AN ABSTRACT OF THE THESIS OF

Ani Anna Elias for the degree of Master of Science in Forest Science presented on March 6, 2009.

Title: Evaluation of Transgenes for Stress Tolerance, Lignin Modification, and Growth Enhancement in Poplar.

Abstract approved:

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Steven H. Strauss

We evaluated genes previously identified from a large scale functional genomics screen for their potential value to help enhance carbon sequestration in planted trees. We used poplar as a model tree species because of its abundant genetic variation, ease of gene transfer, and availability of large databases for genomic, anatomical, physiological, and silvicultural traits. Enhancement of carbon sequestration can be achieved by modifying many aspects of plant growth and development, including rate of growth, stress tolerance, and partitioning of assimilated carbon into compounds that are resistant to rapid decomposition, such as lignin. The selected genes were placed under a constitutive promoter and transferred to poplar via *Agrobacterium* transformation, then clonally propagated and evaluated in the greenhouse.

We tested 15 genes (15 different plasmids constructs) in greenhouse trials. For each construct, we tested 10-15 events (i.e., unique gene insertions into the genome) with 3-8 ramets per event (replicate plants). The traits analyzed included: growth rate, plant morphology, fertilizer response, lignin concentration, and drought, salt, and shade tolerance. Treatment conditions for the abiotic stress treatments were based on preliminary experiments using non-transgenic plants that examined the response of the transformed host poplar (*Populus tremula x P. alba* INRA 717-1B4) to a variety of salt, drought, fertilizer, and shade levels in potted plants. The abiotic treatment levels identified and used in the transgenic trials were -2 MPa soil moisture potential for drought tolerance; 275 mM saline solution for salinity tolerance; 60% shade or 40% light

for shade tolerance; and 0, 300, 600, and 1,200 ppm NPK fertilizer rates for fertilizer efficiency. Constructs were considered to give preliminary evidence for an effect of interest when at least two events showed a similar modified phenotype of biological interest *and* the individual events were statistically significant ($P < 0.05$) or substantially different from controls; or, all events pooled were significantly different from controls.

We tested eight constructs for improved interaction of plants with their abiotic environment. Drought construct #13 (*At* glycosyl hydrolase protein) exhibited an increase in biomass accumulation and growth in 4 out of 12 events. The salinity tolerance constructs (*At* hydrolase and *Zea mays* SF16 calmodulin-like family protein) provided no detectable improvement in tolerance. Both of the constructs for shade tolerance (*At* zinc finger protein and *At* salicylic acid carboxyl methyltransferase) imparted shade tolerance characteristics, including lower SR and taper. Construct #11 (*At* zinc finger protein) imparted significantly higher relative growth rate (RGR) for 2 out of 9 events. Despite the lack of fertilizer x event interactions, fertilizer efficiency construct #16 (*At* myb protein) imparted strong and significant growth enhancement.

We screened and scored two constructs (construct #41 – *At* ARR (Arabidopsis response regulator) protein and construct #44 – *At* DEAD/DEAH box gene, DNA/RNA helicase) for their ability to increase lignin accumulation in stems and roots via the Weisner reaction. Accumulation of lignin for the transgenic events was higher in stem tissues compared to root tissues, and caused a significant increase in the concentration of lignin in stems, with both constructs. Growth was also modified by both constructs, but in one case it was increased (construct #41) and in the other it was reduced (construct #44).

We evaluated five constructs for growth modification. Of the constructs tested for stature or growth rate modification, only #7 (*Triticum aestivum* bHLH family protein) showed a significant effect of interest. Twenty percent (2 out of 10) of the events in construct #7 showed a significant increase in growth, RGR, and biomass accumulation.

The analyses provide evidence that several transgenes—all of unknown physiological function and derived from other plant species—can provide increased growth, biomass accumulation, and/or RGR, but that the results are highly variable and event-dependent. This indicates that these genes may be useful in promoting carbon sequestration from

planted or wild stands, but require extensive testing to identify useful candidate constructs and genes, even at the primary stage of greenhouse analysis.

Evaluation of Transgenes for Stress Tolerance, Lignin Modification, and Growth Enhancement in Poplar

by

Ani Anna Elias

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Ani Anna Elias, Author

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DEDICATION

I dedicate this thesis to all young and ambitious scientists working on any form of plant system.

Even if the open windows of science at first make us shiver after the cozy indoor warmth of traditional humanizing myths, in the end the fresh air brings vigor, and the great spaces have a splendor of their own. ~Bertrand Russell, *What I Believe*, 1925.

Evaluation of Transgenes for Stress Tolerance, Lignin Modification, and Growth Enhancement in Poplar

CHAPTER 1: OVERVIEW OF THESIS

GENERAL RESEARCH GOALS

Several aspects of tree growth and development can influence the capacity for carbon sequestration by stands of trees. These include changes in growth rate, interactions with abiotic elements, partitioning of assimilated carbon, and production of compounds such as lignin that are recalcitrant to decomposition. In this project, we evaluated 15 different genes affecting traits that included abiotic interaction, plant growth, and lignin accumulation. Depending on the effects of these genes on carbon uptake and metabolism in the field, and the production ecosystems in which modified trees are used, they may influence carbon sequestration of planted forests.

This project was funded by the Department of Energy, and was part of an intensive collaboration with the plant biotechnology company Ceres (ceres.com). Together with Ceres, we selected from a number of genes identified in their ongoing large-scale genomics program - as capable of modifying traits of interest in tree crops. In short, their screens involved over-expressing large numbers of genes in Arabidopsis, which can complete its life cycle in one month and requires very little room in a growth chamber, and observing plant phenotypes. Our laboratory then transformed these same genes into poplar, verified gene presence via molecular analysis, and tested their effects on poplar growth and abiotic stress tolerance in the greenhouse. For the purpose of this thesis, we describe 15 genes from a total 38 under study in the larger research collaboration. The thesis focuses on developing the methods for greenhouse trials, and the results of the subsequent trials with respect to a variety of growth, chemistry, and abiotic stress tolerance traits. The core goal of the study was to test if genes identified in other species could impart similar phenotypic effects in a model tree, poplar.

ORGANIZATION OF THESIS

The thesis begins with a general literature review in areas relevant to the research, provides three chapters of research results written in general manuscript style, and ends with a general conclusion section about the lessons from conducting these studies and suggestions for future work. Each manuscript has only a brief discussion of the results as the genetic identity and mechanisms of physiological function of the genes tested is either unknown, or only partially known, due to the novelty of the genes and because many of these details are considered proprietary information of Ceres. We believe that for scientific publication the data will require statistical verification via a larger study, including analysis of gene expression and a more detailed physiological analysis of plant phenotypes. Thus, the manuscript style is primarily intended as a pedagogic exercise in scientific writing; they are not manuscripts being readied for publication.

CONTRIBUTIONS TO THESIS

There were a number of persons in our laboratory and at the collaborating company Ceres who contributed work and ideas to make this thesis possible. The plasmid constructs and genes, as well as funding under a subcontract for a joint grant from the Department of Energy, were produced by Ceres, who also provided the primers for verification of transformation, and help in interpretation of data and troubleshooting. Transformation of genes into poplar, regeneration of transgenic plants, and PCR verification were performed by Faculty Research Assistant Charleson Poovaiah in our laboratory. Our Laboratory Manager, Cathleen Ma, supervised the laboratory and greenhouse activities, and our Program Manager Elizabeth Etherington dealt with the budget and related financial issues for the project. Dr. Steve Strauss, the Principal Investigator of this study, gave guidance, and support in conducting the experiments and writing the thesis. A number of high school and undergraduate student helpers aided in propagation, greenhouse management, plant measurements, and transformation. The focus of my work was in executing the preliminary greenhouse studies, the experiments with transgenic plants, and quantitative data analysis.

CHAPTER 2: SCIENTIFIC BACKGROUND

RATIONALE FOR STUDY

Carbon sequestration refers to the capture and storage of carbon from the atmosphere. The two major sinks for carbon storage are terrestrial ecosystems and oceans. Other benefits that are expected from ecosystems that store substantial carbon include improved soil fertility and water retention, improved water quality due to filtration and reduced erosion, reduced air pollution as a result of enhanced uptake of volatile pollutants, and enhanced biological diversity due to improved productivity and niche complexity.

Forest ecosystems are important carbon sinks in terrestrial ecosystems. The popularized concept of trees as 'carbon sinks'(Liski et al. 2000) further increases public appreciation of the importance of forest ecosystems, and the role of trees and forests in mitigating local and global climate change. Efforts that promote carbon mitigation by forests include afforestation, reforestation, reduction of fire intensity and/or frequency, and silvicultural methods that avoid soil disturbance (e.g., tillage) and erosion.

Abiotic stresses are major limits to reforestation and carbon mitigation by forests. They limit the area of living forests and reduce carbon uptake and storage. Abiotic stresses inhibit activities of anabolic enzyme pathways, denature protein and membrane structures needed for photosynthesis, reduce stomatal conductance and thus carbon fixation, and increase premature leaf senescence that truncates growing periods (Benzel and Reuvini 1994; Munns 2002). These same physiological phenomena also reduce agricultural crop yield and quality.

Abiotic stresses can also cause extended ecological changes to wild or planted forest ecosystems (Bernier et al. 1989). Long term processes that increase salinity, reduce soil nutrition quality, reduce plant cover, and increase soil erosion can harm linked aquatic ecosystems via changes in water quality and siltation, with major effects on aquatic organisms. If breeding or biotechnology can produce trees with improved tolerance of abiotic stresses, these ecological changes can be slowed or possibly reversed.

Lignin is a major component of wood, and thus a major carbon sink in trees (Morohoshi and Kajita 2001); it accounts for approximately 30% of carbon sequestrated in terrestrial plant material (Humphreys and Chapple 2002). Lignin also provides key physiological roles in plant development and adaptation. It stiffens wood, providing structural integrity for support of stems and branches; it is water resistant, allowing long distance water transport through wood and fibers; and it serves as a key barrier to pathogens and herbivores (Rogers and Campbell 2004). It also is the main chemical barrier to pulping and fermentation of wood to produce paper and ethanol. Thus, new genetic tools to modify lignin amount may have a variety of uses in promoting tree growth or the value of trees for providing wood, paper, or energy products.

FUNCTIONAL GENOMICS

Genomics is the large-scale study of the structure and/or function of genes (Attwood 2000). The genome of an organism usually refers to its haploid set of nuclear chromosomes, though it can also refer to both the nuclear and organelle genomes (Leitch and Bennett 2003). Functional genomics refers to large-scale characterization of the effects, or mechanisms, by which genes act (Collinge et al. 2008). In addition to providing data on large numbers of genes in single experiments, genomic approaches facilitate the identification of gene networks that control complex developmental and economically valuable traits (Varshney and Tuberosa 2007).

A key element of functional genomics is the bioinformatic comparison of new genes to those in public databases that have similar sequences. This enables at least preliminary assignments of gene function for a majority of genes (Holtorf et al. 2002). When comparative information on chromosomal position, gene expression, and phenotypes of mutants are also available, advanced assignments of gene function are usually possible.

In trees, where high heterozygosity, long generation times, and inbreeding depression make the production of homozygous loss-of-function mutants impractical, genetic transformation provides an important tool. It allows the suppression of specific genes, or groups of similar genes, via RNA interference (RNAi) so that their roles in tree development can be studied (Hannon 2002) . It also allows specific genes to be mis- or

over-expressed by fusion to a heterologous promoter. In trees, it is desirable to employ field trials when studying transgenic mutants so that the organismal functions of genes, effects on wood structure and chemistry (Kumar and Fladung 2003), and maturation and flowering (Brunner et al. 2004; Brunner and Nilsson 2004), can be studied in a normal physiological environment. Stringent regulations and long rotations, however, have limited the use of field tests as a research tool. A partial exception is activation tagging, where large plantings of mutants have been screened in field environments for two to three years (Busov et al. 2005). There are a number of other approaches to the high-throughput characterization of gene function, including random insertional or activation mutagenesis; proteomics and metabolomics (to characterize proteins and small molecules); and microarrays for characterizing RNA transcription (Busov et al. 2005; Holtorf et al. 2002).

FOREST BIOTECHNOLOGIES

Overview

Because of their long generation times, intolerance of inbreeding, and the difficulty of clonal propagation from elite genotypes, forest tree breeding is a slow and costly process that has progressed only one or a few generations from wild progenitors (Zobel and Talbert 1984). Biotechnologies, by facilitating cloning, shortening generation times, introducing new traits into proven genotypes, and reducing costs of selection, therefore hold considerable promise for accelerating breeding progress (Pena and Seguin 2001).

The discovery of restriction endonucleases led to the possibility of analyzing DNA more effectively, as well as providing the capability to cut different DNA molecules so that they could later be joined together to create new recombinant DNA fragments. This capability, plus the capacity to insert these new DNA molecules into cells and regenerate modified plants, gave rise to genetic transformation and genetic engineering. In addition to its commercial value, it is a powerful method for studying gene function and regulation (Walker and Rapley 2002).

Substantial advances have been made in producing genetic maps in some forest-tree species, notably *Populus* and many conifers. This enables the use of DNA-marker-

assisted selection (MAS) to speed or reduce costs of selection (Tzfira et al. 1998). MAS can be substantially improved with current methods for identification of SNPs (single nucleotide polymorphisms) and association genetics (AG) that more effectively identify the genes responsible for quantitative traits (Pijut et al. 2007). AG studies greatly enhance our understanding of the architecture of complex traits in large non-structured populations in trees, which can facilitate the development of better offspring through breeding (Neale and Savolainen 2004).

Goals and rationale

Biotechnology has been defined in a number of ways. We define biotechnology as the use of any kind of technology to modify the genetics or physiology of organisms for some purpose (Tzfira et al. 1998). It includes plant tissue culture to promote vegetative propagation, clonal forestry, and gene transfer; grafting for propagation, manipulation of maturation state, or genetic selection; use of chemicals to modify pest loads, stature, and reproductive development; molecular biology for identification of genes, proteins, and small molecules; and genomics to catalog entire sets of genes and states of transcription and translation. All of these technologies are essential platforms for molecular tree breeding, using either molecular genome markers or genetic engineering (Nehra et al. 2005).

The branch of biotechnology known as genetic engineering manipulates genetic information encoded, usually in the form of nucleic acids and genes, to achieve specific goals. These include production of useful proteins in microorganisms, plants and animals; generation of transgenic plants and animals with new management/quality traits; and accelerated basic research about organism physiology and adaptation, making use of genes as physiological probes and experimental perturbations.

The changes made to plants through genetic engineering can be categorized into three broad areas: input, output, and biorefinery traits. Input traits help producers by lowering the cost of production, improving crop yields, or reducing the cost or environmental impacts of agronomic practices such as pest control. Output traits provide direct value to consumers by enhancing the quality of food or fiber products. These include nutritionally

enhanced foods, food with improved taste, and trees with enhanced amenity traits such as the removal of allergenic or nuisance tissues (e.g., pollen, fruits), or improved beauty (form, flowers, fall colors) or resilence in urban environments. Biorefinery traits include production of therapeutic proteins, fibers, and biodegradable plastics (Vines 2001).

Tree biotechnology

Tree biotechnology has the potential for overcoming some of the limitations to conventional tree breeding (Boerjan 2005; Campbell et al. 2003). The breeding of forest trees for tree improvement is a slow process, primarily due to the long generation period typical of most forest trees. Over-expression of genes such as *LEAFY* and *FT* can induce very early flowering in trees, though it has not yet been shown to be reliable in all genotypes, or to produce normal progeny when very young plants are induced (Rottmann et al. 2000). The out-breeding reproductive system of forest trees also limits conventional forest tree breeding, in that it is difficult to isolate and concentrate desirable traits, as well as heterotic potential, via inbreeding. Genetic engineering, by the production and insertion of dominant alleles into diverse genotypes, can speed the process of tree domestication (Bradshaw and Strauss 2001). Genetic engineering also enables phenotype modification through the introduction of foreign genes obtained from sexually incompatible species such as bacteria. The resulting transgenic trees are able to express new traits such as pest and herbicide resistance that can provide significant commercial value (Poupin and Arce-Johnson 2004). The first commercial genetically engineered forest trees in the world, grown in China, contain modified forms of insect tolerance genes from a bacterium (Hoenicka and Fladung 2006).

Advanced methods for genome analysis and identification of novel molecular markers have been used for research, and to a limited extend for tree improvement. Construction of genetic linkage maps enables the genetic dissection of polygenic traits. Molecular markers (RFLPs, RAPDs, AFLPs, STSs, SNPs and microsatellites) have been widely used to produce dense genetic linkage maps (Cervera et al. 2001; Tabor et al. 2000). Such maps describe both the relative order of genetic markers along each chromosome and their genetic distances from one another (Gilmartin and Bowler 2002).

These enable the statistical dissection of complex inherited traits and the identification of QTL, which decomposes continuous traits into their qualitative components (Confalonieri et al. 2003). These markers potentiate MAS for tree improvement (Bradshaw and Foster 1992), but to date have primarily been used for genetic fingerprinting to improve germplasm control, and to understand levels of diversity, phylogeography, and mating systems in breeding and conservation programs (Ribaut and Hoisington 1998).

Poplar as model tree

Poplars, comprised largely of cottonwoods and aspens (*Populus* L.), represent a group of approximately 30 species that are largely native to the temperate zones of the Northern hemisphere. Some of these species are cultivated by forestry organizations, and many are cultivated by farmers for a diversity of uses (Gordon 2001). Fast growth, ease of vegetative propagation, the narrow form of some varieties, and its suitability as a feed for animals have made *Populus* one of the first domesticated tree species. Its flexibility in adapting to different environmental conditions is also a key factor in promoting the rapid increase of poplar plantations with the onset of modern industry (Confalonieri et al. 2003). Poplars also provide a range of ecological services such as bioremediation, biofiltration, nutrient cycling, and carbon sequestration (Brunner et al. 2004).

Due to its small genome size, availability of routine transformation systems, saturated genetic maps, fast growth, ease of vegetative propagation, extensive genomic resources, and versatility in adaptation to the physical environment, poplars are widely regarded as the model tree for genomics and biotechnology (Tuskan et al. 2006; Tuskan et al. 2003). Poplar was the third plant species to have a high quality reference genome sequence produced (Tuskan et al. 2006; http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html).

Environmental and social concerns over GMO plants

Genetically modified organisms (GMOs) can have positive and negative social and ecological effects, depending on the specific products, the environments in which they are found, and how they are used. Extensive research has suggested that GMOs could provide positive benefits in agriculture, forestry, aquaculture, bioremediation, and

environmental management. The record from the currently used GM crops, which are planted on a vast scale and in more than two dozen countries, suggest extensive benefits in the form of reductions in cost, pesticide use, and food toxin levels (Phipps and Park 2002). The spread of some kinds of GMO crops into the environment, however, may produce negative impacts, such as harm to wild insects from insect tolerance genes (Snow et al. 2005). However, it is unknown if these direct harms are substantive in comparison to those from conventional breeding and agricultural practice, especially when all of the environmental impacts and economic benefits from agriculture are considered. Despite the potential of GMOs to improve the reliability and quality of the world food supply, the public and the scientific community have raised concerns about the environmental and food safety of GM crops (Nap et al. 2003), and generally suggest a cautious approach, especially for genes that encode ecologically novel traits such as exotic forms of pest or herbicide tolerance (Halford and Shewry 2000). Unfortunately, the hostile reaction by the developed world to GMOs has had large impacts on its uptake by the developing world, which is in desperate need of improved crop varieties of all types to feed its own public, and as sources of export earnings (Paarlberg 2001).

Among forest biotechnologies, GMOs have given rise to the greatest public controversy. MAS has been of no particular concern as it is considered a supplement to traditional breeding. However, clonal forestry has engendered some concerns and even regulations in some countries because of the risks of extensive “monocultures” of single genotypes, with potential for damage from biotic and abiotic agents (Tigerstedt and Niskanen 2002).

The main issue for GM forest trees relates to the wide extent of possible gene flow, and thus for dispersal into wild and non-GMO forest lands, and possible non-target effects on organisms there (DiFazio et al. 2004). Regulations, laws, and marketplace mechanisms such as certification systems strongly constrain research and commercial application of genetically engineered trees (Brunner et al. 2007). In addition to these factors, another major barrier in commercialization of GM trees is the uncertainty regarding public acceptance. It has been argued that the technology is too new and poorly understood, and that introduction of foreign genes into the forest environment would be

damaging and irreversible (Kaiser 2001). However, there are also cogent arguments that the basic technology is not only safe, but essential, especially as a tool for dealing with major threats to forest health from exotic organisms and climate change, and the pressing need to produce more wood and biofuels on a limited land base (Sederoff 2007).

There are also significant ethical concerns about the use of foreign and human modified genes dispersing into wild or feral lands. These concerns are reflected in the Forest Stewardship Council's (FSC) ban on all GMO trees in the field, even for research (Strauss et al. 2001a). FSC is the largest international green certifying organization for forestry. The social concerns are also reflected in the strict regulations for field research and commercial uses in most of the world. However, some researchers have argued that the ecological concerns of current GM tree crops are similar in magnitude to those facing breeders and managers on a routine basis, especially when exotic species and hybrids are used, such as for poplars and eucalypts in many locations around the world (Strauss et al. 2001b). There is little question that the level of social concern and regulation over GM trees and crops is highly asymmetrical when compared to intensive forms of conventional breeding; both appear to impose significant levels of risk but also benefit.

ABIOTIC STRESS

Overview of stress responses

Salinity and drought are the most important abiotic stresses that affect crop production in forestry and agriculture. The reduction of soil water potential they induce brings about a rapid reduction in leaf water content and potential, which in turn reduces stomatal conductance, photosynthesis, cell expansion, cell division, and thus growth (Blackman and Davies 1984; Chen et al. 1997; Cheng et al. 1997). Plant growth is thereby limited by these osmotic stresses, and they restrict the location where specific crops can be grown, and the extent of crop cover.

Typically, abiotic stresses affect root growth to a lesser degree than shoot growth (Hsiao and Xu 2000). Even under a mild water deficit, shoots may stop growing completely while roots continue to grow. There are also associated changes in carbon partitioning that favor root over shoot system development (Sharp and Davies 1979).

This behavior makes adaptive sense in that continued root growth allows the plant to continue searching for water and nutrients, increasing the possibility of above growth recovery (Weele et al. 2000). Severe water stresses, however, also lead to a reduction of water potential in the root zone and impede water acquisition and below ground growth (Bohnert and Sheveleva 1998).

Salinity stress reduces the plant's ability to imbibe water, which also slows growth. Salinity stress can cause hyper-ionic and hyper-osmotic effects, resulting in membrane disorganization, increases in reactive oxygen species (ROS), and metabolic toxicity (Hasegawa et al. 2000). A high salt content in soil may lead to its permeation into the transpiration stream, eventually injuring cells in transpiring leaves. This may further reduce growth, an effect known as the salt-specific or ion-excess effect of salinity (Munns 2002; Munns 2005).

An excess or deficit of required nutrients can adversely affects plant growth. Fertilization acts as a growth supplement for plants, helping to provide ample nutrients so that elements such as nitrogen and phosphorous are not major limiting factors to yield. Fertilization commonly increases leaf area and thus total photosynthetic area (Dana 2001; Hubbard et al. 2004). Excessive fertilization can also damage plants, causing sequential chlorosis and necrosis. An excess of elemental ions in plant cells damages many enzymes required for photosynthesis, as well as other essential metabolic processes such as pigment and fatty acid synthesis (Fernandes and Henriques 2008). Nutrient deficits often lead to chlorosis, where nutrients from older leaves are transferred by the plant to newer growth, causing yellowing, leaf abscission, and reduction of photosynthetic leaf area (highnutrients.com 2006-07). Plant tissues are commonly measured for nutrient status to help guide the timing and concentration of fertilization treatments.

Light is often a limiting factor in plant communities as a result of intra- and inter-specific competition. Shade leads to modifications in plant morphology, and changes in the concentration of chlorophyll, and thus to photosynthesis. Shade-grown leaves exhibit an increase in photosynthetic light use efficiency to maximize light capture. Leaf mass per unit area (LMA) decreases with a decrease in irradiance for broad-leaved species (Feng et al. 2004). Height growth is increased, especially in shade-intolerant species, in

an attempt to out-compete neighboring plants and improve light interception (Kennedy et al. 2007). Shade-tolerant plants have photosynthetic mechanisms that enable efficient capture and use of limiting irradiance, but generally have lower rates of photosynthesis at high irradiance than shade-intolerant plants (Khan et al. 2000; Loach 1970).

Physiological mechanisms of abiotic stress tolerance

Plants respond to abiotic stresses via a series of physiological, cellular, and molecular adjustments. These responses include stomatal closure, repression of cell growth and photosynthesis, activation of respiration, and accumulation of osmolytes and proteins (Caruso et al. 2002; Shinozaki and Yamaguchi-Shinozaki 2007; Shinozaki et al. 2003). Osmotic stress disrupts cellular functions, which leads to the generation of oxygen-radical species (Bohnert and Jensen 1996). Reductions of photosynthetic activity, accumulation of organic acids and osmolytes, and changes in carbohydrate metabolism are typical physiological and biochemical responses (Valliyodan and Nguyen 2005).

During drought conditions, plants suffer from cellular dehydration and frequently from thermal damage due to their inability to dissipate heat via transpiration (Henckel 1964). Physiological and biochemical changes at the cellular level associated with drought stress include turgor loss, changes in membrane fluidity and composition, changes in solute concentration, and adverse protein-protein and protein-lipid interactions (Chaves et al. 2003). Drought causes reductions in photochemical quenching, photosystem II (PSII) antennae transfer efficiency (Fv'/Fm'), and light-adapted PS II yield (phiPSII) (Rami et al. 2004). Drought tolerant plants often shed leaves during dry periods and enter dormancy (Dimmitt 1997).

Integration of different response mechanisms of cells, tissues, and organs is required for survival. To survive salt stress, cells must continuously protect themselves against oxidative damage to the membranes caused by reactive oxygen species (Jouve et al. 2003). Other mechanisms include ion homeostasis, synthesis of osmoprotectants, water transport, and long distance response scavenging (Borsani et al. 2003; Hasegawa et al. 2000). Biochemical strategies are also used in saline stress survival, including selective accumulation or exclusion of ions, control of ion uptake by roots and transport into

leaves, compartmentalization of ions at the cellular and whole-plant levels, secretion of sodium, synthesis of compatible solutes, changes in photosynthetic pathways, alteration in membrane structure, induction of antioxidative enzymes, and induction of plant hormones (Parida and Das 2005; Shuyao et al. 2005). Several small metabolites, typically sugars, amino acids, and inorganic acids, play critical roles in salt stress tolerance (Hanson et al. 1994).

Shade tolerance is achieved through a combination of morphological and physiological adaptations (Givnish 1988). Plants grown under shade exhibit a highly efficient photon capture mechanism, achieved through a high content of chlorophyll. Shade-grown leaves exhibit increased photosynthetic light use efficiency and effective quantum efficiency of photosystem II. They also display a decreased photosynthetic light saturation point and dark respiration compared with leaves grown in full sun (Marler 2004). Plants minimize carbon loss by reducing both respiration and construction cost of supporting as well as assimilation tissues (Givnish 1988). Under shade, plants exhibit increased photosynthetic light use efficiency and decreased biomass loss by minimizing whole plant respiration (Walters and Reich 2002). Specialized adaptations to shade also include an increase in shoot:root ratio (SR) and height:diameter ratio (HD). As light increases, plants allocate more biomass to the above ground structures, especially leaves, to increase photosynthetic leaf area (Hees and Clerkx 2003; Robakowski et al. 2004; Wang et al. 1994).

Genes identified for abiotic stress tolerance

Biosynthetic pathways for sugars, amino acids, and inorganic acids are often modified in response to plant stress, and the genes that control them have been extensively characterized and used to modify stress tolerance (Cushman and Bohnert 2000; Zhu 2000). Drought tolerance has been improved by manipulating genes encoding osmo-protectants and protective protein accumulation. For example, regulation of drought tolerance was achieved by manipulation of 9-cis-epoxycarotenoid dioxygenase and its orthologs, key enzymes in abscisic acid biosynthesis in *Arabidopsis* (Iuchi et al. 2001; Wan and Li 2006). Mannitol accumulation, and constitutive over expression of genes

encoding Na^+/H^+ antiporters, result in saline stress tolerance. The gene encoding mannitol-1-phosphate dehydrogenase (*mtl*D) caused mannitol accumulation in plant tissues and enhanced salt tolerance (Hu et al. 2005). Increase in aldehyde dehydrogenase (ALDH) activity, and regulation of the genes involved in abscisic acid (ABA) and proline biosynthesis, can enhance osmotic stress tolerance. Antiquitin-like proteins ALDH7 from soybean and its homologs improved tolerance to salinity and drought stress. This was achieved by scavenging toxic aldehydes and thus reducing lipid peroxidation, in *Arabidopsis* and tobacco in the greenhouse (Rodrigues et al. 2006). Increase in the concentration of ABA in roots helped maintain root growth and increase root hydraulic conductivity; both leading to an increase in water uptake, and thereby postponing the development of water deficiency in shoots (Liu et al. 2005). The over-expression of both glyoxalase I and II enzymes conferred improved salt tolerance in crop plants in the greenhouse (Singla-Pareek et al. 2003). Improved biomass productivity and water use efficiency under water deficit was exhibited by transgenic wheat that constitutively expressed the barley *HVA1* gene under field conditions (Bahieldin et al. 2005).

Transgenic modification of transcription factor expression has been shown to be effective in enhancing osmotic stress tolerance, a likely result of the consequent up-regulation of many downstream genes (Hsieh et al. 2002; Jaglo-Ottosen et al. 1998). For example, *Arabidopsis thaliana* transcriptional activator CRT/DRE binding factor 1 and 3 (*CBF1* and *CBF2*) genes, when expressed in cucumber, elevated salinity tolerance in greenhouse experiments (Tawfik and Grumet 2003). Increased vacuolar sequestration of sodium ions and compartmentalization of Na+ ions appear to provide efficient mechanisms for salt tolerance in plants. Over-expression of wheat vacuolar Na^+/H^+ antiporter gene *TNHX1* and H^+-PPase *TVP1* in *Arabidopsis* increased transcription of salt and drought tolerance (Brini et al. 2007). *AtSOS1* from *Arabidopsis thaliana* has been shown to encode a plasma membrane Na^+/H^+ antiporter with significant sequence similarity to plasma membrane Na^+/H^+ antiporters from bacteria. The over-expression of *SOS1* improved salt tolerance in *Arabidopsis*, demonstrating that improved salt tolerance can be attained by limiting Na^+ accumulation in plant cells. Similar results were observed for Na^+/H^+ antiporters *SOD2* from *Schizosaccharomyces pombe* and *nhaA* from

Escherichia coli when overexpressed in *Arabidopsis* and rice. Over-expression of *NHX1*, a vacuolar Na^+/H^+ antiporter, and its orthologs in *Arabidopsis*, tomato, rice, maize and barley, increased salinity tolerance (Yamaguchi and Blumwald 2005). Over-expression of dehydration-responsive element-binding protein-encoding genes resulted in enhanced tolerance to drought and high-salt stress in *Arabidopsis* and tobacco (Chen et al. 2006).

A major goal of current functional genomics programs is the identification of genetic regulators that, based on the gene expression patterns observed, appear to coordinate plant responses to complex stresses. These can then be used in transgenic research to study their value for modifying stress responses. The promoters and co-regulated networks of genes also discovered in transcriptome studies can reveal the complex transcriptional networks and downstream elements they interact with (Sreenivasalu et al. 2007).

LIGNIN BIOLOGY AND MODIFICATION

Lignin is a complex and heterogeneous cell-wall-bound phenolic polymer that plays fundamental roles in the development and physiology of woody plants. It is a major structural component of plant cell walls, and confers strength, rigidity, and impermeability to water (Kubitzki 1987; Saxena and Stotzky 2001). In woody plants, a large proportion of photosynthetically assimilated carbon is channeled to lignin synthesis. Along with cellulose and hemicellulose, lignified cell walls comprise the major components of plant biomass, and are thus a large reservoir of stored carbon (Boudet et al. 2003; Tuskan et al. 2006).

Lignin is predominantly deposited in the secondary cell walls of vascular plants, where it provides rigidity to the cell vessels and forms a physico-chemical barrier against microbial attack (Chen et al. 2001). Modification of lignin content in trees can alter their defense mechanisms against invading pathogens. Lower lignin content tends to make trees to become easier to attack and more palatable for leaf-eating and stem-boring insects, large herbivores, and microbial and other faunal communities (Halpin et al. 2007). However, modest reductions in lignin content, or alterations in lignin chemistry, may provide commercial benefits. This includes increased pulping efficiency and

reductions in mill effluents during paper production (Rastogi and Dwivedi 2008). The livestock industry regards lignin as a barrier to nutrient digestibility by animals; thus is interested in reduced lignin forages.

Lignin is primarly derived from the dehydrogenative polymerization of three different cinnamyl alcohols: *p*-coumaryl, coniferyl, and sinapyl alcohol, which give rise to the lignin precursors (Chen et al. 2001). In angiosperms, lignin is composed of two major monomeric phenolic constituents: guaiacyl (G) and syringyl (S) units. The degree of S to G subunits dictates the degree of lignin condensation (Zubieta et al. 2002). By modifying genes that encode enzymes along the lignin biosynthetic pathway, genetic alteration of both lignin quantity and quality has been achieved. Antisense suppression of the gene encoding 4-coumarate CoA ligase (4CL) reduced lignin content, while over-expression of the gene encoding coniferaldehyde 5-hydroxylase (CAld5H) increased lignin monolignols (Hancock et al. 2006). Down-regulating the expression of cinnamyl alcohol dehydrogenase (CAD), caffeoyl –CoA O-methyltransferase (CCoAOMT), and caffeic acid O-methyltransferase (COMT) in the monolignol biosynthesis pathway reduces the lignin content of wood. This may be useful for the pulp and paper industry, as suggested from field and greenhouse trials in poplar (Chen et al. 2001; Pilate et al. 2002). Over-expression of *EgMYB2*, a transcriptional activator from *Eucalyptus* xylem, resulted in an increase in secondary wall thickness, as well as alteration of the lignin profiles, in tobacco (Goicoechea et al. 2005). A new gene belonging to the C3H family was found in *Ginkgo biloba* that codes for p-coumarate 3-hydroxylase, whose over-expression can lead to increased flux through the monolignol biosynthesis pathway with consequent accumulation of phenylpropanoids and changes in lignin composition (Liu et al. 2008). Down-regulation of transcriptional repressors of lignin biosynthesis genes can lead to an increase in lignin composition. As the genes that encode repressors of lignin deposition are repressed, they allow the increased expression of lignin biosynthetic genes. This was shown for *AtMYB4*, a known repressor of some of the genes involved in lignin biosynthesis (Rogers et al. 2005).

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CHAPTER 3: EVALUATION OF GENES FOR MODIFYING ABIOTIC INTERACTIONS

ABSTRACT

Eight constructs, selected from a previous large scale genomic screens in *Arabidopsis* and *Zea*, were tested for improved abiotic stress tolerance when overexpressed in transgenic poplar. We first developed abiotic treatment conditions for the poplar genotype from preliminary stress experiments, then imposed those stress conditions on an average of 10 transgenic events and 5 ramets per event, and compared them to transgenic and non-transgenic controls in the greenhouse. The tested traits included responses to drought, salinity, shade, and fertilization. The drought and salinity constructs were screened under two different phases – growth during stress and capacity for recovery from severe stress.

Evidence for a benefit from transgene insertion was rare and varied widely among constructs. Construct #15 (*Arabidopsis thaliana* (*At)* DNA binding protein) exhibited improved performance during the stress phase based on its higher level of relative volume growth rate (RGR). Another drought construct, construct #13 (*At* glycosyl hydrolase protein), showed a consistent increase in overall growth performance by the end of both phases. The transgenic poplars containing the two salinity tolerance constructs tested (*At* hydrolase as well as *Zm* SF16 calmodulin-like family protein) showed no significantly different or consistent improvements. Both of the constructs tested for shade tolerance (*At* zinc finger protein and *At* salicylic acid carboxyl methyltransferase) exhibited increased tolerance based on a reduction in shade-induced shoot extension and an increase in biomass. Fertilizer response efficiency was screened over four levels of fertilizer application for one construct (*At* myb protein), but no statistically significant event x fertilizer interactions were observed.

INTRODUCTION

Osmotic stresses such as drought and salinity limit plant growth and biomass accumulation, as well as extent of crop production. These stresses also reduce the potential for carbon sequestration in terrestrial ecosystems, adversely affect the quantity and quality of the crop produced, and thus have substantial socio-economic

impacts. There have been numerous instances where genetic engineering approaches have been used to improve plant tolerance to stress in research trials, and some products are nearing commercial application (Halford 2006).

Light is one of the most important environmental factors in determining plant growth, morphology, and the transition from vegetative to reproductive phases (Endo et al. 2005). A reduction in light availability affects both absolute growth, and causes large changes in allocation of carbon among plant organs. Plants generally adapt to shade by increasing their height growth relative to stem girth and root development (Messier et al. 1999) — traits that are often deleterious to yield, plant stability, and stress tolerance in densely grown crops and trees. Therefore, we screened for genes that might attenuate this response.

Due to financial constraints, space availability in the greenhouse, lack of sufficient ramets, and the necessity to assess a large number of constructs in this project, we restricted the evaluation of osmotic stress and shade tolerance constructs only to the comparison of controls and transgenics under stress conditions. However, for the intensive screening for selected significant events in the second phase of this project, we will be doing a comparison over stress and non-stress conditions as well.

Fertilization generally results in an increase in plant growth and changes in allocation of biomass, and there is often substantial genetic variation in fertilization response (Newton and Amponsah 2006). However, both excess and inadequate fertilization can produce deleterious responses (Ringrose and Neilson 2005). Both the ability to grow well in the presence of low fertility, and the ability to respond strongly to added fertilizer, are desirable in different environments.

The genes we evaluated were first identified by large-scale screens in model plant species, primarily Arabidopsis. Arabidopsis is the model plant for genomics and molecular biology due to its small size, rapid life cycle, transformability, and extensive genomic resources. It was therefore used in prior work by our collaborator Ceres (www.ceres.com) to screen thousands of novel or poorly known candidate genes, with only the most outstanding continuing to further evaluation in crop models, such as rice and poplar. Thus, the organism-level physiological functions of most of the genes we studied are unknown.

Poplar was chosen as a secondary test species because of its economic importance as a forest and potential biofuels species, and also because it is widely considered the model woody species for genomics and biotechnology. Poplar is considered a 'bridge species' – a vehicle with which the growing knowledge of Arabidopsis and other model annual plants can be extended to help understand the function of trees, and accelerate their breeding and genetic engineering (Brunner et al. 2004). Its ease of transformation and vegetative propagation, and its rapid growth, were particularly important for the current study.

This work appears to be the first to test whether a variety of novel genes, identified in physiologically and phylogenetically distinct species based on a rapid genomics screen, could produce useful improvements to abiotic stress treatments in a woody plant. The greenhouse tests we conducted are considered a primary screen; promising transgenic events within constructs will undergo more intensive screens, using larger sample sizes and more treatments, in future work. We report a modest and highly variable level of benefit from the eight transgenes tested.

MATERIALS AND METHODS

Transformation, selection, and acclimatization of plants

The selected genes from *Arabidopsis* and *Zea* were transferred into poplar for evaluation of their phenotypic effects in young trees in a greenhouse environment. Of the eight genes selected, three were for improved drought tolerance, two each for improved salinity tolerance and shade tolerance, and one for nutrition efficiency. We evaluated all of these genes for their effects on biomass accumulation and allocation, and tested the appropriate constructs for tolerance to drought, salt, or nutrient stress.

We transformed the plasmid constructs provided by Ceres into *Agrobacterium tumefaciens* strain, *AGL-1*, using a freeze-thaw protocol. All genes were driven by the constitutive, proprietary 326 promoter (Ceres, unpublished data), and had an OCS terminator sequence. This vector contained the selectable marker encoding resistance to the Basta herbicide (active ingredient glufosinate-ammonium), that was used in the regeneration medium to select transgenic tissues. The selectable marker gene *bar* was driven by the p28716 promoter and had an OCS terminator. All genes were transformed into hybrid poplar INRA 717-IB4 (*Populus tremula x alba*), essentially

as reported previously (Filichkin et al. 2005). Basta was added to callus and shoot regeneration media at 10 mg/L. Polymerase chain reaction (PCR) using gene-specific primers were used to verify transgene presence, along with negative controls.

We created and propagated nine to fifteen transgenic events (i.e., a unique insertion of a gene into the plant genome) for each construct, based on the independent origins of transgenic shoots (i.e., one shoot per explant taken). We micro-propagated these events via cuttings to produce ramets (replicates). On producing four or more ramets per event, we transferred the plants to potting media, acclimated them at high relative humidity for several weeks, and then began greenhouse screening. Plants were grown in 15 cm (10,839 cm^3) or 21 cm (16,443 cm^3) diameter pots in the greenhouse trials.

Preliminary greenhouse experiments

We conducted preliminary experiments to identify suitable abiotic treatment levels for 717-IB4. Non-transgenic plants were grown at a range of levels based on the literature, and a stress level that moderately reduced growth, but did not kill the plants. Experiments were started after four to five weeks of growth in the greenhouse, using a completely randomized design (CRD) for all the greenhouse screens. For all the screens, we then gathered weekly growth measurements such as shoot height (cm), collar diameter (cm), average leaf size (cm^2), and number of leaves on plants. We defined shoot height as the distance from ground level (inside the pots) to the shoot tip. Shoot diameter (collar diameter) was measured using Vernier calipers at five cm from the soil level. We chose two leaves – one fully opened from mid-height of the plant and one emerging leaf from the top of the shoot, to measure leaf size index, based on the longest vertical (parallel to main leaf vein) and horizontal (perpendicular to main vein) axes of leaves. Leaf size index, proportional to mean single leaf area, was the simple product (length x breadth). We calculated the total leaf size index by multiplying leaf size index by the total number of leaves present on the plant. After harvest, we collected the roots, shoots, and leaves and dried them in an oven at 55 ^{0}C for approximately three weeks. Dry weights were taken separately for all of these plant parts. In addition to size measurements, for drought and salinity tolerance we also considered the survival rate of plants under different treatment

conditions. Plants with visible shoot re-initiation during the recovery phase were considered as survivors.

Drought tolerance

We conducted a first experiment by withholding water for different time periods. After the stress period, plants were re-watered and survival recorded. We selected 40 plants and divided them into four different groups and subjected them to 0, 5, 10, and 15 days without water. After the recovery phase of 15 days, re-growth capacity was assessed.

All of the plants subjected to our first preliminary drought screen survived. We therefore performed a second preliminary study by varying the soil moisture content, and thus the soil water potential, of the potting mixture by removing the plants' water supply for a fixed period (Verslues et al. 2006). We determined the gravimetric moisture content of the soil and deduced the level of soil moisture potential at which there would be no water available for plants (i.e., the wilting point), by air drying 20 soil samples to different moisture levels, testing the soil moisture potential using a water potentiometer, and observing the onset of wilting. The wilting point occurred at a soil water potential below -1.0 MPa (Figure 3.1). For the soil drying experiments, we selected three treatment conditions just above the wilting point (-2 MPa, -6 MPa, and -10 MPa) and one control treatment (0 MPa). We maintained the soil moisture potential during the stress phase at the target levels with the aid of a water potentiometer. Soil samples were collected from a depth of approximately three cm below the surface of the soil in the pots; soil moisture content was maintained by partial watering or withholding of water. During the subsequent recovery phase, the soil water potential was maintained at 0 MPa.We used 12 plants per treatment and included a stress period of 15 days followed by a recovery period of 15 days with full watering for all plants. We measured growth variables throughout the experiment, and then survival rate during the recovery period.

Salt tolerance

We conducted this experiment by irrigating plants with saline water (NaCl solution) during the stress phase. We gradually increased the saline concentration of the

irrigating water to avoid sudden death of cells (Verslues et al. 2006). During the recovery period, following the stress period, we watered the plants with non-saline water.

The treatment conditions were based on both the final concentration and rate of increase in saline solution. There were 12 plants per treatment condition. All of the plants were initially treated with 50 mM saline water. The rate of increase in concentration was 25, 50, and 75 mM of saline solution per week for the respective treatment (Figure 3.2); the final targeted saline concentrations for irrigation were 125, 200, and 275 mM, respectively. We irrigated control plants with non-saline water throughout the experiment and subjected all plants to irrigation on alternate days. We measured shoot height, collar diameter, mean leaf size, total number of live leaves on the plant, and total leaf size of the plant. During the recovery phase, we recorded survival rate and calculated the root and shoot dry weights after the final harvest.

Nutrition status

Our laboratory routinely uses 1,300 ppm of 20-10-20 peat-lite fertilizer (Jack's professional, J R Peters, Allentown, PA) for the poplar experiments in the greenhouse. We therefore selected fertilizer concentrations as multiples of this basic rate. The treatments consisted of irrigating plants with half the concentration of fertilizer solution (650 ppm), the identical concentration of fertilizer solution (1,300 ppm), or twice the concentration of fertilizer solution (2,600 ppm), while control plants remained unfertilized. Twelve plants were subjected to each treatment rate over five weeks, after which we measured shoot height, collar diameter, mean leaf size, total number of leaves, and total leaf size of all the plants. After harvest, we measured dry weights of shoots, leaves, and roots.

Shade tolerance

Shade nets were used to construct a shade tent in the greenhouse, from which plants were given either 30% shade (70% light), 60% shade (40% light), or full light in controls (Figure 3.24). We grew 13 plants per treatment condition and measured shoot height, collar diameter, internode length, and after harvest the dry weight of above and below ground plant parts. We calculated volume index (height x diameter^2) and

taper ratio (ratio of height:diameter^2, HD) as a measurement of height response to shading.

Transgenic experiments

Plant materials and measurements

There were three drought tolerance constructs, two salinity tolerance constructs, two shade tolerance constructs, and one nutrition efficiency construct for testing abiotic interactions (Table 3.2). We selected treatment conditions based on the results of the preliminary experiments described above. For all experiments, at least three ramets were screened per event and treatment condition. Both non-transgenic and transgenic "empty vector" controls were employed; however, because they were never significantly different the two were pooled for all analyses. Empty vector plants had the same vector inserted, but lacked the entire abiotic gene locus (promoter through terminator).

We used 21 cm diameter pots for the drought, salinity, and nutrition response experiments. Events were arranged in a CRD and the selected treatment condition was randomly assigned to them. During the experimental period pots were also randomly re-arranged every week or two to reduce variation due to distance from light source, competition from neighboring plants, border effect, and ventilation. For fertilizer efficiency constructs, four rates of fertilization were applied to the events. Ramets from each event were randomly selected and assigned one of these rates. Ramets from all the events which were treated with the same fertilizer rate were placed together as a 'block' for the efficiency of application, intentionally confounding 'blocks' and fertilizer rates for this preliminary screen. This was necessitated by a lack of sufficient ramets for a full block design, our arrangement of plants in the same greenhouse room to provide a high degree of homogeneity, re-randomizing the pots within 'blocks' during the experiment to improve precision for estimating event effects, and because we planned to re-evaluate any events that looked promising with a fully replicated, randomized design in a second round of intensive studies. The transgenic events had three to five ramets per treatment, and controls had eight to nine plants per treatment. We used high pressure sodium (HPS) bulbs for all our greenhouse experiments, with a photoperiod of 16 hours of light and eight hours of dark. The temperature inside the

greenhouse was controlled and monitored by a Wadsworth EnvireSTEP controller. Constructs used for particular traits, events under each construct, screening period, and temperature inside the greenhouse are summarized in Table A14 in Appendix I. We measured shoot height, collar diameter, volume index, mean single leaf area, and dry weights of shoot, leaves, and roots after harvest. We calculated shoot:root ratio (SR ratio) from the root and shoot dry weights (shoot dry weight / root dry weight). In addition, we measured the length of the longest root.

We calculated relative volume growth rate (RGR) for each ramet from the final and initial volume index as RGR = $(\ln W_2 - \ln W_1)/(t_2 - t_1)$, where W_1 and W_2 are plant dry weights at times t_1 and t_2 (Hoffman and Poorter 2002). We estimated mean single leaf area from the average of two fully expanded leaves in the mid-crown using a leaf area meter (LI-3100 area meter, LI-COR Inc. Lincoln, NE).

Statistical analysis

We performed the statistical analysis using SAS version 9.2. We first tested the data for homogenity of variance and normality by plotting residuals, transformed variables that deviated from normality and homogeneity of variance, then performed analysis of variance (ANOVA) for each variable to test the significance of difference in means among events. We used the Proc Mixed procedure at a 5% significance level. If the overall F-value for the main effects were significant, we compared all possible pairs of means using the Bonferroni correction. Then, we grouped all the transgenic events and compared this transgenic group against control by performing a t-test.

The main a priori questions were: 1) Are constructs under same trait significantly different from each other?; 2) Are transgenic events significantly different from one another and/or the controls?; 3) Are transgenics as a group significantly differ from controls?; 4) Are fertilizer rates significantly differ from each other; and 5) Is there any interaction between fertilizer rates and events? The statistical null hypotheses tested were: 1) There is no difference between constructs under same trait; 2) There is no difference between events including controls; 3) There is no difference between transgenics as a group and control; 4) There is no difference in different fertilizer rates; and 5) There is no interaction between fertilizer rates and events.

Three examples of the basic ANOVA structure used to test the hypotheses are given in Table 3.1. For the multiple-construct example, two constructs with 16 events (including control) each are given. There are five ramets for each of these events. Constructs are considered as fixed effect and events are considered as a random effect to test the first null hypothesis, while both of the effects are considered as fixed to test the second and third null hypotheses. Events are nested inside constructs, and ramets are nested inside events and constructs. For single constructs, an example of a construct with 16 events (including controls) are given. Event is the only effect tested for hypotheses two and three, for which events are considered as fixed effects to test these hypotheses. The fertilizer efficiency construct was tested as a two-factor model. There were 13 events (including control), and four fertilizer rates. Events, fertilizer rates, and their interactions were considered as fixed effects for this two-factor model.

Mathematical models for these basic ANOVA structures are given below:

1) Mathematical model for multiple constructs was $Y_{ijk} = \mu + \alpha_i + \beta_{j(i)} + \varepsilon_{k(ji)}$, . In this model, Y_{ijk} is the observed response variable for the k^{th} ramet in the j^{th} event in i^{th} construct; μ is the overall mean of the response for all constructs; α_i is the fixed effect of i^{th} construct; $\beta_{j(i)}$ is the fixed effect of j^{th} event within i^{th} construct for testing hypothesis 1 (this effect is treated as a random effect for testing hypothesis 2); $\varepsilon_{k(ij)}$ is the random error term for the k^{th} ramet in the j^{th} event within i^{th} construct. ε_{ijk} adds variability to the value of Y_{ijk}; $\varepsilon_{ijk} \sim N(0,\sigma^2)$, $\varepsilon_{k(ij)}$, and $\varepsilon_{i'j''k'}$ are independent.

2) Mathematical model for single construct was $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$. In this model, Y_{ij} is the observed response for the j^{th} ramet in the i^{th} event; μ is the overall mean of the response variable of all the plants under one construct; α_i is the fixed effect of i^{th} event; ε_{ij} is the random error term for the j^{th} ramet within the i^{th} event. ε_{ij} adds variability to the value of Y_{ij} ; $\varepsilon_{ij} \sim N(0,\sigma^2)$, ε_{ij} , and $\varepsilon_{i'j'}$ are independent.

3) Mathematical model for two-factor single construct is $Y_{ij} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ij}$. In this model, Y_{ij} was the observed response for a plant in the i^{th} event in the j^{th} fertilizer rate; μ is the overall mean of the response variable of all the plants under the construct; α_i is the fixed effect of i^{th} event; β_j is the fixed effect for j^{th} fertilizer rate; γ_{ij} is the fixed effect for interaction for i^{th} event on j^{th} fertilizer; ε_{ij} is the random error term for the j^{th} fertilizer and the i^{th} event. ε_{ij} adds variability to the value of Y_{ij} ; $\varepsilon_{ij} \sim N(0,\sigma^2)$, ε_{ij} , and $\varepsilon_{i'j'}$ are independent.

In addition, to summarize results from the large number of variables measured, we conducted principal component analysis (PCA) using Proc Princomp via the SAS (Cary, NC) software package. We used principal component scores, which are linear combinations of all the variables used in the analysis, as a way of summarizing our response variables in order to reduce dimensionality and the potential for Type I errors. We used the event (including control) means' correlation matrix for PCA, to emphasize genetic covariance rather than the environmental covariance among traits.

RESULTS

Preliminary greenhouse experiments

Drought tolerance

Although the drought treatments all reduced growth, only total leaf size (canopy area) differed among the different drought levels (Figure 3.3). Therefore, we considered the survival rate of plants during the recovery phase as the criterion for selecting one of the treatments. Forty-two percent of the plants survived, showing flushing buds and developing new leaves, for plants subjected to -2.0 MPa soil water potential. By contrast, only 8% of the plants under the -6.0 MPa soil water potential survived, and none of the plants under the -10.0 MPa soil water potential survived. We therefore selected -2.0 MPa for evaluating transgenic plants.

Salinity tolerance

Plant height was not differentially affected by the treatment; however, plants did show a progressive response, mainly in their leaf retention and morphology. Plants irrigated with 275 mM of saline solution developed shriveled leaves and showed a small reduction in collar diameter. Towards the end of the stress phase, leaves became necrotic and were shed in large numbers, reducing total leaf size (canopy area) (Figure 3.4A). Total biomass was reduced by approximately 50% at 275 mM, mainly due to reductions in total leaf size and shoot weight (Figure 3.4B). With the exception of the 275 mM treatment for which 58% of plants were killed, all the plants in other treatments survived. These results, combined with literature studies showing that poplar hybrids can be tolerant to saline concentrations around 275 mM (see discussion). We selected this level as the tolerance level for our transgenic studies.

This phase was followed by a recovery phase, in which we irrigated the plants with normal tap water.

Nutrition status

All of the fertilizer-treated plants showed a higher rate of biomass accumulation in comparison to the control. There was a significant increase in both shoot height and collar girth (Figure 3.5), and there were no obvious toxic effects due to fertilization. However, there were also no significant differences in growth seen between the different fertilization levels. To avoid possible toxicity that might occur in the transgenic experiments, we therefore avoided the 2,600 ppm level for the transgenic screening experiments, and applied three levels (300, 600 and 1,200 ppm) of fertilization rate to better examine responses to low fertilizer.

Shade tolerance

The objective of the experiment was to identify the lowest light condition in which plants showed tolerance by decreased HD ratio, SR ratio, and possibly increased volume (volume index). Although we did not observe a statistically significant difference due to the treatment conditions, we did see a trend of increased SR ratio for the lowest light treatment (40%), and a decrease of the HD ratio as the experiment progressed (Figure 3.6). We therefore chose the lowest light condition (40% light or 60% shade) as the treatment for screening transgenic plants.

Transgenic greenhouse experiments

Drought tolerance

We screened three constructs for drought tolerance: construct #13 (OSU-13 - *At* glycosyl hydrolase), construct #14 (OSU- 14 - *At* putative stricosidine synthase), and construct #15 (OSU-15 -*At* DNA binding protein) (Table 3.2).

We started the stress treatment after the plants had adapted to greenhouse conditions, approximately four weeks after transplanting to large pots (Figure 3.7). The screening started with a stress phase, followed by a recovery phase. The effect of stress treatment on leaves was significant; it included necrosis and drooping (Figure 3.8). During the subsequent recovery phase, new buds and leaves flushed on the

plants. Some plants retained viable leaves throughout the stress and recovery periods (Figure 3.9).

We screened constructs #13 and #14 simultaneously due to their simultaneous transformation, propagation, and integrated greenhouse experimental design. Compared to control plants, none of the transgenic events showed significant increases in growth or biomass accumulation (Table 3.3). However, the growth performance of construct #13 was superior to that of construct #14 (Figures 3.10). Under construct #13, approximately half of the events showed greater volume growth than controls, whereas for #14 approximately one-third showed greater volume growth.

The first two principal components explained over 80% of the variation in growth traits (Table 3.4). PC1 was strongly and similarly influenced by shoot height, collar diameter, volume index, and dry weights, whereas PC2 seemed to primarily summarize a dimension of negative association between root length and other growth variables. ANOVA results with PC1 were very similar to that with the growth variables (Table 3.3), whereas PC2 had no statistically significant effect (data not shown). The growth superiority of construct #13 over #14, and the strong correlation (0.97) between volume index and PC1, are illustrated in Figures 3.11 and 3.12.

Transgenic events showed a highly significant difference for several growth traits, as well as for PC1, under construct #15. In addition, transgenics as a group were significantly different compared to controls in height, though not in diameter or volume (Table 3.3). Nearly all the transgenic events were smaller compared to the control for all three traits (Figure 3.13). However, the RGR of 6 out of 11 events was uniformly higher even though it did not reach statistical significance (Figure 3.14). The negative effect of the construct compared to controls was also demonstrated in Figure 3.15, where PC1 and PC2 were plotted; PC2 was similar to that for constructs #13 and #14 in its contrast between growth traits and root length (Table 3.4). There was a significant increase in above ground biomass for construct #15 compared to the control at the end of the experiment (Figure 3.16).

Salinity tolerance

We tested two constructs, #18 (OSU-18- *At* hydrolase) and #19 (OSU-19- Z.mays SF16 calmodulin-like family protein) (Table 3.2).

The phenotypes of plants undergoing salinity treatment are shown in Figure 3.17, where the common phenotype of upward curling (and subsequently abscission) of leaves are visible. Some ramets, however, did not show curling, and retained green leaves even during the entire stress phase (Figure 3.17C). Unfortunately, because these phenotypes were so variable and leaves often dropped rapidly before scoring of all plants was possible, we were unable to statistically analyze this trait.

Because the experiments were conducted at different times, we performed ANOVA separately for each construct. The transgenic events under construct #18, as a group, failed to show significantly improved growth based on any measure. However, some transgenic events were significantly different from each other (Table 3.5). The height growth of the transgenic population appeared to be substantially improved, with 13 out of 14 events taller than the control (Figure 3.18). During the recovery phase, only four of the transgenic events showed a higher RGR.

Similar to the drought constructs, PC1 explained overall biomass accumulation, was strongly correlated to volume index (Figure 3.19), and accounted for more than half the variance; PC2 represented root length and to a lesser extent leaf dry weight and represented 22% of the variance; and PC3 mainly represented leaf dry weight (12% variance; Table 3.6). Considering both PC1 and PC2 it is clear that only a single transgenic event had substantially improved overall growth (Figure 3.20), though we considered this construct of interest for further evaluation as events J and H both showed strong (though not statistically significant) improvement of height growth (Figure 3.18).

For construct #19, there was little evidence that the transgenics as a group differed from controls, with the exception of root mass and RGR recovery after stress (Table 3.5). Root mass was lower for the transgenics compared to controls for all events (Figure 3.21). However, there was clearly significant variance among events for several traits. Taper ratio was higher for the large majority of the transgenic plants, though diameter was little affected (Figure 3.22). One of the events was significantly less tapered compared to the other events. The PC values had similar meanings to that

for construct #18, with PC1 (71% variance) largely representing variance in plant mass and PC2 (19% variance) largely reflecting variance in root length and root mass. PC1, by summarizing variation in both root and shoot mass, shows most clearly the depressing effect of the transgene on growth — where all events had lower PC1 values, and most had lower PC2 values (Figure 3.23).

Shade tolerance

We screened two constructs, #11 (OSU-11- *At* zinc finger protein) and #12 (OSU-12- *At* salicylic acid carboxyl methyltransferase) (Table 3.2) under shade nets (Figure 3.24).

All of the events under construct #11 were shorter, with two of them being significantly shorter based on multiple range testing (Figure 3.25); most events had a higher RGR, one significantly so (Figure 3.26; Table 3.7). ANOVA indicated a lack of overall transgene effect for any trait other than RGR, but a significant event effect for final height. PC1 (57%) was largely related to overall plant mass, while PC2 (26%) reflected a dimension of negative association between root length and weight with shoot size. Interestingly, in addition to reduced height, the transgene appears to have produced a distinctive change in morphology, as reflected in their predominantly higher values for PC2 (Figure 3.27).

Construct #12 produced plants that were variably reduced in stature, an effect that was most dramatic for volume (Figure 3.28) and RGR (Figure 3.29). The reduction in RGR was also apparent in comparison to construct #11 (Figure 3.30). All of the transgenic events in construct #12 showed curling of the leaves (Figure 3.31), accounting for an associated reduction in mean single leaf area and significant transgenic effect (Figure 3.32, Table 3.7). Events were also highly significant for many other traits, despite a lack of an overall transgenic effect (Table 3.7). PC1 (69%) again summarized total plant mass, whereas PC2 summarized a dimension with an inverse association of root length to plant mass. Similar to construct #11, PC2 suggested that this transgene had a substantially altered morphology in this dimension (Figure 3.33). When the effects of constructs #11 and #12 on plant architecture are compared, it appears that #11 may predominantly influence shoot taper, and #12 may predominantly influence shoot:root allocation (Figure 3.34). In both cases, the

transgenes appear to cause changes in the desired directions (less shoot vs. root growth, and less height vs. diameter growth, in response to shading).

Nutrition efficiency

One construct, #16 (OSU-16 – *At* myb protein) was studied, but in this case we assessed growth at a variety of treatment conditions rather than in a single condition. Four fertilizer treatments were studied: 0, 300, 600, and 1,200 ppm.

Transgenic events were highly significant effects for most of the traits studied, as was fertilizer level for many traits (Table 3.9). Fertilizer level had a significant effect on growth only at the highest level, and mainly for final volume and shoot mass (Figure 3.35). Although the majority of transgenic events had superior diameter, volume (Figure 3.36), and shoot dry weight (Figure 3.37A), there were no statistically significant event- or transgenic pool-fertilizer treatment interactions (Table 3.9). Taper ratio was smaller in the majority of transgenics (Figure 3.38) and statistically significant at event and transgenic pool levels, but again did not interact with genotype (Table 3.9). RGR was significantly lower for the transgenic pool vs. control (Figure 3.39). PC1 (54% variance) again reflected total plant mass, whereas PC2 through PC4 (~10% variance each), reflected specific plant attributes and complex associations with other traits, but predominantly root length, diameter, and root dry weight, respectively. The majority of transgenics had higher PC1 values, reflecting their faster growth in a number of traits; PC2, reflecting root length, was mostly smaller in the transgenics (Figure 3.40).

DISCUSSION

Experimental variation

The major goal of the greenhouse experiments was to provide a highly precise system such that small genetic effects could be detected with a modest degree of replication. It is also largely free of the costs and constraints of obtaining regulatory approval for outdoor growth of transgenic plants. Unfortunately, despite strong efforts to minimize experimental error by clonal replication, reinsertion of genes into the same poplar clone, and re-randomization of pot locations in the greenhouse during the

experiments, error variation was substantial in most of these experiments. Because this problem was common to other experiments, it is discussed under conclusions.

In addition, the effects of stress treatments may have depended on plant size in a manner that amplifies variance, for example if the faster growing plants suffered stress faster or more severe forms of stress by using up moisture or fertilizer in their pots sooner; or if roots of fast growing plants reached the limits of pots sooner and began desiccating earlier. This may explain why PC2 often seemed to contrast plant size and root length. It is also possible that the extensive handling of plants when they were re-randomized induced significant physiological stress (Braam and Davis 1990), in addition to the stress and physiological changes applied through treatment conditions. Pronounced differences between the plants were often apparent in the size of the seedlings, and these differences may have caused the observed tendency for RGR to be high in small plants, as RGR generally declines with increasing plant size (Evans 1996; Rey et al. 2001). The increased RGR exhibited by small seedlings is often due to increased photosynthetic activity associated with lower self-shading or lower respiration losses as a result of their lower above ground biomass (Bruhn et al. 2000; Lima et al. 2005). Finally, because of the difficulty of maintaining a precise level of stress by selective watering in our drought experiments, we may have imposed additional error variance via our stress treatments. Although we aimed for -2.0 MPa inside the pots, our measurements showed that we actually imposed a range of potentials, from -1.5 to -2.5 MPa, which could have significant consequences for plant growth. For the salinity constructs, it was possible that the frequent irrigation of pots using 275 mM saline solution aided in the accumulation of ions inside the potting mixture, which could have varied between the pots and thus resulted in the variation in growth seen between ramets within events.

Experimental system

The greenhouse experimental system is only a rough indicator of how plants might fare in field environments. The rate of drought, salinity, and nutrition stress is often much slower in the field compared to pots, as plants gradually exhaust available moisture and nutrients during extensive root growth (Bradshaw and Foster 1992; Krizek 1985). Pots provide a notoriously unnatural environment for root growth,

especially for trees like poplar that show rapid shoot and root elongation. On the other hand, changes in stress levels due to wind, storms, and temperature swings are far greater in the field, and can alter plant health and physiology dramatically. In addition, we used a specialized potting mixture which is very different from the soils that poplars would see under field conditions. Finally, we conducted our experiments throughout the year, where in spite of greenhouse light and temperature controls, it is obvious that light intensity, temperature, and photoperiod vary significantly, especially between summer and winter. This variation is likely to have affected the phenotypic expression of the transgenes studied. Field evaluations are clearly a required next step for any constructs that show promise.

Our experimental system and preliminary data were comparable to those seen in other studies of poplar. *P. popularis* plants maintained a higher growth rate, high photosynthetic rate, and no leaf loss, while *P. italica* showed all of these symptoms at -2. 2 MPa (Chen et al. 1997). This is comparable to the soil water potential of -2.0 MPa that we applied in our transgenic drought tolerance experiments. The poplar hybrid *P. pyramidalis* x *P. tomentosa* showed 78% survival under 250 mM of saline solution during *in vitro* screening experiments (Watanabe et al. 2000). Because young *Populus* plants show higher tolerance in *in vitro* conditions than do older plants, we expected that our young plants in the greenhouse environments might also show higher tolerance. This expectation was roughly supported by our preliminary screening results, where 275 mM was successfully employed to screen transgenic plants for salinity tolerance.

Transgene effects on target traits

Despite high experimental error and the complex multigenic nature of stress resistance traits (Valliyodan and Nguyen 2005), notable results were seen with some constructs that warrant additional research. Despite a lack of genotype x fertilizer interaction, the strong response to nutrition observed for construct #16 suggests that the transgene may be a useful for improving nutrient use efficiency. One event showed particularly strong and significant growth performance. In future work, an examination of chlorophyll content present in these leaves would be helpful in explaining the increase in growth and presumably, photosynthetic efficiency.

Measurements of the presence of nitrogen and phosphorous in plant tissues of these events would help to explain if they are more efficient in nutrient uptake.

The changes in plant form observed for the two shade tolerance constructs suggest that they may be useful. Reduction in light availability for plants triggers shade avoidance responses including stem elongation (Cregg et al. 1993; Endo et al. 2005); these are often undesirable in crop systems. Specialization for shade can also include an increase in SR and HD ratios, since plants allocate more biomass to the above ground structures, especially leaves, as light increases (Hees and Clerkx 2003; Robakowski et al. 2004; Wang et al. 1994). Therefore, shade tolerant plants should have lower SR and HD ratios producing shorter, more taper stems. Shade tolerance construct #11 gave rise to reduced height growth in all transgenic events as desired. It also led to a desired reduction in HD ratio, whereas shade construct #12 led to a reduction of SR ratio under shade.

One-third of the events from construct #13 showed much more rapid growth and biomass accumulation under drought and recovery than controls and other events under construct #14. Drought tolerance construct #15 demonstrated uniformity in events with smaller plant size and higher RGR during stress phase. They also showed high taper. Most of the events from salinity construct #18 showed very strong height growth and one of them displayed increased biomass accumulation and growth enhancement at the end of the experiment. Construct #19 produced a nearly uniform increase of diameter and uniformly less taper after the experiment, suggesting it also may be a useful gene for modification of tree form.

These are all preliminary results, and await rescreening with larger numbers of replicates, and at several environmental treatment levels including non-stress conditions, to confirm or refute these results. Given the severe effects on leaf form and abscission we observed under drought and salinity stress, we also believe that lower levels should also be included during screens. In addition, measurement of electrical conductivity and pH to avoid ion accumulation in pots may also be useful. If transgene effects are confirmed, a logical next step would be to investigate the effects on plant anatomy and physiology. This might include studies of vascular conductivity and osmotic potential. A reduction in vessel lumina, associated with an increase in cell wall strength, was observed in poplar as a response to osmotic stress (Junghans et

al. 2006)—an effect that varies with the season and time of stress application (Arend and Fromm 2007). Osmotic stress decreases net assimilation rate and photosynthetic rate, and increases organic osmolyte content (Hale et al. 2005). Many other potential physiological parameters could be studied, depending on the gene and trait modification observed. These measures would also help to predict the the kinds of stresses under which these transgenes might be beneficially employed.

CONCLUSIONS

Only some of constructs studied showed promising results. Drought tolerance constructs #13 and #15 demonstrated increased biomass accumulation and RGR in stress and recovery phases. Salinity tolerance constructs #18 and #19, however, performed similar to control plants in stress and recovery phases. Shade tolerance properties were exhibited by both of the shade constructs – #11 and #12. In addition, one out of 12 transgenic events studied showed significantly higher growth performance. Although there was no interaction of event with fertility level, most events showed an increased rate of growth in nutrition efficiency construct #16.

The objective of increased in biomass accumulation and, thus, increased carbon allocation in tree shoot and roots, was exhibited by some of these constructs. The constructs also demonstrated their ability to affect growth and morphology in stressed environments. As hoped, our work has identified several promising candidate genes for further evaluation.

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Table 3.1. ANOVA tables used for basic data analysis. Type 3 tests of fixed effects for traits with:

A) Multiple constructs.
B) Single construct.
C) Two-factor.

A

Effect		Df	
Total (N)		n-1	159
Construct (C)		c-1	1
Event (E)	E(C)	c(e-1)	30
Ramet (R) (Error)	R(EC)	ec (r-1)	128

i. Constructs were compared by taking construct as fixed effect (F stat $_{(1,128)}$)

ii. Events under constructs were compared by taking constructs and events as fixed effects (F stat $_{(30,128)}$)

B

Effect		Df	
Total (N)		n-1	79
Event (E)	E	e-1	15
Ramet (R) (Error)	R	r-1	4
	R.E	(r-1)(e-1)	60

i. Events under single constructs were compared by taking event as fixed effects (F stat $_{(15, 79)}$)

C

Effect		Df	
Total (N)		n-1	259
Event (E)	E	e-1	12
Fertilizer (F)	F	f-1	3
Event*Fertilizer	EF	(e-1)(f-1)	36
Ramet (R) (Error)	R	r-1	4
	R.EF	(r-1)(ef-1)	204

i. All effects are fixed; events (F stat $_{(12, 208)}$), fertilizer (F stat $_{(3, 208)}$), and event*fertilizer interaction (F stat $_{(36, 208)}$)

Table 3.2. Genes in constructs for abiotic interactions.

Code ID	Target trait	Protein encoded
OSU-11	Shade tolerance	At zinc finger protein
OSU-12	Shade tolerance	At salicylic acid carboxyl methyltransferase
OSU-13	Drought tolerance	At glycosyl hydrolase
OSU-14	Drought tolerance	At putative stricosidine synthase
OSU-15	Drought tolerance	At DNA binding protein
OSU-16	Nutrition efficiency	At myb protein
OSU-18	Salt tolerance	At hydrolase
OSU-19	Salt tolerance	Z.mays SF16 calmodulin-like family protein

Table 3.3. Variables used for ANOVA and their statistical significance for drought constructs.

	Constructs #13 and #14			Construct #15	
Variable	**Construct**	**Event**	**Transgenic pool**	**Event**	**Transgenic pool**
Initial height (cm)	*	*	-	**, #	*
Final height (cm)	**	*	-	**, #	*
Initial diameter (cm)	**	-	-	*	-
Final diameter (cm)- Ŧ	**	-	-	**	-
Initial volume index (cm^3) - ŧ	*	-	-	**	-
Final volume index (cm^3) - ŧ	**	*	-	**, #	-
Root length (cm)	*	-	-	-	-
Shoot dry weight (g)- Ŧ,ŧ	-	*	-	**	-
Root dry weight (g)- Ŧ	-	-	-	**	-
Leaf dry weight (g)- Ŧ,ŧ	-	-	-	-	-
PC1	*	-	*	**, #	-
Taper ratio _stress - Ŧ	-	-	-	-	-
Taper ratio _recovery - Ŧ	-	-	-	-	-
SR – Ŧ,ŧ	-	-	-	*	*
RGR_stress	NT	-	-	-	*
RGR_recovery	NT	-	-	-	*

- = No significance; * = P- value <0.05; ** = p-value <0.01; # = some transgenic events differ from control

Ŧ = log transformed data for constructs #13 and #14; ŧ = log transformed data for construct #15

NT = Not tested

Construct = tested with control included under each construct

Transgenic pool = tested with controls excluded from transgenic and pooled

Table 3.4. **Eigenvectors (multiplied by 100) for variables on event means.**

	Construct #13 and #14			Construct #15	
Variables	**PC1**	**PC2**	**PC3**	**PC1**	**PC2**
Final height (cm)	39.5	-18.9	-8.7	44.9	-11.4
Final diameter (cm)	37.7	-3.0	22.2	44.7	-21.6
Final volume index (cm^3)	40.3	-3.4	-5.6	45.1	-20.9
Root length (cm)	27.9	11.6	85.7	16.6	92.2
Shoot dry weight (g)	39.8	-1.8	-27.5	45.2	0.5
Root dry weight (g)	37.8	-8.5	-3.7	40.4	21.4
Leaf dry weight (g)	38.0	-4.3	-33.0	-	-
% variance	*71.0*	*11.0*	*8.0*	*77.0*	*16.7*

Table 3.5. **Variables used for ANOVA and their statistical significance for salinity constructs.**

	Construct #18		Construct #19	
Variable	**Event**	**Transgenic pool**	**Event**	**Transgenic pool**
Initial height (cm)	**	-	-	-
Final height (cm)	**	-	*	-
Initial diameter (cm)- Ŧ	-	*	-	-
Final diameter (cm)	*	-	**,#	-
Initial volume index (cm^3) - Ŧ	-	*	*	-
Final volume index (cm^3)	*	-	-	-
Root length (cm)	-	-	-	-
Shoot dry weight (g)- SQ	**	-	-	-
Root dry weight (g)- ŧ	-	-	**#	*
Leaf dry weight (g)- Ŧ	*	-	_	_
PC1	*	-	*	-
Taper ratio_stress- Ŧ	-	-	**,#	-
Taper ratio_recovery- Ŧ,ŧ	-	-	-	-
SR- Ŧ,ŧ	-	-	*	-
RGR_stress - NP	-	-	-	-
RGR_recovery - NP	-	-	-	*

SQ = square root transformed for construct #18
NP= Non-parametric test for both construct #18 and #19;
Ŧ – log transformed for #18; ŧ – log transformed for #19
Other symbols explained in table 3.3

Table 3.6. **Eigenvectors (multiplied by 100) for variables on event means.**

	Construct #18			Construct #19	
Variables	**PC1**	**PC2**	**PC3**	**PC1**	**PC2**
Final height (cm)	44.5	11.0	-9.6	47.0	-1.6
Final diameter (cm)	44.3	-11.1	0.7	44.1	-26.4
Final volume index (cm^3)	47.4	-4.9	1.3	44.7	-29.4
Root length (cm)	3.5	64.9	-40.3	23.3	76.8
Shoot dry weight (g)	37.4	-3.3	-41.9	43.1	-22.2
Root dry weight (g)	44.4	9.6	16.1	38.1	45.2
Leaf dry weight (g)	16.9	30.1	79.2	-	-
% variance	*52.0*	*21.9*	*12.4*	*71.1*	*19.4*

Table 3.7. **Variables used for ANOVA and their statistical significance for shade constructs.**

	Construct #11		Construct #12	
Variable	**Event**	**Transgenic pool**	**Event**	**Transgenic pool**
Initial height (cm)- Ŧ	-	-	*	-
Final height (cm) - Ŧ	**,#	-	**,#	-
Initial diameter (cm)	-	-	*	-
Final diameter (cm)	-	-	**, #	-
Initial volume index (cm^3)- SQ	-	-	*	-
Final volume index (cm^3) - Ŧ	-	-	**,#	-
Root length (cm)	-	-	-	-
Mean single leaf area (cm^2)	**	-	**,#	*
Shoot dry weight (g)	-	-	**	-
Root dry weight (g) – Ŧ,ŧ	-	-	**	-
Leaf dry weight (g)	-	-	**,#	-
PC1	-	-	**,#	-
Taper ratio	-	-	-	-
SR- ŧ	-	-	-	-
RGR	**,#	**	**,#	**

Ŧ – log transformed for construct #11; ŧ – log transformed for construct #12; SQ – square root transformed for construct #12
Other symbols explained in table 3.3 and 3.5.

Table 3.8. Eigenvectors (multiplied by 100) for variables on event means.

	Construct #11		Construct #12	
Variables	**PC1**	**PC2**	**PC1**	**PC2**
Final height (cm)	32.9	-36.3	35.2	5.2
Final diameter (cm)	32.3	-41.2	34.9	-7.3
Final volume index (cm^3)	39.4	-30.7	35.0	-13.3
Shoot dry weight (g)	42.6	16.0	34.3	-15.5
Leaf dry weight (g)	42.3	4.7	34.4	-14.0
Root dry weight (g)	33.3	46.8	33.9	-15.7
Mean single leaf area (cm^2)	34.3	1.9	32.2	-0.8
Root length (cm)	20.7	59.7	26.8	39.7
% variance	*57.0*	*26.5*	*68.7*	*11.4*

Table 3.9. Variables used for ANOVA and their statistical significance for nutrition efficiency construct #16.

Variable	**Event**	**Treatment**	**Event *treatment**	**Transgenic pool**	**Treatment**	**Transgenics *treatment**
Initial height (cm)	-	-	-	**	-	-
Final height (cm)	-	*	-	-	-	-
Initial diameter (cm)	**,#	-	-	**	-	-
Final diameter (cm)	**,#	-	-	**	-	-
Initial volume index (cm^3)	**,#	-	-	**	-	-
Final volume index (cm^3)	**,#	**	-	*	-	-
Root length (cm)	-	-	-	*	-	-
Mean single leaf area (cm^2)	-	-	-	-	-	-
Shoot dry weight (g)	**,#	**,#	-	**	-	-
Root dry weight (g)- ∓	-	-	-	*	-	-
Leaf dry weight (g)	*	*	-	-	-	-
PC1	**,#	-	-	*,#	-	-
Taper ratio - ∓	**,#	-	-	**	-	-
SR- ∓	-	**,#	-	-	-	-
RGR	**,#	*	-	**	-	-

Symbols explained in table 3.3 and 3.5.

Table 3.10. Eigenvectors (multiplied by 100) for variables on event means.

Variables	PC1	PC2	PC3	PC4
Final height (cm)	42.0	18.8	6.8	-31.5
Final diameter (cm)	27.2	-19.7	64.8	-40.4
Final volume index (cm^3)	46.2	-4.2	1.4	0.1
Shoot dry weight (g)	45.9	-5.7	0.1	3.3
Leaf dry weight (g)	40.9	9.0	-15.9	14.4
Root dry weight (g)	30.9	-21.0	7.1	77.2
Mean single leaf area (cm^2)	24.2	-5.2	-72.9	-32.4
Root length (cm)	6.5	93.1	11.5	12.2
% variance	*54.1*	*13.2*	*12.5*	*9.2*

Figure 3.1. **Soil moisture release curve (absolute values of MPa are given).**

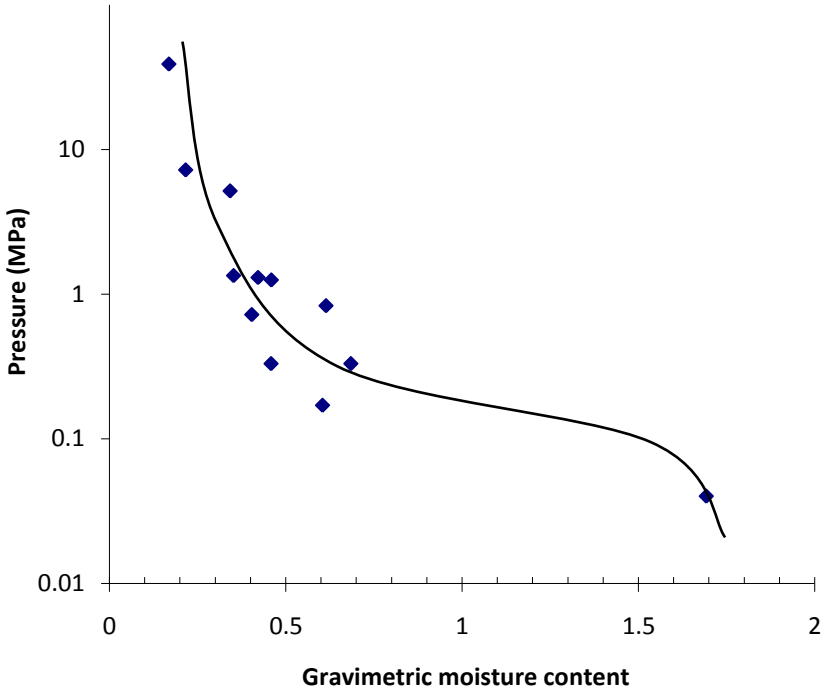


Figure 3.2. **Experiment regime for preliminary salinity tolerance screening.**

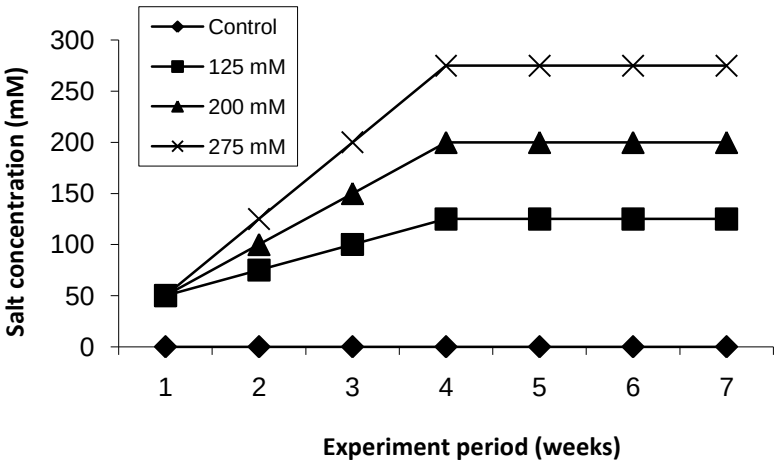
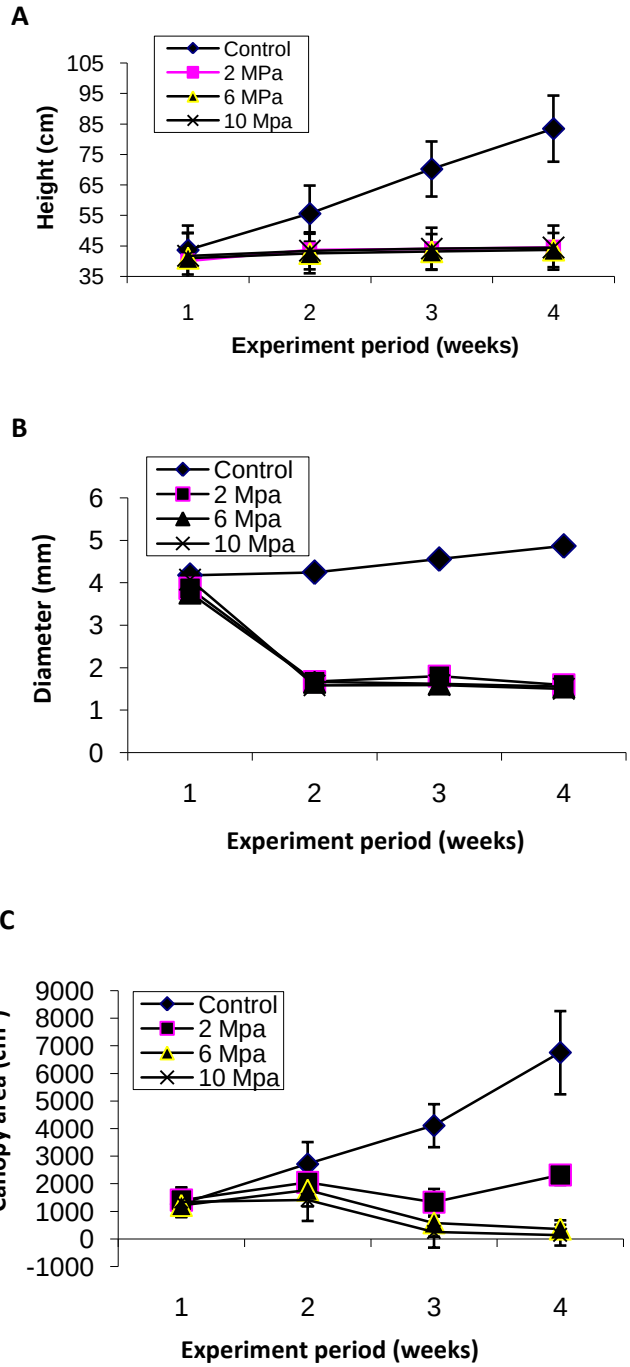


Figure 3.3. Results of preliminary greenhouse drought experiment.

A) Height

B) Diameter

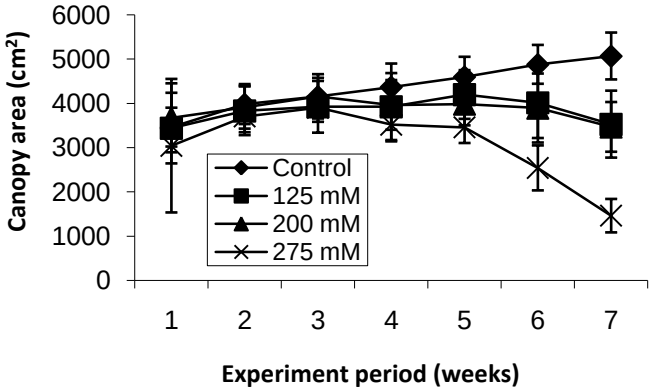
C) Canopy area



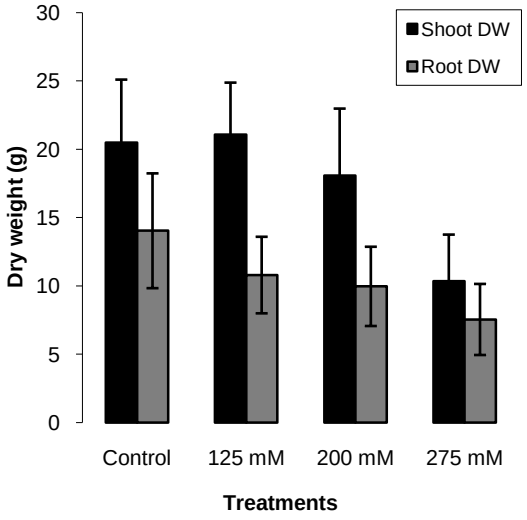
Data is represented as mean ± standard deviation

<u>Figure 3.4.</u> Results of preliminary greenhouse salt tolerance experiment.

A) Canopy area



B) Shoot and root dry weights



Data is represented as mean ± standard deviation

Figure 3.5. **Result of preliminary greenhouse fertilization response experiment.**

A) Height

B) Diameter

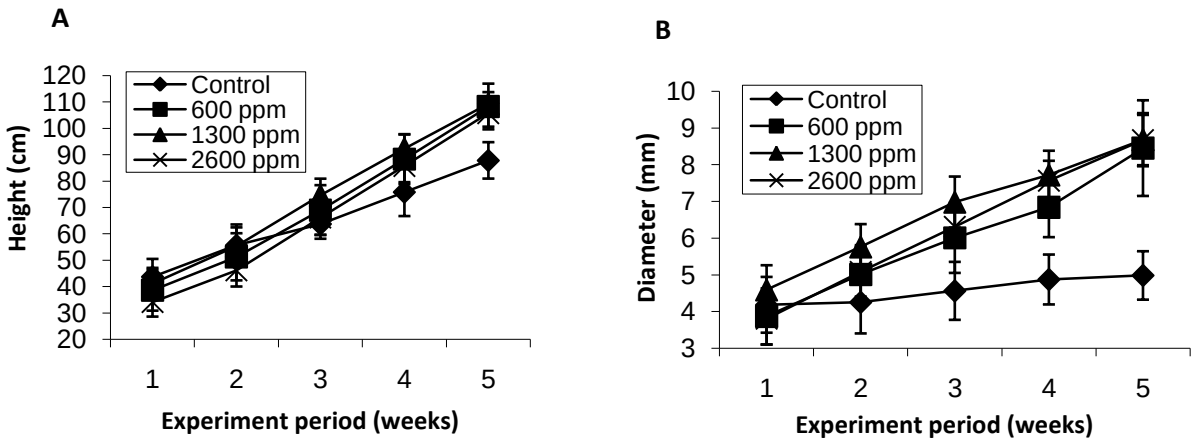


Figure 3.6. **Taper (height to diameter^2) ratio for shade tolerance preliminary screening.**

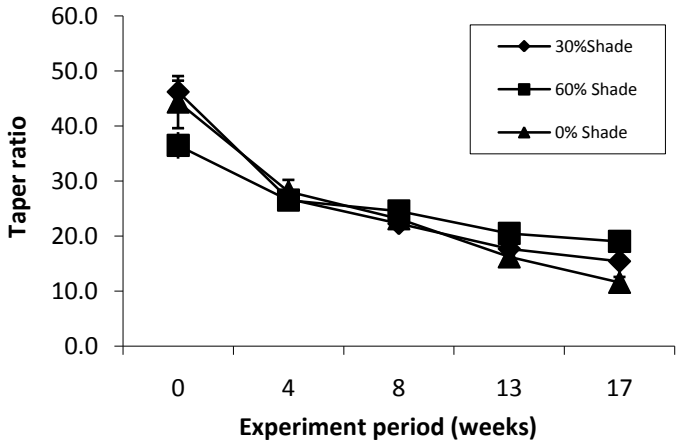
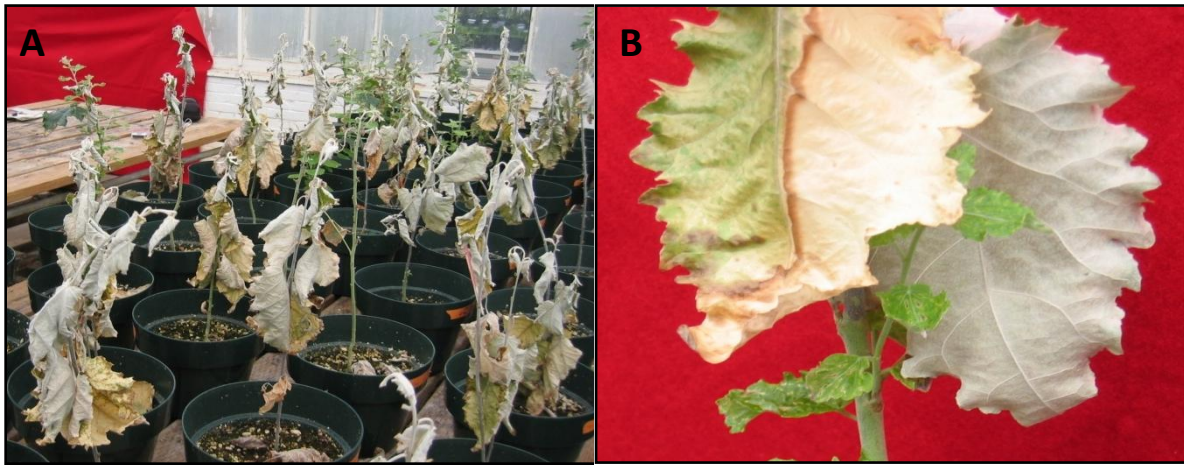


Figure 3.7. **Drought constructs during screening in greenhouse.**

A) Before stress treatment
B) At the beginning of stress phase (under -2 MPa of soil moisture)

Figure 3.8. **Effect of drought stress on**

A) General plant health
B) Leaf health



<u>Figure 3.9.</u> Drought tolerance constructs during recovery phase.

A) General plant health

B) Sprouting buds

C) Sprouting fresh leaves and retained old leaves

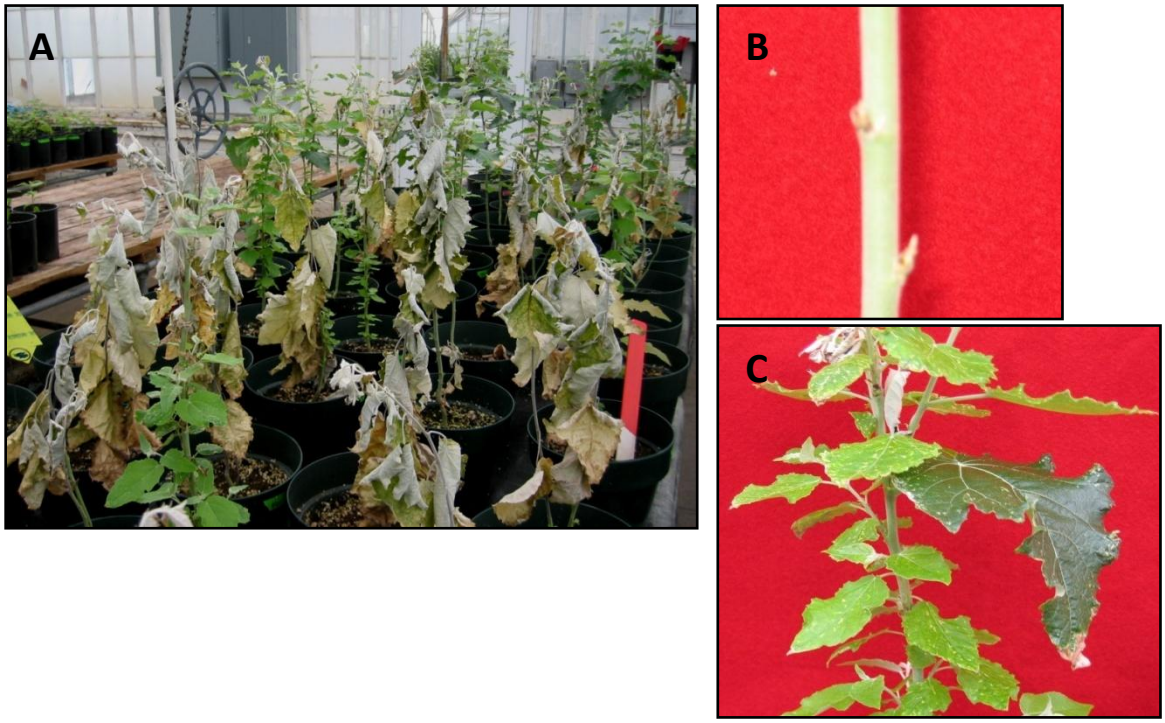
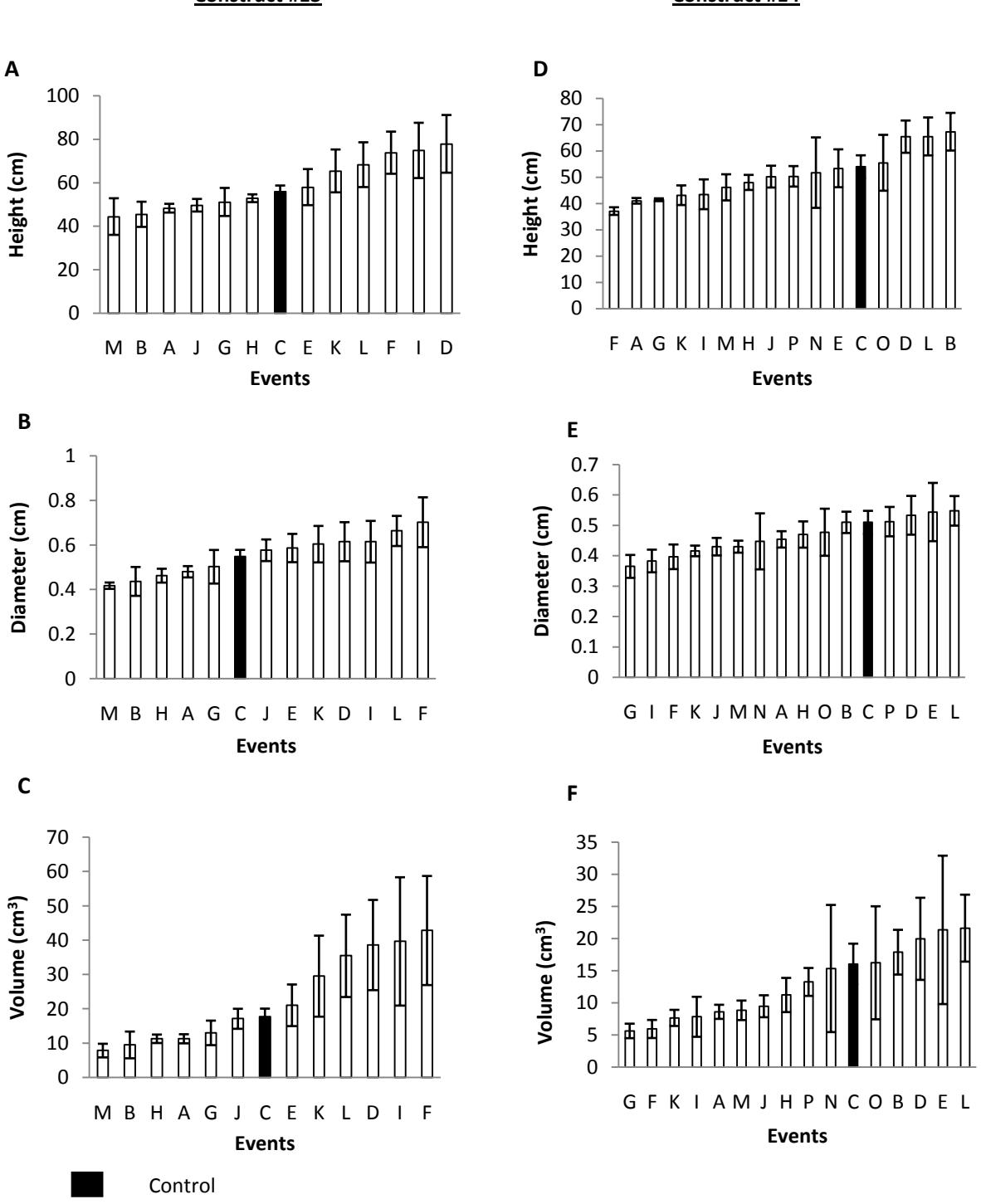


Figure 3.10. Growth comparison between drought tolerance constructs #13 and #14.

A and D) Height

B and E) Diameter

C and F) Volume



Data is represented as least square mean ± standard error

Figure 3.11. **PC1 vs PC2 for drought constructs #13 and #14.**

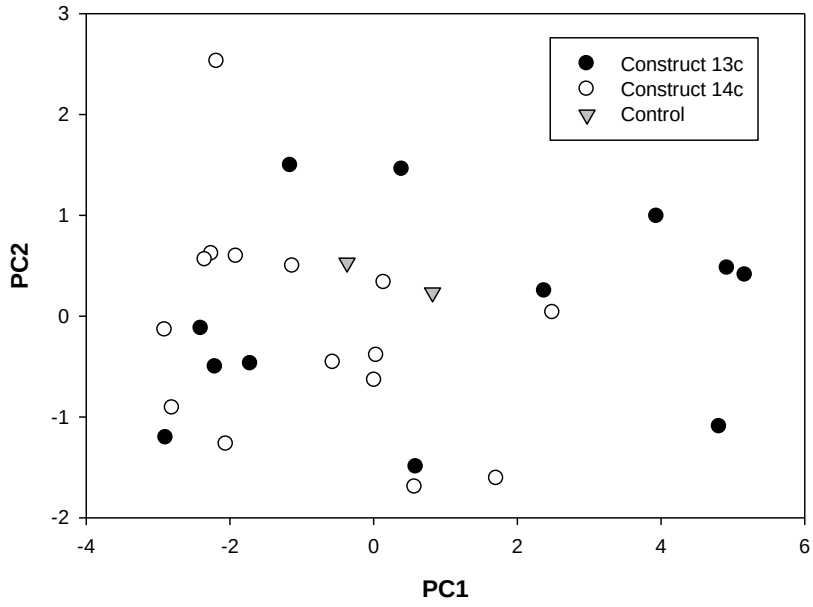


Figure 3.12. **PC1 vs volume for drought construct 13 after experiment period.**

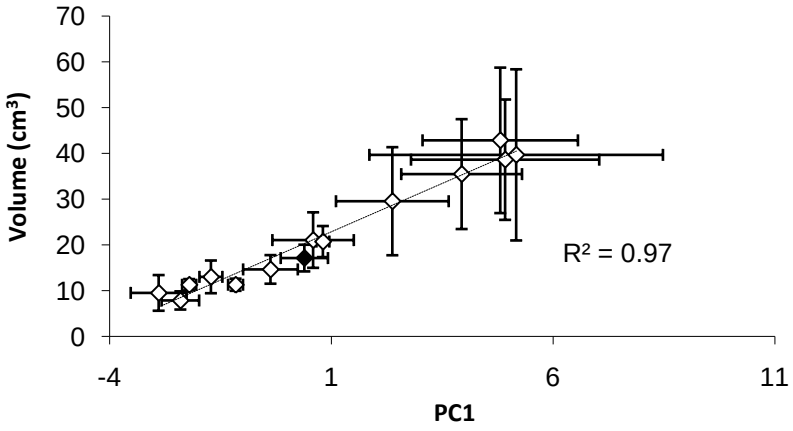
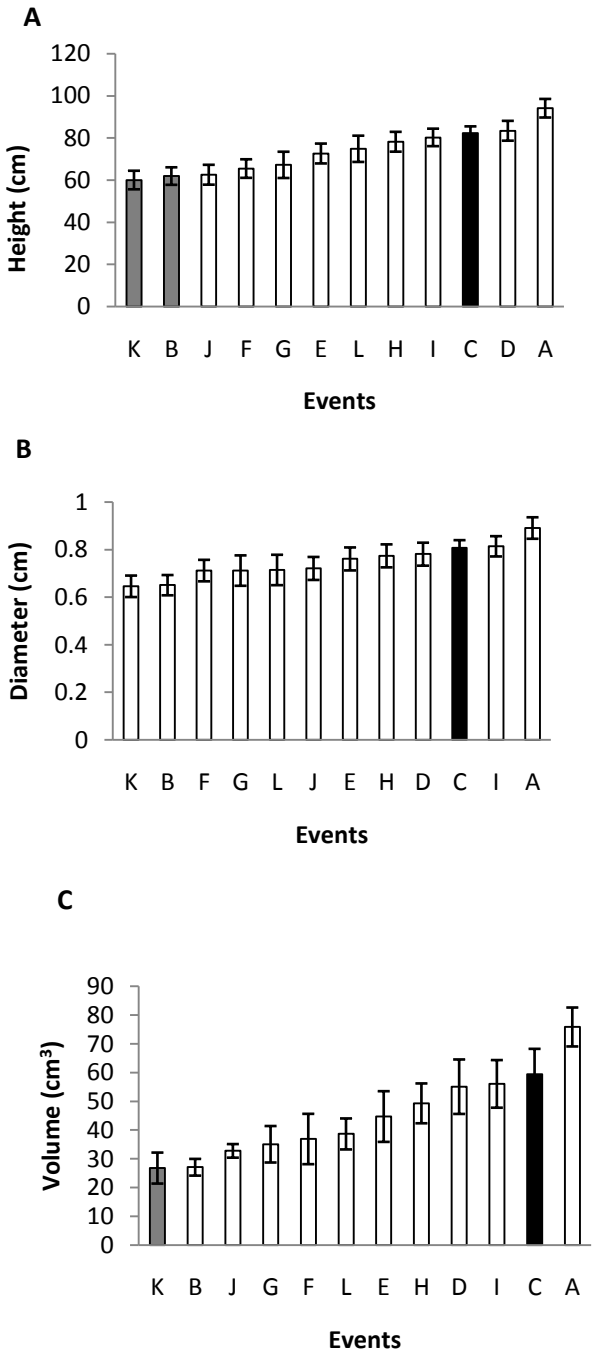


Figure 3.13. Growth comparison between drought tolerance constructs #15.

A) Height
B) Diameter
C) Volume



Data is represented as least square mean ± standard error

Figure 3.14. **Relative volume growth rate (RGR) during stress phase for drought construct #15.**

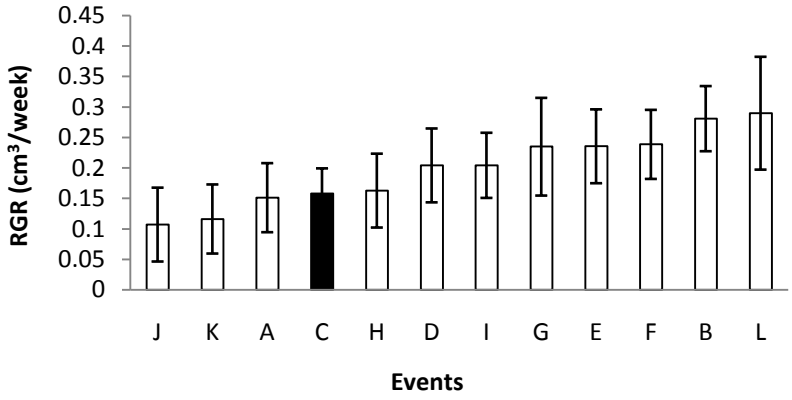


Figure 3.15. **PC1 vs PC2 for drought construct #15.**

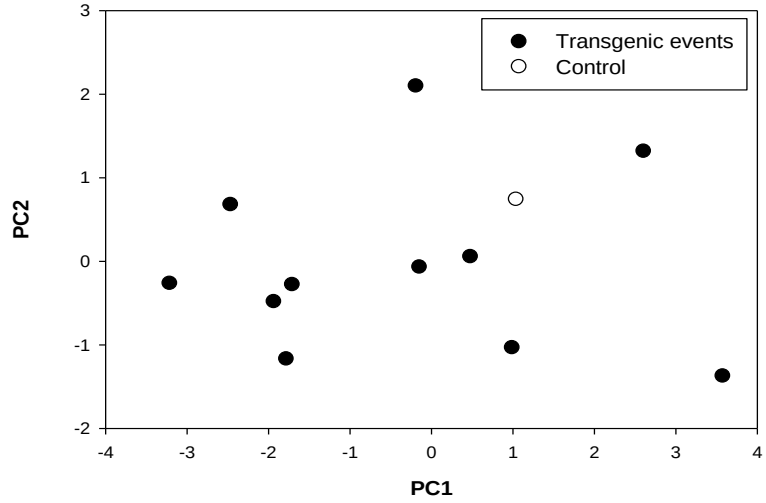


Figure 3.16. **Shoot:root ratio for drought construct #15.**

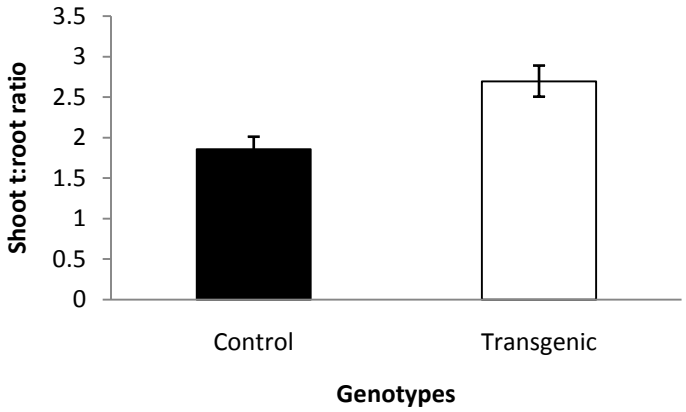


Figure 3.17. **Salinity constructs undergoing screening in greenhouse.**

A) General health
B) Curly leaves
C) Healthy leaves

Figure 3.18. **Variation in height among events in salinity tolerance construct #18.**

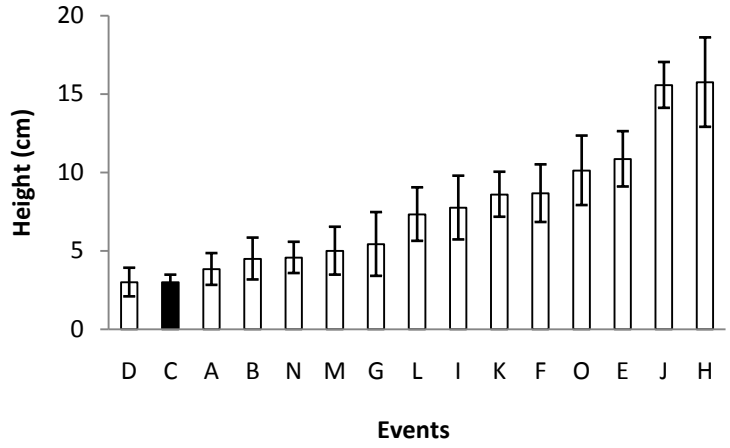
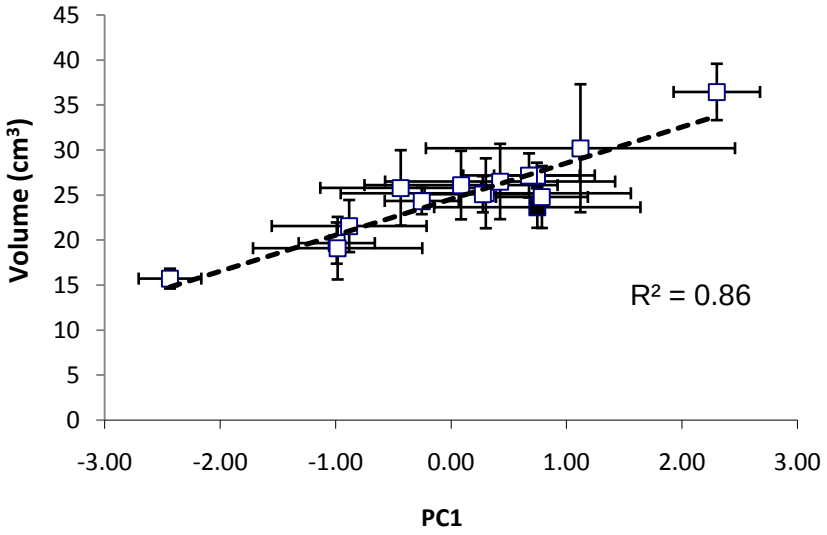


Figure 3.19. **PC1 vs volume for salinity construct #18.**



Control

Data is represented as least square mean ± standard error

Figure 3.20. **PC1 vs PC2 for salinity tolerance construct #18.**

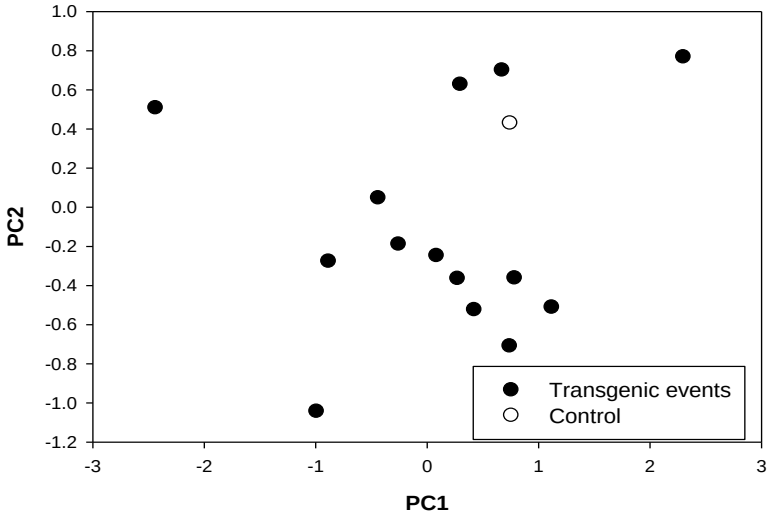
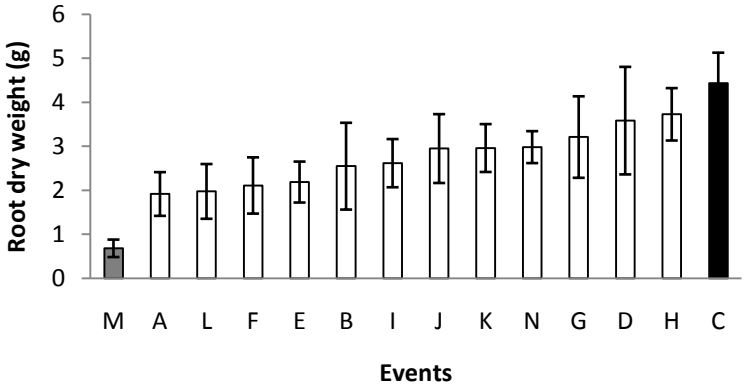


Figure 3.21. **Variation in root dry weight among events for salinity construct #19.**

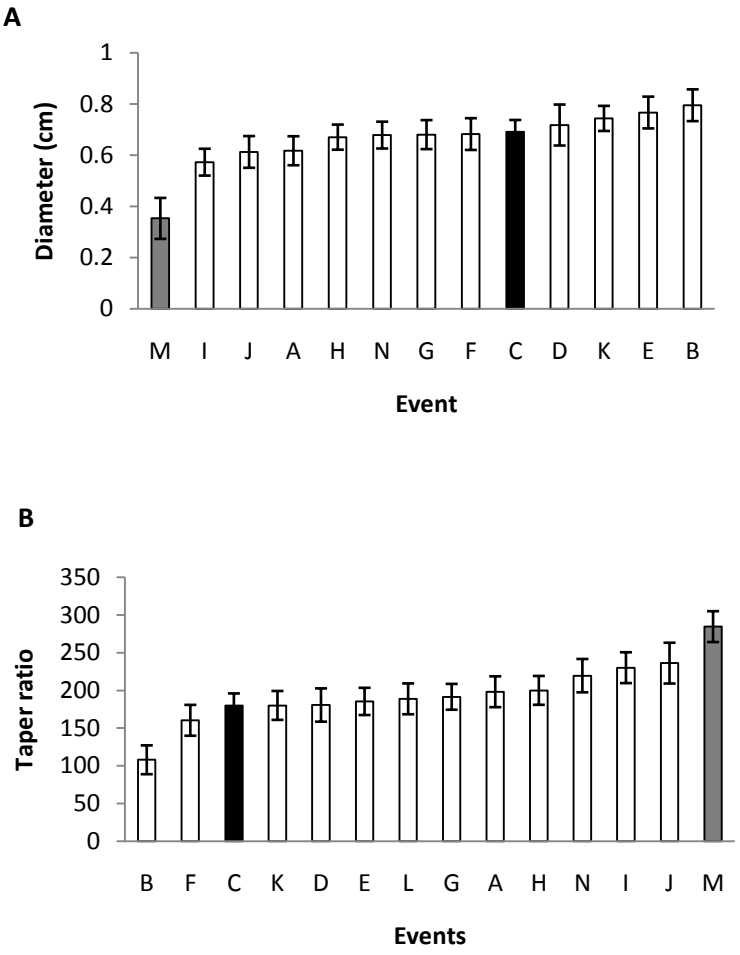


Control
Significant event
Data is represented as least square mean ± standard error

<u>Figure 3.22.</u> Comparisons among events in salinity tolerance construct #19.

A) Collar diameter

B) Taper ratio



Control

Significant event

Data is represented as least square mean ± standard error

Figure 3.23. **PC1 vs PC2 for salinity tolerance construct #19.**

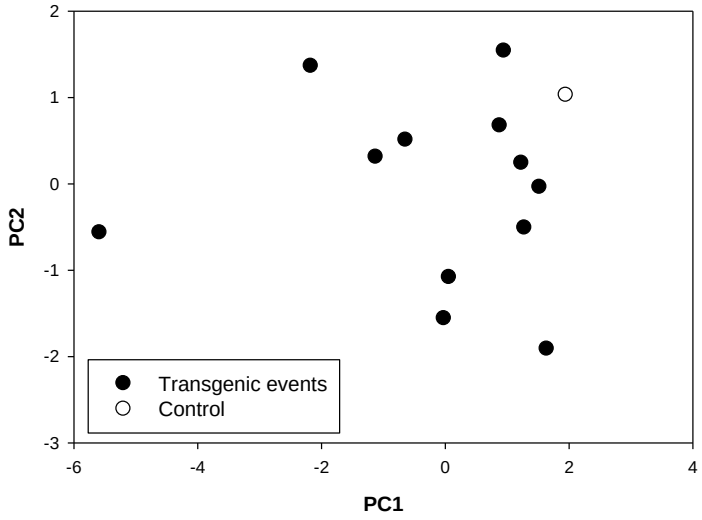


Figure 3.24. **Shade tolerance constructs under shade nets in greenhouse.**

Figure 3.25. **Variation in height among events for shade construct #11 (untransformed data).**

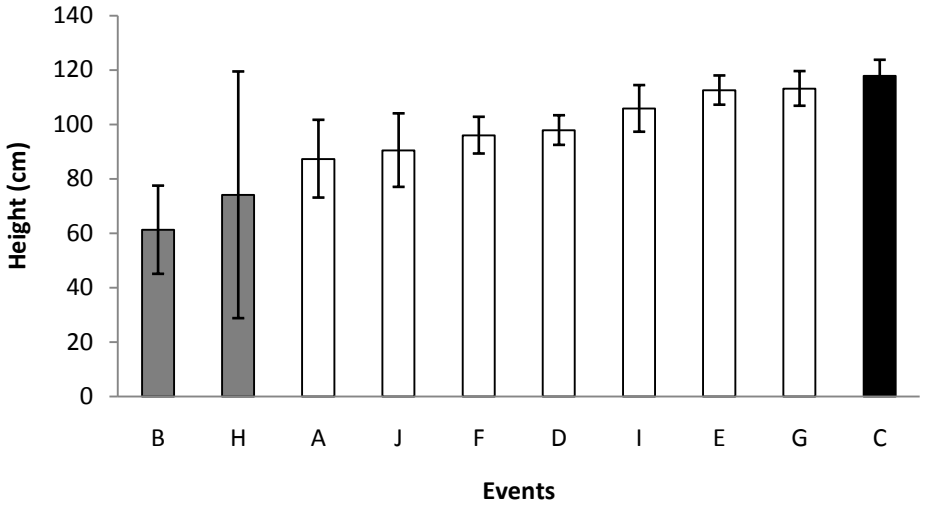


Figure 3.26. **Variation in RGR for shade tolerance construct #11.**

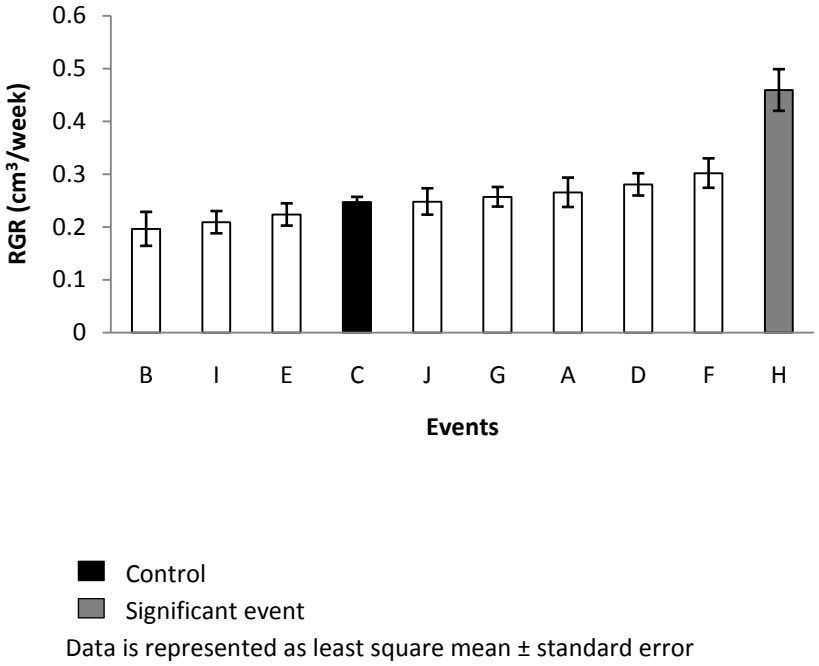
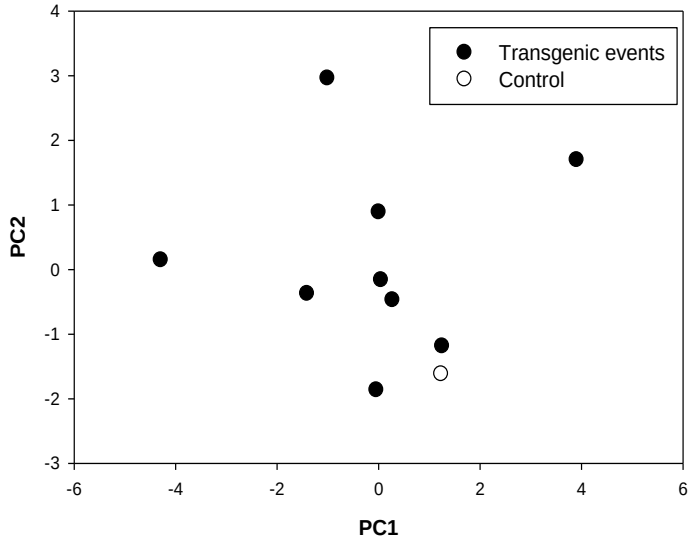
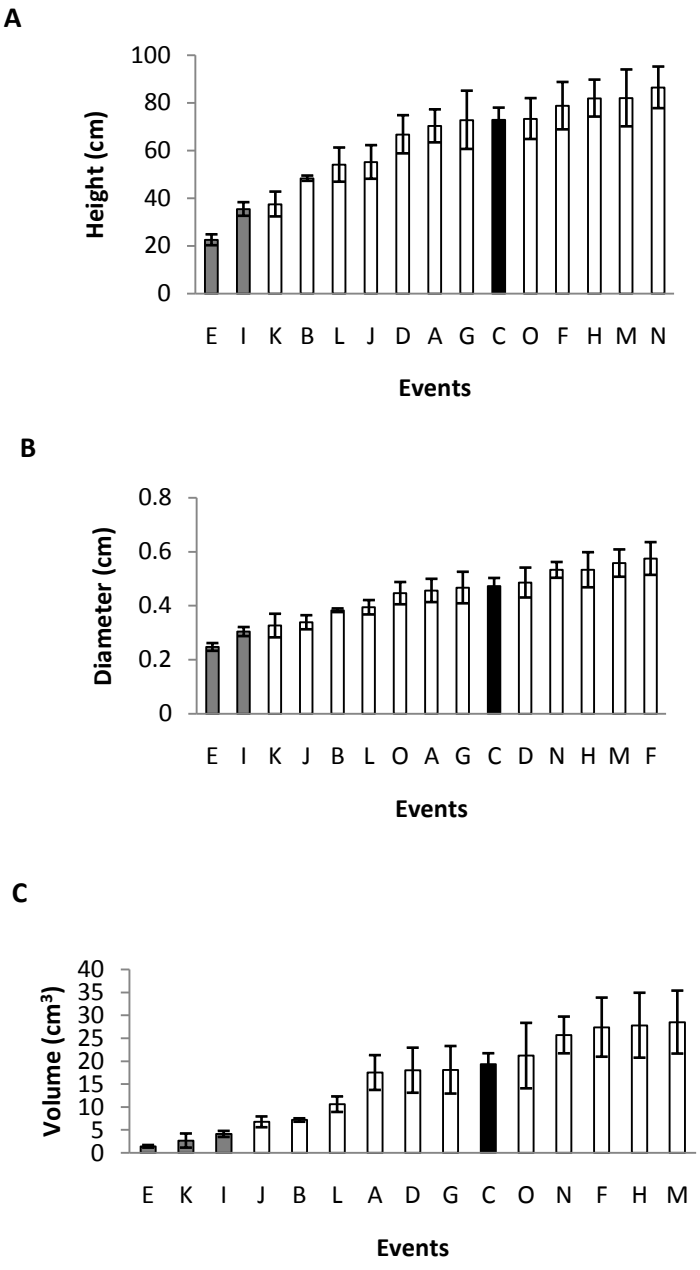


Figure 3.27. **PC1 vs PC2 for shade tolerance construct #11.**



<u>Figure 3.28.</u> Growth comparison among events for shade tolerance construct #12.

A) Height
B) Diameter
C) Volume



Data is represented as least square mean ± standard error

Figure 3.29. **Variation in RGR for shade tolerance construct #12.**

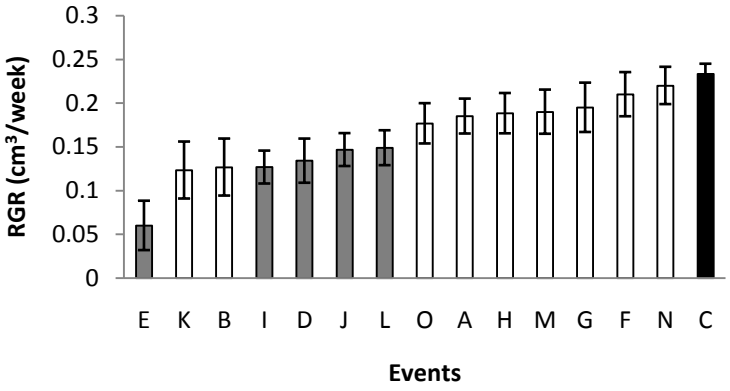
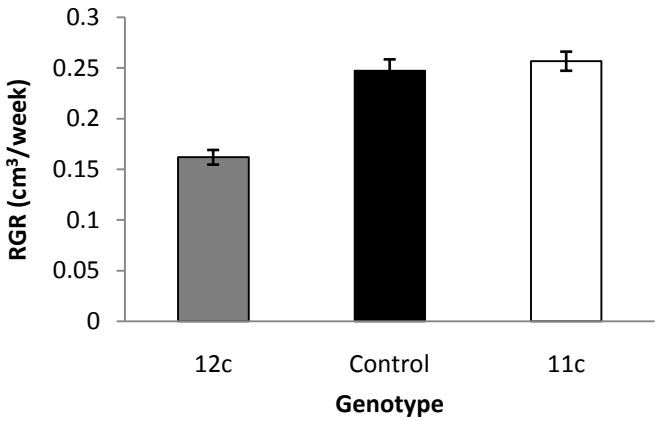


Figure 3.30. **Variation in RGR among shade constructs.**



Data is represented as least square mean ± standard error

Figure 3.31. **Leaf curling in shade tolerace construct #12.**

Figure 3.32. **Variation in mean single leaf area for shade tolerance construct #12.**

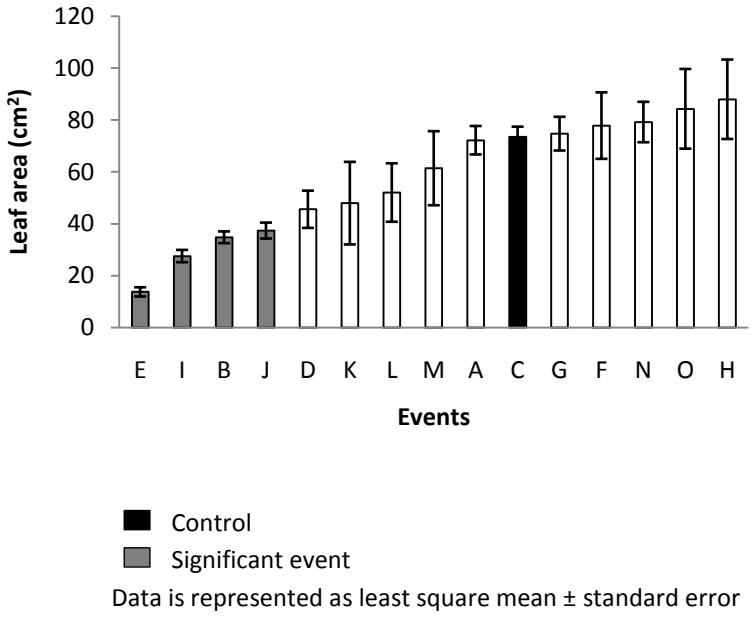


Figure 3.33. PC1 vs PC2 for shade tolerance construct #12.

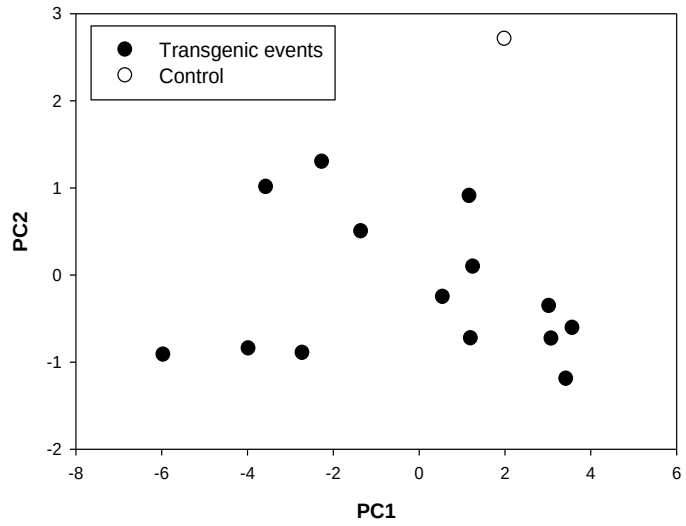
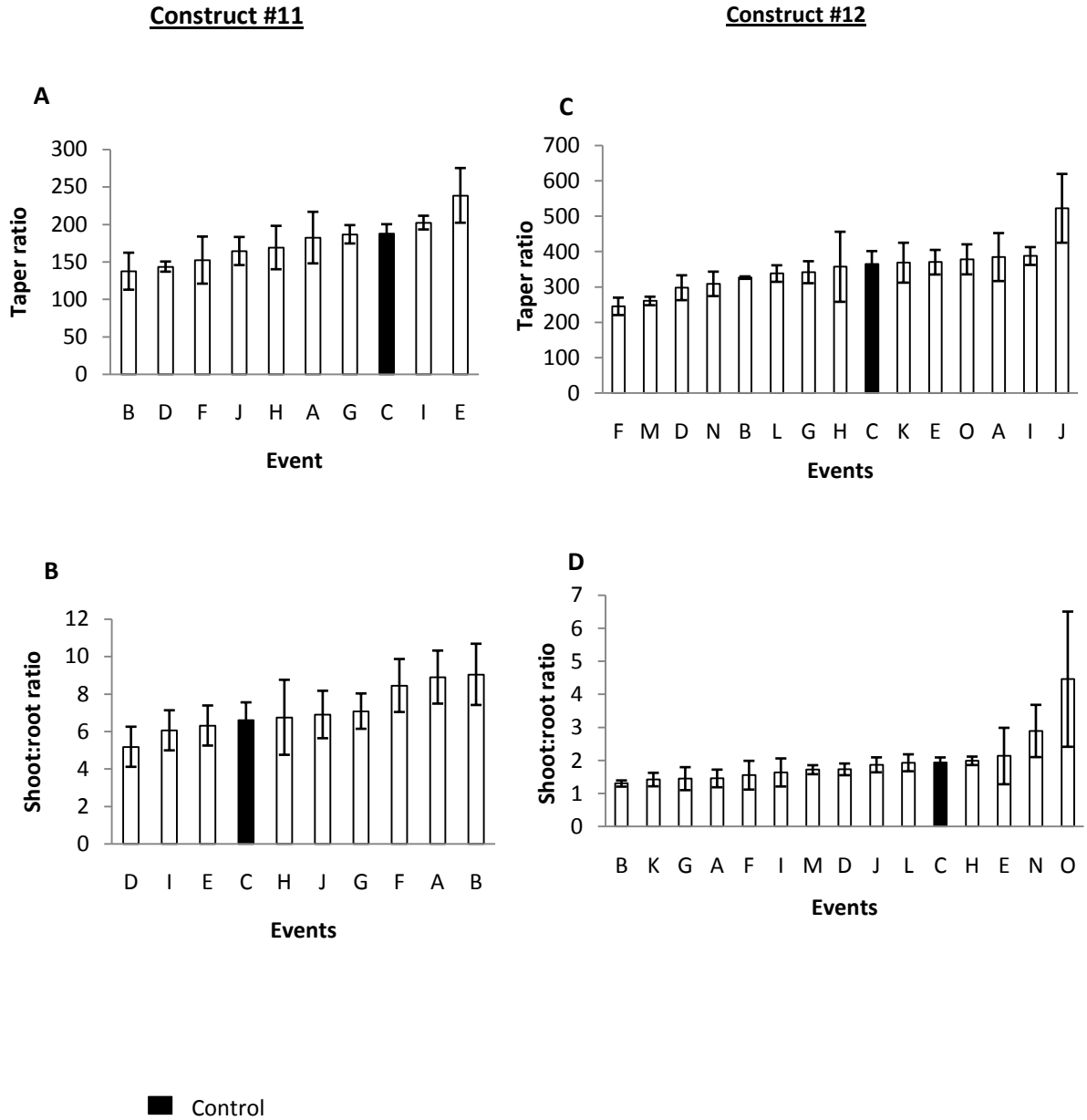


Figure 3.34. Comparison of taper ratio and shoot:root ratio for shade constructs.

A and C) Taper ratio
B and D) Shoot:root ratio



Data is represented as least square mean ± standard error

Figure 3.35. **Effective fertilization rate on nutrition efficiency construct #16.**

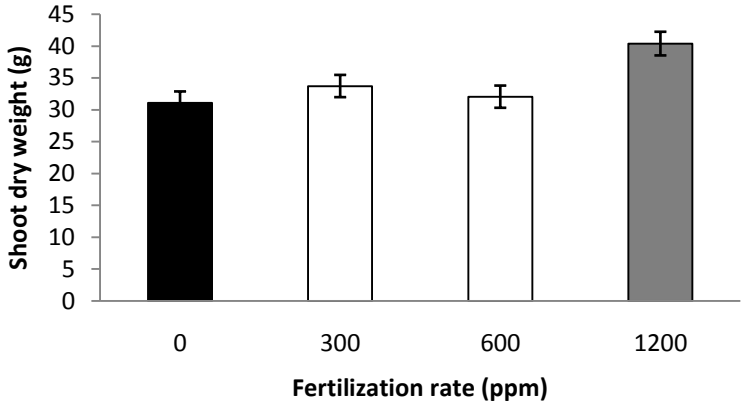


Figure 3.36. **Growth comparison among events for nutrition efficiency construct #16.**

A) Diameter
B) Volume

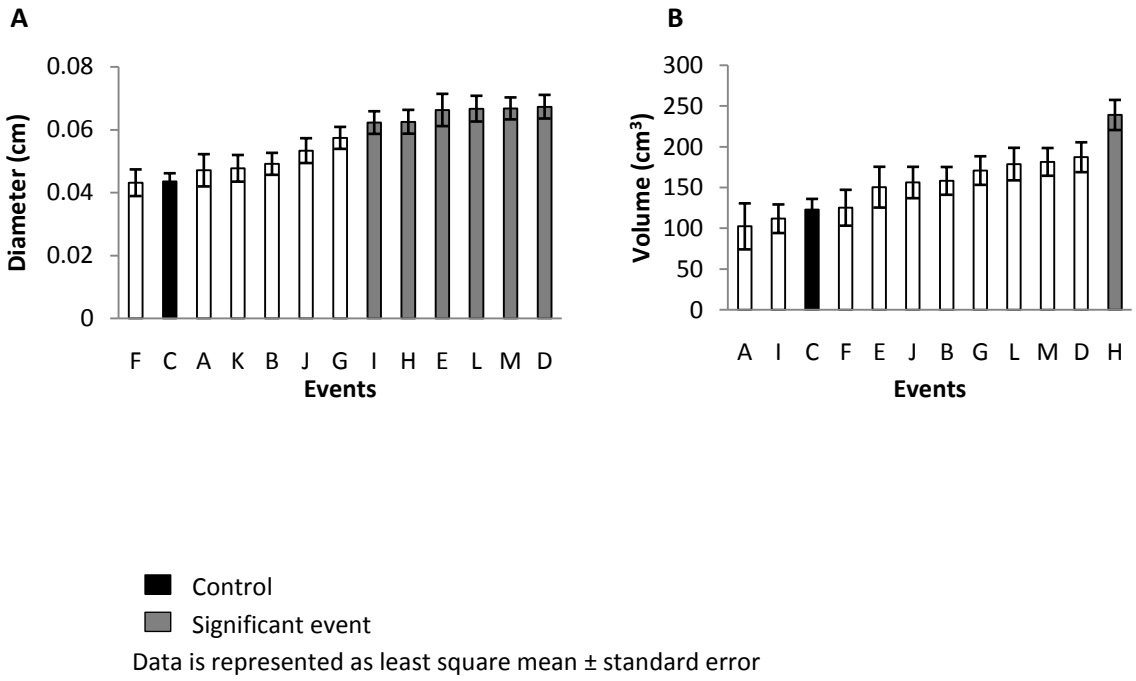


Figure 3.37. **Variation of shoot dry weight among events for nutrition efficiency construct #16.**

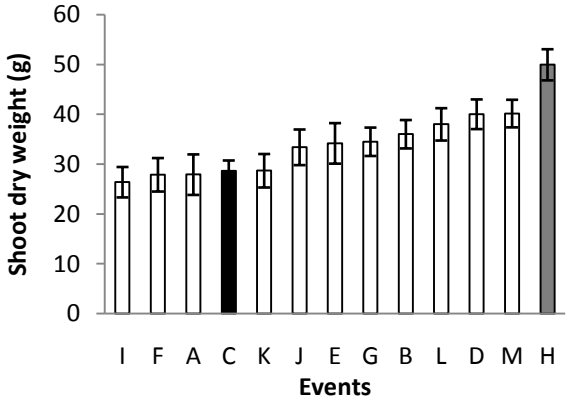
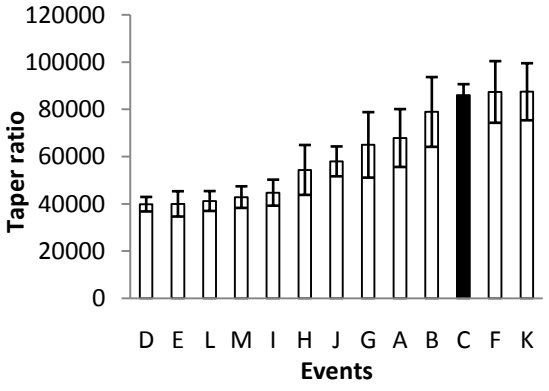


Figure 3.38. **Variation of taper ratio among events for nutrition efficiency construct #16.**



Control

Significant event

Data is represented as least square mean ± standard error

Figure 3.39. Variation in RGR for nutrition efficiency construct #16.

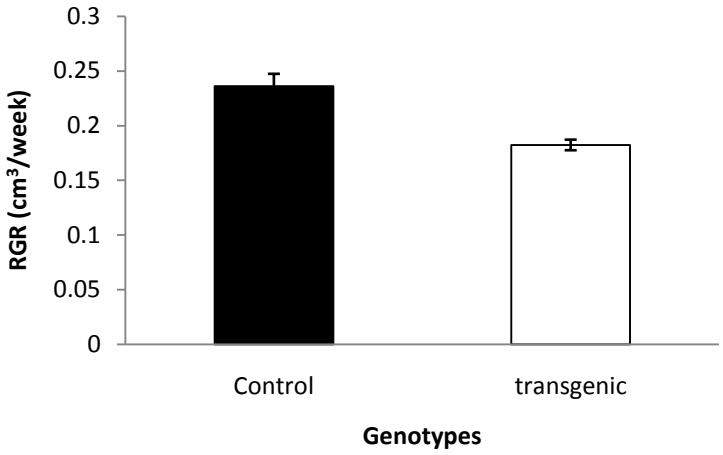
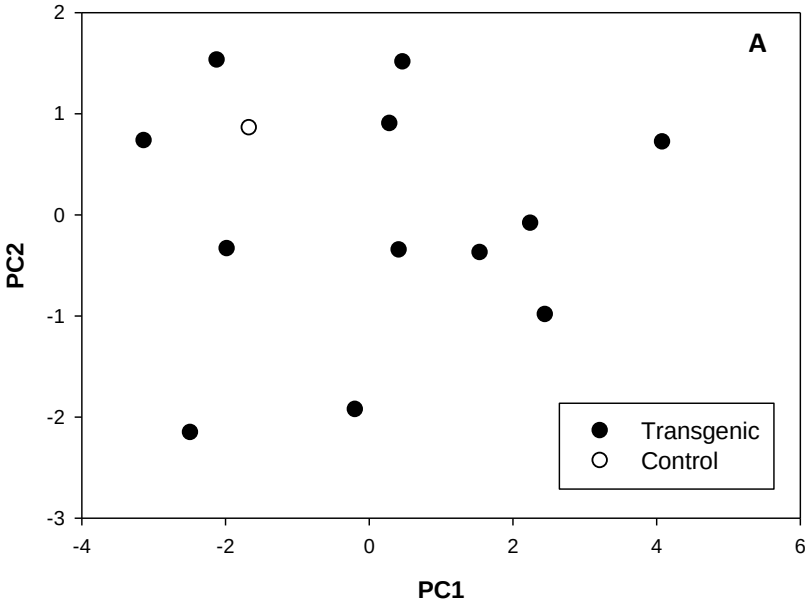
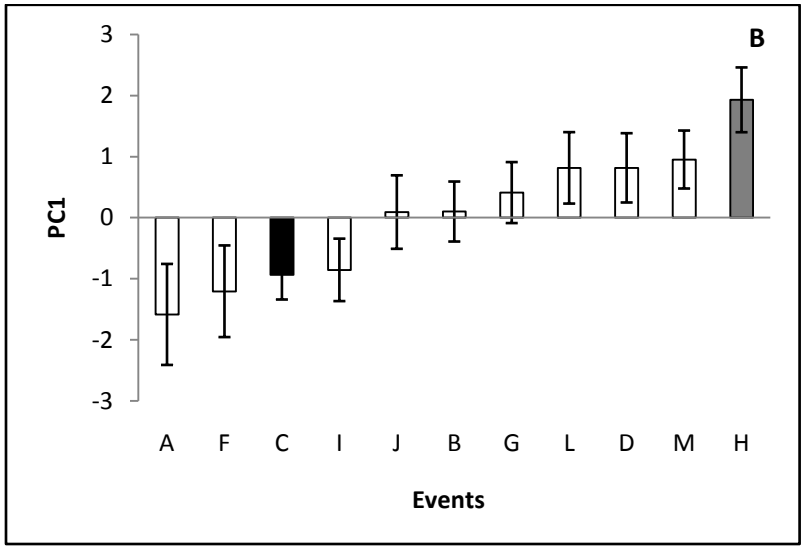


Figure 3.40A. **PC1 vs PC2 for nutrition efficiency construct #16.**

Figure 3.40B. **Variation in PC1 for construct #16.**





Control

Significant event

Data is represented as least square mean ± standard error

CHAPTER 4: EVALUATION OF GENES FOR MODIFYING LIGNIN ACCUMULATION

ABSTRACT

We evaluated the effect of two genes derived from *Arabidopsis* on lignin accumulation in transgenic poplar. We transformed these genes, driven by the constitutive cauliflower mosaic virus 35S promoter, using *Agrobacterium* as a gene transfer agent. Transformed poplar seedlings were evaluated in randomized greenhouse trials. The transgenic poplars showed changes in lignin concentration based on phloroglucinol staining of stems and roots, and one construct caused an increased rate of plant biomass growth.

INTRODUCTION

Lignin is a major component of the cell walls of plants, and accounts for 15-36% of the dry weight of wood (Whetten and Sederoff 1995). The biosynthesis of lignin is a key adaptation for plant mechanical support, water transport, and provides defense against pests and pathogens (review in Chapter 2). Changes in lignin concentration can also affect carbon sequestration in planted forests; lignin accounts for approximately 30% of the annual terrestrial organic carbon fixed by the biosphere (Battle et al. 2000).

Lignin has been recognized for its negative impact on forage quality, paper manufacturing, and cellulosic ethanol production. Thus, there have been many efforts to genetically engineer changes in lignin content in diverse crops (Li et al. 2008). In this study, we investigated the effect of two genes (construct #41 – *At* ARR (*Arabidopsis* response regulator) protein and construct #44 – *At* DEAD/DEAH box gene, DNA/RNA helicase) selected from *Arabidopsis* for their effect on lignin content of the stems and roots of young poplars. Our results suggest that both of these genes may provide useful tools for modulating lignin content in poplar and other tree species.

MATERIALS AND METHODS

Transformation, sub-propagation, and plant measurements

We transformed binary vectors into 717-IB4 poplar hybrids via *Agrobacterium*, and the resulting transgenic plants were sub-propagated and eventually transferred to the greenhouse for screening. The source of transgenic plants, method of transformation, controls, and propagation were the same as described in Chapter 3.

We measured shoot height, collar diameter, mean single leaf area, root length, and dry weights (shoot, root, and leaf), after four weeks of growth in the greenhouse. We calculated volume index, taper ratio, SR ratio, and RGR as previously described (Chapter 3). The number of events for each construct, screening period, and average temperature inside the greenhouse are given in Table A14. We used five to eight ramets per transgenic event and nine to ten ramets for controls. The day/night length was same as described in Chapter 3.

Sectioning and staining

On harvesting the transgenic plants from the greenhouse, we separated stem and root sections, then cut a 3.75 cm-long stem section from 7.5 cm above the ground, washed the roots of the plant, and cleaned off soil particles. An approximately 3.75 cm-long section was also collected from the part of the longest root closest to the stem. We fixed and preserved these sections in 5 volume% formalin – 5 volume% acetic acid – 90 volume% ethanol (FAA) solution and stored them at 5 ^{0}C until analysis.

We cut 20 µm transverse sections from these sections with a sliding microtome. After mounting them on a glass slide, we performed the Wiesner reaction (Xu et al. 2006) by pouring 2 to 3 drops of 1% phloroglucinol-ethanol solution on these sections, followed by one drop of 35% HCl. The stained sections were then covered by a cover slip and viewed under a light microscope.

The simplicity of this staining procedure has led to its general use as an indicator of cell wall lignification. The Wiesner reagents react with coniferyl and sinapyl aldehyde units in lignin, producing a purple-red coloration of the cell wall. Photographs of the stained sections were scored later for intensity using the scoring

system shown in Figure 4.1. Scoring was given from one to four based on the intensity of staining in the ascending order, where four stands for the highest lignin staining, and one stands for the lowest. Staining intensity is proportional to the concentration of aldehyde lignin.

Statistical analysis

The events were arranged in a completely randomized design in the greenhouse for each of the construct as described in Chapter 3. We performed statistical analysis using SAS version 9.2. A priori questions were: 1) Are the events (including control) significantly differ in growth and lignin scoring? and 2) Are transgenics as a group significantly differ from control in growth and lignin scoring? The statistical null hypotheses tested were: 1) There is no difference between events (including control), and 2) There is no difference between transgenic groups and control. Growth of the events as well as the lignin scoring was tested for these hypotheses. The Proc Mixed procedure in SAS was used for testing hypothesis one for growth based on a completely randomized design statistical model. On finding a significant F-statistics for events, multiple comparisons was performed using Bonferroni correction, and then transgenics were pooled together and compared to control. We performed a multivariate analysis for the growth variables using Proc Princomp. The eigenvalues for PCA were similar in describing the variables in different dimensions as described in Chapter 3. We used the single construct model for ANOVA (Chapter 3). Fisher's exact test was used for testing hypotheses one and two for lignin scoring.

RESULTS

We screened two constructs, #41 (OSU 41c – *At* ARR protein) and #44 (OSU-44c – *At* DEAD/DEAH box gene, DNA/RNA helicase) (Table 4.1).

For construct #41, both events and transgenic plants as a group were significant sources of variance for several growth traits (Table 4.2). The height, stem dry weight, leaf dry weight, and SR ratio were higher than the control in all of the events (Figures 4.2 through 4.4). For two events, these differences were significant based on the

Bonferroni range test. Root biomass was also significantly higher for the transgenic pool compared to the control (Table 4.1), as was PC1 (62% variance), an indicator of overall growth (Table 4.3, Figure 4.5). PC2 (15% variance), reflective of a vector with an inverse association of volume and dry weight (Table 4.3), was substantially lower for transgenics compared to controls.

RGR showed the inverse pattern, with all of the transgenic events having lower valuesthan the control, and RGR was statistically significant both for events and pooled transgenics (Figure 4.6). Mean single leaf area was also consistently smaller in the transgenics compared to the controls, though this difference was statistically significant only among events and not for transgenic group (Figure 4.7).

We analyzed the lignin scoring for the stem and root cross-sections produced via the Weisner reaction. Transgenic plants stained more darkly in roots for all but one event (Figure 4.8 and 4.9A), and for stems stained more darkly in all events (Figure 4.9B, C). The scores showed that stem sections stained more densely than did root sections, suggesting higher lignin concentration in stems (Figure 4.9A vs. B). The differences in staining intensity of stem sections was also statistically significant in the transgenic pool vs. control based on Fisher's exact test ($P<0.05$, Figure 4.9C).

For construct #44, the transgenic plants were significantly smaller in size at the beginning of the screening period, and this trend continued throughout the greenhouse experiment (Table 4.2). By the end of the screening period, the control and transgenic plants were similar in height (Figure 4.10), but most transgenics had lower stem biomass and reduced total leaf mass, a result that was statistically significant only at the event level, but not at the transgenic pool level (Table 4.2; Figure 4.11). The transgenic events also tended to have a smaller single leaf area, a result that was also significant only at the event level (Figure 4.12, Table 4.2). The smaller sizes of the transgenic events were also reflected in PC1 (55% variance), whose values were larger for the control than for all but two transgenic events (Table 4.3, Figure 4.13).

Weisner stain intensity in plant stems was significantly higher for the transgenic events ($P <0.01$) (Figure 4.14), with all but three events above the control for

construct #44. We also noticed darker staining in stem sections compared to root sections (data not shown), as observed in construct #41.

DISCUSSION

Although only a small number of events were available in these constructs, their effects on lignin content and growth were clear. Of most interest was that although both genes caused increased Weisner staining, in one case plant growth was improved and in the other case it was retarded. The effects of lignin modification are known to have a wide variety of effects in plants, including substantial physiological disturbances when modification is strong. Strong reduction in lignin content has resulted in tobacco and poplar with smaller stems, reduced root biomass, and collapsed xylem structure (Coleman et al. 2008; Pincon et al. 2001). Changes in leaf, root, and stem growth, including longer internodes, have been associated with a reduction in lignin content and increase of cellulose content in poplar (Hu et al. 1999; Hu et al. 1998). Interestingly, other studies using the same kinds of transgenic poplars have shown a lack of substantial alteration of growth rate and biomass accumulation as a result of lignin reduction, showing that lignin modification can have variable effects depending on environment and method of analysis (Hancock et al. 2007; Zhong et al. 2000). In addition to reconfirmation of these results and chemical analysis of lignin composition in future work, it would be of interest to characterize the cellular and physiological effects of these modifications. It would also be of interest to expose these plants to physiological stresses such as low water potentials and bending, preferably under field conditions, to assess the extent of any pleiotropic effects these two distinct kinds of genetic alterations may have caused.

CONCLUSIONS

Construct #41 gave rise to larger plants with an increased rate of biomass accumulation and lignin content, whereas construct #44 gave rise to shorter plants with lower biomass deposition and significantly higher lignin content. These results

show that these genes could be useful tools for modifying rate of carbon accumulation, as well as for understanding how lignin accumulation affects growth.

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Table 4.1. Genes in constructs for lignin modification.

Code ID	Target trait	Protein encoded
OSU-41	Modified lignin	*At* ARR (Arabidopsis response regulator) protein
OSU-44	Modified lignin	*At* DEAD/DEAH box gene, DNA/RNA helicase

Table 4.2. Variables used for ANOVA and their statistical significance for lignin constructs.

	Construct #41		Construct #44	
Variable	**Event**	**Transgenic pool**	**Event**	**Transgenic pool**
Initial height (cm)	-	-	**,#	*
Final height (cm)	**,#	-	**	-
Initial diameter (cm)	-	-	-	*
Final diameter (cm)	-	-	-	-
Initial volume index (cm^3)	-	-	*#	**
Final volume index (cm^3)	*	**	-	-
Root length (cm)	-	-	-	-
Shoot dry weight (g)	**,#	**	**,#	-
Root dry weight (g)	-	*	-	-
Leaf dry weight(g)	**,#	**	**,#	-
Mean single leaf area (cm^2)	*,#	-	**,#	-
PC1	*	**,#	*	-
Taper ratio	-	-	-	-
SR	**,#	**	-	-
RGR	**,#	**	-	-

Symbols explained in table 3.3 and 3.5.

Table 4.3. Eigenvectors (multiplied by 100) for variables on event means.

	Construct #41			Construct #44		
Variables	**PC1**	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**
Final volume index (cm3)	26.4	74.7	56.1	42.9	-29.3	-13.6
Mean single leaf area (cm^2)	41.5	-28.3	-14.8	41.0	-29.2	23.7
Leaf dry weight (g)	33.5	47.0	-73.5	28.7	60.7	8.9
Shoot dry weight (g)	46.5	-20.0	26.6	40.8	40.7	1.6
Root dry weight (g)	47.3	-31.6	19.3	32.1	36.6	-0.8
Root length (cm)	45.4	1.2	-12.3	-8.8	-3.3	87.9
Final height (cm)	-	-	-	39.5	-27.9	27.0
Final diameter (cm)	-	-	-	36.4	-28.7	-26.6
% variance	*61.9*	*15.1*	*11.1*	*55.4*	*23.0*	*12.8*

Figure 4.1. **Lignin scoring for lignin constructs #41 and #44.**

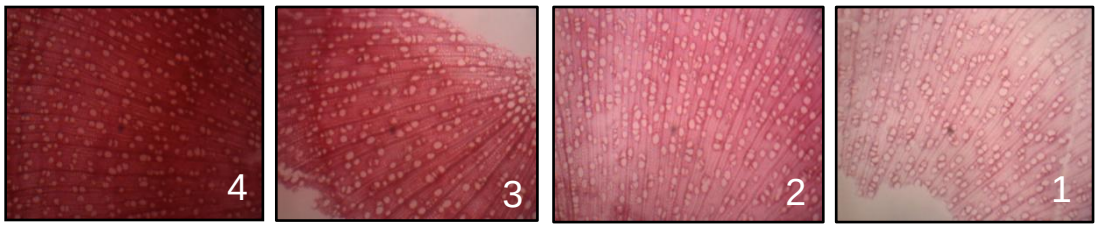
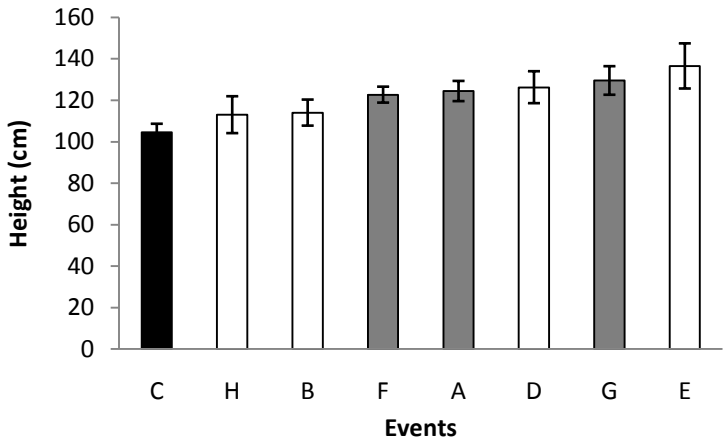


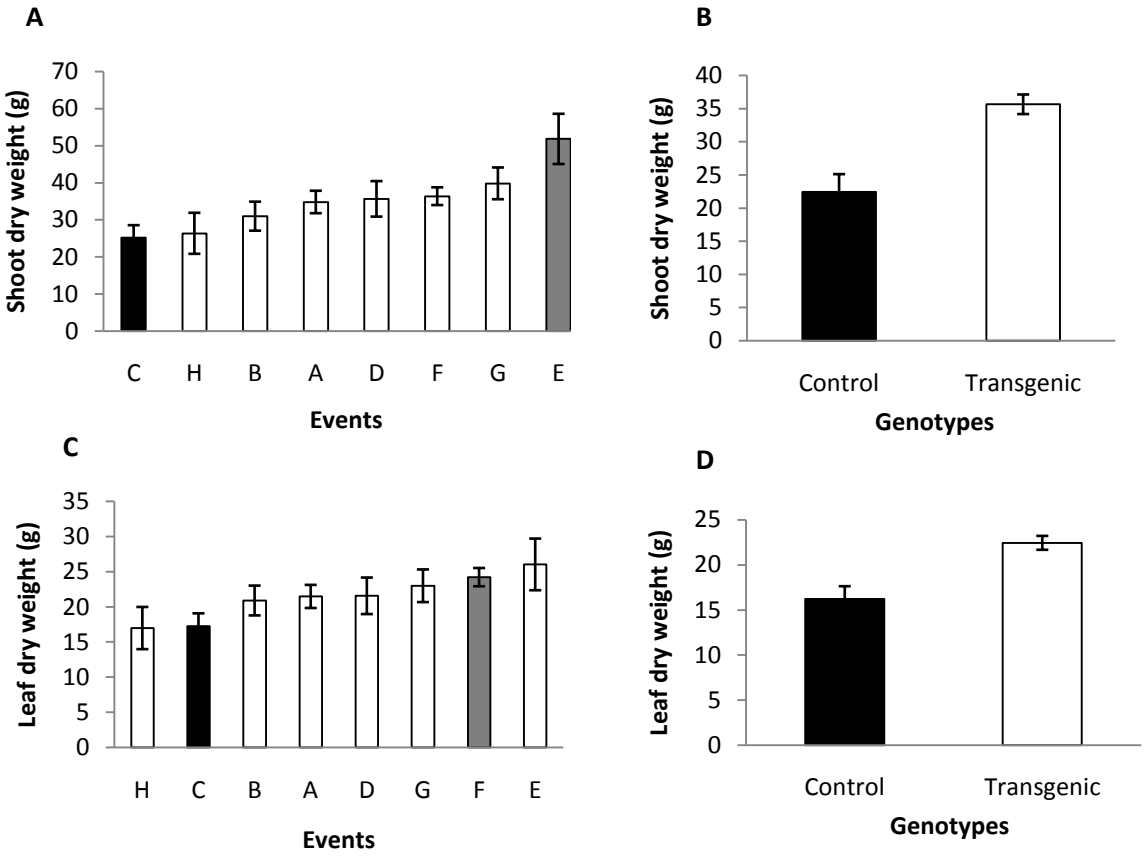
Figure 4.2. **Variation in height among events for lignin construct #41.**



Control
Significant event
Data is represented as least square mean ± standard error

Figure 4.3. **Growth comparison among events in lignin construct #41.**

A and B) Shoot dry weight
C and D) Leaf dry weight



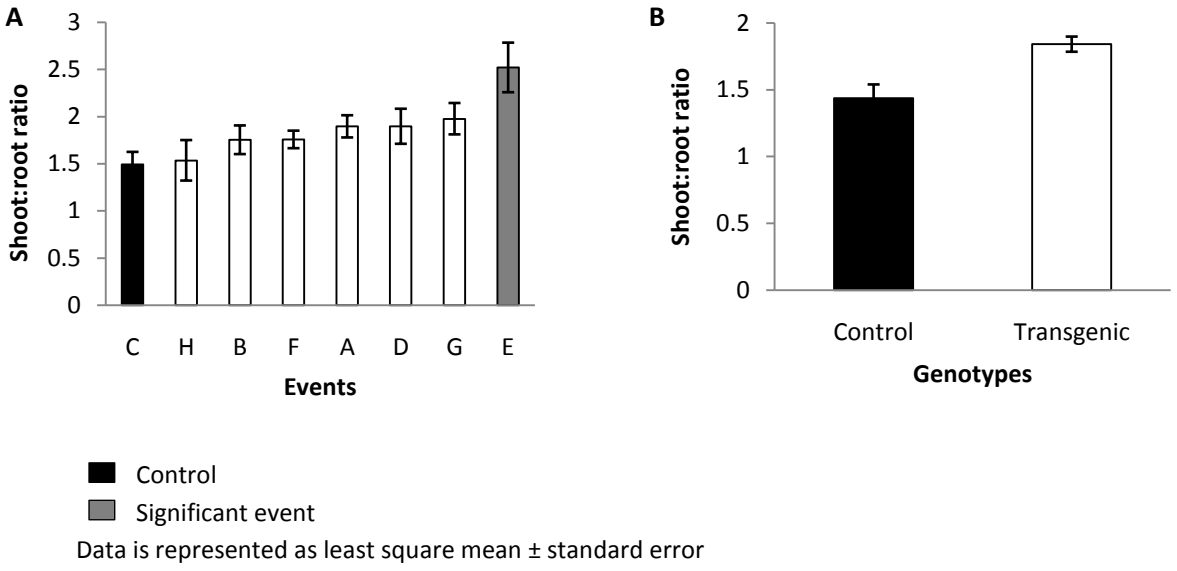
Control
Significant event

Data is represented as least square mean ± standard error

Figure 4.4. **Variation in shoot:root dry weight ratio for lignin construct #41.**

A) Events

B) Genotypes



Control

Significant event

Data is represented as least square mean ± standard error

Figure 4.5. **PC1 vs PC2 for lignin construct #41.**

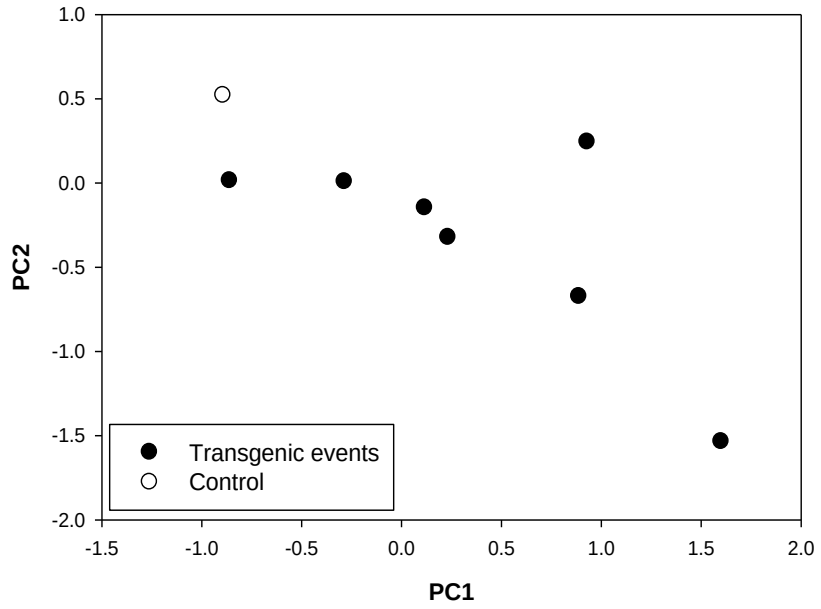


Figure 4.6. Variation in RGR for lignin construct #41.

A) Events

B) Genotypes

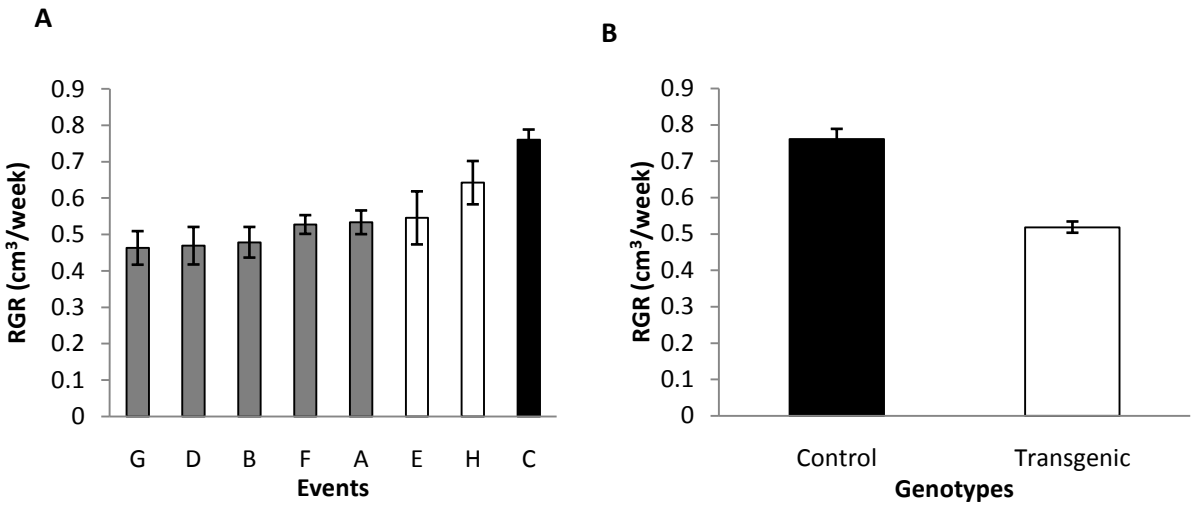
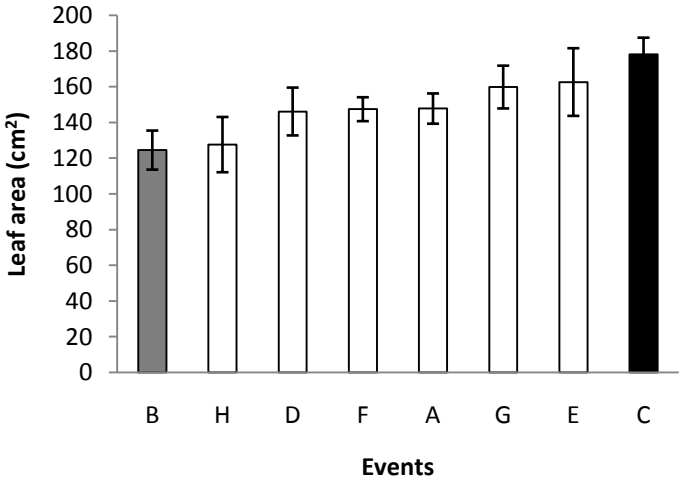


Figure 4.7. Variation in mean single leaf area for lignin construct #41.



Control

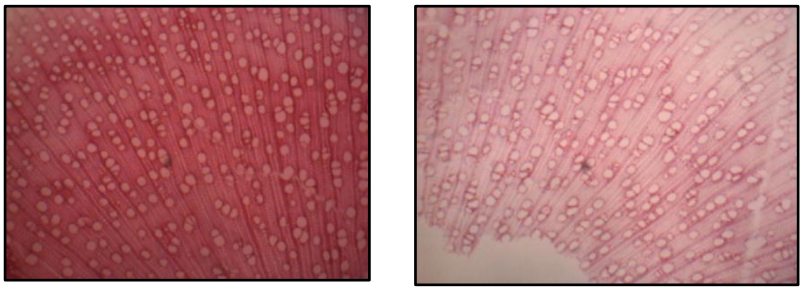
Significant event

Data is represented as least square mean ± standard error

<u>Figure 4.8.</u> Variation in lignin staining.

A) Controls
B) Transgenics

A



B

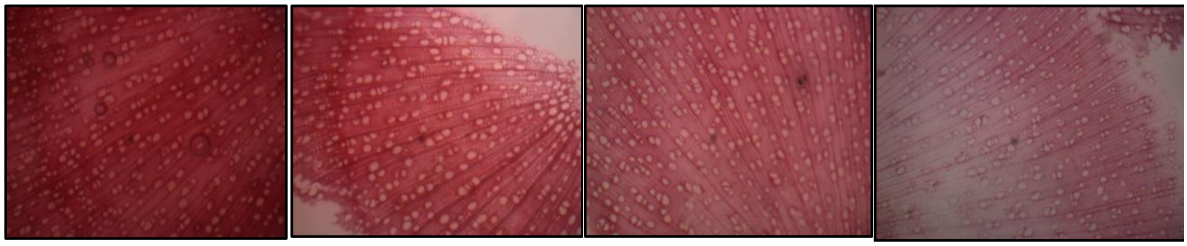
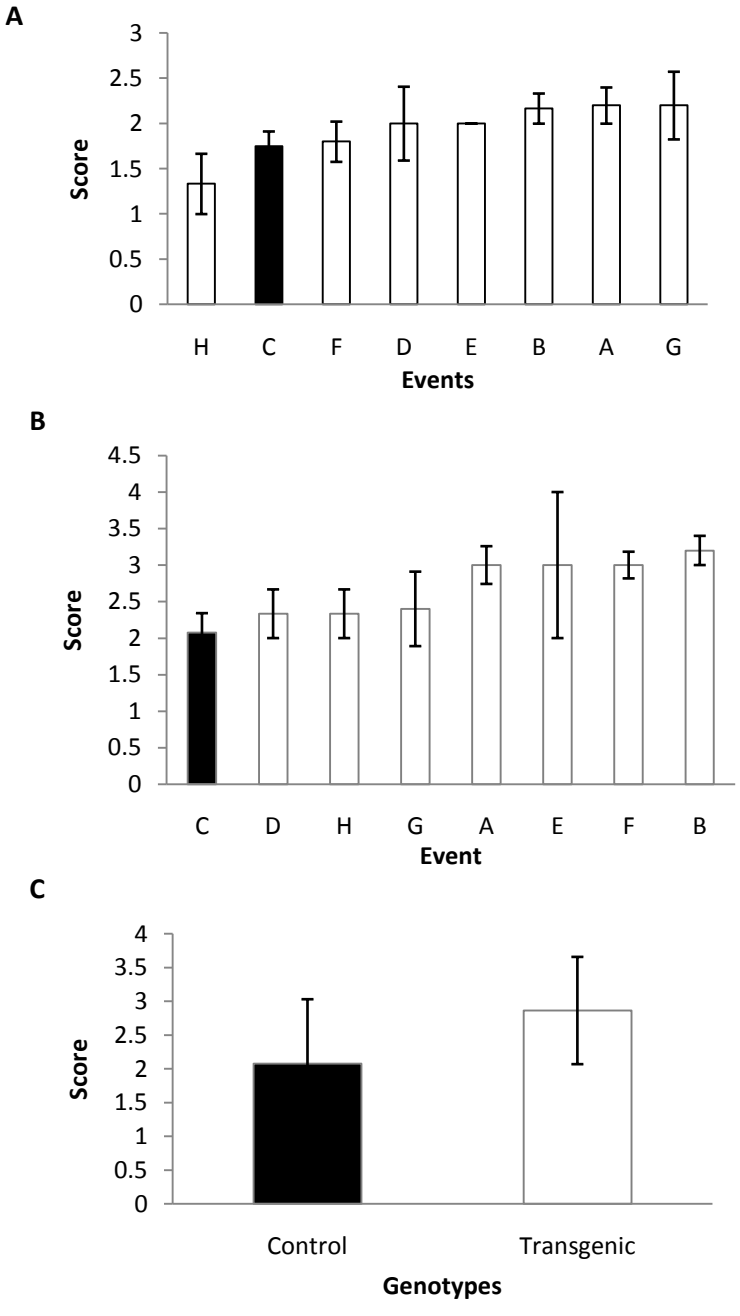


Figure 4.9. Variation in lignin score for lignin construct #41.

A) Root
B) Stem
C) Stem in genotypes



Data is represented as least square mean ± standard error

Figure 4.10. **Variation in height among events for lignin construct #44.**

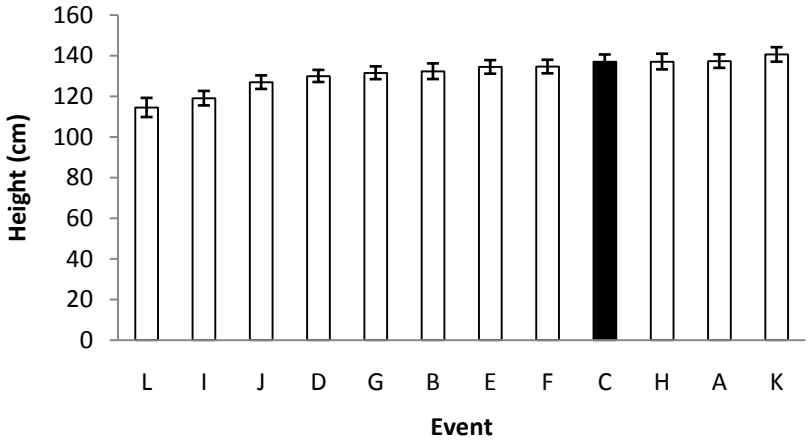
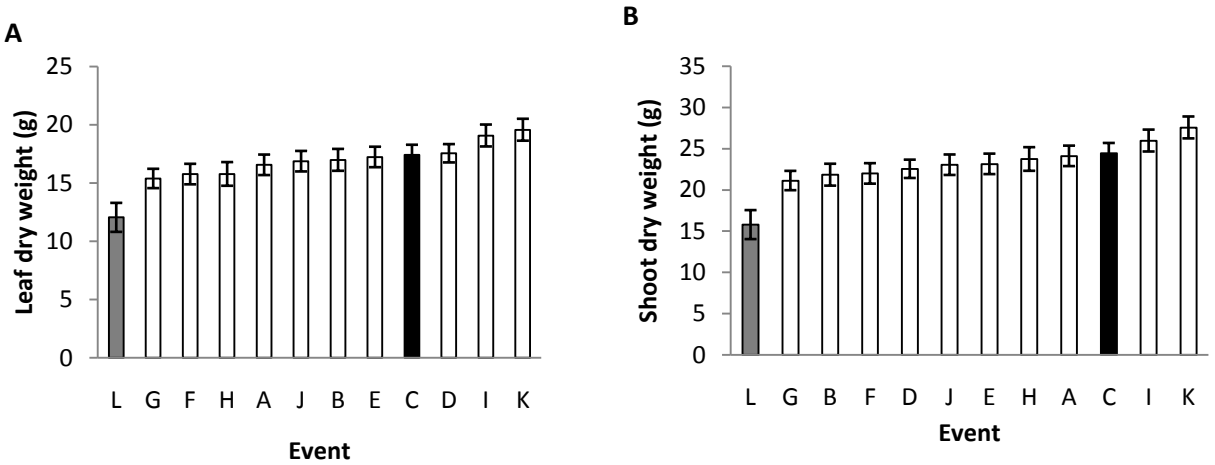


Figure 4.11. **Growth comparison among events for lignin construct #44.**

A) Leaf dry weight

B) Shoot dry weight

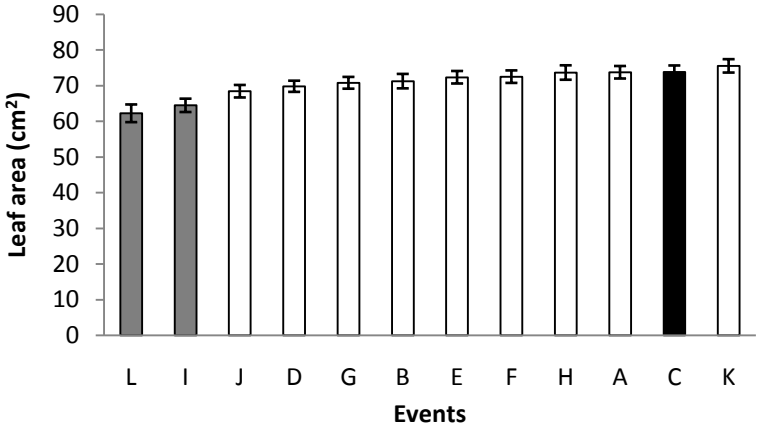


Control

Significant event

Data is represented as least square mean ± standard error

Figure 4.12. Variation in mean single leaf area for lignin construct #44.



Control
Significant event
Data is represented as least square mean ± standard error

Figure 4.13. PC1 vs PC2 for lignin construct #44.

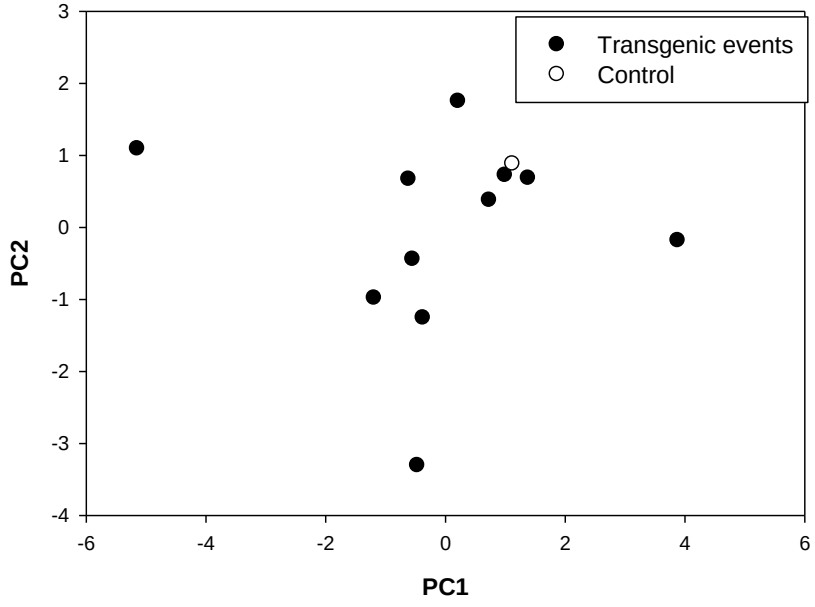
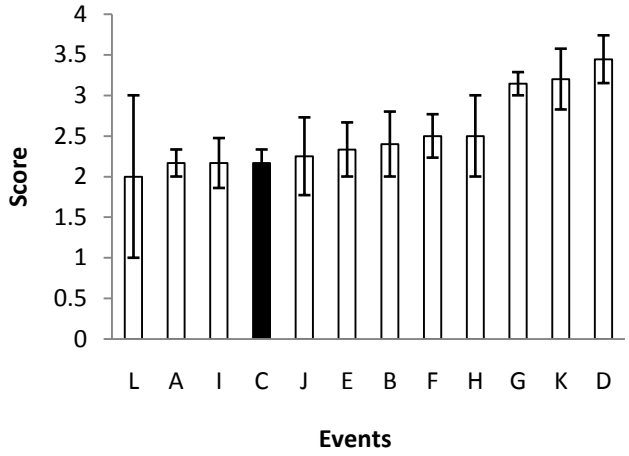


Figure 4.14. Variation in lignin scoring in stem for lignin construct #44.



Control

Data is represented as least square mean ± standard error

CHAPTER 5: EVALUATION OF GENES FOR MODIFYING GROWTH RATE

ABSTRACT

We tested five gene constructs for their influence on growth rate in poplar. The genes were selected from *Arabidopsis* and *Triticum,* placed under the control of the strong 326 promoter, then inserted into poplar using *Agrobacterium*-mediated transformation. Regenerated plants were propagated and studied in a randomized greenhouse trial, where we measured final size, stem and root growth, and for one gene looked for evidence of modified (precocious) flowering. The three constructs designed for stature modification had no significant effect on poplar growth or morphology. Two other constructs, construct #7 (*Triticum aestivum* bHLH family protein) and construct #10 (*Arabidopsis thaliana* expressed protein) significantly affected the growth rate and biomass accumulation.

INTRODUCTION

Genetic improvement to increase growth rate is an important goal for nearly all forest tree breeding and biotechnology programs (Tzfira et al. 1999). Rapid growth can also enhance carbon sequestration benefits from planted forests, which can take the form of enhanced soil and above ground carbon, or marketed products such as lumber. Genetic engineering approaches, for example by over-expressing genes involved in the biosynthesis of growth hormones (Eriksson et al. 2000), might be able to complement or accelerate traditional breeding for improved growth. However, very few genes have been evaluated for growth benefits in trees to date. In the current study we report on the effects of five genes that modified growth or stature in model organisms, but have not been previously studied in any tree species. We report that some of these genes did lead to modified growth, and thus may provide useful tools for tree biotechnology.

MATERIALS AND METHODS

Transformation, sub-propagation, and plant measurements

The sources of the plant material, method of genetic transformation, control genotypes, and steps in propagation, acclimatization, and transfer to the greenhouse were conducted as described in Chapter 3. The number of events used for each construct, the screening period, and average temperature inside the greenhouse were summarized in Table A14. We used four to ten ramets per transgenic event and 5 to 20 ramets for control. The measured growth variables were shoot height, collar diameter, mean single leaf area, root length, and dry weights (shoot, root, and leaves). The calculated variables included volume index, taper ratio, SR ratio, and RGR, as explained in Chapter 3.

Statistical analysis

We used a completely randomized design statistical linear model for each construct in the greenhouse. The priori questions were 1) Are the events (including control) significantly differ in growth? and 2) Are transgenics as a group significantly differ from control in growth? The statistical null hypotheses tested were 1) There is no difference between events (including control), and 2) There is no difference between transgenic groups and control. We used Proc Mixed for testing hypothesis one for growth. On finding a significant F-statistic for events, multiple comparisons was performed using the Bonferroni correction to determine the specific events whose means were significantly different from that of control, and then transgenics were pooled together and compared to the control using a t-test. We performed a multivariate analysis for the growth variables using Proc Princomp. The rationale and calucation of eigenvalues for PCA were as described in Chapter 3. We used the single construct model for ANOVA, and PCA for multivariate analysis (Chapter 3).

RESULTS

Stature modifying constructs

We evaluated three constructs – #4, #5, and #6 (OSU -4, OSU-5, and OSU-6) – that were hypothesized to modify plant stature (final size) (Table 5.1). The transgenic events were a significant source of variance for growth measurements only for #4, where they also had a significant effect on taper ratio and RGR (Table 5.2). Contrary to our expectations (Table 5.1), most of the transgenic plants had a higher taper than did the controls (Figure 5.1), though not significantly so for the transgenics as a group. Their PCs suggested that overall mass was largely unchanged by the gene (PC1, 65% variance, $P>0.05$), but that, parallel to the results for taper ratio, transgenics tended to have shorter roots, and greater height and shoot mass (PC2, 16% variance; Table 5.7; Figure 5.2). For constructs #5 and #6, the transgenic plants as a pool generally did not differ from controls for growth rate traits, with the exception of root dry weight and taper ratio for #5. The transgenics tended to have lesser root mass and lower taper, though the effects were small and no individual events stood out (Figure 5.3).

Growth rate modifying construct

There were statistically significant differences among events and transgenic pools for a number of growth traits (Table 5.4), and all of the events were taller and had higher RGR than controls (Figure 5.4). Two of the 10 transgenic event means exceeded control means for several traits based on the Bonferroni test (shoot height, diameter, and volume). However, the construct had highly variable effects on taper ratio (Figure 5.5) and root mass, and produced statistically significant changes in both directions for root mass (Figure 5.6). PC1 (74% variance) suggested little overall effect of the gene on total plant mass or volume (Table 5.5, Figure 5.7), but PC2 (14% variance) suggested an independent dimension where the transgenes tended to impart shorter roots relative to diameter.

Flowering modifying construct

We transformed construct #10 (OSU-10), a gene that had acted to delay or completely repress flowering in *Arabidopsis* (Table 5.1). Transgenic events were a significant source of variance for a number of traits, but the transgenic pool mean had no significant difference despite an increase in mean single leaf area (Table 5.6, Figure 5.8). The significant difference among events in root mass were obvious after harvest (Figure 5.9), where the majority of transgenic events had higher root mass than controls, despite showing no consistent effect on shoot dry weight (Figure 5.10). PC1 (62% variance) supported the lack of a consistent difference in plant mass (Table 5.7, Figure 5.11). However, PC2 (16% variance) suggested that transgenics had greater root lengths in relation to other plant attributes. The gene had no apparent effect on reproductive development; like most other poplars at this age, no floral structures were formed.

DISCUSSION

The transgenes tested had clear effects on plant morphology, though they were complex and generally not what was predicted based on prior studies. Some of the effects may have been a consequence of transformation itself, though we do not think this is likely given that the non-transgenic controls and transgenic empty-vector controls never showed a difference for any trait, and somaclonal variation has rarely been observed in our laboratory in poplar (Meilan et al. 2001; Strauss et al. 2004). Construct #7, which appears effective in increasing RGR, shoot height, and root dry weight, appears worthy of further study. The results from the other constructs were either not statistically significant, or their effects were so unpredictable in direction that they do not appear worthwhile as tools to obtain specific kinds of genetic modifications.

CONCLUSIONS

Five constructs were screened in the greenhouse for growth modification traits. The three constructs designed for stature modification had no significant effect on poplar

growth or morphology. Two other constructs, #7 (*Triticum aestivum* bHLH family protein) and #10 (*Arabidopsis thaliana* expressed protein) significantly affected growth rate and biomass accumulation . These constructs warrant further study as tools for modifying carbon uptake and for analysis of natural controls on growth rate.

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Strauss, S.H., A.M. Brunner, V. Busov, C. Ma and R. Meilan 2004. Ten lessons from 15 years of transgenic *Populus* research. Forestry. 77:455-465.

Tzfira, T., V. Alexander and A. Altman 1999. *rol*-gene expression in transgenic aspen (*Populus tremula*) plants results in accelerated growth and improved stem production index. Trees. 14:49-54.

Table 5.1. Genes in constructs for abiotic interactions.

Code ID	Target trait	Protein encoded
OSU-4	Reduced stature and branch angle, increased branching	At Ethylene binding, AP2 domain protein
OSU-5	Increased stature and reduced branching	At Glycine-rich protein
OSU-6	Reduced stature, decreased branch angle	At p450(CYP71A12)
OSU-7	Higher growth rate	Triticum aestivum bHLH family protein
OSU-10	Delayed or no flower	At expressed protein

Table 5.2. Variables used for ANOVA and their statistical significance for stature constructs.

	Construct #4		Construct #5		Construct #6	
Variable	**Event**	**Transgenic pool**	**Event**	**Transgenic pool**	**Event**	**Transgenic pool**
Initial height (cm)	*	-	-	-	-	-
Final height (cm)- SQ6	*	-	-	-	-	-
Initial diameter (cm)	**	-	-	-	-	-
Final diameter (cm)	**	-	-	-	-	-
Initial volume index (cm^3)-Ŧ,ŧ	**	-	-	-	-	-
Final volume index (cm^3)- ŧ	**	-	-	-	-	-
Root length (cm)- ŧ	-	-	-	-	-	-
Shoot dry weight(g)	-	-	-	-	-	-
Root dry weight (g)- SQ4, ŧ	-	-	-	*	-	-
Leaf dry weight (g)	-	-	-	-	-	-
Mean single leaf area (cm^2)	NT	NT	-	-	*	-
PC1	-	-				
Taper ratio	**	-	-	**	-	-
SR- Ŧ,Ţ	-	-	-	-	-	-
RGR	*	-	-	-	-	-

Ŧ = log transformed for construct #4; ŧ = log transformed for construct #6; Ţ= log transformed for construct #5; SQ4 = square root transformed for construct #4; SQ6 = square root transformed for construct #6;

Other symbols explained in table 3.3 and 3.5.

Table 5.3. Eigenvectors (multiplied by 100) for variables on event means for reduced stature construct #4.

Variables	PC1	PC2
Final height (cm)	36.7	21.5
Final diameter (cm)	39.6	14.6
Final volume index (cm^3)	42.4	13.1
Root length (cm)	-1.0	70.7
Shoot dry weight (g)	42.8	-5.6
Root dry weight (g)	41.4	-1.8
Leaf dry weight (g)	40.7	-23.9
% variance	*64.9*	*16.2*

Table 5.4. Variables used for ANOVA and their statistical significance for higher growth rate construct #7.

Variable	Event	Transgenic pool
Initial height (cm)	*	**
Final height (cm)	**,#	-
Initial diameter (cm)	*	-
Final diameter (cm)	**,#	*
Initial volume index (cm3)	**,#	*
Final volume index (cm3)	**,#	*
Root length (cm)	-	-
Shoot dry weight (g)	**	-
Root dry weight (g)	**,#	-
Leaf dry weight (g)	**	-
Mean single leaf area (cm^2)	*	*
PC1	*	-
Taper ratio - ₸	**,#	-
SR	-	-
RGR	**,#	**

Symbols explained in table 3.3 and 3.5.

Table 5.5. **Eigenvectors (multiplied by 100) for variables on event means for higher growth rate construct #7.**

Variables	PC1	PC2
Final height (cm)	39.7	3.3
Final diameter (cm)	36.5	-24.7
Final volume index (cm^3)	39.3	-8.2
Root length (cm)	7.0	91.3
Shoot dry weight (g)	39.9	-7.8
Root dry weight (g)	38.4	-1.5
Leaf dry weight (g)	37.9	-3.4
Mean single leaf area (cm^2)	31.7	30.1
% variance	*73.9*	*13.9*

Table 5.6. **Variables used for ANOVA and their statistical significance for flowering construct #10.**

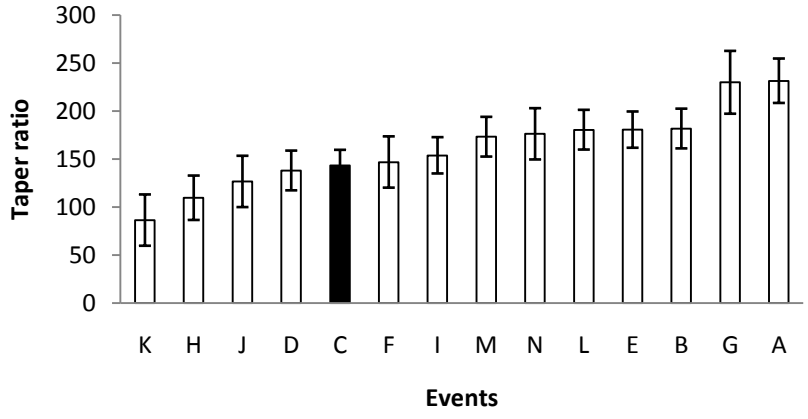
Variable	Event	Transgenic pool
Initial height (cm)	*	-
Final height (cm)	*	-
Initial diameter (cm)	*	-
Final diameter (cm)	-	-
Initial volume index (cm^3)	*	-
Final volume index (cm^3)	-	-
Root length (cm)	*	-
Shoot dry weight (g)	**	-
Root dry weight (g)	**, #	-
Leaf dry weight (g)- ₸	**	-
Mean single leaf area (cm^2)- ₸	-	*
PC1	*	-
Taper ratio	**	-
SR	-	-
RGR	-	-

Symbols explained in table 3.3 and 3.5.

Table 5.7. Eigenvectors (multiplied by 100) for variables on event means for flowering modification construct #10.

Variables	PC1	PC2	PC3
Final height (cm)	39.1	-34.9	-1.7
Final diameter (cm)	38.2	0.8	61.1
Final volume index (cm^3)	44.6	-17.9	34.9
Root length (cm)	11.0	85.6	19.9
Leaf dry weight (g)	35.6	29.8	-50.8
Root dry weight (g)	39.9	-10.1	-44.3
Shoot dry weight (g)	45.2	11.9	-10.3
% variance	*62.0*	*16.1*	*10.8*

Figure 5.1. **Variation in taper ratio for stature construct #4.**



Control

Data is represented as least square mean ± standard error

Figure 5.2. **PC1 vs PC2 for reduced stature construct #4.**

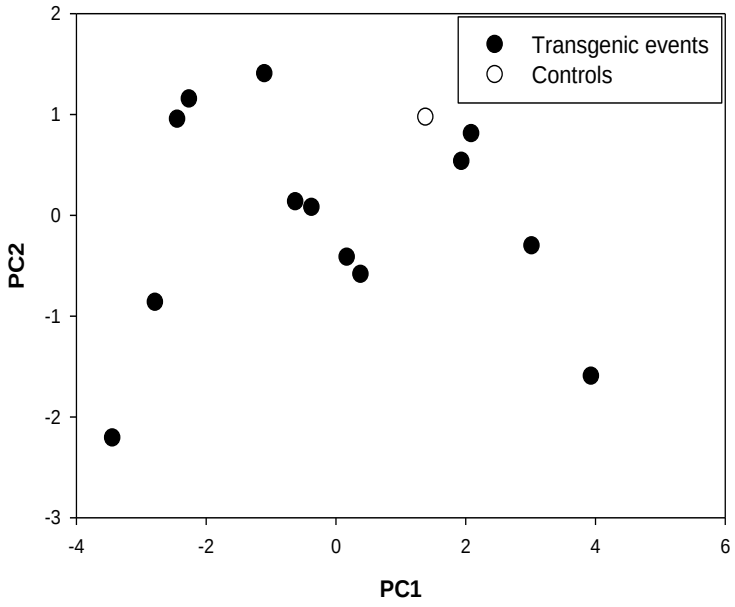
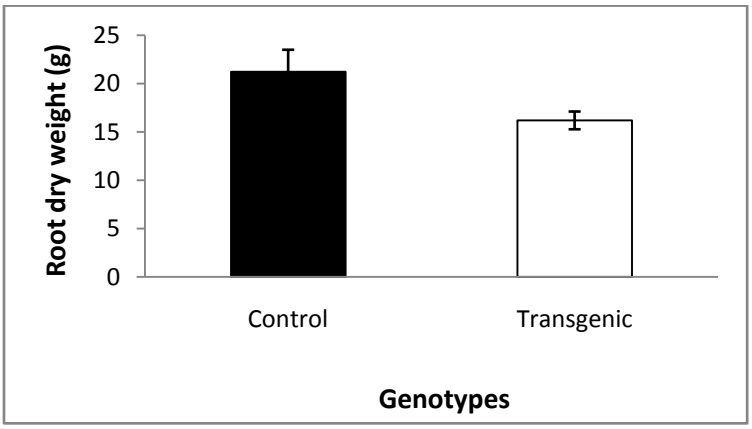


Figure 5.3. Growth comparison for increased stature construct #5.

A) Root dry weight
B) Taper ratio

A



B

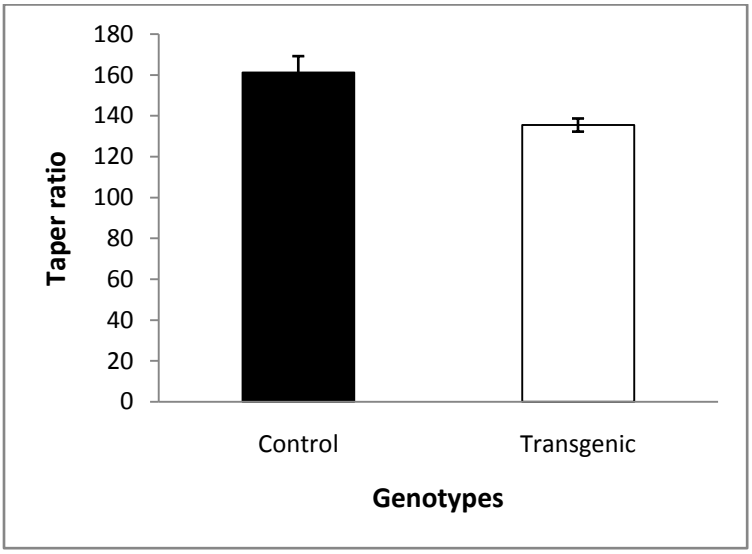
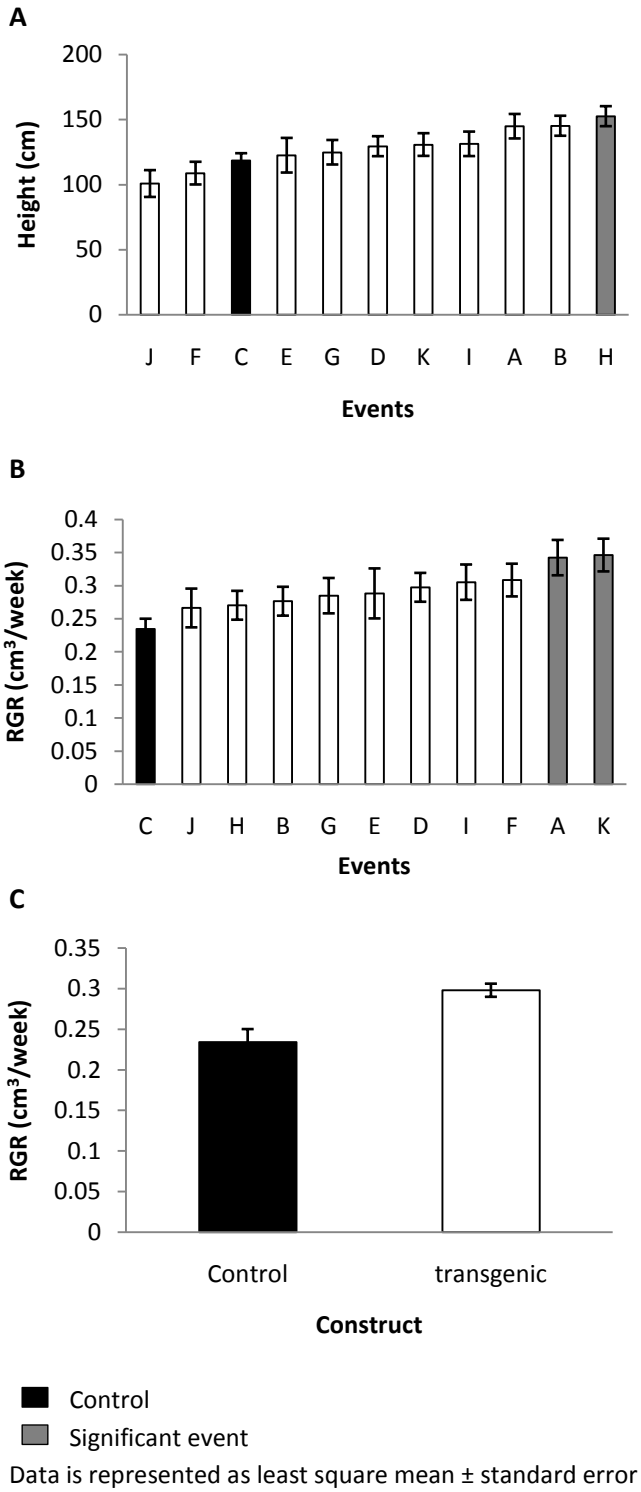


Figure 5.4. Growth comparison for higher growth rate construct #7.

A) Height
B) RGR
C) RGR between genotypes



Data is represented as least square mean ± standard error

Figure 5.5. Variation in taper ratio for higher growth rate construct #7.

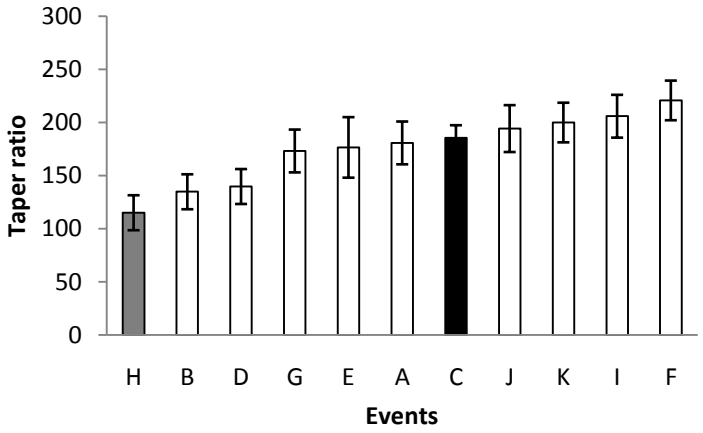
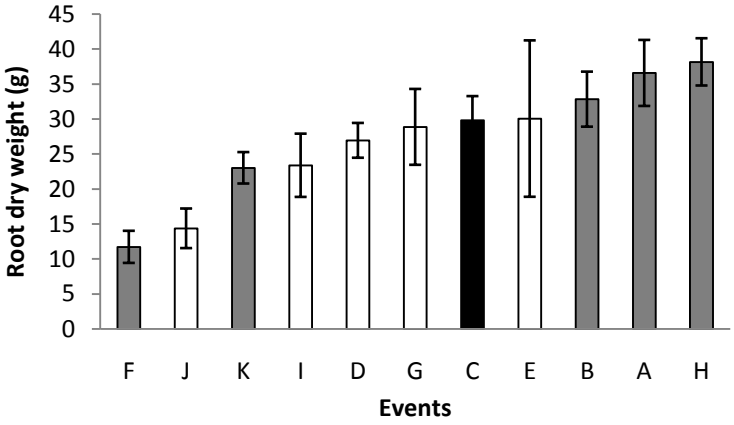


Figure 5.6. Variation in root dry weight for higher growth rate construct #7.



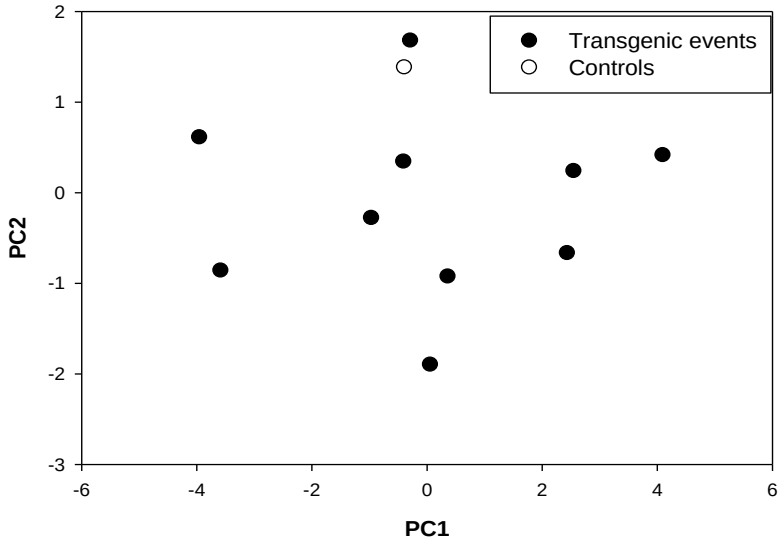
Control
Significant event
Data is represented as least square mean ± standard error

<u>Figure 5.7.</u> PC for higher growth rate construct #7.

A) PC1 vs PC2

B) PC1 vs volume

A



B

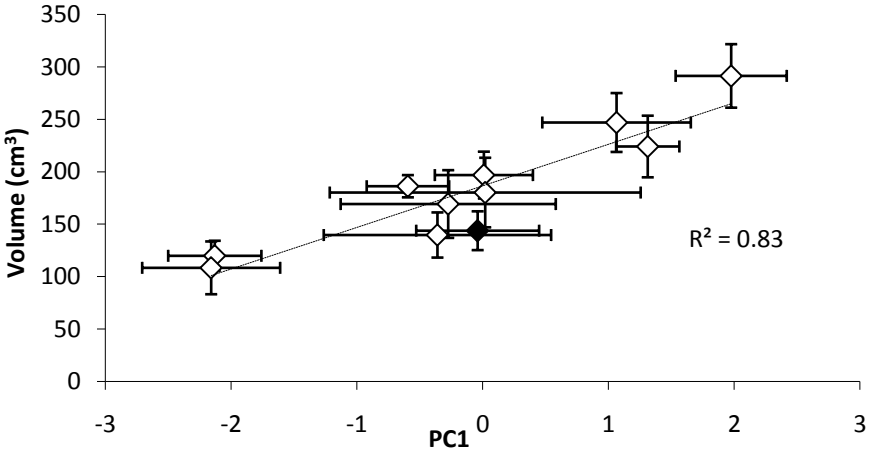


Figure 5.8. **Variation in mean single leaf area for flowering modification construct #10.**

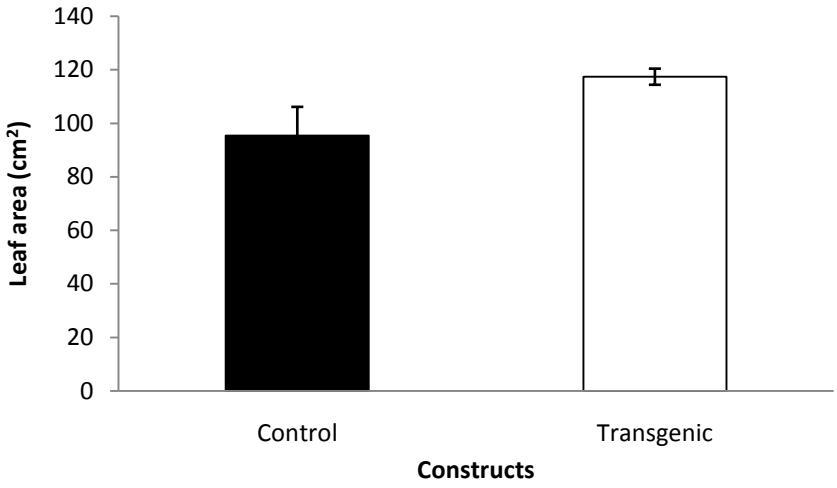
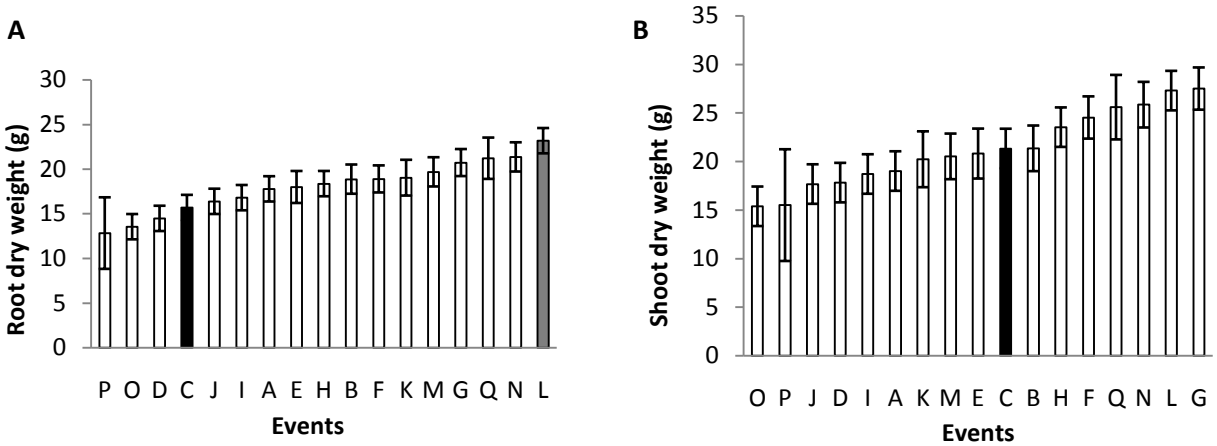


Figure 5.9. **Variation in root biomass between genotype flowering modification construct #10.**

Figure 5.10. Growth comparison for flowering modification construct #10.

A) Root dry weight

B) Shoot dry weight

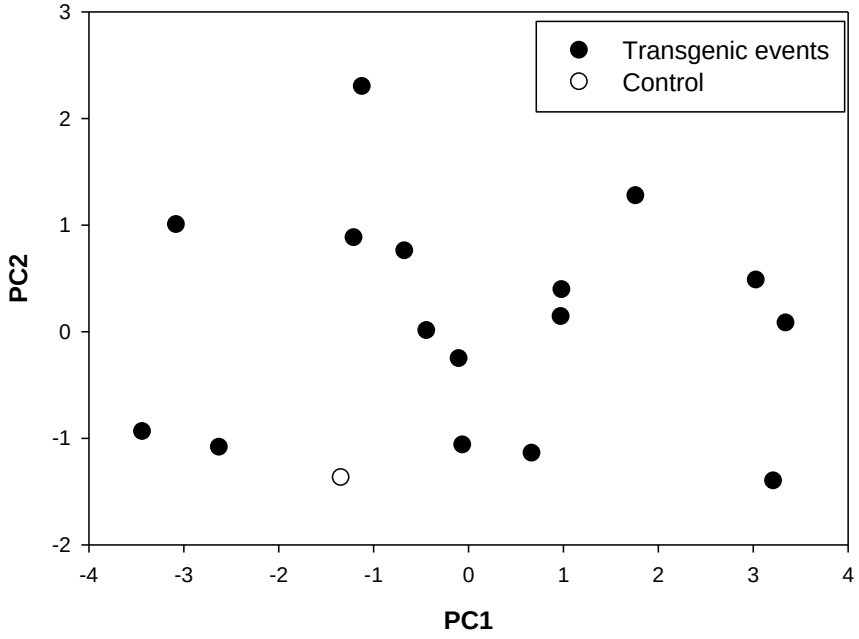


Control

Significant event

Data is represented as least square mean ± standard error

Figure 5.11. PC1 vs PC2 for flowering modification construct #10.



CHAPTER 6: CONCLUSIONS

Overview of results and suggestions for future work with candidate genes

Results from these preliminary greenhouse trials suggested that some genes from a phylogenetically distant species can be successfully transferred to trees for enhancing similar kinds of complex traits. However, the results were highly variable, and many genes gave no useful or consistent changes in phenotype. The constructs that we believe are most worthy of further studies are shade tolerance construct #11, lignin constructs #41 and #44, and growth rate modification construct #7. Construct #11 showed shade tolerance and tree form modifications in the desired direction, in addition to biomass accumulation. However, these results need to be confirmed by testing the constructs across shade and non-shade conditions. Although only a small number of events were available for the lignin modification constructs, their effect on increasing the stem lignin content and altering the growth were consistent. However, it would be desirable to verify these results with more events and a larger number of ramets per event. A chemical analysis should also be performed to support the results from the Weisner staining we conducted. Construct #7 showed a strong and significant increase both in relative growth rate as well as in biomass accumulation, thus is of great interest; those two traits were often negatively correlated in other constructs. The two significant events from this construct should be subjected to intensive screening with more ramets to confirm these results. Gene expression analysis should be performed for all re-tested constructs to provide further support for the results, and to insight into the nature of the cause-effect relationship between gene function and the phenotype.

Options for extension and improvement of study design

One reason for the highly variable nature of the results could be the use of heterologous genes and promoters (from different species), that have not coevolved to interact with poplar proteins and regulatory DNA sequences. It would therefore be of interest to compare the results we observed to those using the orthologous poplar genes, with or without native poplar promoters. Events within each construct showed significant

differences among themselves, as is commonly observed in transgenic studies in all organisms due to the uncontrolled site of chromosomal insertion. This underlines the desirability of developing an efficient site-specific integration system for plants. Although this has been demonstrated in rice and Arabidopsis, it has never been shown to function in poplar, and its efficiency is nonetheless too low for routine use.

To help understand the precision of our experimental methods, we calculated the coefficient of variation (CV) among ramets within events for all constructs, using volume index—which is highly sensitive to environmental conditions—for study. We also did this for two other experiments recently performed in our laboratory (control test1 and 2), to help benchmark our own results (Table A15; Figure A4). Control test 1 consisted of constructs with genes (promoter::coding region) *GA20ox: GA20ox* and *RGL1-1: GA20ox*, and control test 2 consisted of *CES: GA20ox*, *βexp: GA20ox*, and *CES: GA20ox+Lip:gai*. CV ranged from about 20 to 100%, with most of our trials below or similar to the other greenhouse trials recently conducted. However, the two drought constructs #13 and #14 that were tested in a single experiment seemed to be outside of the normal range shown by the other constructs. However, our third drought construct #15 did not show a similar high level of variation, nor was it as severely affected in its growth rate as was the other two constructs (Table A15). This suggests that the more stringent drought treatment might have caused more variable ramet effects, perhaps depending more on their initial size (and thus water depletion rate), that might affect the largest plants more, and thus induce greater variance. Similar results can be seen in the two shade tolerance constructs, where the much more poorly growing construct had a much larger CV. These size effects may indeed be biological rather than experimental, but methods to more carefully control stress at levels that vary depending on plant size may be warranted for more detailed physiological studies. This might include more frequent checking of moisture content and electrical conductivity (EC) in a larger number of sentinel pots, or perhaps in every pot, to reduce the experimental error seen for osmotic stress constructs. Other solutions such as increases in the number of ramets, reducing the stress intensity, and changing the stress intensity based on season (for example, applying less intense stress during summer) may help in reducing experimental error. Changes in stress treatments might also be

warranted, such as the use of hydroponics culture and osmotica such as polyethylene glycol (PEG) or mannitol; that are used in many studies to homogenize osmotic stress (Blum; Zhu et al. 2006).

The physiology of plants may have been affected by the re-randomization of pots in our experiments. Re-randomization is expected to help in reducing border and neighbor effects, as well as variation due to distance from light source. However, plant handling is also known to induce a cascade of physiological changes, which may affect growth rate and entry of plants into dormancy. It would be useful to study this effect, and thus to inform the structure of future studies. The results from CV suggest it did not markedly improve precision, compared to the control studies where no such randomization was made (Figure A4). To finish this project, our laboratory is conducting intensive secondary screens of the most promising events, considering the recommendations mentioned above.

Summary conclusions

We suggest three major results and suggestions from this study:

1. Some of the foreign genes transferred to poplar were effective in enhancing the target traits. The greenhouse screening method used, though perhaps costly and imprecise, was clearly successful in fulfilling the overall aim of the project to identify useful genes in a tree based on large scale genomic studies of phylogenetically distant plant species.
2. The level of experimental error was substantial, and was clearly amplified by our stress treatment conditions. Although we had conducted many preliminary experiments, we suggest that before undertaking further large scale greenhouse screens it would be worthwhile to re-evaluate options for improved control of stress levels, and of factors such as plant rotation as means to improve precision.
3. The limitations of our study underline that the promising genes require reconfirmation in the greenhouse. They also require field evaluation—and anatomical, physiological, chemical, and gene expression analyses—to confirm and understand their mode of action, and thus to guide possible applications.

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APPENDIX –I

ACCESSORY TABLES AND FIGURES

Tables A1 to A13 represent legends used in graphs for event names for respective constructs. Control event ID is "C" for all constructs.

Table A1. **Drought construct #13.**

Events	ID
10~1	A
10~3	B
10~4	D
10~6	E
11~1	F
11~2	G
12~6	H
13~1	I
13~2	J
13~3	K
13~4	L
13~5	M

Table A2. **Drought construct #14.**

Events	ID
13~3	A
14~1	B
14~2	D
3~1	E
4~10	F
4~5	G
4~6	H
4~7	I
4~8	J
5~10	K
A~1	L
A~13	M
A-15	N
E~1	O
J-1	P

Table A3. **Drought construct #15.**

Events	ID
B-5-3	A
B-5-4	B
C-1-1	D
C-2-1	E
E-3-3	F
E-4-2	G
G-1-1	H
G-3-1	I
G-3-3	J
G-3-5	K
H-2-1	L

Table A4. **Salinity construct #18.**

Events	ID
13~1	A
4~1	B
4~10	D
4~11	E
4~12	F
4~13	G
4~14	H
4~15	I
4~2	J
4~3	K
4~5	L
4~8	M
5~1	N
5~2	O

Table A5. Salinity construct #19.

Events	ID
3~1	A
3~10	B
3~11	D
3~2	E
3~3	F
3~4	G
3~6	H
3~7	I
4~1	J
4~9	K
7~1	M
7~2	N

Table A6. Shade construct #11.

Event	ID
1~14	A
1~2	B
2~1	D
3~4	E
6~3	F
9~1	G
9~11	H
A~2	I
c~3	J

Table A7. Shade construct #12.

Events	ID
18-1	A
2~11	B
2~13	D
3~2	E
4~1	F
4~11	G
4~12	H
4~17	I
4~2	J
4~4	K
4~9	L
7~17	M
B-1	N
D-1	O

Table A8. Nutrition efficiency construct #16.

Events	ID
2,3~1	A
4~3	B
5~1	D
5~11	E
5~2	F
5~3	G
5~6	H
7~17	I
7~5	J
7~6	K
A~1	L
A~2	M

Table A9. Lignin construct #41.

Events	ID
1~12	A
1~13	B
1~16	D
2~5	E
3~1	F
3~4	G
3~7	H

Table A10. Lignin construct #44.

Events	ID
1~10	A
1~11	B
1~4	D
1~6	E
1~7	F
1~8	G
1~9	H
2~4	I
2~5	J
2~6	K
A~4	L
A-3	N

Table A11. Stature construct #4.

Events	ID
1~1	A
1~22	B
1~7	D
3~1	E
3~3	F
4~23	G
4~35	H
5~1	I
5~2	J
5~3	K
5~6	L
6~1	M

Table A12. Higher growth rate construct #7.

Events	ID
4~10	A
4~12	B
4~15	D
4~18	E
4~2	F
4~3	G
4~5	H
4~6	I
4~7	J
5~1	K

Table A13. Flowering modifications construct #10.

Events	ID
1~2	A
1~3	B
1~4	D
1~5	E
1~6	F
1~7	G
1~8	H
3~1	I
6~1	J
6~12	K
6~2	L
6~3	M
7~1	N
7~2	O
7~6	P
A~5	Q

Table A14: Traits, number of events, screening period, and average temperature for constructs screened in the greenhouse.

Trait and construct	# events	Screening period	Temp (^{0}C)
Drought tolerance (#13)	12	June-July, 2007	24.40
Drought tolerance (#14)	15	June-July, 2007	24.40
Drought tolerance (#15)	12	June-July, 2008	25.12
Salinity tolerance (#18)	14	June-July, 2007	24.40
Salinity tolerance (#19)	12	January-March, 2008	23.29
Shade tolerance (#11)	9	February-April, 2008	23.64
Shade tolerance (#12)	14	August-October, 2007	24.27
Nutrition efficiency (#16)	12	December,07-January,08	22.24
High lignin (#41)	7	July-August, 2008	25.48
High lignin (#44)	11	August-October, 2008	25.02
Reduced stature (#4)	13	June-July, 2007	24.40
Increased stature (#5)	11	March-April, 2008	24.16
Reduced stature (#6)	12	August-September, 2007	24.73
Higher growth rate(#7)	10	October-December, 2007	22.83
Flowering modification (#10)	16	July-August, 2008	25.48

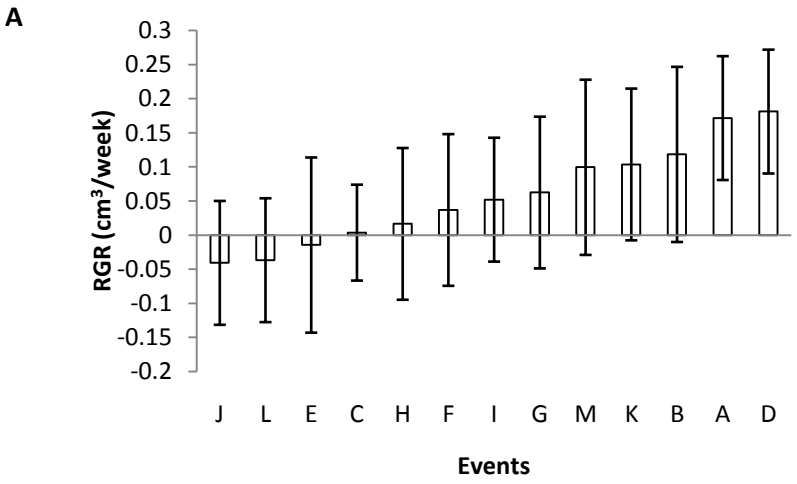
Table A15. Coefficient of variation (CV) for variance among ramets within events for volume index. MSE is mean square error from ANOVA.

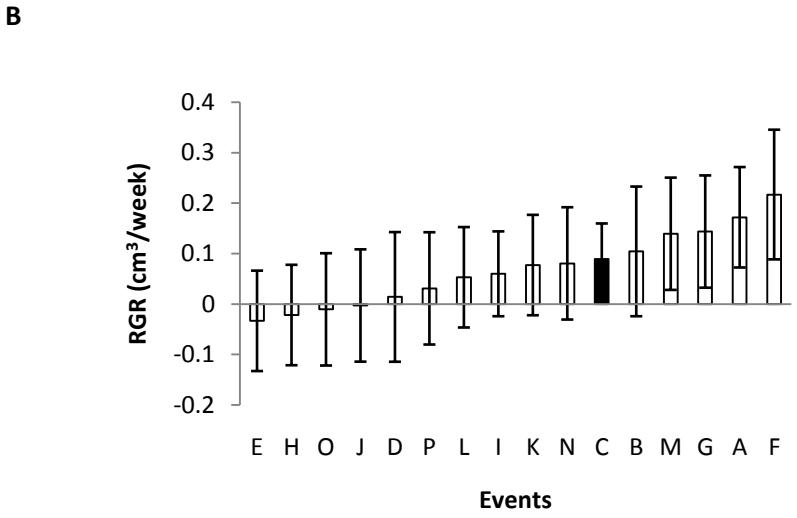
Constructs and traits	CV (%)	√MSE	Mean Volindex
#13 & #14 (drought tolerance)	97.45	17.46	17.91
#15 (drought tolerance)	54.00	25.11	46.50
#18 (salinity tolerance)	33.30	8.29	24.90
#19 (salinity tolerance)	55.13	23.93	43.40
#11 (shade tolerance)	49.54	31.81	64.22
#12 (shade tolerance)	65.69	10.77	16.41
#16 (fertilizer efficiency)	50.18	78.65	154.78
#41 (lignin accumulation)	32.92	31.99	97.18
#44 (lignin accumulation)	23.57	32.32	137.18
#4 (stature modification)	47.31	14.10	29.8
#5 (stature modification)	30.90	5.92	19.18
#6 (stature modification)	41.23	20.50	49.70
#7 (growth rate modification)	37.89	69.82	184.27
#10 (flowering modification)	32.82	24.75	75.42
Control test 1 (C1)	61.12	25.41	41.58
Control test 2 (C2)	41.36	40.70	98.40

<u>Figure A1.</u> Variation in RGR for drought tolerance constructs during stress phase.

A) Construct #13

B) Construct #14





<u>Figure A2.</u> RGR for drought tolerance constructs during recovery phase.

A) Construct #13
B) Construct #14
C) Construct #15

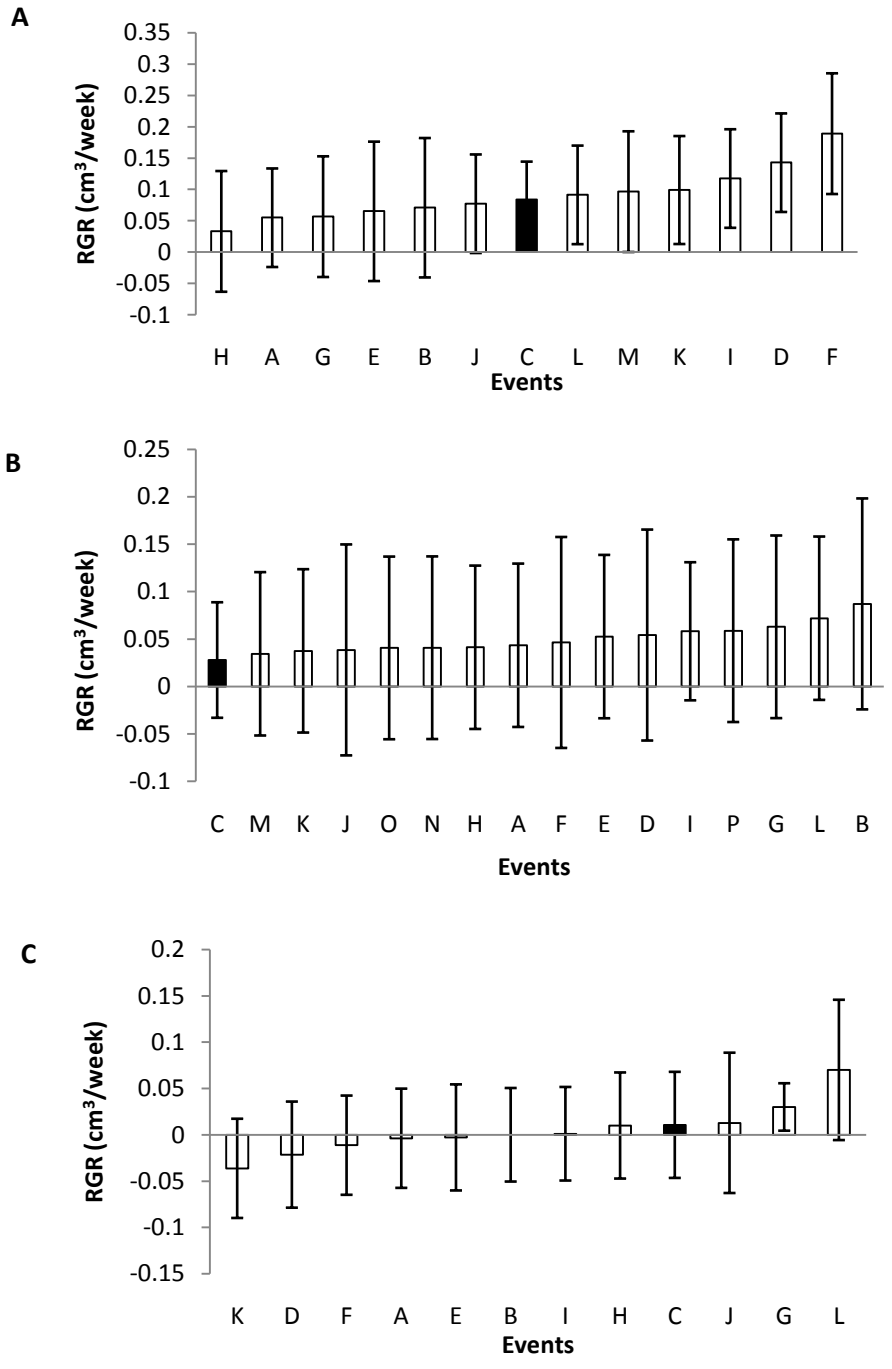


Figure A3. **RGR for nutrition efficiency construct #16.**

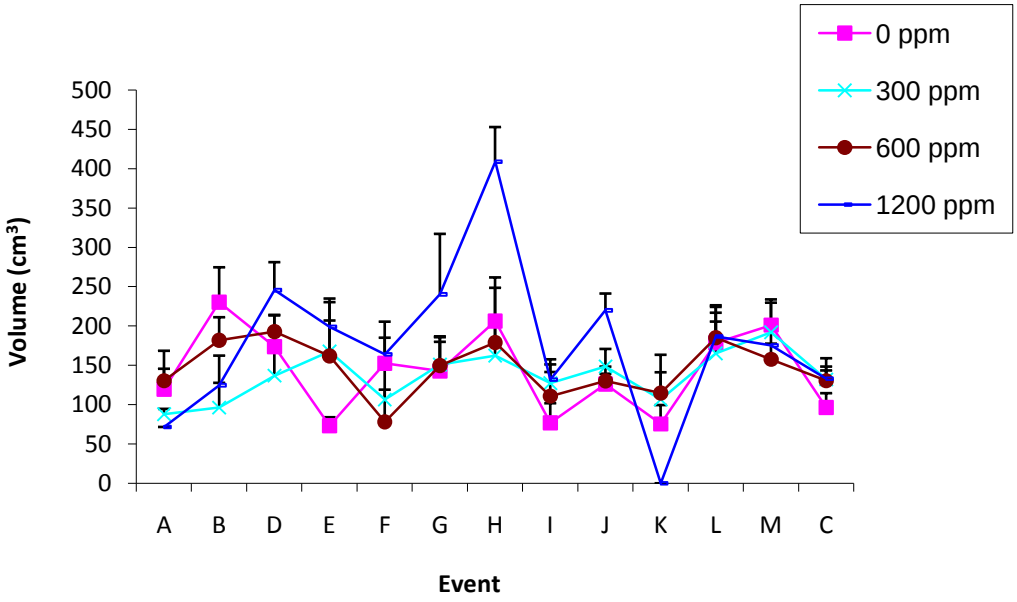
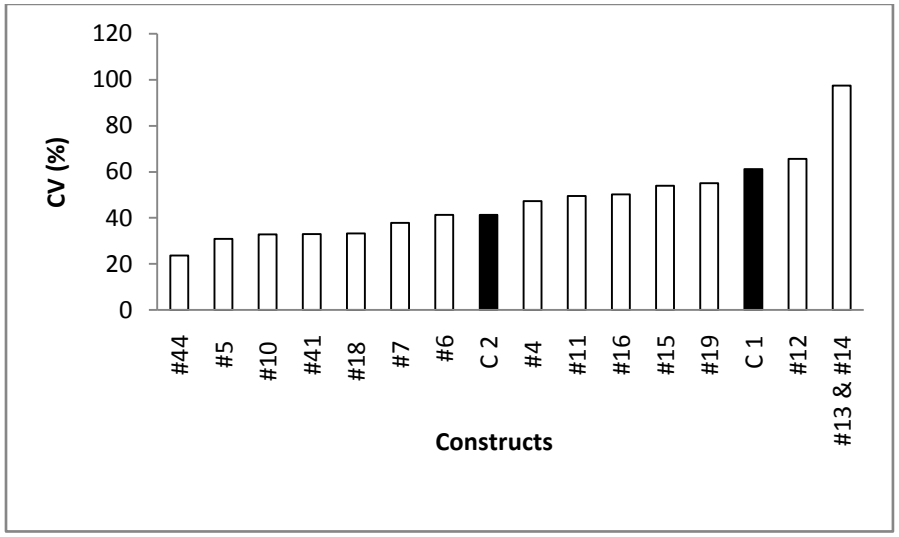


Figure A4. **Comparison of coefficient of variation (CV%) among constructs.**



Constructs and traits
#13 & #14 (drought tolerance)
#15 (drought tolerance)
#18 (salinity tolerance)
#19 (salinity tolerance)
#11 (shade tolerance)
#12 (shade tolerance)
#16 (fertilizer efficiency)
#41 (lignin accumulation)
#44 (lignin accumulation)
#4 (stature modification)
#5 (stature modification)
#6 (stature modification)
#7 (growth rate modification)
#10 (flowering modification)
Control test 1 (C1)
Control test 2 (C2)

APPENDIX II

Examples of SAS programs used for testing the effect of constructs

Program 1: Analysis of variance (ANOVA) for single construct.

```
data drought15;
input event$ ramet h1 d1 v1 h2 d2 v2 h3 d3 v3 h4 d4 v4 shdrwt rtdrwt lfdrwt rtlen;
lshdrwt=log(shdrwt);
sqrtshdrwt=sqrt(shdrwt); ….
if event='Control' then plant='Control';
if event='G-3-1' then plant='Transgenic';
if event='B-5-4' then plant='Transgenic';
if event='G-1-1' then plant='Transgenic';…..
datalines;
Control          3.00     52.00   …….
          …….0.64        27.00; run;

%macro check(response);
title "&response -anova";
proc mixed data=drought15;
class event;
model &response=event/ outpm= pred S;
lsmeans event/cl diff alpha=0.0006;
run;
title "&response - test of variance";
proc gplot data=pred; plot resid*pred=event/vref=0; run;
title "&response - test of normality";
proc univariate data=pred plot normal;
var resid; probplot resid/normal (mu=0 sigma=est); run;
%mend;
%check(h1);
%check(h2);
%check(h3);
%check(h4);…..

%macro check(response);
title "&response -anova";
proc glm data=drought15;
class plant;
model &response=plant;
run;
%mend;
```

Program 2: ANOVA for multiple constructs.

```
%macro check(response);
title "&response -anova";
proc mixed data=drought;
class construct event;
model &response=construct/ outpm= pred S;
random event(construct);
lsmeans construct /cl diff alpha=0.008;
run;
proc mixed data=drought;
class construct event;
model &response=construct event(construct) / outpm=pred S;
lsmeans construct / cl diff alpha = 0.008;
lsmeans event(construct)/cl diff alpha=          0.00007;
title "&response - test of variance";
proc gplot data=pred; plot resid*pred=event/vref=0; run;
title "&response - test of normality";
proc univariate data=pred plot normal;
var resid; probplot resid/normal (mu=0 sigma=est); run;
%mend;
```

Program 3: ANOVA for fertilizer construct.

```
%macro check(response);
title "&response -anova";
proc mixed data=nutrition;
class event treatment;
model &response=event treatment event*treatment / outpm= pred S;
lsmeans event/cl diff alpha=0.0006;
lsmeans treatment /cl diff alpha=0.008;
lsmeans event*treatment / cl diff alpha=0.001;
run;
title "&response - test of variance";
proc gplot data=pred; plot resid*pred=event/vref=0; run;
title "&response - test of normality";
proc univariate data=pred plot normal;
var resid; probplot resid/normal (mu=0 sigma=est); run;
%mend;
```

Program 4: Principal component analysis (PCA).

```
proc princomp data=lignin out=lignin_components;
var vol la leafdry stemdry rootdry rtlen;
run;
```

Program 5: Fisher's exact test.

```
proc freq data=scoring;
tables scoring*event/chisq exact;
run;
```