Cd. *Populus* clones showed significant variability whereas willow clones showed no

variability in leaf Cd accumulation (Zacchini *et al.*, 2009). Large biomass and higher leaf Cd contents were reported among *Populus* clones in response to Cd treatment (Utmazian *et al.*, 2007). *In vitro* screening of *Populus* clones resulted in significant variability among the clones for leaf Cd accumulation (Di Lonardo *et al.*, 2011). The variation observed in leaf Cd contents among the genotypes in this study and all the studies discussed above suggested the potential for enhancing the phytoextraction component of phytoremediation.

QTL

Although we detected 16 QTL in this study, these occurred at only four different chromosomal locations. Co-localization of QTL for total dry weight and dry weight components was not surprising, given that these traits were highly intercorrelated and were co-segregating in the pedigree. However, it is potentially more interesting and informative that dry weight under control and Cd conditions mapped to different QTL, and had opposing relationships to the Cd response QTL (C-Cd dry weight). Dry weight under control conditions was positively correlated with Cd effect and allelic effects were in the same direction for these traits when they were co-located, suggesting that these QTL were driven by the size of plants under control conditions (i.e., large plants showed more sensitivity to the Cd treatment). In contrast, dry weight under Cd conditions was negatively correlated with Cd effect, and allelic effects were opposite for these traits at co-located QTL, suggesting that Cd tolerance at this locus was associated with growth in the presence of Cd irrespective of performance under control conditions. The correlations among collocated traits could be the result of underlying genes with pleiotropic effects, positive selection, and/or linkage disequilibrium due to genes that are tightly linked (Novaes *et al.*, 2009; Wullschleger *et al.*, 2005).

QTL for different traits may also be co-located even when they are not correlated (Wu *et al.*, 1997; Zhang *et al.*, 2006). For example, in this study leaf dry weight Cd and leaf dry weight C-Cd were mapped to the same position on LG XVI, but were not significantly correlated. The same was observed between root dry weight Cd and root dry weight C-Cd and between total dry weight Cd and total dry weight C-Cd (Tables 2.3 and 2.4). This could be due to a single gene or a regulatory element having pleiotropic effects on these traits and/or different genes within the QTL interval that might be independently controlling these traits, as one QTL encompasses hundreds of candidate genes (Novaes *et al.*, 2009; Wullschleger *et al.*, 2005). Identification of different QTLs on different linkage groups under control and Cd conditions for total dry weight and root dry weight suggested that the genes governing those traits were differentially controlled (Rae *et al.*, 2006), and that the control exerted by these QTLs or genomic regions was dependent on Cd availability. Finally, it is important to note that QTL identified in this study account for a relatively small proportion of the total variation in Cd responses. Furthermore, since the population used for the current study was relatively small, QTL effects were probably overestimated (Beavis and Paterson, 1998), so, much work remains to identify further mechanisms of Cd tolerance in *Populus*.

Identification of the underlying QTLs for Cd contents should shed some light on candidate genes for Cd accumulation. It would be interesting to see whether the QTLs underlying Cd tolerance and Cd accumulation coincide, even though the correlations between these traits were not significant. Bert *et al.*, (2003) reported that Cd accumulation as a complex trait and that Cd tolerance and Cd accumulation segregate independently in *Arabidopsis halleri*. In contrast, Cd accumulation and tolerance were reported be segregating together *Thlaspi caerulescens* populations (Escarre *et al.*, 2000; Lombi *et al.*, 2000). There is lack of clear

evidence regarding the genetic control of these two traits. Potential candidate genes could be identified from a comparative study of the Cd tolerant and Cd accumulating plants and the co-located QTLs.

Candidate genes from QTL and microarray analyses

Transcriptional responses to experimental stimuli are one possible indicator of gene function. We therefore used microarray analyses to gain further insights into the potential roles of the many candidate genes within the QTL intervals. Of the 1571 genes contained within the Cd response QTL intervals, only 12 showed differential expression under Cd treatment. Among these were homologs of AT3G59140.1 (also known as *Arabidopsis thaliana* MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 14), which were significantly overexpressed in the Cd tolerant genotype (1-183) relative to the control after 72 h of treatment. Two closely-related sequences matching this gene occur in the Cd tolerance QTL on LG III, presumably the result of a recent tandem duplication. This gene belongs to the MRP (multidrug resistance-associated protein) subfamily of the superfamily of ATP Binding Cassette (ABC) transporters, which has been implicated in Cd sequestration (Klein *et al.*, 2006). ABC transporters facilitate the translocation of chelates including glutathione with Cd and Pb by ATP-driven processes (Klein *et al.*, 2003; Kolukisaoglu *et al.*, 2002; Lu *et al.*, 1998; Martinoia *et al.*, 2002; Tommasini *et al.*, 1998). Interestingly, expression of AtMRPs 3, 4, 6, 7 and 14 were upregulated by Cd in *Arabidopsis* (Bovet *et al.*, 2003; Kim *et al.*, 2007; Kolukisaoglu *et al.*, 2002). Wojas *et al.* (2009) demonstrated the role of AtMRP7 in Cd tolerance and vacuolar sequestration in tobacco. Many yeast mutant complementation studies have demonstrated the role of AtMRPs in Cd tolerance, and these MRPs could be used as targets of genetic engineering to increase Cd tolerance and

accumulation (Wojas *et al.*, 2009). This MRP is therefore an excellent candidate for further functional characterization in *Populus*.

The remaining genes with differential expression in the QTL intervals have no known role in Cd homeostasis or transport. Four of these genes showed significant up-regulation, and five showed down-regulation (Table 2.7). Six of these have no known function for their closest homologs in model plant species. Interestingly, the gene that was most strongly up-regulated (12.3 fold) in response to 24-h Cd treatment in both genotypes encodes a putative membrane protein. The predicted protein sequence contains an NHL-repeat domain which is a 6-bladed propeller structure characteristic of a large number of proteins across all major kingdoms (Chaudhuri *et al.*, 2008), including 35 predicted proteins in *Populus* and 30 in *Arabidopsis*. For example, the beta propeller is a prominent feature of receptor proteins like TolB, which is involved in antibiotic translocation in *E. coli* (Kleanthous, 2010) and of phosphate-chelating phytases found in a wide range of bacteria and fungi (Lung *et al.*, 2005). The vast majority of plant proteins containing this domain have no known function, though some may be involved in alkaloid biosynthesis (Ma *et al.*, 2006). Although no known connection with Cd tolerance has been demonstrated, it is intriguing that this protein is localized in cell membranes and is known to function in pore formation in microbes. Functional characterization could well lead to the identification of a novel metal tolerance mechanism in *Populus* and other plants.

Candidate genes from the QTL intervals

It is quite possible that genes responsible for interspecific differences in Cd tolerance would not show transcriptional responses that would be detectable in our experiments. In fact, some candidate genes with potential roles in Cd tolerance were present in the QTL intervals, but did not show differential expression in the microarray experiment. These include CAD2

(CADMIUM SENSITIVE 2), HMA5 (HEAVY METAL ATPase5), CHX18 (cation/hydrogen exchanger 18), GST10 (Glutathione S-Transferase1), and GPX6 (Glutathione peroxidase 6) (Table 2.7).

Phytochelatins (PCs) are one of the major chelators produced by plants upon exposure to heavy metals. These peptides chelate heavy metals and facilitate their sequestration in vacuoles, thus limiting heavy metal toxicity (Koprivova *et al.*, 2002). Phytochelatins are synthesized from glutathione by phytochelatin synthase in the presence of heavy metal ions (Grill *et al.*, 1986; Rauser, 1999). Phytochelatins are induced most strongly by Cd, and plants without phytochelatins showed hypersensitivity toward Cd (Howden *et al.*, 1995b) and other heavy metals. The Cd–PC complexes are stored primarily in the root vacuoles, though translocation to shoots also occurs (Kim *et al.*, 2007; Saathoff *et al.*, 2011). The transporter responsible for vacuolar sequestration of Cd–PC complexes has yet to be definitively identified in plants, but ABC-transporters like the MRPs have been implicated in plants (Klein *et al.*, 2006; Wojas *et al.*, 2009) and yeast (Mendoza-Cozatl *et al.*, 2010). CADMIUM SENSITIVE 2 is a γ -glutamylcysteine synthetase, which is involved in glutathione biosynthesis, a precursor for phytochelatins (Howden *et al.*, 1995a). In *Arabidopsis*, *cad2-1* seedlings were more sensitive to Cd than wild-type plants, presumably because the mutants were unable to synthesize phytochelatins (Howden *et al.*, 1995a; Howden *et al.*, 1995b). In this study, the presence of CAD2 in the Cd tolerance QTL on LG III suggests a role for this gene in Cd tolerance in *Populus*.

Populus contains three homologs of HMA5 (Heavy Metal ATPase5) (Migeon *et al.*, 2010), including the one present within the LG III QTL. Some members of this family have been shown to transport Cd and enhance Cd tolerance when overexpressed. For example, HMA 1

through 4 transport Zn/Cd/Pb/Co in Arabidopsis and other plants (Cobbett *et al.*, 2003).

AtHMA4 enhanced Cd and Zn tolerance, transport, accumulation, and root-shoot partitioning when overexpressed in tobacco (Siemianowski *et al.*, 2011). Furthermore, over-expression of HMA3 enhanced Cd accumulation and tolerance in rice, (*Oryza sativa*) (Miyadate *et al.*, 2010). Although HMA5 is not known to transport Cd (Andres-Colas *et al.*, 2006), it is possible that the specificities of these transporters are different in *Populus*, especially since the expression profiles of the putative homologs differ markedly between *Populus* and Arabidopsis (Migeon *et al.*, 2010). This gene is therefore a viable candidate for functional characterization for Cd transport in *Populus*.

Another gene from the QTL interval on LG III has homology to CHX18, a cation transporter that belongs to a group of vacuolar membrane proteins in the cation exchanger (CAX) subfamily of proteins. These transport cations utilizing an H⁺/ATPase pump that generates proton gradients across the vacuolar membrane, and are involved in calcium transport and metal homeostasis (Hirschi *et al.*, 2000; Mei *et al.*, 2009; Migeon *et al.*, 2010). CAXs have a broad range of metal substrates in plants, including significant roles in transporting Cd in roots and shoots (Korenkov *et al.*, 2009). Overexpression of CAX genes enhances Cd tolerance and accumulation in Arabidopsis (Hirschi *et al.*, 2000; Mei *et al.*, 2009; Migeon *et al.*, 2010), tobacco (Korenkov *et al.*, 2007) and petunia (Wu *et al.*, 2011).

A third gene from the QTL interval on LG III has homology to GST10, which belongs to the theta group of the superfamily of glutathione S-transferases (GSTs). GSTs are broadly important in protection against and detoxification of harmful xenobiotics, including heavy metals (Brentner *et al.*, 2008; Dixon *et al.*, 2005). GPX6 (glutathione peroxidase), together with reduced glutathione, reduces hydrogen peroxide and other organic peroxides to water. AtGPX6 was

upregulated under Cd exposure in *Arabidopsis* (Sarry *et al.*, 2006), and is strongly induced under a wide range of abiotic stresses (Milla *et al.*, 2003). Thus, homologs of AtGSTT1 and AtGPX6 might contribute to Cd tolerance in *Populus* through glutathione-mediated oxidative stress tolerance.

Conclusions

This study is the first to map QTL loci for Cd tolerance in the commercially and ecologically important genus *Populus*. This is an important initial step toward enhancing Cd tolerance in this genus for phytoremediation applications, either through future marker-assisted selection strategies, or through genetic engineering. We refined the list of potential candidate genes from the QTL intervals using whole genome microarray analysis. This revealed putative metal transporters and genes of unknown function that can now be further characterized for their role in Cd transport and vacuolar sequestration. Further work should be geared toward establishing finer-scale associations for these genes by assaying polymorphisms in association populations within *P. trichocarpa* and *P. deltoides*, and by assaying informative candidate polymorphisms that differentiate these species. Furthermore, expression data should be gathered for a larger number of genotypes, and QTLs should be validated in different genetic and environmental backgrounds under both greenhouse and field conditions. Ultimately, this research could lead to the development of heavy metal tolerant and/or hyperaccumulating *Populus* clones for reclaiming and remediating the extensive marginal lands contaminated with heavy metals.

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CHAPTER III

QUANTITATIVE TRAIT LOCI FOR GROWTH, BIOMASS AND PHENOLOGY TRAITS IN POPULUS UNDER GREENHOUSE AND FIELD CONDITIONS

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Authors' contribution

Brahma Reddy Induri wrote the manuscript and performed the greenhouse experiments and Cd assays. Wellington Muchero and Gerald Tuskan contributed toward QTL mapping, QTL analysis and provided text describing those methods. Tongming Yin and XinYe Zhang contributed towards mapping. Gancho Slavov contributed to the statistical analysis. Stephen DiFazio directed the project and contributed toward the writing.

Abstract

A pseudo-backcross pedigree of *Populus trichocarpa* and *P. deltoides* (Family 52-124) was examined under greenhouse and field conditions to quantify the phenotypic variation and to identify the genetic basis of growth, biomass distribution and phenology traits. Broad-sense heritability (H^2_{BS}) values ranged from 0.18 to 0.39 in the greenhouse, and from 0.33 to 0.68 and 0.50 to 0.78 in Years 1 and 2 in the field, respectively. The lower heritabilities in the greenhouse might be due to competition among genotypes, which were grown at tight spacing in relatively small hydroponic containers. Quantitative trait locus (QTL) analysis revealed a total of 15 QTLs under greenhouse conditions at a logarithm of odds (LOD) ratio ≥ 2.5 , accounting for an average of 6.1% of phenotypic variation per QTL. We also detected 62 QTLs in the first growing season and 52 QTLs in the second growing season under field conditions (LOD ≥ 3.0), accounting for 4.4% and 5.1% of the phenotypic variation on average, respectively. Many biomass traits were intercorrelated and consequently shared QTL positions within the greenhouse and field studies. There was some correspondence between QTL measured in the two years of the field study, but little correspondence with QTL detected in other studies with this and other *Populus* pedigrees, indicating substantial genotype x environment interactions for these traits. There was also a weak relationship between biomass and phenology traits based on correlations and colocation of QTL. Allometric analyses suggested changes in biomass distribution with increase in age, from the first to second growing season. The findings reported here provide further insights into mechanisms of genetic control of growth, phenology, and crown architecture, and will therefore contribute toward the development of *Populus* as an important model system and as a crop for production of fuel, fiber, wood, and phytoremediation.

Introduction

Breeding for high biomass has long been a major goal in forest tree improvement. Hybrid *Populus* breeding programs have been focused in part on developing renewable feedstocks for biofuel production (Marron *et al.*, 2010 b). *Populus* is one of the fastest growing temperate trees and can sequester more carbon over a longer period of time compared to annual crops, and is also ecologically important, often playing the role of a foundation species in different ecosystems (Whitham *et al.*, 2008). *Populus* is regarded as one of the best suited species for short rotation coppicing (Laureysens *et al.*, 2003). In fact, *Populus* is already being grown in intensively managed plantations and its biomass is used as a source of wood fiber and as an energy source (Scarascia Mugnozza *et al.*, 1997). Exploration of new methods to enhance commercial *Populus* biomass production and elucidation of the effects of genetics and environment on biomass production are of immediate importance to make short rotation forestry an economically viable option (Marron *et al.*, 2010 b; Updegraff *et al.*, 2004).

Allometry describes the relationships of one plant component to another in morphology, physiology and anatomy, and it also reveals any major shifts in biomass distribution to various plant growth components including height, diameter and number of sylleptic branches at different growth stages. These relationships among traits can be studied by using an allometric power equation (McMahon, 1973; Schmidt-Nielsen, 1984). The pattern of carbon allocation and partitioning to roots and shoots determines the competitive ability of a genotype in a particular environment (Aerts *et al.*, 1991; Wullschleger *et al.*, 2005). Despite its importance, there have been a very few studies that have investigated allometric relationships in *Populus* (Wullschleger *et al.*, 2005; Marron *et al.*, 2010 a; Karim and Hawkins, 1999; Dillen *et al.*, 2007; Dillen *et al.*, 2009). Although the exact mechanisms are not understood, a genetic basis was expected for these

changes in size in different organisms (Oldham *et al.*, 2000). The genetic basis of aboveground and belowground biomass distribution has been examined in various *Populus* studies (Scarascia Mugnozza *et al.*, 1997; Karim and Hawkins, 1999; Dickson *et al.*, 1998; Pregitzer *et al.*, 1990; Heilman *et al.*, 1994). Five *Populus* clones upon exposure to elevated CO₂ and O₃ demonstrated significant differences in their carbon allocation patterns to the above-ground and below-ground plant parts within and among genotypes (Dickson *et al.*, 1998). In another study, four *P. trichocarpa* x *P. deltoides* *Populus* clones were studied for growth and biomass traits for four years and they exhibited significant clonal variation in their biomass and biomass allocation patterns to leaf and root components in different seasons of growth (Scarascia Mugnozza *et al.*, 1997). Another study using two *Populus* genotypes with differing phenology and morphology demonstrated significant variation in biomass distribution to roots and shoots (Pregitzer *et al.*, 1990; Isebrands and Nelson, 1983). It is difficult to draw robust inferences on biomass distribution from these studies because most of them used a small number of genotypes (Wullschlegel *et al.*, 2005). As a result there is an urgent need to not only study phenotypic variation in total and fractional biomass distribution but also to elucidate its genetic basis, using a large number of genotypes (Poorter and Nagel, 2000).

Genotype by environment (G x E) interactions determine the robustness and repeatability of genotypic control across different environments (Marron *et al.*, 2010 a). The presence of a G x E interaction implies that the effects of genotypes and environment are not statistically additive (Lynch *et al.*, 1998) such that the same genotypes respond differently in different environments. Estimation of the performance of a genotype is difficult with the presence of a G x E interaction in changing environments (Marron *et al.*, 2010 a) as it is difficult to deconvolute the main effects of genotype and environment unless trials are replicated across environments. It is therefore

important to measure quantitative trait locus (QTL) in multiple environments and years to determine the robustness of the associations, which will inform the range of scenarios in which these loci are relevant. Many studies in *Populus* have employed interspecific hybridization and QTL analysis to elucidate the genetic basis of quantitative traits (Wu, 1998; Bradshaw and Stettler, 1995). Such studies, coupled with the availability of *Populus* whole genome sequence (Tuskan *et al.*, 2004) facilitate not only the understanding of the genetic basis of complex traits but also the identification of candidate genes for quantitative characters including total and fractional biomass distribution (Wullschleger *et al.*, 2005; Ingvarsson, 2005; Grattapaglia *et al.*, 2009).

Some studies have identified QTL for biomass related traits in *Populus* including bud burst and bud set (Bradshaw and Stettler, 1995; Frewen *et al.*, 2000), branching (Bradshaw and Stettler, 1995), osmotic potential (Tschaplinski *et al.*, 2006), growth and biomass (Wullschleger *et al.*, 2005), rooting capacity (Zhang *et al.*, 2009), yield in bio-energy plantations (Rae *et al.*, 2008; Rae *et al.*, 2009; Wu *et al.*, 1998), leaf biomass productivity traits (Bunn *et al.*, 2004), and sylleptic branch number and stem volume (Zhang *et al.*, 2006). The present study is one of the first to explore growth and biomass distribution in *Populus* using a large number of genotypes under greenhouse and field conditions. The main objectives of the current study are (1) characterization of the extent of genetic and phenotypic variation in different growth, biomass distribution, and phenology traits in a pseudo-backcross *Populus* pedigree under field and greenhouse hydroponic conditions; (2) estimation of allometric relationships among individual plant biomass components under field and greenhouse hydroponic conditions; (3) identification of QTLs, and thereby the genetic basis for growth and fractional distribution of biomass under

greenhouse and field conditions; and (4) comparison of QTLs across greenhouse and field conditions and among years in the field.

Material and Methods

Mapping population used for greenhouse and field studies

An interspecific mapping population was developed at the University of Minnesota Natural Resources Research Institute by hybridization of *P. trichocarpa* clone 93-968 from western Washington and *P. deltoides* clone ILL-101 from Southern Illinois. The resulting F1 hybrid, 52-225, was in turn backcrossed to *P. deltoides* clone D-124 from Minnesota. The resulting full-sib progeny from the ‘pseudo-backcross’ pedigree (family 52-124) were used for the current study.

Greenhouse hydroponic study

This manuscript describes QTL for untreated cuttings from a larger experiment to map QTL for Cd-tolerance (Induri *et al.*, 2012). Cuttings of approximately 15 cm length and 1 cm diameter were pre-rooted in a non-randomized design with four adjacent cuttings per clone. Rooting dates were recorded on all genotypes at 2 to 3 day intervals. Groups of 8 rooted cuttings were transferred to containers with 20 L Johnson’s solution of 0.1X strength in a completely randomized design with 2 ramets per genotype and 504 total cuttings (63 containers; Figure 3.1a). Observations were made on root volume (by water displacement), shoot length (from the base of the shoot to the base of the apical bud), cutting diameter, shoot diameter (2.5 cm from the base of the shoot), photosynthetically active radiation (PAR), root collar diameter (right above the origin point of the uppermost root), and rooted length of each cutting (length of cutting occupied by roots) at 30 and 50 days after transfer to experimental containers. At the conclusion of the experiment, roots and shoots were harvested separately into paper bags and dried in an oven at 60 °C for 6 days. Dry weights were recorded for leaves, roots, and shoots. Cutting dry weights were also recorded and were used as a covariate in the data analysis.

Field study

Cuttings for 755 genotypes from Family 52-124 were procured from the University of Minnesota and the University of Florida and were rooted and grown in containers from March to April of 2006 in the Department of Biology greenhouse at West Virginia University. A plantation was established in a field that had previously been used for hay production at the West Virginia University Agronomy Farm (GPS coordinates from Google Earth: latitude: 39°39'37"N and longitude: 79°54'19"W). The field was prepared by disking and tilling during May and June of 2007 and was sprayed with glyphosate shortly before planting. Trees were planted in July 2007 at 2 m x 2 m, with two ramets per genotype in an interlocking block design during early July of 2007. A moisture gradient was apparent in north-south and east-west directions in the field due to a moderate elevational gradient that roughly corresponded to rows and columns. The plantation was drip irrigated as needed and was surrounded by an electric fence to prevent incursion by deer (Figure 3.1b). The plantation was thinned in December 2008 by removing every other tree in a diamond pattern. All remaining trees were harvested in January of 2010.

Growth and phenology traits in the field study

Phenotypes were measured on all trees (742 genotypes with ≥ 2 replicates) in December 2008 and on the trees remaining after thinning (170 genotypes with ≥ 2 replicates and 360 genotypes unreplicated) in January, 2010. It should be noted that height and diameter observations were taken on standing trees in 2008 whereas these observations were taken on harvested trees in January 2010. Height (cm) was recorded from base of the stem to the tree apex. Stem diameter (mm) was recorded at 20 cm above the tree base. Internode length (cm) was measured for five consecutive internodes in the zone where consecutive internode lengths varied the least (typically 15-20 nodes below the tree apex). Number of nodes without branches

(counting from the tree apex) was recorded. Apical dominance was measured as the distance from the base of the highest branch to the tree apex. Number of sylleptic branches was recorded for the 2008 and 2009 growing seasons. Stem volume was calculated assuming a conical stem shape (Marron *et al.*, 2006).

Bud set of the terminal leader was scored during weekly surveys between August 1 and September 18 of 2008 as the first date on which a closed bud was observed (Figure S.3.1). Bud flush was recorded on the date when the first fully unfolded leaf was observed (Howe *et al.*, 2000) at the beginning of the 2008 and 2009 growing seasons based on biweekly surveys. Julian dates were used for the statistical analyses of bud flush and bud set.

Statistical Analysis

Both greenhouse and field data were analyzed by analysis of variance using a mixed model based on Type IV sum of squares using SAS JMP, version 8.0. The data were tested for normality for each trait separately using the Shapiro-Wilk test (Shapiro and Wilk, 1965) and transformations were performed wherever necessary to correct for heteroscedasticity. Differences between means were considered significant for $P < 0.05$ and highly significant for $P < 0.01$ by using a reverse selection procedure.

The transformed data for all the greenhouse traits was analyzed by fitting a mixed model using Restricted Maximum Likelihood and a reverse selection procedure. The full model for greenhouse traits was:

$$Y = \text{Genotype} + \text{Rooted length of each cutting} + \text{Cutting dry weight} + \text{PAR} + \text{Error}$$

The full model for field traits was:

$$Y = \text{Genotype} + \text{Row} + \text{Column} + \text{Row}^2 + \text{Column}^2 + \text{Error}$$

where, *Genotype* was the only random effect in the model and the remaining variables were fixed effects. The row and column positions were squared and included in the model to account for a nonlinear gradient (White *et al.*, 2007), presumably due to soil moisture. Variance components of genotype and environment were estimated using REML (Howe *et al.*, 2000) and broad sense heritabilities were calculated from these variance components. The response of each genotype for all the traits was estimated using Best Linear Unbiased Predictors (BLUPs) (Coles *et al.*, 2010), which provided input data for QTL analysis.

The response variables for the greenhouse study were total dry weight, root dry weight, leaf dry weight, stem dry weight, root volume, shoot length, shoot diameter (mm), leaf number, internodes and rooting dates. Fractional biomass distributions including the ratios of total biomass to its individual components, root volume, height, diameter, leaf number and internodes were also estimated. The response variables for the field study were height year 1, height year 2, diameter, sylleptic branch number, apical dominance, average internode length, stem volume, nodes without branches, bud flush and bud set. Height:diameter ratios were calculated for both years separately. Height, diameter and internode length were compared across greenhouse and field conditions. Finally, greenhouse rooting data was compared with the phenology data from the field. A list of traits and abbreviations is provided in Tables 3.1 and 3.2.

Construction of linkage map

A SNP genetic map with 3,559 markers previously described in Slavov *et al.*, (2012) was used in this study. The SNP were selected based on 32X Illumina sequences of the *P. trichocarpa* and *P. deltoides* parents of this pedigree (clones 93-968, ILL-101, and D-124). SNP loci were selected that were homozygous in all three parents and different between species, thereby maximizing the chances of informative segregation in the backcross. An Illumina

Infinium® assay was devised for each locus using polymorphism information for flanking sequences. SNP were selected to maximize coverage of the genome and also to enhance assembly of sequence scaffolds that were not previously mapped. The map was constructed based on SNP segregating among 692 individuals from the 52-124 pedigree. SNPs were named according to the scaffold carrying the SNP and the physical position of each locus. The map was comprised of 19 linkage groups corresponding to the haploid number of chromosomes in *Populus* and encompassed 3,054 cM in length with a genome-wide average marker distance of 0.86 cM. Individual linkage groups ranged in size from 98.7 to 379.2 cM and genome coverage was estimated at 91%.

QTL analysis

The Multiple-QTL Model (MQM) mapping package in MapQTL 6.0 (Van Ooijen, 2009) was used to identify QTL intervals using Best Linear Unbiased Predictors (BLUPs) determined as described above as input data. Logarithm Of Odds (LOD) significance thresholds were calculated empirically for each trait and experiment using 1000 permutations at the 0.05 significance level (Churchill and Doerge, 1994). QTL were declared significant when they met the genome-wide significance threshold in at least two independent measurements for each trait.

Results

There was substantial phenotypic variation for all traits measured in the greenhouse (Table 3.2, Figure 3.2) and in each year of the field study (Table 3.3, Figure 3.3). Backcross offspring showed transgressive segregation, exceeding the extremes of parental traits and even those of the F_1 for all traits measured in the field study (Table 3.3, Figure 3.3). Parental trees were not included in the greenhouse study due to inadequate rooting.

Broad-sense heritability (H^2) of greenhouse traits ranged from 0.18 to 0.39 (Table 3.2) and was generally lower than that of field traits, which ranged from 0.33 to 0.68 in Year 1 and 0.50 to 0.78 in Year 2 (Table 3.4). Stem dry weight and abscised leaf number showed highest heritability ($H^2 = 0.39$) whereas shoot diameter showed the lowest heritability ($H^2 = 0.18$) under greenhouse conditions. All dry weight components showed comparable heritability estimates under greenhouse conditions. In the field study, shoot diameter again showed lowest heritabilities in Year 1 ($H^2 = 0.23$) whereas sylleptic branch number showed the lowest heritability ($H^2 = 0.35$) in Year 2. There was a considerable difference in the heritability estimates for shoot diameter and shoot length in Year 1 (0.23 and 0.55 respectively) and the difference narrowed in Year 2 (0.42 and 0.47 respectively). Bud flush showed highest heritabilities in Year 1 ($H^2 = 0.64$) and Year 2 ($H^2 = 0.71$) of the field trial respectively, whereas bud set showed a heritability of 0.57 in the only season (2008) it was recorded and was lower than the bud flush heritability reported in both seasons. Bud flush heritability was relatively higher in the second growing season ($H^2 = 0.71$) than the first growing season ($H^2 = 0.64$) (Table 3.4).

Correlations among growth and biomass traits in greenhouse and field studies

In the greenhouse study, all measured growth and biomass traits except rooting dates showed significant phenotypic and genetic correlations (Table 3.5). Total dry weight showed the highest correlations with its components including root dry weight, stem dry weight and leaf dry weight. Growth traits including shoot length, shoot diameter, leaf number, number of nodes, root volume and stem volume showed significant correlations among themselves and also with the biomass traits. Rooting dates showed no correlations with either growth or biomass traits (Table 3.5).

In the field study, there were significant positive phenotypic and genetic correlations between height and diameter in 2008 ($r = 0.82$) and 2009 ($r = 0.76$) (Figure 3.4A, 3.4B; Table 3.6). Apical dominance and height showed a weak correlation of 0.18 in 2008 whereas they showed a strong positive correlation of 0.51 in 2009 (Figure 3.4C, 3.4D; Table 3.6). It was interesting to observe strong correlation between total height in 2008 and 2009 (Figure 3.5A), whereas there was no correlation between height growth during the 2008 and 2009 field seasons (Figure 3.5B). Shoot length and shoot diameter of the greenhouse study showed significant positive correlations with height growth, apical dominance and bud flush of 2009 season of the field study, but no correlations were observed among these traits in 2008 (Table 3.7). Most of the greenhouse traits showed no correlations with 2008 field study traits.

Correlations with Phenology

There was poor correlation between rooting in the greenhouse and bud flush and bud set in 2008. However, there was a significant correlation of 0.29 between rooting and 2009 bud flush (Table 3.7). Similarly, there was no correlation between height and bud flush of 2008 ($r = 0.03$, Table 3.6; Figure 3.6A) whereas there was a significant positive correlation between height and bud set of 2008 ($r = 0.46$; Table 3.6; Figure 3.6B). Bud flush of 2008 had a positive genetic

correlation with bud flush of 2009 ($r = 0.29$; Figure 3.6C) and the heritabilities were very high across both seasons for these traits. No correlation ($r = 0.06$) was observed between bud flush and bud set in 2008 (Table 3.6; Figure 3.6D). Finally, sylleptic branching had significant positive correlations with bud set but not with bud flush of both the growing seasons (Table 3.6).

QTL in Greenhouse study

A total of 15 QTLs were identified for the traits including total dry weight, root dry weight, stem dry weight, leaf dry weight, shoot length, shoot diameter, root volume, stem volume, leaf number, number of nodes and rooting dates in the greenhouse hydroponic study, at a LOD ratio of ≥ 2.5 (Table 3.8). The QTL for total dry weight and its components including root dry weight, stem dry weight and leaf dry weight were localized to positions 122.4 cM and 152.3 cM on linkage group III. Leaf number and shoot diameter were mapped to positions 19.8, 33.3 and 54.1 cM on linkage group XIX. No QTLs were detected for shoot length whereas three QTLs were detected for shoot diameter on LG XIX and XV at LOD ratio of ≥ 2.5 . Two QTLs were identified for shoot length:shoot diameter ratio on LG I (at position 89.5 cM) and LG XVIII (at position 45.9 cM). The QTL detected for shoot diameter at position 19.8 cM on LG XIX had the highest LOD score value among all the greenhouse traits studied. Two QTLs were detected for leaf scar number on LG X at position 151.92 and LG VI at position 143.68 cM and a single QTL was detected for number of nodes on LG V at position 184.3 cM. The percent variation explained by each of the QTL ranged from 5.4% to 8.5% averaging 6.1% across all the traits.

These QTLs were localized to six independent positions within the genome after accounting for co-localization (e.g., Figure 3.6) and correlations among phenotypic traits. Total dry weight and its components were intercorrelated (Table 3.5) and co-localized to linkage group III, although at two different positions (Table 3.8). In contrast, growth traits including shoot

length and shoot diameter showed significant phenotypic and genetic correlations with biomass traits (Table 3.5) but their QTLs were not co-localized (Table 3.8). No QTLs were co-localized for traits that did not show significant correlations in the greenhouse study.

Field study QTL

In the first growing season, 62 QTLs were detected at a LOD of ≥ 3.0 for the 14 traits studied. The percent variation explained by each QTL ranged from 2.1% to 9.7% and averaged 4.4% across all QTLs (Table 3.9). In the second growing season for all the 13 traits studied, 52 QTLs were detected at a LOD of ≥ 3.0 . The percent variation explained by each QTL ranged from 3.1% to 13.6% and averaged 5.1% across all QTLs. Across both growing seasons, plant height had common QTLs on linkage groups IV, VI, IX, and XI with the QTLs on IV and VI being localized to the same positions in both years. Height, height growth, stem volume, diameter, nodes without branches and bud flush had QTLs that were shared between the growing seasons 2008 and 2009 (Table 3.9). Several QTLs were co-localized and correlated in both growing seasons of the field-study (Table 3.6 and 3.9). Some traits, including bud flush 09 and Sylleptic branching 08 were co-localized to LG V at position 84.74 cM but not inter-correlated (Tables 3.7 and 3.9). One of the QTLs for bud flush and apical dominance in 2009 was co-localized to LG VI at a position of 72 Cm but these are not inter-correlated.

Phenology

Six QTLs were detected for bud flush in 2008 whereas in 2009 only five QTLs were detected. Bud flush QTL which occurred on linkage groups II, VI and XI were consistent across years, suggesting the importance of these loci in their genetic control of bud flush. Four QTLs were detected for bud set. Although bud flush and bud set QTLs were both detected on linkage groups VI and XI, their positions are not overlapping (Table 3.9). In 2008, bud flush QTL on LG

II had the highest percent variation explained (PVE 8.4%) and bud set QTL on LG VI had the highest PVE of 8.7%. Percent variation explained by bud flush in both field seasons was similar (Table 3.9).

Comparison of QTL locations across greenhouse and field studies

No QTLs were detected for shoot length and stem volume under greenhouse conditions whereas more than 5 QTLs were identified for these traits under field conditions across both years. Shoot diameter had completely different QTLs under greenhouse (LG XIX and XV) and field conditions (LG I, IV, VI). Under field conditions, shoot diameter QTLs were located to the same positions on linkage groups IV and VI in 2008 and 2009 whereas in 2009 it had an additional QTL on LG I. QTL for number of leaf nodes in the greenhouse and nodes without branches in the field were localized to the same positions on LG V in 2009 but not in 2008 (Table 3.8 and Table 3.9). Height to diameter ratio had one QTL localized to the same position on linkage group XVIII under greenhouse conditions and 2009 of the field study whereas in 2008 all QTLs were localized to LG IV (Table 3.9).

Table 3.1. List of traits measured in the field trial.

Field Trait	Description
Hgt 08	Height at the end of 2008, mm
Hg1 08	Height growth until 2008, mm
Hg2 08	Height growth in 2008, mm
Dia 08	Diameter of stem in 2008, mm
Adl 08	Apical dominance in 2008, mm
SB 08	Sylleptic branch number in 2008
NWB 08	Nodes without branches in 2008
INL 08	Internode length in 2008, mm
BF 08	Bud flush in 2008
BS 08	Bud set in 2008
BF 09	Bud flush in 2009
Hgt 09	Height at the end of 2009, mm
Hg3 09	Height growth in 2009, mm
Adl 09	Apical dominance in 2009, mm
Dia 09	Diameter of stem in 2009, mm
SB 09	Sylleptic branch number in 2009
NWB 09	Nodes without branches in 2009
INL 09	Internode length in 2009, mm

Table 3.2. Clonal means, variance components, and heritabilities for all measured traits in the greenhouse hydroponic study. Values in parentheses are standard deviations.

Greenhouse trait	Mean (SD)	Range	Vg	Vp	H ² _{BS}
Stem dry weight, g	1.63 (1.17)	7.39	0.23	0.6	0.39
Leaf dry weight, g	3.56 (2.29)	13.05	0.16	0.5	0.32
Root Dry Weight, g	1.14 (0.89)	7.34	0.2	0.58	0.35
Total Dry weight, g	6.32 (4.14)	24.98	0.17	0.47	0.36
Root volume	1.63 (0.95)	5.8	0.05	0.23	0.23
Number of Leaves	19.71 (6.91)	67	0.04	0.1	0.36
Shoot Length, cm	42.80 (12.03)	76.9	0.04	0.11	0.37
Shoot diameter, cm	0.48 (0.14)	0.99	0	0.01	0.18
Number of Leaf Scars	4.40 (2.95)	16	0.16	0.41	0.39
Number of Nodes	23.68 (6.43)	67	12.25	32.92	0.37
Stem volume, cm ³	61.85 (77.77)	741.15	1.16	3.11	0.37
Rooting date, Julian	144.65 (3.02)	12	2.89	9.17	0.31
Root/Leaf+Shoot, g	0.23 (0.14)	1.64			
Root/Leaf, g	0.34 (0.21)	2.17			
Leaf/Total dry weight, g	0.57 (0.07)	0.62			
Stem/Total dry weight, g	0.25 (0.06)	0.51			
Root/Total dry weight, g	0.18 (0.07)	0.60			
Leaf+Shoot/Total dry weight, g	0.82 (0.07)	0.60			
Root volume/Total dry weight, g	0.33 (0.30)	4.22			
Leaf no/Total dry weight, g	4.97 (5.65)	73.98			
Shoot Length/Total dry weight, g	9.78 (7.44)	91.28			
Shot diameter/Total dry weight, g	0.13 (0.28)	5.55			
Shoot length/Diameter, cm	91.17 (19.28)	1776.93			
Shoot Length/nodes number	1.89 (0.39)	2.53			
Nodes number / Shoot Length	0.56 (0.15)	1.18			

Vg = genetic variance, Vp = phenotypic variance, H²_{BS} = broad-sense heritability.

Table 3.3. Mean and standard deviation (SD) estimates for all the measured traits at the end of 2008 and 2009 in the field.

Field Trait	2008				2009			
	52-124	T ^a	D ^b	TD ^c	52-124	T	D	TD
Height, cm	324.33 (73.41)	432.33 (36.22)	297.66 (47.48)	425 (21.2)	502.44 (93.91)	631	512.2 (23.48)	641
Height growth, cm	261.75 (60.46)	367.66 (40.50)	239.33 (42.30)	333 (18.38)	212.95 (56.87)	259	228.8 (23.29)	263
Diameter, cm	3.54 (1.38)	52.66 (4.16)	28.5 (7.39)	68.5 (13.43)	6.1 (1.49)	76	50.2 (6.68)	92
Apical Dominance, cm	170.88 (73.17)	136.33 (4.50)	148.33 (42.76)	216 (111.72)	179.93 (66.16)	272	205.6 (53.04)	274
Sylleptic branches #	6.39 (8.36)	41 (4.58)	4.33 (4.27)	10 (9.89)	2.51 (4.05)	0	1 (1.41)	0
Bud Flush	108.56 (2.94)	110 (0)	110 (2.12)	110 (0.71)	113.42 (2.82)	112 (1.73)	115.5 (1.22)	111
Bud Set	243.64 (8.05)	262 (0)	249.6 (5.50)	248.5 (9.19)				
Height/Diameter, cm	90.94 (43.08)	8.21 (0.31)	10.77 (1.66)	6.29 (0.92)	84.73 (14.06)	8.30	10.31 (1.07)	6.96
Stem volume, m ³	28.94 (44.73)	11071 (6203)	693 (657)	16964 (10303)	557.15 (797.11)	23310 (11515)	143703	228035
Nodes without branches	45.48 (17.73)	30.5 (14.85)	21	24	43.37 (13.21)	42	50.2 (19.56)	48
Internode length, cm	22.8 (4.8)	33 (8.49)	26	31	51.89 (10.4)	83.8	48.76 (13.37)	63.2

T - *Populus trichocarpa*, genotype 93-968, female grandparent

D - *Populus deltoides*, genotypes - ILL-101(male grandparent) and D-124 (male parent)

TD - Female parental genotype - 52-225, F1 hybrid of *Populus trichocarpa* (93-968) x *Populus deltoides* (ILL-101)

Table 3.4. Heritability estimates for field traits measured in 2008 and 2009 growing seasons.

Field Trait	2008			2009		
	Vg	Vp	H ² _{BS}	Vg	Vp	H ² _{BS}
Height, cm	2272.50	4155.30	0.55	3670.10	7767.11	0.47
Height 1, cm	290.32	556.91	0.52			
Height 2, cm	1245.80	2719.20	0.46	1576.20	3009.15	0.52
Diameter, cm	0.44	1.91	0.23	0.82	1.97	0.42
Apical dominance, cm	1948.20	5327.2	0.37	1896.91	4257.02	0.45
Sylleptic branch number	0.46	0.88	0.53	0.26	0.74	0.35
Bud flush	5.48	8.62	0.64	5.61	7.90	0.71
Bud set	35.51	62.10	0.57			
Nodes without branches	88.35	296.30	0.30	65.43	171.76	0.38
Average internode length, cm	6.77	21.01	0.32	52.45	106.98	0.49
Stem volume, cm ³	1.31	2.52	0.52	0.78	1.79	0.44

Vg = genetic variance, Vp = phenotypic variance, H²_{BS} = broad-sense heritability.

Table 3.5. Phenotypic (below diagonal) and genetic correlations (above diagonal) among greenhouse traits estimated by REML method. All the correlations were significant except with rooting dates.

Trait	Total dry weight, g	Root dry weight, g	Leaf dry weight, g	Stem dry weight, g	Root volume	Leaf number	Shoot length, cm	Shoot diameter, cm	No. of nodes	Stem volume, cm ³	Rooting dates
Total dry weight, g		0.92	0.98	0.95	0.74	0.62	0.83	0.67	0.52	0.82	0.04
Root dry weight, g	0.89		0.86	0.84	0.78	0.56	0.72	0.63	0.50	0.72	0.07
Leaf dry weight, g	0.98	0.80		0.92	0.70	0.61	0.80	0.64	0.49	0.79	0.04
Stem dry weight, g	0.95	0.80	0.89		0.69	0.60	0.88	0.66	0.52	0.88	0.03
Root volume	0.79	0.81	0.72	0.75		0.57	0.61	0.64	0.52	0.60	0.09
Leaf number	0.51	0.47	0.49	0.48	0.52		0.58	0.57	0.76	0.58	0.09
Shoot length, cm	0.78	0.65	0.75	0.79	0.65	0.48		0.58	0.46	1.00	0.05
Shoot diameter, cm	0.74	0.62	0.71	0.76	0.70	0.55	0.72		0.53	0.57	0.03
No. of nodes	0.56	0.50	0.53	0.56	0.58	0.90	0.59	0.65		0.46	-0.02
Stem volume, cm ³	0.75	0.65	0.70	0.80	0.61	0.36	0.78	0.58	0.44		0.05

Table 3.6. Phenotypic (below diagonal) and genetic (above diagonal) correlations among shared genotypes of growing seasons 2008 and 2009 estimated by REML method (Correlations of $> |0.5|$ are in bold).

Trait	Hgt 08	Hg2 08	Dia 08	Adl 08	SB 08	BF 08	BS 08	BF 09	Hgt 09	Hg3 09	Adl 09	Dia 09	NWB 09	INL 09
Hgt 08		0.95	0.65	0.33	0.27	0.04	0.52	-0.05	0.72	0.05	-0.03	0.37	0.25	-0.05
Hg2 08	0.95		0.59	0.34	0.28	0.01	0.44	0.02	0.68	0.11	0.07	0.30	0.15	0.01
Dia 08	0.82	0.78		0.09	0.26	0.07	0.27	-0.09	0.49	0.01	0.14	0.16	-0.01	0.19
Adl 08	0.18	0.16	-0.01		-0.42	0.08	0.11	-0.02	0.11	-0.09	0.03	-0.17	-0.12	-0.07
SB 08	0.41	0.44	0.47	-0.46		-0.10	0.22	-0.05	0.33	0.10	0.02	0.29	0.08	0.12
BF 08	0.03	0.01	-0.02	0.04	-0.09		0.09	0.29	-0.03	-0.01	-0.18	0.19	0.01	0.07
BS 08	0.46	0.39	0.26	0.09	0.20	0.06		-0.02	0.42	0.12	-0.07	0.18	0.03	0.09
BF 09	0.01	0.02	0.03	0.02	-0.03	0.54	0.00		-0.18	0.66	-0.20	-0.05	-0.13	0.55
Hgt 09	0.78	0.74	0.68	0.04	0.42	-0.01	0.39	-0.07		0.16	0.05	0.40	0.33	-0.04
Hg3 09	0.10	0.09	0.13	-0.13	0.18	-0.05	0.08	-0.11	0.66		0.37	-0.26	-0.39	0.60
Adl 09	0.12	0.12	0.09	0.10	-0.08	0.06	0.12	0.01	0.51	0.69		-0.64	-0.66	0.36
Dia 09	0.80	0.76	0.86	0.01	0.47	-0.01	0.27	0.02	0.76	0.27	0.16		0.82	-0.59
NWB 09	0.07	0.04	0.00	0.12	-0.17	0.10	0.18	0.02	0.34	0.48	0.83	0.05		-0.65
INL 09	0.18	0.21	0.19	0.01	0.12	-0.03	-0.08	0.01	0.39	0.45	0.40	0.25	0.07	

Table 3.7. Genetic correlations between selected traits measured in the greenhouse and those measured in the field.

Trait	Hg 08	Adl 08	Dia 08	BF 08	BS 08	SB 08	NWB 08	Stem		Hgt3 09	Adl 09	Dia 09	NWB 09	SB 09	BF 09	Stem Vol 09
								Vol 08	Hgt 09							
Total dry wt	-0.08	-0.09	0.05	0.07	-0.19	0.05	-0.10	0.06	-	0.27	0.26	-0.32	-0.16	-0.23	0.27	-0.32
Root volume	0.01	-0.13	0.06	0.00	-0.11	0.18	-0.16	0.01	0.00	0.21	0.19	-0.06	0.07	-0.10	0.14	-0.09
Shoot length	-0.04	-0.09	0.05	-0.03	-0.13	0.04	-0.19	0.00	-	0.49	0.25	-0.20	-0.04	0.04	0.48	-0.15
Shoot Dia	0.07	-0.03	0.10	-0.02	-0.12	0.15	-0.11	-0.03	0.05	0.27	0.13	-0.10	0.06	0.06	0.25	-0.06
Rooting date	-0.01	0.03	-0.08	0.02	-0.07	0.02	0.01	-0.09	-	0.00	-0.25	0.16	0.10	0.08	0.29	0.09
Stem volume	-0.04	-0.09	0.05	-0.03	-0.13	0.04	-0.19	0.00	-	0.50	0.24	-0.20	-0.03	0.05	0.49	-0.14
No. of nodes	0.08	-0.18	0.13	-0.02	-0.03	0.21	-0.11	0.23	0.14	0.30	0.48	-0.30	-0.16	-0.14	-0.03	-0.19

Table 3.8. QTLs identified for traits measured in the greenhouse study.

Trait	Group	Position	Locus	LOD	$\mu_{ac}\{00\}$	$\mu_{bd}\{00\}$	Variance	PVE
TotalDryWeight	III	152.3	scaffold_3_16851717	2.64	0.061091	-0.08324	0.084995	5.7
RootDryWeight	III	122.478	scaffold_3_14318461	2.5	0.073528	-0.07959	0.101916	5.4
LeafDryWeight	III	152.3	scaffold_3_16851717	2.72	0.0562	-0.07874	0.071885	5.9
LeafStemDryWeight	III	152.3	scaffold_3_16851717	2.8	0.062729	-0.08616	0.084829	6.1
Leaf number	XIX	54.186	scaffold_19_12422289	2.91	0.030627	-0.04136	0.018999	6.4
Shoot diameter	XIX	19.806	scaffold_19_5105586	3.99	0.008026	-0.0075	0.000644	8.5
	XIX	33.368	scaffold_19_9728106	2.75	0.006631	-0.00633	0.000662	6
	XV	38.717	scaffold_15_4994116	2.55	-0.0691	0.06798	0.072453	6
Leaf scar number	X	151.927	scaffold_10_18548092	2.54	0.089935	-0.04969	0.072464	6
	VI	143.684	scaffold_6_20157326	2.65	0.006165	-0.00459	0.000471	5.8
Root/Total dry weight	VI	143.684	scaffold_6_20157326	2.71	0.00384	-0.00285	0.000178	5.9
	III	141.784	scaffold_3_16087018	2.83	0.380513	-0.37807	2.19789	6.1
Shoot length/Shoot diameter	I	89.561	scaffold_1_6501526	2.6	0.365827	-0.3622	2.20923	5.7
	XVIII	45.939	scaffold_18_325052	2.91	0.246306	-0.41434	1.59344	6.3
Number of nodes	V	184.437	scaffold_5_23503568	2.53	0.503554	-0.6767	5.542	5.9

Table 3.9. QTLs identified for traits measured in the field study in both growing seasons.

Trait	Group	Position	Locus	LOD	mu_ac{00}	mu_bd{00}	Variance	PVE
Height 08	IV	5.097	scaffold_4_539917	8.86	-12.4838	7.21805	1397.11	6
	VI	170.543	scaffold_6_22731802	11.45	10.9703	-10.3438	1372.18	7.6
	IX	36.294	scaffold_9_5575904	4.68	-6.1561	7.66471	1438.24	3.2
	X	128.917	scaffold_10_16314664	5.63	8.95735	-6.29537	1428.73	3.8
	XI	27.816	scaffold_11_4270068	10.6	-9.65271	10.8943	1380.33	7.1
	XVII	103.138	scaffold_17_2127487	3.28	-4.83744	6.8266	1452.27	2.3
Height 1 2008	VI	167.788	scaffold_6_22478252	14.71	4.52118	-3.98709	168.212	9.7
	VI	138.759	scaffold_6_19467988	12.08	4.39226	-3.37623	171.308	8.1
	VIII	85.323	scaffold_8_6983625	5.87	3.72855	-1.90747	178.857	4
	X	137.43	scaffold_10_17119908	7.76	3.9005	-2.43972	176.528	5.2
	XI	28.725	scaffold_11_4391888	8.75	-3.0119	3.62018	175.319	5.9
	XVII	50.95	scaffold_17_10534691	3.33	-1.63984	2.51484	182.043	2.3
Height 2 2008	I	287.688	scaffold_1_32522298	3.91	-4.49619	4.23294	690.695	2.7
	IV	5.097	scaffold_4_539917	9.49	-9.07566	5.00129	664.463	6.4
	VI	170.543	scaffold_6_22731802	6.24	5.54396	-5.42779	679.623	4.2
	IX	37.105	scaffold_9_5626994	4.29	-4.20565	4.94805	688.884	2.9
	X	128.917	scaffold_10_16314664	3.7	4.92252	-3.65717	691.674	2.5
	XI	27.816	scaffold_11_4270068	7.5	-5.7644	6.24692	673.693	5.1
ADL 2008	VI	93.217	scaffold_6_9191531	3.6	5.75624	-4.07339	940.702	2.5
	VIII	92.892	scaffold_8_7564001	4	-5.94868	4.52761	938.125	2.7
	XII	88.155	scaffold_12_10815212	4.96	5.61847	-5.84526	931.868	3.4
	XIV	70.526	scaffold_14_6437075	8.78	7.72848	-7.38385	907.476	5.9
Diameter 2008	IV	5.097	scaffold_4_539917	7.17	-0.07944	0.088421	0.127069	4.8
	VI	174.227	scaffold_6_23030368	5.27	0.100331	-0.03753	0.128738	3.6
INL_Average_08	XIV	144.07	scaffold_14_16355453	3.52	-0.30099	0.287576	2.26806	3.7
SB_2008	IV	12.993	scaffold_4_841806	4.05	-0.13994	0.072707	0.264128	3.6

NWB_2008	V	84.744	scaffold_5_8368828	3.57	0.098261	-0.08867	0.265278	3.2
	V	134.183	scaffold_5_19064721	4.72	0.101012	-0.11439	0.26253	4.2
	VIII	93.333	scaffold_8_7639312	6.88	0.150769	-0.10892	0.25744	6
	XI	39.056	scaffold_11_5313640	3.62	-0.08636	0.101889	0.265168	3.2
	VI	194.608	scaffold_6_24869186	3.03	1.04447	-0.84172	29.5028	2.9
	VIII	125.204	scaffold_8_10399900	3.19	-0.95654	0.984878	29.4582	3.1
	XIV	70.526	scaffold_14_6437075	7.27	1.43941	-1.46042	28.3033	6.9
StemVolume_08	I	288.385	scaffold_1_35185480	3.13	-0.12865	0.137588	0.803634	2.2
	IV	5.097	scaffold_4_539917	11.98	-0.33645	0.199423	0.755741	8
	VI	125.882	scaffold_6_17149154	4.78	0.187804	-0.14094	0.794476	3.3
	VI	170.543	scaffold_6_22731802	10.44	0.251122	-0.22835	0.76385	7
	IX	36.294	scaffold_9_5575904	3.15	-0.11399	0.153531	0.80352	2.2
	X	131.207	scaffold_10_16522436	4.51	0.195742	-0.12646	0.795961	3.1
	XI	6.866	scaffold_11_1001189	8.2	-0.21456	0.212588	0.775836	5.5
Height08_Diameter08	XVII	103.138	scaffold_17_2127487	3.73	-0.11745	0.174586	0.800335	2.6
	XI	32.982	scaffold_11_4596463	6.39	-0.17371	0.203955	0.785687	4.3
	IV	4.106	scaffold_4_413415	8.18	0.012609	-0.00762	0.001604	5.5
	IV	14.655	scaffold_4_1012241	6.96	0.011311	-0.00725	0.001617	4.7
	IV	16.281	scaffold_4_1136937	5.94	0.010469	-0.00672	0.001629	4
	IV	26.219	scaffold_4_2141909	4.88	0.009165	-0.00632	0.001641	3.3
	IV	37.637	scaffold_4_3083849	3.04	0.006595	-0.00548	0.001662	2.1
Height_2_Diameter08	I	19.943	scaffold_1_4955139	3.58	-0.0073	0.006922	0.002	2.5
	IV	5.097	scaffold_4_539917	5.52	0.01128	-0.00709	0.001973	3.8
SB08_Height_2	V	84.744	scaffold_5_8368828	3.06	0.092839	-0.08285	0.274388	2.7
	V	134.183	scaffold_5_19064721	4.17	0.096878	-0.10882	0.271632	3.7
Height_2009	VIII	93.333	scaffold_8_7639312	7.5	0.15948	-0.11519	0.263561	6.6
	I	282.516	scaffold_1_33177048	4.05	-9.47752	9.58832	2081.61	4.2
	IV	14.655	scaffold_4_1012241	7.53	-16.5644	10.0882	2006.75	7.6
	VI	170.543	scaffold_6_22731802	5.53	11.2733	-10.9064	2049.48	5.7
	IX	67.7	scaffold_9_8279027	6.3	-10.2941	13.4856	2033.02	6.4
	XI	0	scaffold_21_497649	4.6	-10.0985	10.1893	2069.59	4.7

Height_3_2009	I	181.85	scaffold_938_3145	3.42	-7.05738	5.14313	963.52	3.5
	IV	55.632	scaffold_4_5102638	3.92	-6.60328	6.16863	958.496	4
	IV	109.523	scaffold_325_19478	5.56	-8.15728	7.03828	942.088	5.7
	IX	89.174	scaffold_9_10082291	6.02	-6.62042	9.16188	937.393	6.2
	XIX	25.865	scaffold_19_5993152	3.47	6.50063	-5.4804	963.026	3.6
ADL_2009	I	183.564	scaffold_1_23137681	6.14	-7.43715	9.62648	1082	6.3
	I	271.48	scaffold_140_37688	4.19	-6.63733	7.49234	1104.42	4.3
	IV	109.523	scaffold_325_19478	3.17	-6.58307	5.84169	1116.35	3.3
	V	198.414	scaffold_5_24593616	4.73	-6.77641	8.23049	1098.13	4.9
	VI	72.119	scaffold_6_6832007	3.61	7.26364	-5.86994	1111.29	3.7
	IX	89.174	scaffold_9_10082291	3.21	-5.13363	7.34274	1115.89	3.3
Diameter_09	I	282.976	scaffold_1_33071595	3.53	-0.15219	0.142121	0.568755	3.7
	IV	15.408	scaffold_4_1035770	13.89	-0.37238	0.216432	0.509842	13.6
	VI	170.543	scaffold_6_22731802	5.39	0.181068	-0.18033	0.557729	5.5
SB_2009	IX	86.651	scaffold_9_10004054	3.5	0.068048	-0.0834	0.066394	7.9
NWB_2009	I	183.564	scaffold_1_23137681	4.03	-1.12756	1.49312	38.9034	4.2
	IV	144.732	scaffold_1702_2558	3.81	-1.41686	1.15881	38.9948	4
	V	183.323	scaffold_5_23438659	3.23	-0.99382	1.36019	39.2351	3.4
	VI	118.261	scaffold_6_12847732	3.08	1.27957	-1.01408	39.2974	3.2
	VI	193.621	scaffold_6_24683679	9.16	2.08266	-1.80576	36.8333	9.3
	IX	89.174	scaffold_9_10082291	4.12	-1.09784	1.56216	38.8641	4.3
INL_AVG_2009	I	271.48	scaffold_140_37688	3.27	-1.10682	1.22734	38.8129	3.4
	IV	80.025	scaffold_4_7989212	4.83	-1.52631	1.32036	38.1757	5
	IV	49.104	scaffold_4_4314064	3.02	-1.1946	1.06623	38.9126	3.1
	VI	194.721	scaffold_6_24769044	4.26	-1.30381	1.35664	38.4066	4.4
StemVolume09	I	282.516	scaffold_1_33177048	4.13	-0.11783	0.127885	0.371504	3.9
	IV	14.655	scaffold_4_1012241	9.59	-0.23815	0.145529	0.352406	8.8
	VI	170.543	scaffold_6_22731802	6.01	0.149612	-0.14562	0.364815	5.6
	VI	174.551	scaffold_6_23095856	3.64	0.120093	-0.11103	0.373244	3.5
	IX	64.973	scaffold_9_8062670	4.38	-0.10795	0.146873	0.37058	4.1
	X	137.43	scaffold_10_17119908	3.32	0.138169	-0.08668	0.374399	3.2

	XI	6.866	scaffold_11_1001189	5.18	-0.1412	0.134011	0.367733	4.9
Height09_Diameter09	IV	4.106	scaffold_4_413415	5.79	2.06476	-1.1014	39.4368	5.4
	VIII	135.75	scaffold_8_11454391	4.46	-1.56814	1.12125	39.944	4.2
	IX	37.105	scaffold_9_5626994	3.65	-1.12313	1.28764	40.2598	3.5
	XVIII	39.954	scaffold_18_992938	4.44	1.12325	-1.55706	39.9534	4.2
SB09_Height_3	V	183.323	scaffold_5_23438659	4	0.094182	-0.15488	0.175913	7.8
	VI	188.849	scaffold_6_24260292	3.38	-0.13781	0.090763	0.178075	6.7
	IX	93.908	scaffold_9_10462781	4.16	0.10801	-0.14202	0.175324	8.1
Height_3_Diameter09	VIII	135.75	scaffold_8_11454391	4.17	-0.98338	0.703588	16.7837	4
SB_09_Height_09	V	183.323	scaffold_5_23438659	3.2	0.066591	-0.10665	0.107426	6.3
	VI	188.849	scaffold_6_24260292	3.96	-0.11315	0.077602	0.10576	7.7
	IX	93.908	scaffold_9_10462781	3.69	0.080885	-0.10165	0.10636	7.2
Bud flush 2008	II	38.102	scaffold_2_5202086	12.76	0.652289	-0.51542	3.66859	8.4
	III	81	scaffold_3_10635121	4.15	0.307358	-0.36683	3.89213	2.8
	VI	54.714	scaffold_6_5114896	6.07	0.433755	-0.37717	3.84123	4.1
	VI	93.77	scaffold_6_9237051	4.48	0.38736	-0.31418	3.88312	3
	XI	89.739	scaffold_11_14473403	4.82	0.333877	-0.39266	3.87378	3.3
	XV	114.235	scaffold_15_13816694	4.68	-0.43828	0.289442	3.87806	3.2
Bud set 2008	VI	167.513	scaffold_6_22536804	12.53	1.51405	-1.36257	21.6349	8.7
	IX	36.294	scaffold_9_5575904	3.79	-0.6827	0.928099	23.058	2.7
	X	133.923	scaffold_10_16760641	8.69	1.48674	-0.95847	22.2478	6.1
	XI	23.072	scaffold_11_3651079	6.87	-1.01565	1.1353	22.5469	4.9
Bud flush 2009	II	7.943	scaffold_2_2534655	3.85	0.455254	-0.42298	4.62901	4
	V	81.1	scaffold_5_8062017	4.57	-0.49298	0.461702	4.59415	4.7
	VI	63.34	scaffold_6_5742508	3.91	0.462762	-0.42215	4.62619	4.1
	VII	96.69	scaffold_7_2740574	3.69	0.519462	-0.35457	4.63698	3.8
	XI	91.64	scaffold_11_14742983	3.69	0.393131	-0.47139	4.63658	3.8

A

B

Figure 3.1. Overview of A) Greenhouse hydroponic study and B) Field trial on July 28, 2008, one year after establishment.

Figure 3.2. Distributions of BLUPs for A) Shoot length, B) Shoot diameter C) Shoot length/Shoot diameter and D) Total dry weight in the greenhouse study.

Figure 3.3. Transgressive segregation in 52-124 Family demonstrated by the distributions of BLUPs for A) Plant height 2008, B) Plant diameter 2008 C) Plant height 2009, D) Plant diameter 2009 in the field study.

Fig 4

Figure 3.4. Scatter plots showing correlations among the measured growth traits within the same year in the field study.

Figure 3.5. Scatter plots showing correlations among the measured traits across different years in the field study.

Figure 3.6. Scatter plots showing correlations among the measured phenology traits and relationships to height in the field study.

Figure 3.7. Co-localization of QTLs as demonstrated by superimposition of QTL peaks observed for total dry weight (blue colored) and root dry weight (red colored) at a LOD threshold of 3.0.

Figure S 3.1. Appearance of a terminal vegetative bud in the family 52-124 trial shortly after bud set.

Discussion

Phenotypic variation across environments and growing seasons

Extensive variation was observed in Family 52-124 for all studied traits under both greenhouse and field conditions. Considerable differences were observed among the genotypes in their performance across environments, as indicated by the poor correspondence between performance in the greenhouse and field, and also across years as shown by the poor correspondence in productivity between 2008 and 2009 (Figure 3.5B).

It is apparent from Figure 3.3 that transgressive segregation was observed in the progeny, because the phenotypes observed in the progeny were beyond the range of the parental phenotypes. This is commonly observed in *Populus* hybrids. For example, transgressive segregation was observed in *Populus* family 331, an F₂ inbred family that uses the same *P. trichocarpa* parent as family 52-124 (Rae *et al.*, 2008). Similarly, most drought response traits exhibit transgressive segregation in family 331 (Street *et al.*, 2006). In an unrelated *Populus* pedigree, all studied traits including growth and wood chemical content showed transgressive segregation (Zhang *et al.*, 2006). However, in another *Populus* pedigree, there was negative transgressive segregation for rooting traits, in which the backcross hybrids had values less than the parental trait values (Zhang *et al.*, 2009).

Heritabilities

All traits in both study environments showed moderate to high heritabilities for growth and biomass traits, as has been observed in other studies in *Populus* (Marron *et al.*, 2010 b; Dillen *et al.*, 2007; Marron *et al.*, 2006). Surprisingly, heritability estimates were generally higher in the field study compared to the greenhouse study. This suggests that environmental effects were stronger in the greenhouse than in the field. One possible reason is that the intensity

of the artificial lights was not uniform across the experiment, as reflected by PAR measurements on each of the containers used in the study (data not shown). Although PAR was used as a covariate in the model, this did not account for all of the variation contributing to differential performance of ramets within a genotype. Furthermore, competition between genotypes was likely more severe in the greenhouse experiment due to close spacing of the plants, thereby accentuating environmental effects. In contrast, there was little obvious interaction among ramets in the field study due to adequate spacing in the first growing season, and thinning prior to the second growing season. Finally, because the greenhouse experiment was much shorter duration than the field study, it is likely that the effects of variation among vegetative cuttings had a stronger effect in the former.

The heritability estimates were comparatively higher in the second growing season relative to the first growing season. The differences between years could be due to differential establishment effects in 2008 and also local micro-environmental conditions, which may have varied between years (Marron *et al.*, 2010 a). The hypothesis of establishment and production phases in *Populus* (Stanton, 2001) may partially explain why different genotypes perform differentially across years, as demonstrated by several *Populus* species and hybrids (Afas *et al.*, 2008). C-effects, the variation that results due to the physiological preconditioning of the cuttings of different genotypes during propagation, could also explain some of the above discrepancies in the correlations in the first growing season compared to the second growing season (Dillen *et al.*, 2009; Lerner, 1958). Another possible cause of the differences could be the larger population size in the pre-thinning population measured in 2008, although there is no inherent reason to believe that this would lead to lower heritability of the measured traits, as the

sample size was quite large in both years (742 replicated genotypes in 2008 and 170 replicated genotypes in 2009).

Phenology

Bud set and bud flush were under strong genetic control in this field study across both the growing seasons, as has been observed in other replicated clonal field trials in hybrid *Populus* (Marron *et al.*, 2010 b). Spring bud flush appears to be under very strong genetic control in *Populus* with up to 98% of the phenotypic variation attributable to genetic control (Marron *et al.*, 2010 b; Bradshaw and Stettler, 1995; Frewen *et al.*, 2000). Different populations of *P. trichocarpa* trees showed bud set heritabilities of 0.71 and 0.81 in previous experiments (Howe *et al.*, 2000). In fact, bud phenology traits often have the among highest heritabilities among all measured traits in forest trees, indicating that these traits are less affected by the microenvironmental changes and show relatively low genotype x environment interaction on a local scale (Howe *et al.*, 2000).

Correlations

Bud flush and bud set are important adaptive traits that could determine the length of the growing season (Howe *et al.*, 2000). Temperature plays a major role in determining bud flush timing whereas bud set timing is affected by a multitude of factors and their interactions including short days, cold days, soil moisture and soil nutrient status (Howe *et al.*, 2000). Length of the growing season is influenced by the timing of bud flush and bud set. Specifically, the amount of biomass produced will be proportional to the length of the growing season, assuming minimal frost damage. Growing season length in turn is determined by vegetative phenology. In general, bud set but not bud flush determines the duration of growing season (Dillen *et al.*, 2009) and thus has more direct relationship to biomass produced by plants. The timing of bud flush and

bud set helps the plants avoid unfavorable growth conditions (Dillen *et al.*, 2009). Delayed bud set was found to be genetically associated with increased height growth in some *Populus* studies (Riemenschneider *et al.*, 1992; Rohde *et al.*, 2011) but not others (Marron *et al.*, 2010 b). We also detected very poor phenotypic and genetic correlations between growth traits and bud flush, but we detected significant positive phenotypic and genetic correlations between growth traits and bud set (Table 3.6).

We also detected no relationship between bud flush and bud set dates. This is in contrast to other studies, which have found both positive (Howe *et al.*, 2000; Eriksson *et al.*, 1978; Rehfeldt, 1992 b) and negative (Marron *et al.*, 2010 b) correlations between these traits in *Populus* and other trees. Similar to the current study, bud flush and bud set were poorly correlated ($r = -0.12$) in a *Populus* study of family 822, which also uses clone 93-968 as the *P. trichocarpa* parent (Frewen *et al.*, 2000). However, QTLs for bud flush and bud set were localized to completely different positions in our study, whereas some bud flush and bud set QTL have been colocalized in previous studies (Frewen *et al.*, 2000). Bud flush and bud set can show strong correlations under growth chamber conditions, but this can be attributed to the photoperiodic component of the response rather than to the complex interplay of photoperiod and temperature that typifies a natural setting (Howe *et al.*, 2000). The poor correlation between bud flush and bud set under field conditions could be explained by a lack of control over temperature at the beginning and end of the season, which might differentially affect bud set and bud flush of different genotypes.

QTLs

The current study focused on identifying the genomic regions governing growth, biomass and phenology traits across two experiments and also whether these genomic regions were

consistent across years. The number of QTLs identified in this study for the studied traits suggested the polygenic control and complex nature of these traits, as has been observed previously (Marron *et al.*, 2010 b; Wullschleger *et al.*, 2005; Bradshaw and Stettler, 1995; Rae *et al.*, 2008; Rae *et al.*, 2009; Novaes *et al.*, 2009). Co-locating QTLs were observed for a majority of growth and biomass traits. In the greenhouse study, several QTLs were co-located (Figure 3.6) and correlated suggesting the pleiotropic nature of these QTLs (Marron *et al.*, 2010 b). The QTLs governing total dry weight and its dry weight components were all co-located and were highly correlated, suggesting that they represent the same genetic mechanisms and are driven by allometric relationships among biomass components.

Sylleptic branching increases the leaf area index by increasing light interception area of the plant, thus eventually influencing the growth and productivity of hybrid *Populus* clones (Ceulemans *et al.*, 1990). Sylleptic branching shows plasticity and the degree of syllepsis could vary greatly from genotype to genotype (Dillen *et al.*, 2007; Wu *et al.*, 1998). Sylleptic branching contributes to stem growth and shows remarkable genetic variation than other growth traits when growth conditions are not limiting (Dillen *et al.*, 2007). Sylleptic branching is under moderate to strong genetic control and has been included in *Populus* ideotype breeding (Rae *et al.*, 2004). Sylleptic branching was found to be positively associated with radial growth and biomass productivity in *Populus* (Marron *et al.*, 2010 b; Wu *et al.*, 1998; Marron *et al.*, 2006; Rae *et al.*, 2004). However, a weaker genetic correlation between syllepsis and growth traits has also been observed in previous studies (Rae *et al.*, 2008). In the current study, sylleptic branching had moderate heritability (0.35 to 0.53) and significant positive correlations with height, height growth and diameter. The correlation between height and apical dominance of 2008 was very low and depended on number of sylleptic branches. Two groups of genotypes

could be observed in the color coded figure (Fig 4C) with genotypes in red having sylleptic branches and the genotypes in blue without any sylleptic branches. This supports the importance of this trait in predicting the growth and biomass productivity of *Populus* clones.

Relation between biomass QTLs and phenology QTLs

The QTLs governing height across both growing seasons were localized to the same positions on linkage groups IV, VI, IX and XI suggesting that the genetic control of this trait did not change with increase of plant age. Even though these traits were poorly correlated, it was interesting to note that the height growth QTLs identified in 2008 and 2009 were localized to LG I, IV and IX albeit to different positions. Although there are a few other QTLs governing plant height on other linkage groups, these QTLs suggested the importance of these linkage groups in the genetic control of this trait. Diameter also had QTLs localized to the same positions on linkage groups IV and VI across both growing seasons, indicating that genetic control of these traits was consistent over time. The trend of constant QTLs across years provides an opportunity to genetically manipulate these traits in genetic improvement trials and other related research investigations including biofuel research and carbon sequestration. For traits including sylleptic branching, bud flush and others, the QTLs changed across years suggesting that the genetic control of these traits changes as the plants mature.

Comparisons between current study and previously reported QTLs

Some QTL reported in the current study were also detected in the past in other families of T x D hybrids of *Populus*. Plant height and plant diameter had a QTL on linkage group IV under field conditions in the current study and also in other studies of *Populus* family 331, which shares one of the grandparents, 93-968 (Rae *et al.*, 2007). Similarly, the QTL for plant height in the current field study was localized to the same linkage group VI in another field study of

Populus family 331 (Rae *et al.*, 2008). Finally, it was interesting to note that QTLs for stem volume were detected on linkage groups I, IV, VI, IX, X and XVII in the current study and also in family 331 (Rae *et al.*, 2007). Furthermore, the QTL for plant height under field conditions were localized to the common linkage groups of X and XI in the current study and yet another related field study of family 331 (Wu *et al.*, 1998). Interestingly, there was virtually no overlap with the major pleiotropic biomass QTL detected in a greenhouse study with family 52-124 previously (Novaes *et al.*, 2009), providing further evidence of poor transferability between greenhouse and field studies.

The QTL for sylleptic branch number were localized to linkage groups IV, V and VIII in the current study and also in another related field study of family 331 (Rae *et al.*, 2008). The QTLs detected for sylleptic branch number on LG IX and XI were detected only in this study and not in the past studies.

Four of the bud flush QTLs identified in this study on linkage groups II, III, VI and XI were also detected in another study of *Populus* family 331 (Bradshaw and Stettler, 1995). Bud flush QTLs on linkage groups VI, VII and XI were also shared across the current study and with another *Populus* study of family 822, which also shares a grandparent with family 52-124 (Frewen *et al.*, 2000). Two bud flush QTLs detected on LG II and LG III were also identified by past bud flush QTL studies although the *Populus* families were different (Marron *et al.*, 2010 b; Bradshaw and Stettler, 1995). The bud flush QTLs detected on LG VI and XI in the current study were also localized to the same linkage groups in other studies involving families 331 and 822 (Bradshaw and Stettler, 1995; Frewen *et al.*, 2000). Bud set QTLs on linkage groups VI and X also were located to the same linkage groups in family 822 (Frewen *et al.*, 2000).

Conclusions

We identified QTLs for growth, biomass and phenology traits under greenhouse and field conditions in the interspecific pseudo-backcross family 52-124 of *Populus*. This was one of the first studies to use an outcrossed tree pedigree of this size to identify the underlying genomic regions for phenotypes under field and greenhouse conditions and across multiple years. Shared growth, biomass and phenology QTLs across years, families, pedigrees and environments suggested the robustness of some of these QTLs in governing these traits. Phenology traits were highly heritable and QTLs were highly consistent across studies and families, demonstrating the appropriateness of these traits for in-depth genetic and molecular studies, and their value as targets for breeding and selection. Genetic manipulation of these traits with consistent QTLs provides opportunities to modify the growth and biomass allocation patterns in *Populus* thereby facilitating breeding for genotypes improved in paper, pulp, above/below ground carbon sequestration and biofuels applications. Further research on identifying the candidate genes from these consistent QTLs will enhance understanding of the mechanisms underlying the genetic control of these traits. Nevertheless, QTL studies do have certain limitations. Although many QTL were identified in this study, multiple and repeated experiments are required to test the repeatability of these QTLs across families, pedigrees, environments and genetic backgrounds. Furthermore, the identified QTL intervals encompass hundreds of genes and further research will be required to unearth the actual genes controlling the traits.

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CHAPTER IV

INFLUENCE OF Fe ON Cd TOXICITY ON GROWTH AND BIOMASS TRAITS OF Cd TOLERANT AND Cd SUSCEPTIBLE GENOTYPES OF *POPULUS*

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Authors' contribution

Brahma Reddy Induri wrote the manuscript and performed the greenhouse experiments and Cd assays. Danielle Ellis and Gancho Slavov contributed towards statistical analysis. Stephen DiFazio directed the project and contributed toward the writing.

Abstract

Cadmium (Cd) affects a wide range of physiological processes in plants, and disturbance of plant mineral homeostasis, especially of iron (Fe), is an important symptom of Cd toxicity. Fe is an essential micronutrient used for plant growth and metabolism. In this study, Cd and Fe interaction was demonstrated in *Populus* by changing the external Cd and Fe status of a hydroponic nutrient medium. Two *Populus* clones with differential responses to Cd were examined for Cd tolerance at Fe deficient (0 μM) and high Fe (100 μM) levels. There were significant differences between genotypes within different treatment combinations for total dry weight, leaf dry weight, shoot dry weight and root dry weight. Cd toxicity was more severe on the Cd-susceptible genotype than the Cd-tolerant genotype under both Fe conditions. Furthermore, the symptoms of Cd toxicity were reduced under high Fe conditions for both genotypes for all the growth and biomass traits studied, although the effect was stronger for the Cd-tolerant genotype. The effects of Cd-Fe interaction were more pronounced on the Cd susceptible genotype 182, compared to the Cd tolerant genotype 1-183. Examining Cd uptake, translocation and distribution patterns in the leaf, shoot and root tissues should reveal further insights on Cd-Fe interaction mechanisms in *Populus*.

Introduction

Cadmium (Cd) is a widespread toxic heavy metal pollutant that poses a serious threat to human health (Shao *et al.*, 2007). Cd enters food chain from contaminated soils thorough plant uptake. In plants, symptoms of Cd toxicity include leaf roll, chlorosis and reductions in root and shoot growth, even at minute concentrations (Milone *et al.*, 2003; Almeida *et al.*, 2007). Cd disrupts the structure of many proteins by acting on sulphhydryl groups (Vanassche and Clijsters, 1990) and is reported to change the permeability of membranes by altering lipid composition (Ouariti *et al.*, 1997). Apart from affecting the ion and water balance of plants, Cd has been shown to cause reduced plant growth by affecting various physiological processes including photosynthesis, respiration, nitrate assimilation, metabolite accumulation and enzyme activity (Sanità di Toppi and Gabbrielli, 1999; Smeets *et al.*, 2005; Solti *et al.*, 2008; Das *et al.*, 1997; Sanita Di Toppi *et al.*, 1998). Cd decreases the photosynthetic activity of plants by disturbing chloroplast ultrastructure (Sandalio *et al.*, 2001) and altering stomatal closure. Cd has also been shown to affect the electron transport system, interact with antioxidative defense systems and induce lipid peroxidation (Smeets *et al.*, 2005; Rodriguez-Serrano *et al.*, 2009). Cd interferes with the uptake, distribution and metabolism of other mineral nutrients in plants (Shao *et al.*, 2007; Rodriguez-Serrano *et al.*, 2009; Liu *et al.*, 2003), most notably Fe (Solti *et al.*, 2008; Sandalio *et al.*, 2001; Fodor *et al.*, 2005; Siedlecka and Krupa, 1999; Zhang *et al.*, 2002; Wallace *et al.*, 1992).

Although abundant in the Earth's crust, the essential micronutrient Fe is mostly unavailable for uptake by plants as it is mostly in insoluble ferric form, especially in alkaline and neutral soils and the Fe status of the plant environment determines one of the two strategies that plants use for Fe uptake (Shao *et al.*, 2007). All higher plants except the grasses utilize strategy I to assimilate iron. This involves reducing the ferric form to ferrous form before uptake by a

plasma-membrane bound redox system (Schmidt, 2003). In grasses, a complex involving phytosiderophores, the plant-borne high affinity Fe (III) chelators, is formed with ferric iron before it is translocated by a transporter specific to this complex (Shao *et al.*, 2007). The specific Fe transporters used in strategy I can also transport Cd (Lombi *et al.*, 2002; Thomine *et al.*, 2000; Rogers *et al.*, 2000).

Cd may compete with other divalent ions including Ca, Zn and Fe for binding sites in the root apoplast and transporters at the plasma membrane (Fodor *et al.*, 2005; Clemens, 2001). Cd accumulates in roots in large quantities and inhibits root to shoot Fe translocation (Mendoza-Cozatl *et al.*, 2008) resulting in higher Fe levels in cucumber (Fodor *et al.*, 1996) and mungbean (Liu *et al.*, 2000) roots. Cd treatment causes Fe deficiency in *Populus* (Solti *et al.*, 2008; Fodor *et al.*, 2005; Sarvari *et al.*, 2001) and in other plants (Siedlecka and Krupa, 1999; Wallace *et al.*, 1992; Siedlecka and Krupa, 1996). Cd can also inhibit Fe (III)-chelate reductase, the inducible enzyme under Fe deficient conditions in roots (Alcantara *et al.*, 1994; Chang *et al.*, 2003). Iron can also affect the intensity of Cd toxicity, with higher Fe mitigating Cd toxicity and Fe deficiency raising Cd toxicity levels (Morrissey and Guerinot, 2009). Such effects have been demonstrated in maize (Meda *et al.*, 2007) and in soybean (Smith *et al.*, 1985). Up-regulation of a high affinity Fe transporter under Fe deficiency increased Cd uptake and sensitivity (Besson-Bard *et al.*, 2009; Nakanishi *et al.*, 2006). Also, Cd accumulation decreased by 5 fold upon knocking out the Fe transporter, IRT1 in *Arabidopsis* compared to the wild-type (Vert *et al.*, 2002), demonstrating the role of this iron transporter in Cd uptake. Although Fe is required for growth and metabolism of plants, it can also be toxic to plants at high levels (Siedlecka and Krupa, 1996; Kabata Pendias, 1992). Under excess Fe levels, Fe was toxic to the bean plants and

also increased the toxic effects of Cd whereas under moderate Fe levels Fe reduced Cd translocation to leaves by immobilizing the Cd on the roots (Siedlecka and Krupa, 1996).

We are currently investigating mechanisms of Cd tolerance in the model tree genus *Populus*. We have conducted a whole-genome microarray (Ellis *et al.*, Unpublished) using two genotypes with extreme responses for Cd tolerance in a greenhouse hydroponic study conducted to detect QTL for Cd tolerance (Induri *et al.*, 2012). We identified several Fe homeostasis genes that were up-regulated in this study suggesting that the genotypic differences in Cd tolerance were partially due to differences in Fe homeostasis (Ellis *et al.*, Unpublished). More specifically, we hypothesized that the up-regulation of Fe homeostasis genes in the susceptible genotype might have resulted in increased Cd uptake, thereby resulting in greater toxicity.

Here, we seek to directly test this interaction by conducting a hydroponic study with a Cd tolerant and a Cd susceptible genotype of *Populus* exposed to different levels of Cd (0 and 25 μM) and Fe (0 and 100 μM). It was hypothesized that Cd stress under Fe deficiency conditions would decrease the growth and biomass of plants, especially in the susceptible genotype. It was further hypothesized that higher Fe concentration in the hydroponic nutrient medium would reduce Cd toxicity in *Populus* with the reduction being stronger in the Cd susceptible genotype.

Material and Methods

Plant material

Two genotypes, a Cd tolerant genotype (1-183) and a Cd susceptible genotype (182) were selected from a Cd tolerance screening study of 252 genotypes from the 52-124 Family of *Populus* conducted by Induri *et al.* (2012). Dormant cuttings were collected from West Virginia University Agronomy Farm prior to bud break and were stored at 4°C for 5 weeks to meet chilling requirements. This facilitated uniform rooting of the cuttings, and enabled selection of plants that were of approximately uniform in size at the beginning of the experiment.

Greenhouse hydroponic experiment

Cuttings of approximately 15 cm length and 1 cm diameter were dipped in 1:10 dilution of the commercial liquid rooting hormone solution (Dip N Grow 1% IBA, 0.5% NAA) for 15 seconds, surrounded by foam stoppers and plugged into 3 cm holes at 8 cm spacing in the lids of polyethylene boxes. Approximately 5 cm of the base of the cuttings was submerged incontinuously aerated 0.1X Johnson's solution (Siddique *et al.*, 1990). The nutrient solution was composed of 400 μM NH_4NO_3 , 400 μM KNO_3 , 200 μM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 100 μM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 μM K_2HPO_4 , 20 μM KCl , 25 μM H_3BO_3 , 0.5 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2 μM $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.5 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 μM CoCl_2 20 μM Fe-Na EDTA. One primary shoot was maintained per cutting (Figure 4.1). For greenhouse growth conditions and other details of hydroponic methods, see Induri *et al.* (2012).

This study had two levels of Cd (0 and 25 μM) and 2 levels of Fe (0 and 100 μM). Once the cuttings developed roots and shoots, they were transplanted to smaller polyethylene boxes arranged in a randomized block design (Table 4.1 and Figure 4.1). The experiment contained 16 polyethylene containers of size 24 cm x 12 cm x 38cm. Each container had four plants of a single

genotype at 9 cm spacing and contained 7L of the 0.1X Johnson's solution described above. The solutions were changed every third day throughout the experiment to maintain nutrient and oxygen levels and to avoid algal growth. An aliquot of hydroponic solution samples was collected from each box in all the treatment combinations just prior to changing solutions to determine the available Cd concentration. These solution samples were filtered using Whatmann number 44 filter paper. Cd contents were determined by ICP-OES (Inductively Coupled Plasma – Optical Emission Spectra) at the Virginia University Plant analysis lab.

Harvesting followed by Cd and Fe treatments

Treatments were applied after 35 days of growth. Cd treatments consisted of 25 μM CdCl_2 . Fe was supplied as 100 μM Fe-Na EDTA. Iron starvation was imposed by excluding Fe from the micronutrient solution described above. The Cd and Fe concentrations for this experiment were selected based on a pilot experiment that revealed substantial phenotypic variation in this family without excessive mortality at these selected concentrations. Fresh Cd and Fe were added each time solutions were changed. The plants were exposed to Fe and Cd treatments for a period of 14 days before harvest.

Observations were taken before and after treatment on plant height (from the base of the shoot to the base of the apical bud), cutting diameter, shoot diameter (2.5 cm from the base of the shoot), leaf number, number of leaf scars, photosynthetically active radiation, (PAR), root collar diameter (just above the origin point of the uppermost root), and rooted length of each cutting (place occupied by the roots throughout the length of a cutting). Plants were also rated twice on a scale of 1 to 4 (with 0 = no damage, 1 = <25% damage, 2 = 25-50% damage, 3 = 50-75% damage and 4 = 75-100% damage or dead plant) and the average rating was used for statistical analyses. Roots and shoots were harvested separately into paper bags and dried in an oven at 60

°C for 6 days. Dry weights were recorded for leaves, roots, and shoots separately and total dry weights were calculated by adding all these components. Cutting dry weights were also recorded and were used as a covariate in the data analysis.

Minteq- species composition analyses

As the measurements of total dissolved ions of Fe, Cd and EDTA will not provide the exact amount of these ions accessible for plant uptake, speciation analyses of Cd, Fe and EDTA were performed using a geochemical speciation program, Visual MINTEQ (Vasyukova *et al.*, 2012; Gustafsson, 1999; Allison *et al.*, 1991). Analyses were performed to estimate the total free ions of Fe⁺⁺, Fe⁺⁺⁺ and Cd⁺⁺ ions in the hydrponic solution and also to determine if the total ions are present as free or as complexed species with other ions at different pH ranges and at different concentrations of Cd, Fe and EDTA.

Statistical analysis

Data was analyzed by mixed-model analysis of variance (ANOVA) based on type IV sum of squares using SAS JMP, version 10.0. Response variables were plant height difference (i.e., height after treatment – height before treatment), leaf number difference, shoot diameter difference, average damage rating, total dry weight, root dry weight, leaf dry weight, shoot dry weight and rooted length of cutting. The data was tested for normality for each trait separately by Shapiro-Wilk test (Shapiro and Wilk, 1965) and no variables required transformation. Three-way nested ANOVA was used for analyzing growth traits but because of limited number of cuttings for each genotype, individual plants were considered as independent replicates for analysis of dry weights, which could be considered pseudo-replication (Hurlbert, 1984). However, the effect of box was not significant ($p > 0.05$, data not shown), so this approach is justified for analysis of dry weights.

Results

Cd content of hydroponic solutions

According to geochemical speciation analyses, at the average hydroponic solution pH of 5.2, 99% of the Cd ions were free and <1% was complexed with EDTA and other anions. At the same pH, most of the Fe was chelated with EDTA.

Solution samples collected at the time of solution changes (after 3 days of exposure) contained 61% more Cd in the high Fe treatment compared to the low Fe treatments. There was no detectable Cd in solutions that did not have Cd added (Figure 4.10).

Normalized Effects of Cd and Fe

The effects of Cd and Fe were apparent when subtracting trait values before treatment application from those measured following treatment (Table 4.2; Figures 4.2 and 4.4). Under Fe starvation, Cd caused reduced growth and higher damage for both genotypes, though as expected the effects were stronger for the susceptible clone (number 182) (Table 4.2; Figures 4.2 and 4.4). This suggested that Cd effects were increased under Fe starvation. In contrast, the effect of Cd was greatly diminished under high Fe for both genotypes. All the main effects were significant in the ANOVA analysis except the Cd effect for shoot diameter difference (Table 4.4).

Furthermore, the Cd x Fe interaction was significant for leaf number difference and average damage rating but not for height difference and shoot diameter difference. Fe x Genotype was also significant for leaf number difference (Table 4.4). Cd x Fe x Genotype interaction was significant only for shoot diameter difference among all the growth and biomass traits studied (Table 4.4).

Biomass traits - Average trait values

Similar trends were observed for rooted length (Figure 4.5) and the raw biomass measurements at the termination of the experiment. The two genotypes showed differences in total dry weight and its components including leaf dry weight, shoot dry weight and root dry weight at different treatment combinations (Table 4.3; Figures 4.6 to 4.9). The average trait values were lower under low Fe - high Cd conditions for total dry weight (Figure 4.6) compared to the other treatment combinations. The effect was particularly pronounced for leaf dry weight (Figure 4.7), although roots and shoots also showed substantial reductions under high Cd and low Fe.

The genotype main effect was not significant for any of the biomass traits, whereas the Cd effect was significant for all biomass traits (Table 4.4). The Fe effect was significant or at least exhibited trends towards significance for all biomass traits except rooted length. None of the interactions between Cd, Fe and genotype were significant for the biomass traits except Cd x Genotype interaction for root dry weight.

Table 4.1. Lay-out of the Cd-Fe interaction experiment.

Genotype	Cd	Fe	Table #	Row	Column
182	0	H	2	2	1
182	0	H	3	3	1
182	0	L	1	5	1
182	0	L	2	1	1
182	25	H	3	6	1
182	25	H	3	4	2
182	25	L	2	3	1
182	25	L	3	2	2
1-183	0	H	3	5	1
1-183	0	H	1	3	2
1-183	0	L	2	5	1
1-183	0	L	1	3	1
1-183	25	H	2	5	2
1-183	25	H	2	2	2
1-183	25	L	3	2	1
1-183	25	L	2	4	2

Table 4.2. Treatment effects averaged over ramets. Effects are calculated as the difference in trait values measured before treatments were imposed subtracted from the final trait values. Numbers in parentheses are standard deviations.

Clone	Cd	Fe	Height difference, mm	Leaf number difference	Shoot diameter difference, mm	Damage rating
1-183	0	H	156.87 (26.47)	5.37 (3.02)	1.78 (1.07)	0.00 (0.00)
1-183	0	L	173.87 (48.92)	6.00 (2.20)	0.82 (1.38)	0.00 (0.00)
1-183	25	H	130.62 (26.92)	3.37 (3.16)	1.33 (0.61)	1.06 (0.50)
1-183	25	L	97.75 (61.81)	2.12 (3.36)	1.46 (0.73)	2.50 (0.53)
182	0	H	156.50 (25.34)	6.12 (2.53)	1.15 (0.48)	0.25 (0.46)
182	0	L	132.00 (46.42)	4.25 (1.98)	0.93 (0.49)	1.18 (1.28)
182	25	H	102.37 (45.81)	3.75 (3.41)	1.06 (0.57)	2.25 (0.85)
182	25	L	55.12 (42.31)	-3.25 (3.92)	0.18 (0.39)	3.50 (0.71)

Table 4.3. Leaf dry weight, Shoot dry weight, Root dry weight and Total dry weight averaged over ramets. Numbers in parentheses are standard deviations.

Clone	Cd	Fe	Leaf dry weight,g	Shoot dry weight,g	Root dry weight,g	Total weight,g	Rooted length,mm
1-183	0	H	5.33 (0.78)	1.70 (0.16)	2.17 (0.43)	9.21 (1.27)	62.00 (31.16)
1-183	0	L	5.04 (1.58)	1.70 (0.54)	1.65 (0.36)	8.40 (2.38)	78.00 (28.15)
1-183	25	H	5.70 (2.48)	1.60 (0.57)	1.97 (0.78)	9.28 (3.80)	56.00 (16.83)
1-183	25	L	3.43 (1.11)	0.95 (0.44)	0.93 (0.45)	5.32 (1.97)	53.00 (36.96)
182	0	H	5.73 (2.60)	1.60 (0.80)	2.47 (1.07)	9.80 (4.45)	72.25(15.65)
182	0	L	4.96 (1.51)	1.46 (0.29)	2.73 (0.73)	9.16 (2.30)	69.50 (18.98)
182	25	H	4.00 (1.63)	0.98 (0.38)	1.01 (0.38)	5.99 (2.22)	57.00 (14.58)
182	25	L	1.26 (0.50)	0.48 (0.12)	0.67 (0.19)	2.42 (0.62)	21.00 (6.56)

Table 4.4. Summary of results of ANOVA analyses. ‘p’ values are from the ‘F’ test. Corresponding ‘p’ values for interaction terms are in parentheses. Significant ‘p’ values are in bold.

Trait	Genotype	Cd	Fe	Significant interactions	R ² Adjusted
Height difference	0.0083	< .0001	0.0380	None	0.42
Leaf number difference	0.0423	< .0001	0.0018	Cd*Fe (0.0187), Fe*Genotype (0.0061)	0.49
Shoot diameter difference	0.0117	0.4154	0.0188	Cd*Fe*Genotype (0.0318)	0.16
Average damage rating	< .0001	< .0001	< .0001	Cd*Fe (< 0.0001), Cd*Fe*Genotype (0.0073)	0.91
Total dry weight	0.2264	0.0020	0.0303	None	0.34
Leaf dry weight	0.1616	0.0125	0.0216	None	0.27
Shoot dry weight	0.0470	0.0015	0.0704	None	0.33
Root dry weight	0.8672	< .0001	0.0825	Cd*Genotype (0.0080)	0.51
Rooted length	0.3988	0.0105	0.4568	None	0.16

Figure 4.1. Cd-Fe experiment setup. A). Overview of hydroponic experiment prior to Cd, Fe treatments. B). Control hydroponic roots before Cd, Fe treatments

Figure 4.2. Effects of Cd and Fe on plant height, calculated as the difference in trait values measured before treatments were imposed subtracted from the final trait values. Error bars represent standard error in this and subsequent figures.

Figure 4.3. Effects of Cd and Fe on leaf number, calculated as the difference in trait values measured before treatments were imposed subtracted from the final trait values.

Figure 4.4. Average damage rating at different levels of Cd and Fe.

Figure 4.5. Rooted length observations at different levels of Cd and Fe.

Figure 4.6. Total dry weight of plants at different levels of Cd and Fe.

Figure 4.7. Leaf dry weight at different levels of Cd and Fe.

Figure 4.8. Shoot dry weight at different levels of Cd and Fe.

Figure 4.9. Root dry weight at different levels of Cd and Fe.

Figure 4.10. Average Cd content of solutions measured after 3 days for different treatment combinations.

Discussion

The present study was aimed at examining the interaction between Cd and Fe by studying the effects of low (0 μM) and high Fe (100 μM) levels in the presence and absence of Cd on growth and biomass traits of a Cd tolerant genotype (1-183) and a Cd susceptible genotype (182) identified in an earlier Cd screening study of *Populus* genotypes. This study demonstrated that the effects of Cd could be mitigated at elevated Fe concentrations, and that the effect differed for the susceptible and tolerant genotypes. This supports our hypothesis, derived from microarray analyses, that differential interactions of Cd and Fe occur in these two genotypes and are possibly related to their different levels of Cd tolerance.

Cd and Fe starvation conditions

Under Cd and Fe deficiency conditions, both genotypes showed no significant differences in the change in height and leaf number during the experiment. This is in contrast to a prior study that showed reduced leaf and root growth, chlorophyll content and photosynthetic rate in *Populus* plants under Cd stress and Fe starvation conditions (Fodor *et al.*, 2005). However, under high Fe and Cd deficiency conditions, both genotypes showed increased, though not significant, growth (Table 4.2). This is consistent with the general trend as Fe is required for growth, photosynthesis and general plant metabolism (Solti *et al.*, 2008). A similar trend was observed for total dry weight and its components including root dry weight, leaf dry weight and shoot dry weight (Table 4.3).

One of the symptoms of Cd toxicity is Fe deficiency (Wallace *et al.*, 1992). This is expected to result in reduced photosynthesis and therefore reduced growth (Sarvari *et al.*, 2001). Cd has previously been shown to reduce *Populus* leaf growth under Fe deficient conditions (Fodor *et al.*, 2005). A significant reduction in Cd toxicity has also been observed in rice plants

at high Fe concentrations, and genotypic variation in this effect was apparent (Shao *et al.*, 2007). Reduced Cd accumulation has also been observed in grains, shoot, leaves and roots when EDTA·Na₂Fe was added to rice plants (Shao *et al.*, 2008). Cd toxicity was also enhanced under Fe deficiency conditions in common bean, and root and leaf Cd accumulation were enhanced (Siedlecka and Krupa, 1996). Finally, a significant reduction of total dry weight and leaf chlorophyll content was observed when tobacco seedlings were exposed to 100 µM Cd but not at 20 µM Cd (Yoshihara *et al.*, 2006). Moreover, Yoshihara *et al.* (2006) observed that the tobacco seedlings appeared similar when they were treated separately with 100 µM Cd and Fe deficiency.

There were no significant interactions detected between Fe and Cd detected for the biomass traits in the present study. However, the main effects were significant for most biomass traits. The failure to detect the interaction may be due to the differential responses of the genotypes, which were accounted for with the genotypic main effects. It may also simply be a case of lack of power to detect the effect.

Decreased Cd effects and Fe effects under high Fe level

Under high Fe, plant height and biomass were similar for the low and high Cd treatments, suggesting that the toxic effects of Cd were mitigated by the high Fe levels. Contrary to our findings, Fe toxicity was observed in bean plants at 1 mmol Fe concentrations (Siedlecka and Krupa, 1996) but the current study showed somewhat enhanced growth in both genotypes, suggesting that interaction between Cd and Fe could be antagonistic or synergistic depending upon on their levels. Analysis of Cd levels in the hydroponic solutions suggested that Cd uptake depended on the level of Fe, with low Fe containers showing increased Cd uptake and high Fe containers showing reduced Cd uptake. Increased root to shoot Cd translocation might have

occurred in the susceptible genotype compared to the tolerant genotype leading to more Cd toxic effects on it compared to the tolerant genotype.

Cd-Fe interaction was stronger on the susceptible genotype compared to the tolerant. The mechanism(s) of Cd-Fe interaction are not clearly understood. In future studies it will be important to explore not only the Cd and Fe uptake and distribution patterns but also Cd immobilization mechanisms in the roots through phytochelatins or non-protein amino acids and/ other chelators (Siedlecka and Krupa, 1999).

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CHAPTER V

GENERAL CONCLUSIONS

Heavy metals released from industrial activity can affect microbial activity and soil fertility and thereby plant growth through several direct and indirect effects (Gu *et al.*, 2007; Durand *et al.*, 2011). Cd is not beneficial to living organisms except for a few marine diatoms (Lane and Morel, 2000). Cd also enters the food chain and can cause various disorders in humans, including lung and prostate cancer and leukemia. The traditional methods of remediation are expensive and ineffective, and disturb the natural topography of the soil. Phytoremediation, the use of plants to remove these pollutants, is an emerging technology that is not only cost-effective but also environmentally friendly. Phytoremediation is 10 times cheaper than the traditional engineering methods (Pilon-Smits, 2005). The plants store the metal in their harvestable plant parts and the metal can be extracted and recycled after harvest. Metal hyperaccumulators typically have short stature and low biomass, which makes them ineffective in metal uptake and storing in harvestable plant parts. There is a need to develop hyperaccumulators of heavy metals. The present study was one of the initial steps in that direction.

In **Chapter II**, a first of its kind in *Populus*, I examined phenotypic and genetic variation among full siblings from an interspecific pseudobackcross family, and identified QTL and candidate genes for Cd tolerance. Extensive phenotypic variation in Cd tolerance was observed among the genotypes, measured as changes in dry weight. Cd tolerant and Cd susceptible genotypes were identified which could be a valuable source for future selection and breeding programs targeted at physiological dissection of heavy metal tolerance. Heritabilities were low to moderate, suggesting the variation observed among these genotypes was due to genetic variation. QTL identified in control and Cd conditions were localized to different linkage groups which suggested that QTL location was determined by availability of Cd. A total of 16 QTLs were identified for Cd tolerance located on four different chromosomes. These QTL could aid future

investigations of phytoremediation and metal tolerance studies through marker assisted selection of superior genotypes.

Candidate genes identified from the underlying Cd QTL intervals were refined using a microarray study that identified genes that were up- and down-regulated in response to Cd. Some of these genes had previously been reported to have roles in heavy metal transport and/or tolerance. These included a chelator specific gene CAD2, γ -glutamylcysteine synthetase, which was involved in glutathione biosynthesis, a precursor for phytochelatins (Howden *et al.*, 1995), a cation transporter CHX18, that belongs to a group of vacuolar membrane proteins in the cation exchanger (CAX) subfamily (Mei *et al.*, 2009), a detoxifier GST10, which belongs to the theta group of the superfamily of glutathione S-transferases, GSTs (Brentner *et al.*, 2008) and HMA5 (Heavy Metal ATPase5) involved in heavy metal transport and tolerance (Migeon *et al.*, 2010). This suggested to us that Cd tolerance might have resulted from a combination of tolerance mechanisms rather than a single method of tolerance. Several other genes in the QTL intervals showed transcriptional responses, but were not previously known to be involved in Cd tolerance. One of these, the *Arabidopsis thaliana* multidrug resistance-associated protein 14 from the superfamily of ATP Binding Cassette (ABC) transporters was one of the most strongly up-regulated genes in the Cd tolerant genotype. Another, encoding a putative membrane protein containing an NHL-repeat domain, was the strongest up-regulated gene in both the genotypes after 24 hours of Cd treatment. Functional characterization of these candidate genes could unravel novel mechanisms of Cd tolerance in *Populus* and other plants (Induri *et al.*, 2012). These results lay the groundwork for further research in genetic engineering and association genetics to develop Cd tolerant hyperaccumulators that could potentially be used to remediate the extensive marginalized soils polluted with heavy metals, especially Cd.

For my **Chapter III**, I compared the QTLs of growth, biomass and phenology traits of the 52-124 *Populus* pedigree grown under greenhouse and field conditions and across years. Extensive phenotypic variation and transgressive segregation observed in both greenhouse and field trials respectively, suggested heterosis in the pseudo-backcross progeny, which could be used as a source to select better parents for future breeding and hybridization programs. Heritabilities of greenhouse traits were in general lower than those of field traits because of the non-uniform greenhouse conditions and also because of tight spacing between greenhouse plants within a container. Within field traits, heritabilities of 2009 were higher than those of 2008 because of the establishment effect in 2008 plants. Higher heritabilities in general reflected the amount of variation that was attributable to genetic variation and the scope for genetic studies. Heritabilities were highest for bud flush and bud set traits, which suggested that these traits were under the highest genetic control among all the traits studied. High heritabilities, high genetic variation, heterosis and transgressive segregation could be a good source of germplasm for future tree breeding programs in selection of superior hybrid *Populus* genotypes (Marron *et al.*, 2006).

Detection of 15, 62 and 52 QTLs in greenhouse, 2008 and 2009 seasons respectively, suggested that these traits were genetically controlled by multiple QTL on different linkage groups. Identifying QTLs among correlated and co-localized traits will aid in understanding the genetic relationships among these traits and pave the way for coordinated control of these traits through breeding and genetic engineering. Some of the QTLs were co-localized and inter-correlated, suggesting allometric relationships and/or common genetic mechanisms among these traits. QTL makers could be used to select genotypes with robust growth and biomass traits (Kim *et al.*, 2004).

Phenotypic plasticity observed in these studies suggested the ability of *Populus* genotypes in showing varied phenotypes in different environments. Ideally, it would be desirable to have the QTLs from greenhouse traits to be located to the same genomic positions as those under field conditions to avoid extensive field trials for corroborating the results of greenhouse experiments. However, this was only true for a small number of traits, including number of nodes and height to diameter ratio. This suggests that genetic control of these traits did not change across environments and suggested low genotype x environment interaction. Future studies should be directed to validate these findings across environments. Non-significant correlations between phenology and biomass traits need further detailed studies to validate this finding.

QTLs were tested across years to investigate if the genomic regions governing growth, biomass, and phenology traits change as the plant ages. Traits that are stable across years make better targets for genetic modification in tree improvement programs. Some QTLs for traits including plant height and diameter in the field study were localized to the same positions across years whereas for some traits including sylleptic branching, QTLs changed across years.

Investigating the genes contained within the QTL intervals for these traits should shed more light on the actual genetic mechanisms involved in controlling these traits. Employing genetical genomics approaches (Street *et al.*, 2006; Morreel *et al.*, 2006; Dillen *et al.*, 2009) and association genetics (Neale and Savolainen, 2004; Ingvarsson, 2005) should refine the list of candidate genes for these traits. However, because the intervals are so large, there are hundreds of candidate genes for each QTL, so identification of viable candidates is beyond the scope of this study.

As demonstrated in Chapter II, it was not surprising that the microarray study showed significant up-regulation of Fe related genes in the Cd susceptible genotype but not in the Cd tolerant genotype. Past and current literature showed that Cd could disturb Fe metal homeostasis in plants (Besson-Bard *et al.*, 2009; Siedlecka and Krupa, 1996). Cd-Fe interaction has been demonstrated in past studies of *Populus* and other plants (Siedlecka and Krupa, 1996; Solti *et al.*, 2008; Sharma *et al.*, 2004; Shao *et al.*, 2007). Considering this, for my **Chapter IV**, one Cd tolerant and one Cd susceptible genotype were selected from the Cd QTL hydroponic screening study and the effects of this interaction were examined for growth and biomass traits between these two genotypes. The results of this interaction experiment demonstrated that Cd toxicity increased under Fe deficiency conditions, especially in the Cd susceptible genotype. Both genotypes showed positive response under high Fe under Cd stress conditions but high Fe mitigated Cd toxicity effects more in the susceptible genotype than the tolerant genotype. High Fe mitigated the Cd toxicity effects possibly through reduced Cd translocation from roots to shoots. In one such Cd and zinc interaction study (Durand *et al.*, 2011), Cd translocation from roots to shoots appeared to be the limiting factor of Cd toxicity and they suggested that some proteins might be interacting with the metals in the medium thus maintaining metal homeostasis and decreasing their translocation to shoots. Investigating Cd and Fe uptake, translocation and distribution patterns should reveal further insights into their mechanism of interaction. Fe nutrition status of the soil was changed to reduce Cd uptake and accumulation in rice (Shao *et al.*, 2007). As Fe was also classified as a heavy-metal, very high Fe levels in the nutrient medium might have Fe toxicity effects as observed in bean plants (Siedlecka and Krupa, 1996). It should be safe to assume that the interaction of Cd with Fe or any other metal might depend on their concentrations in the nutrient medium rather than by themselves.

High Fe reduced the Cd uptake and accumulation in rice by inhibiting the expression of the high-affinity iron transport system (Shao *et al.*, 2007). Hyper-accumulators could be produced through enhanced expression of high-affinity iron transporters through genetic engineering thereby increasing the Cd uptake and accumulation (Shao *et al.*, 2007). Similar transgenic techniques could be employed in *Populus* to develop Cd hyperaccumulators by over-expressing the Fe transporters. Heavily polluted soils including strip mines could be remediated using these Cd hyperaccumulators with great success, assuming mechanisms for Cd tolerance also exist. My work therefore has been successful in generating numerous hypotheses that are worth pursuing for the improvement of *Populus* as a crop for biofuels production and phytoremediation.

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CURRICULUM VITAE

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RESEARCH EXPERIENCE:

Graduate Research Assistant

August 2006 - August 2007

Department of Biology, West Virginia University.

- Extensive experience in establishing hydroponic experiments.
- Extensive experience in field work including laying out the field, planting, thinning, irrigation, weeding, mulching, spraying pesticides and harvesting

PhD dissertation - Mechanisms of cadmium tolerance in *Populus*

August 2006 - Present

Department of Biology, West Virginia University.

- Identified Quantitative Trait Loci (QTLs) and candidate genes Cd tolerance after screening 252 *Populus* genotypes for Cd tolerance.
- Compared the QTLs of growth and biomass traits in the above hydroponic study with the QTLs of growth and biomass traits from a field study conducted over 2 years.
- Studied the influence of Fe on Cd toxicity and Cd tolerance on growth and biomass traits of Cd tolerant and Cd susceptible genotypes of *Populus*.

MS thesis - Genetic diversity analysis and morphological characterization of landraces of pigeonpea [*Cajanus cajan* (L.)]

August 2003 - November 2005

Department of Botany, Mahathma Phule Krishi Vidyapeeth

- Genetic diversity among 150 genotypes of *Cajanus cajan* (redgram) was assessed, grouped them into 16 genetic clusters and suggested better parents for future hybridization programs.
- These genotypes were also characterized on the basis of morphological characters.

B.S. - Agriculture Extension Worker, Andhra Pradesh, India**September 2002 - December 2002**

- Studied the agronomic practices and socio-economic status of the farmers and suggested sustainable agricultural practices.

EDUCATION:

Degree	Duration	College/ University	Percentage
PhD (Biology)	Fall 2006 – present	West Virginia University, Morgantown, West Virginia, United States of America	Current GPA is 3.90/4.00
M.S. (Cytogenetics & Plant Breeding)	Nov. 2005	Mahatma Phule Krishi Vidya Peeth, Rahuri, Maharashtra, India	Passed out in First class with 84.6%
B.Sc (Agriculture)	Jul. 2003	Agricultural College , Bapatla, Guntur Dist, Andhra Pradesh, India	Passed out in First class with 85.9 %

MANUSCRIPTS:

- **Induri, B.R.**, Ellis, D.R., Slavov, G.T., Yin, T., Zhang, X., Muchero, W., Tuskan, G.A., DiFazio, S.P. 2012. Identification of quantitative trait loci and candidate genes for cadmium tolerance in *Populus*. Tree Physiology 32:626-638.
- **Induri, B.R.**, Slavov, G.T., Yin, T., Zhang, X., Muchero, W., Tuskan, G.A., DiFazio, S.P. 2012. Quantitative trait loci for growth, biomass and phenology traits in *Populus* under greenhouse and field conditions. Tree Genetics and Genomes (In preparation).
- Ellis, D.R., **Induri, B.R.**, Slavov, G.T., DiFazio, S.P. 2012. Transcriptional profiling of cadmium tolerant and susceptible genotypes of *Populus* under cadmium and iron exposure (In preparation).

PRESENTATIONS:

ESA Conference: Oral presentation on the research paper “Mechanisms of cadmium toxicity and tolerance in *Populus*” at the 95th Ecological Society of America Annual Meeting held at Pittsburgh, PA, USA. Presented by S DiFazio.

ACADEMIC ACHIEVEMENTS:

- Secured first class and 1st Rank with distinction in B.S (Agriculture) with 85.9%.
- Leah Williams Graduate Student Summer Scholarship in 2009, Department of Biology, West Virginia University
- Doctoral Student Research Scholarship in 2010, Department of Biology, West Virginia University
- Doctoral Student Travel Grant in 2010, Department of Biology, West Virginia University
- David Fairchild Blaydes Biology Doctoral Dissertation Scholarship, 2012, Eberly College of Arts and Sciences, West Virginia University.

TEACHING EXPERIENCE:

I taught the laboratory sections of the following courses at the Department of Biology, West Virginia University.

Bio 103 (Introductory Biology): Basis biological concepts including DNA structure, Seed germination were covered in this lab. This lab also covered fields of genetics, ecology, organismal biology. I taught this course for **one** semester.

Bio 115 (Principles of Biology): Basic principles of modern biology were covered in this introductory biology course. It also covered aspects including ecology and evolution, organismal biology, and cellular and molecular biology. I taught this course for **two** semesters.

Bio 117 (Introductory Physiology): Different biological principles encompassing the diversity of reproductive, developmental, functional, and integrative mechanisms in plants and animals were discussed in this course. I taught this course for **two** semesters.

Bio 219 (Living Cell): This course primarily dealt with the structure, function, and diversity of cells with an emphasis on gene expression and cellular phenotype including cell chemistry, energetics, and regulation of cell activities. I taught this course for **two** semesters.

Bio 321 (Total Science Experience Lab): Cap-stone biological research experience course incorporating diverse learning experiences that take place in the process of being a research scientist. In this course, students were asked to write grant proposals on two-way interaction studies; carry on their own experiments to test their hypotheses; analyze their results statistically; write a manuscript on their experiments, and present their results in a public forum. As a Graduate Teaching Assistant and as an advisor for 20 students in this course, I reviewed their grant proposals and manuscripts. I taught this lab for **one** semester.

IMPORTANT COURSE WORK:

Genetics

Fundamentals of genetics

Molecular genetics

Biometrical genetics
Cytology – genetics – cytogenetics
Population and quantitative genetics

Plant Breeding

Fundamentals & Advanced courses in Principles of Plant Breeding and Botany
Seed production and certification
Breeding for Field crops & Crop Production
Molecular Biology

Statistics

Statistical methods
Design of experiments
Genetical statistics
Biometry (SAS-JMP)
Statistical genetics

PROFESSIONAL ACTIVITIES:

- Biology Graduate Student Association (BGSA) Vice-President, West Virginia University, 4/2010 – 4/2011. Assisted the student body in organizing social, educational and service activities for graduate students of Biology Department.

LANGUAGES: Fluent in English, Hindi, Marathi and Telugu.