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Drought response of *Populus* transformed with stress response transcription factors

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To the Graduate Council:

I am submitting herewith a thesis written by Alina S. Campbell entitled "Drought response of *Populus* transformed with stress response transcription factors." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

Zong-Ming Cheng, Major Professor

We have read this thesis and recommend its acceptance:

Timothy Tschaplinski, Jennifer Franklin

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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**Drought response of *Populus* transformed with stress
response transcription factors**

A Thesis Presented for
the Master of Science
Degree
The University of Tennessee, Knoxville

Alina Sandra Campbell
August 2010

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Abstract

The economic feasibility of producing biomass-based fuels requires high-yielding feedstocks to supply biomass to biorefineries. *Populus* trees are a potential biomass feedstock due to their high yield, but their high water requirement limits productivity under drought conditions.

The number of genes controlling drought tolerance, and the long generation time for perennial species, slows cultivar development. Accelerated domestication proposes using the sequenced *Populus* genome to quickly incorporate target traits into productive clones by transgenesis.

Six putative drought tolerance transcription factors: DREB2A, DREB2B, AtMYB, AREB1/ABF2, MYB, and NAC, had been previously identified and manipulated in eastern cottonwood (*Populus deltoides*). Three constructs of each gene were transformed into a *P. deltoides* background clone, including constitutive overexpression (OE), drought inducible OE, and knockdown. This greenhouse study examines the effect of these previously transformed constructs on drought tolerance by characterizing leaf abscission, leaf water potential, and growth under drought and well-watered conditions.

AREB1/ABF2 constitutive OE lost significantly fewer leaves under drought than the Vector control, and had one of the lowest rates of leaf loss overall. Both DREB2A inducible OE and AREB1/ABF2 constitutive OE plants were more productive than the Vector control under drought conditions. MYB inducible OE was a productive construct and initially appeared to be drought tolerant. It is possible that this construct experienced xylem cavitation early on due to the severity of drought experienced by the large trees containing this construct.

DREB2A inducible OE, AREB1/ABF2 constitutive OE, and MYB inducible OE were the most productive constructs as well as being likely to confer drought tolerance. Field trials would be the next step, providing a clearer picture of how these constructs would perform under natural conditions.

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Chapter 1

Introduction

Introduction

Concerns over energy availability and cost have placed an emphasis on alternative fuel sources. The economic feasibility of producing biomass-based fuels requires high-yielding feedstocks to supply abundant plant material for biorefineries. *Populus* trees garner considerable attention as a potential feedstock due to their high yield, easy propagation, and available genomic resources. Their range extends across the Northern Hemisphere from the tropics to the Arctic Circle, encompassing diverse environmental conditions. This genus also exhibits substantial variation among adaptive traits (Brunner *et al.* 2004).

Hybrid *Populus* reach productivity targets in moist environments where they're grown with highly managed nutrition. Yet for enough biomass to be produced for widespread adoption, marginal land must be cultivated. The main yield-limiting factor on these lower quality agricultural lands will likely be water availability (Tuskan 1998). A clone's ability to withstand water deficit varies, which can be exploited to breed more tolerant trees. The degree of phenotypic variation has been identified as one of the factors that most influences breeding success (Moose and Mumm 2008). Addressing this constraint will ensure adequate biomass production even under non-ideal conditions (Moose and Mumm 2008).

Breeding technology

Tree domestication

Plant domestication has been described as human-directed evolution (Simmons 1996). It converts a plant with attributes that helped it survive and reproduce in nature, into one with traits more valuable and amenable to human needs. Although numerous crop species have undergone centuries of domestication, trees and other perennial plants capable of becoming a bioenergy feedstock have not benefited in the same way. Commercial *Populus* clones, for instance, only represent one to two generations of genetic improvement.

In addition to the complexity of breeding for quantitative traits, the long generation times of trees slow the process of developing new cultivars through traditional means. The genomic resources available for *Populus* allow for rapid advances, making it possible to create clones incorporating traits that are particularly valuable for a biomass feedstock. Desirable phenotypes include increased stem diameter, reduced proleptic branching, increased sylleptic branching, decreased response to competition, and increased drought tolerance, cold tolerance, and pathogen resistance. Genes involved in several of these traits have been identified and can be used in future accelerated domestication programs. Drought tolerance was chosen as a target trait for this project because of its potential to substantially increase biomass production, elevating productivity even under water deficit. Intense poplar research has produced valuable genetic resources, such as a fully-sequenced genome, high-density genetic maps, and

microarrays that can be used as part of an accelerated domestication program (Tuskan et al 2003).

Molecular markers

Molecular breeding techniques have advanced over the past 20 years. Molecular markers, specific proteins or DNA sequences, enable identification of sites in the genome where genotypes differ from one another. Microarrays can scan the genome for variation, identifying where markers exist (Zhu and Salmeron 2007). The known locations of observable mutations/alterations on the chromosome are used to construct genetic maps (Prince and Ogundiwin 2004). Map resolution depends on the number of markers mapped to different loci. A large genetic marker linkage map has been developed for the *Populus* pedigree being used in this accelerated domestication study (Yin *et al.* 2008).

Genetic maps are available for many crop species. Markers can be used to track traits of interest throughout breeding programs because their proximity to target traits suggests that they will likely be inherited together. Molecular marker technology also enables identification of genomic regions found to affect quantitative traits. These quantitative trait loci (QTL) are segments of DNA that are closely linked to the genes responsible for a particular quantitative trait. The genetic gain possible from QTLs depends on how large their effects are, how stable these effects are across various environments, and how accurately individual QTL positions have been calculated (Moose and Mumm 2008).

Populus genus

Taxonomy and morphological characteristics

Populus and *Salix* are the two genera comprising the Salicaceae family (Dickmann 2001). *Populus* contains roughly 30 species of woody plants, including some with the highest growth rates found among temperate trees. These deciduous or semi-evergreen trees are dioecious, meaning that individual trees are either male or female (Eckenwalder 1996). Male and female flowers are clustered in catkins that are visible early in the spring, before leaves have emerged (Braatne *et al.* 1996). This timing ensures maximum wind pollination (Eckenwalder 1996). Leaf form varies substantially between *Populus* species. They are always simple but shape varies from narrow, lanceolate leaves to broad ovate or heart-shaped leaves (Dickmann 2001).

Most species propagate clonally via root suckers in the wild (Eckenwalder 1996). This is particularly evident in the tendency of aspens (*Populus tremuloides*) to form large groves by sprouting saplings from horizontal roots. Crown and root damage often stimulates root sucker production. Broken branches will root after becoming partially buried in soil (Braatne *et al.* 1996).

Eastern Cottonwood

Eastern cottonwood (*Populus deltoides*) can be found in most states in the United States and throughout Canada and adapts to various soil types (USDA 2002). It reaches a height of 25 to 30 m and 1 to 1.2 m in diameter. This species was named for its cottony, tufted seeds that are dispersed by wind and water. Seeds have high germination rates but are only viable for a couple of weeks (Braatne *et al.* 1996). It is associated with riparian

areas and wetlands but will grow nearly anywhere given adequate precipitation. Due to its fast growth rate, *P. deltoides* has soft, weak wood. It is a commercially important timber species in the Southeastern United States, grown for use as plywood, pulp and material for containers (Dickmann 2001).

Drought tolerance varies significantly among different *Populus deltoides* genotypes (Wullschleger *et al.* 2009). Restoration plantings of *P. deltoides* on strip mine spoils show that it is relatively tolerant of dry sites (USDA 2002). Despite this, ample moisture is needed to maintain the high growth rates characteristic of this genus (Wullschleger *et al.* 2009). Fast growing *Populus deltoides*, and other *Populus* species produce maximum biomass under irrigation or when they're growing in riparian habitats (Wullschleger *et al.* 2009).

Populus drought tolerance

Populus genotypes exhibit various levels of drought resistance. Most mechanisms by which members of this genus achieve resistance fall into the drought avoidance category (described in greater detail in Chapter 2), which is characterized by anatomical and morphological changes allowing plants to decrease water loss through transpiration and increase water uptake (Kikuta 2005). Changes experienced by *Populus* species in response to water deficit include stomatal closure, leaf abscission, increased root growth relative to shoot growth and osmotic adjustment (Marron *et al.* 2002).

Commercial releases of stress resistant crops

Biotic stress tolerance

Breeding resistance to biotic stresses such as insects, fungi, and viruses has been successful, resulting in commercially-available cultivars (Fitch *et al.* 1992; Schuler *et al.* 1998). In contrast to abiotic stresses though, the successful development of crops resistant to biotic stresses was conferred by introducing a single, or a few genes (Moose and Mumm 2008). Papaya seedlings resistant to papaya ringspot virus (PRV) were developed by introducing a PRV coat protein gene (Fitch *et al.* 1992). A similar introduction produced maize resistant to the European corn borer (Schuler *et al.* 1998). Resistance to abiotic stresses such as drought, heat, and cold is more difficult to achieve because they are quantitative traits, controlled by multiple genes (Vinocur and Altman 2005).

Transgenic Populus

Significant biotechnology research is being conducted on forest trees worldwide, but only one country has employed them on a commercial scale (Figure 1.1). Transgenic *Populus* have been commercially released in China, but they are for environmental restoration efforts, as opposed to commercial wood production (Sedjo 2005). *Populus* trees are fed upon by many lepidopteran insects, such as poplar loopers (*Lygris populuata* and *Ectropis crepuscularia*), gypsy moth (*Porthetria dispar*), poplar caterpillar (*Clostera anachoreta*), and fall webworm (*Hyphantria cunea*) (Huang *et al.* 2007). In 1978 China began the “Great Green Wall” project in the western part of the country. It reforested

degraded land as a means of combating desertification by the encroaching Gobi desert (Malagnoux 2007). Given that defoliating insects were impeding the reestablishment efforts, approval was granted in 2000 to release a transgenic *P. nigra* transformed with the Bt toxin gene *CryIAc*. This toxin is effective against both the Lepidoptera and Coleoptera species that feed on the trees. One million of these transgenic trees are thought to cover 30 hectares (Sedjo 2005). A smaller release took place in 2003. In this case *CryIAc* was stacked with *API* (arrowhead proteinase inhibitor) gene and transformed into a hybrid poplar clone (Huang et al 2007).

Objectives

Due to the complexity of breeding for quantitative traits as well as the trees' long generation times, improvement through traditional techniques, such as recurrent selection, would be extremely slow. Available genomic tools would speed up tree domestication, making it possible to develop new varieties in a few years rather than decades. Accelerated domestication proposes using the fully-sequenced *Populus* genome to hasten the addition of target traits into productive clones.

This accelerated domestication experiment will use drought tolerance as a target trait, examining the individual effect of six drought-tolerance transcription factors (DREB2A, DREB2B, AtMYB, AREB1/ABF2, MYB, and NAC) (Table 1.1). Three types of gene constructs, differing in terms of gene expression, had been previously produced. One always overexpresses the gene (constitutive OE), another overexpresses the gene under stress conditions (inducible OE), and the third has reduced expression (knockdown). Two controls had also been produced previously, a vector control and an

Table 1.1 Genes transformed into a Populus deltoides clone

Gene label for experiment	Transcription factor family	Associated Poplar gene model
DREB2A	AP2-EREBP	eugene3.00101772
DREB2B	AP2-EREBP	eugene3.0008067
MYB	MYB	grail3.0033008701
AtMYB	MYB	fgenesh4_pm.C_LG_VIII000421
AREB1/ABF2	bZIP	eugene3.00021164
NAC	NAC	eugene3.00101577

untransformed Wildtype. The Vector control was a clone transformed with an empty vector, containing only a promoter and terminator, but no gene of interest. It accounted for the effects that transformation itself might have had on the phenotype. The untransformed Wildtype was the original background *P. deltooides* clone that had been used in transformation. Similar to the transformed constructs, Wildtype was also propagated through tissue culture. This was to eliminate any potential effects arising from propagation through cuttings instead of tissue culture. The objectives of this study are to 1) determine the effect of gene expression on drought tolerance and 2) assess the genes' impact on productivity.

Drought tolerance assessment

Leaf loss is considered highly indicative of plant susceptibility to drought with drought tolerant plants losing less leaves than drought susceptible plants (T.J. Tschaplinski, personal communication, May 2009). Gene impact on drought tolerance will be determined by observing how the drought treatments influence leaf loss within the same construct. This will be quantified using percent leaf abscission.

Hypotheses:

- *Plants with OE constructs will lose less leaves under drought than control plants
- *Plants with knockdown constructs will lose more leaves under drought than control plants.

Assess productivity

Productivity is an essential agricultural trait and desirable constructs must be able to remain productive under both drought and watered conditions. A *Populus* clone able to withstand water deficit is of little value if it does not produce large amounts of biomass. Relative growth rate (RGR) will be calculated and each transgenic construct will be compared against the Vector Control, which lacks the target gene, to see how these genes and expression types impact productivity.

Hypotheses:

*Plants with inducible OE constructs will have higher RGR than control regardless of treatment.

*Constitutive promoters often have a negative growth impact so compared to control plants, constitutive OE trees will have higher growth under drought but not under irrigated conditions.

*Plants with knockdown constructs have reduced expression and will have lower RGR than control regardless of treatment.

Chapter 2

Literature Review

Plant response to drought

Changes in growth

When faced with water stress, plants undergo morphological and biochemical changes that lead to acclimation then to functional damage and abscission of plant parts as the drought intensifies. Developmental stage strongly determines how drought will impact a plant but initial responses are reductions in photosynthesis and growth, and a shift from shoot to root growth (Praba *et al.* 2009). Unlike other stresses drought isn't sudden, it develops slowly and becomes more severe over time (Munné-Bosch and Alegre 2004). Plants acclimate to slowly declining water availability before tissues dehydrate. The changes that occur during this period improve the plant's water balance. Growth slows during acclimation due to inhibited cell expansion and reduced carbon assimilation (Costa e Silva *et al.* 2004). Shifts in carbon allocation favoring root growth over shoot growth further reduce the amount of aboveground biomass a plant produces. Roots are less drought sensitive than leaves and have the added advantage of increasing plants' access to water.

Hormone levels

Drought causes changes in a plant's hormone levels, increasing inhibitors and reducing growth promoters (Munné-Bosch and Alegre 2004). Cytokinin content is

positively correlated with photosynthesis and chlorophyll composition, but is shown to decrease under drought stress. This reduced cytokinin content doesn't trigger leaf senescence, but it enables its progression. Abscisic acid (ABA), on the other hand, is produced as roots begin dehydrating and accumulates in drought-stressed plants. ABA initiates stomatal closure and the expression of stress-response genes. Studies conducted on rice and wheat plants under drought stress have shown that ABA increases carbon remobilization from senescing leaves to the seeds (Munné-Bosch and Alegre 2004).

Stomatal regulation

Stomatal regulation is one of the most valuable desiccation avoidance mechanisms plants have evolved. It occurs quickly in response to low water availability, at times responding to soil water depletion before there is a measurable change in leaf water status (Lowenstein and Pallardy 1998). Stomatal aperture and closure establish a balance between maximizing CO₂ uptake to drive photosynthesis and reducing the water lost through transpiration (Reigosa Roger and Sánchez-Moreiras 2001). Soil moisture content exerts a stronger influence on stomatal response than does leaf water status (Yordanov *et al.* 2003). As soil begins to dry, ABA is transported from plant roots to the shoot, and signals stomatal closure (Loewenstein and Pallardy 1998). In situations of severe water deficit, survival also depends on a plant's ability to minimize the amount of water lost through the epidermis after stomata have reached minimum aperture (Praba *et al.* 2009).

Although stomatal closure improves a plants ability to cope with water deficit, it also minimizes CO₂ uptake which leads to reduced yield because the plant is not

photosynthesizing at its maximum capacity. There is a negative linear relationship between stomatal closure and yield under drought stress (Praba *et al.* 2009).

Leaf senescence and abscission

Senescence and abscission occur slowly and are essential components of a plant's response to water deficit. These processes alleviate water and nutrient deficits by reallocating nutrients to reproductive organs and eliminating water consumption by older, less productive leaves. In doing so, they protect important bud and cambium meristem tissues. The most visible change that leaves undergo as they senesce is the yellowing that occurs as chlorophyll is degraded. Less apparent changes include alterations in cell ultrastructure (chromatin condensation, thylakoid swelling, plastoglobule accumulation), metabolism (protein degradation, lipid peroxidation), and changes in gene expression (Munneé-Bosch and Alegre 2004). Even once senescing leaves are no longer contributing through photosynthesis, they contain a substantial pool of nutrients, such as lipids and proteins that can benefit other parts of the plant (Munneé-Bosch and Alegre 2004).

Resistance strategies

Drought resistant plants have three main strategies: escape, avoidance, and tolerance (Kikuta 2005). Drought escape is seen in areas with a predictable dry season. It includes a plant completing its life cycle during the moister periods and the development of subsurface water-storing organs in bulbs. Drought avoidance is characterized by anatomical and morphological changes that enable plants to maintain

high water potentials by reducing transpiration and increasing water uptake, but may negatively affect productivity. Transpiration is reduced through rapid stomatal closure, leaf abscission, and leaf rolling in order to prevent water loss. Shifting biomass production from shoot to roots maximizes water uptake by increasing root surface area (Kikuta 2005). Development of a deep taproot is another drought avoidance mechanism, allowing plants to access water deeper in the soil (J.A. Franklin, personal communication, April 2010).

Drought tolerance, on the other hand, ensures that cellular and molecular structures are not damaged even under severe desiccation (Vartania 1996). This can be achieved by maintaining a low osmotic potential (π_o) at full turgor or osmotic adjustment (Tschaplinski *et al.* 1998). Plant cells undergoing osmotic adjustment accumulate solutes in order to reduce their osmotic potential, and thereby reduce cell water potential, which prevents water loss (Taiz and Zeiger 2006). Low osmotic potentials are thought to facilitate a plant's ability to uptake water without the negative impact on aboveground yield that results from morphological acclimation, such as increased root growth or stomatal closure (Tschaplinski *et al.* 1998). Low osmotic potential is considered indicative of drought tolerance and is measured to determine how well a particular plant will be able to withstand water deficit. Low osmotic potential has been reported in clones that maintain growth even under drought conditions (Tschaplinski *et al.* 2006).

Plants decrease turgor pressure under low water conditions, which in turn limits cell expansion and photosynthesis (Altman 2003). Similar to salinity, drought causes plants to experience osmotic stress. A sudden increase in the solute concentration around the cell causes water to be pulled across the membrane and out of plant cells. Osmotic

stress regulates numerous genes that encode the proteins and enzymes responsible for osmotic adjustment.

Stress-induced genes

Reactive oxygen species

Gene expression can change within hours following exposure to drought (Munné-Bosch and Alegre 2004). The genes that are induced help a plant survive and recover from drought (Figure 2.1). Stress-induced genes fall into two categories based on their products: functional molecules and regulatory proteins. Functional molecules improve a plant's ability to tolerate stress and include antioxidants, chaperones, and compatible solutes. Upon exposure to abiotic stress, reactive oxygen species (ROS) form in plant tissues. These are compounds such as superoxide anions, hydrogen peroxide, and hydroxyl radicals that damage cells by oxidizing their components (Corraggio and Tuberosa 2004). Antioxidants act on different cell compartments, scavenging ROS free-radicals to detoxify the plant. ROS scavengers include enzymes such as catalase, superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase as well as non-enzymes, such as ascorbate, glutathione, carotenoids, late embryogenesis abundant (LEAs) proteins, and anthocyanins (Wang *et al* 2003).

A single antioxidant is ineffective at preventing damage; it is the combined effect of numerous ROS scavengers that act as a plant's detoxification system. Alfalfa (*Medicago sativa*) plants engineered to overexpress Mn-SOD experienced less injury following drought stress. SOD is an essential component of oxidative defense systems that works by breaking down two superoxide radicals into hydrogen peroxide and

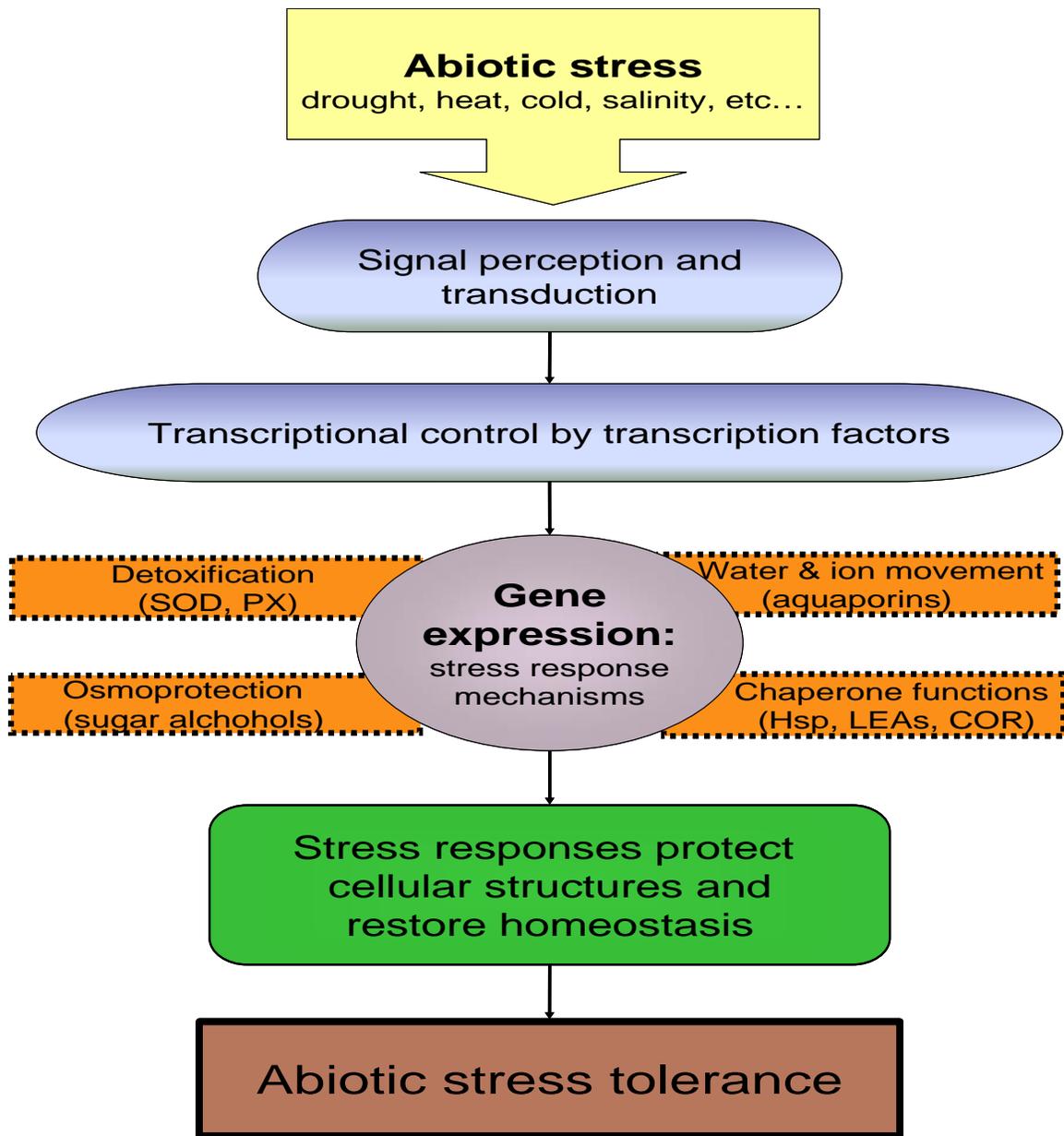


Figure 2.1 Plant response to abiotic stress. Transcription factors control signal transduction, leading to the expression of stress response genes

oxygen. The transgenic alfalfa showed greater membrane stability, photosynthetic efficiency and regrowth following drought stress than non-transgenic plants (McKersie *et al.* 1996).

Chaperone proteins

Some genes involved in stress response encode for chaperone proteins. Chaperones stabilize membranes and proteins by keeping them properly folded and repairing them (Coraggio and Tuberosa 2004). This prevents the enzymes and proteins from malfunctioning, which is often the case under abiotic stress. Though their precise mode of action is unknown, LEAs are thought to benefit plants both by targeting free-radicals and acting as chaperones (Shinozaki and Yamaguchi-Shinozaki 2007). HVA, a LEA gene in barley, has been successfully manipulated to increase salt and drought tolerance. Plants carrying an up-regulated form of the gene that were exposed to stress took longer to show symptoms of damage, had greater yield and recovered more quickly than controls (Coraggio and Tuberosa 2004). Heat-shock proteins (Hsps) and LEAs are two chaperones that have shown success in increasing tolerance. Hsps were initially identified in plants exposed to high temperatures, but they are also induced by dehydration, salt and oxidative stress (Wang *et al.* 2003). Studies involving *Arabidopsis* seedlings constitutively overexpressing Hsps resulted in plants that were more resistant to both drought and salinity (Coraggio and Tuberosa 2004).

Compatible solute accumulation

In addition to proteins and enzymes, compatible solutes are produced in response to osmotic stress. These include the amino acid proline, which is produced by numerous plant species in response to osmotic stress and sugars such as mannitol and trehalose produced by grass species such as *Setaria sphacelata* (Vinocur and Altman 2005). Some compatible solutes, like proline, are present in all plant species while others like glycine betaine are unique to plants with high cold or salt tolerance (Corraggio and Tuberosa 2004). Compatible solutes' primary function is to maintain turgor pressure by accumulating and preventing water from exiting cells with the concentration gradient. They are also known to act as antioxidants and chaperones (Wang *et al.* 2003).

Sugar alcohols (i.e. mannitol) have been extensively targeted in efforts to engineer compatible solute overproduction. Tarczyński *et al.* (1993) transformed tobacco plants with a bacterial gene that produces mannitol 1-phosphate dehydrogenase in order to increase mannitol synthesis and accumulation in plant tissues. They were quite salt resistant, even producing new leaves and roots in the presence of NaCl (Tarczyński *et al.* 1993). These plants had a normal phenotype but pleiotropic effects have been observed in many plants engineered to accumulate sugar alcohols (Wang *et al.* 2003).

Regulatory proteins further regulate gene expression

Regulatory proteins, such as transcription factors (TFs) and enzymes involved in ABA biosynthesis regulate signal transduction and lead to the expression of stress-response genes (Shinozaki and Yamaguchi-Shinozaki 2007). Gene transcription is controlled by the interaction of TFs with specific regulatory sequences in the promoters

of the genes they regulate (Taiz and Zeiger 2006). Transcription factors are organized into several large multi-gene families (Figure 2.2). Those grouped together in a family generally share a similar DNA binding domain (Agarwal *et al.* 2006). Families being studied for stress tolerance include AP2/ERF, bZIP, MYB/MYC, and NAC.

While overexpressing single genes that encode functional compounds often impacts drought tolerance to an extent, much greater results are attained when the single genes encode regulatory proteins. Transforming plants with stress-induced TFs has led to the overexpression of numerous downstream genes (Wang *et al.* 2003). Early experiments with tomato plants constitutively overexpressing a gene encoding for *Arabidopsis* C repeat/dehydration-responsive element binding factor 1 (CBF1/DREB1B) produced tolerant plants but they had a dwarf phenotype (Hsieh *et al.* 2002).

More recent studies have showed that using a stress-inducible promoter can offset negative growth effects seen in plants constitutively overexpressing TFs. An experiment involving tobacco overexpressing the *Arabidopsis* DREB1A gene compared the effect of the CaMV 35S, a strong constitutive promoter, and *rd29A*, a stress-inducible promoter, on growth. Plants containing genes preceded by *rd29A* promoter had less negative growth effects while still producing cold and drought tolerant plants (Kasuga *et al.* 2004).

Osmotic stress signaling

Signal transduction pathways

Stress responses involve many signaling pathways and genes affected by different types of stresses often overlap with one another (Agarwal *et al.* 2006). Over 50% of the

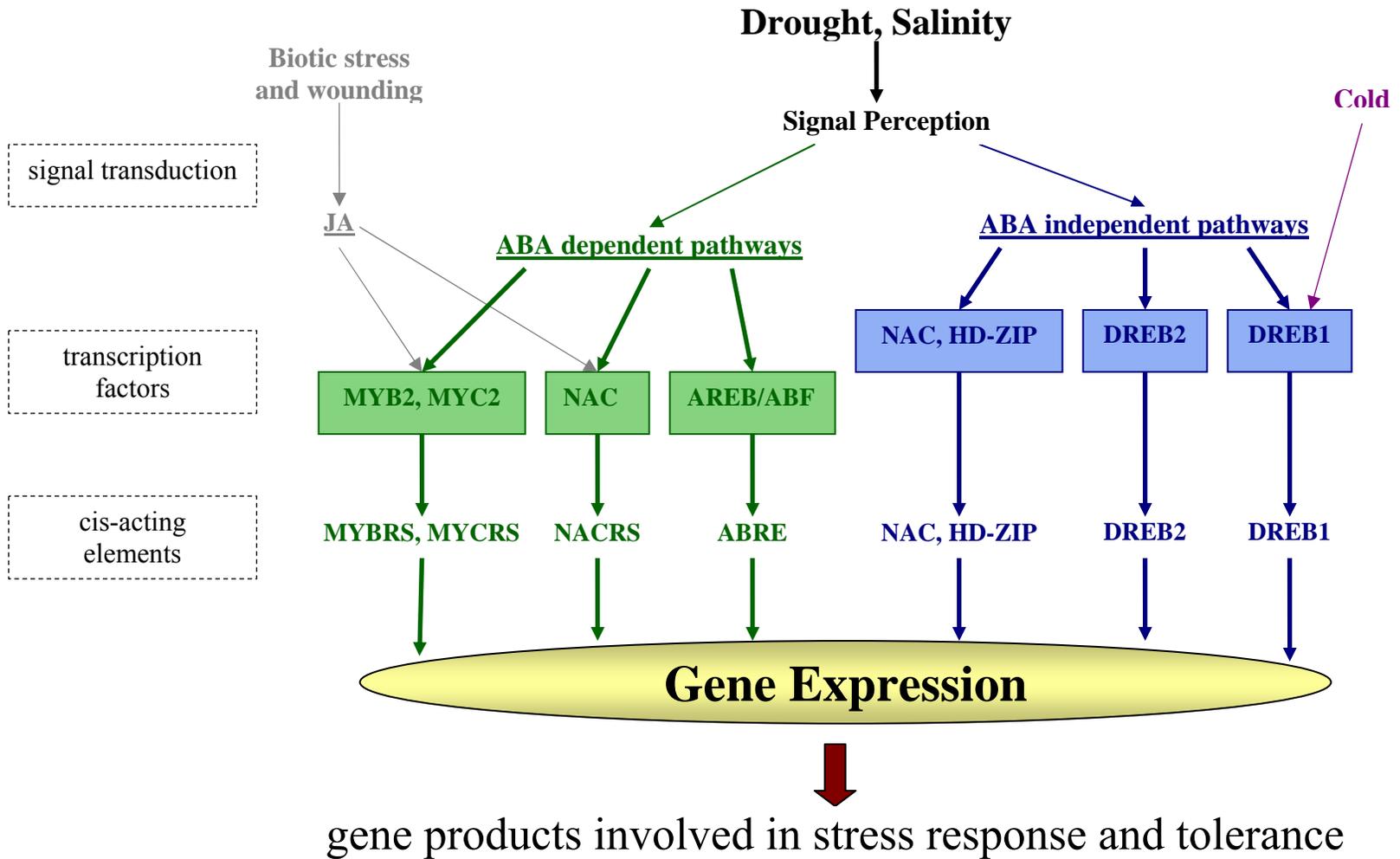


Figure 2.2 Abiotic stress signalling pathways. Adapted from (Shinozaki and Yamaguchi-Shinozaki 2007).

genes up-regulated in *Arabidopsis* following drought treatment were also present in plants treated with salt stress and/or ABA. Drought triggers ABA accumulation in plants, which initiates stomatal closure and the expression of stress-related genes. Several drought-inducible genes are activated by exogenous ABA treatment alone (Figure 2.2) (Shinozaki and Yamaguchi-Shinozaki 2007).

ABA-regulated transcription factors

At least six signal transduction pathways have been identified in drought, salinity and cold stress responses (Figure 2.2). Three of these are ABA-dependent and three are ABA-independent (Shinozaki and Yamaguchi-Shinozaki 2007). Many ABA-regulated genes contain a nucleotide sequence referred to as the ABA response element (ABRE). The basic domain/leucine zipper transcription factor (bZIP), known as ABA-responsive element binding protein 1 (AREB1), binds to this ABRE motif and initiates the expression of downstream genes. The eight genes that were singled out as being potential direct targets of this TF encode for LEAs and regulatory proteins (Fujita *et al.* 2005). MYB/MYC transcription factors also function through the ABA-dependent pathway. They target ABA- or JA-inducible genes as well as alcohol dehydrogenase production. Plants overexpressing both AtMYC2 and AtMYB2 were ABA-hypersensitive and were better able to tolerate osmotic stress (Shinozaki and Yamaguchi-Shinozaki 2007).

NAC transcription factor family

The NAC transcription factor family acts through both ABA-dependent and ABA-independent pathways and includes genes involved in plant development, pathogen response, and salinity tolerance. SNAC2, a rice TF, conferred drought tolerance when overexpressed. Many of the genes up-regulated in these transgenic plants were identified as producing proteins, such as peroxidase, ornithine aminotransferase, heavy metal-associated protein, sodium/hydrogen exchanger, and Hsps (Honghong *et al.* 2008).

DREB/CBF transcription factors

Dehydration-responsive transcription factors (DREB) and C-repeat binding factors (CBF) bind to a dehydration response element (DRE) in the promoters of certain cold and drought responsive genes (Wang *et al.* 2003). These ABA-independent transcription factors were named CBF/DREB1 and DREB2. CBF/DREB1 responds quickly to cold and plants overexpressing it were more resilient to freezing, dehydration, and salinity (Shinozaki and Yamaguchi-Shinozaki 2007). Early research in *Arabidopsis* and rice did not show greater drought tolerance in plants overexpressing DREB2. Later however, a maize homolog was identified and shown to produce two different transcripts. *ZmDREB2A* could be found as *ZmDREB2A-S* and *ZmDREB2B-L*, the former being the transcriptionally active form (Qin *et al.* 2007).

Candidate gene selection

Identification of osmotic potential QTL

To identify genes likely to control drought tolerance in *Populus*, quantitative trait loci (QTL) associated with osmotic potential needed to be located. A study looking at interspecific poplar pedigrees grown in contrasting environments succeeded in identifying two large-effect QTL for osmotic potential (Tschaplinski *et al.* 2006). The study was conducted on F₂ progeny family 331, produced from a cross between black cottonwood (*P. trichocarpa*; T) and eastern cottonwood (*P. deltoides*; D) grandparental clones, resulting in the two TD F₁ hybrid parents (53-246 ♀, 53-242 ♂). They were grown at a dry location in Boardman, OR and either watered daily or every other day (Tschaplinski *et al.* 2006). The same clones were also cultivated in a Clatskanie, OR, a wet site that doesn't require water or nutrient applications. Osmotic potential ranges were relatively narrow in grandparents and parents, but were much larger in the F₂ population. This study identified five QTL for osmotic potential, but few clones in this family had been mapped, meaning the mapping interval was too large for candidate genes to be found.

A similar analysis was conducted for a larger inbred TxD family 822 in Corvallis, OR. It identified two large-effect QTL which explained 43.6% and 32.1% of the phenotypic variation in osmotic potential. The large range of osmotic potential in F₂ individuals facilitated QTL identification and can be used to increase drought tolerance. The *P. deltoides* (D) allele showed a negative influence on osmotic potential, while the dominant *P. trichocarpa* (T) allele had a positive influence. Since low osmotic potential

signals drought tolerance, this indicates that substantial dehydration tolerance (low osmotic potential) was accorded to the F₂ offspring by the *P. deltoides* grandparent.

Narrowing down candidate gene list

After being identified in *Populus*, the stretches of DNA within these large-effect QTLs were searched for known genes. They contained more known genes than could realistically be examined. The candidate gene list was further narrowed down by finding genes up-regulated by water deficit in *Populus* and other species. In *Populus* this was done by subjecting two grandparent clones to drought and using NimbleGen's poplar whole genome microarrays to see which genes indicated up-regulation.

Candidate genes from other species were found by searching scientific literature. Twenty *Arabidopsis* genes were found that were either up-regulated by drought or shown to increase drought tolerance when overexpressed. The protein sequences of these genes were used to search the poplar genome for orthologs associated with the large-effect QTLs previously identified. Orthologs were found for six *Arabidopsis* drought tolerance transcription factors: DREB2A, DREB2B, AtMYB, AREB1/ABF2, MYB, and NAC (Table 2.1). These six genes made up the first round of drought tolerance genes for an accelerated domestication program.

Table 2.1 *Transcription factors selected for use in first round of the accelerated domestication program*

gene	species identified in	function
DREB2A	<i>Populus trichocarpa</i>	Encodes a transcription factor that distinctly binds to DRE/CRT cis elements, these respond to drought and low-temperature stress
DREB2B	<i>Populus trichocarpa</i>	Acts as a trans-acting factor in the signal transduction pathway under drought conditions
MYB	<i>Populus trichocarpa</i>	Encodes a MYB transcription factor possessing an R2R3 MYB DNA binding domain and is known to control the expression of salt- and drought-responsive genes
AtMYB	<i>Arabidopsis thaliana</i>	Encodes a MYB transcription factor possessing an R2R3 MYB DNA binding domain and is known to control the expression of salt- and drought-responsive genes
AREB1/ABF2	<i>Populus trichocarpa</i>	Leucine zipper transcription factor that binds to the abscisic acid (ABA) responsive element (ABRE) motif in the promoter of ABA-inducible genes
NAC	<i>Populus trichocarpa</i>	Encodes a NAC transcription factor whose expression is induced by drought, high salt, and abscisic acid

Chapter 3

Materials and Methods

Plant material

Transgenic constructs

The individual effect of six genes (DREB2A, DREB2B, AtMYB, AREB1/ABF2, MYB, and NAC) on growth, drought tolerance, and metabolism were examined. Each transgenic construct was represented by a single line (Table 3.1). Work regarding identification of candidate genes and transformation was not part of this drought study, it had been done previously. Under a subcontract, Arborgen LLC transformed three constructs of each gene into a *P. deltoides* clone. One construct had a strong, constitutive promoter in front of the gene, resulting in the gene being overexpressed at all times. Another construct used a drought-inducible promoter (RD29A), found to be up-regulated 3000-fold under mild drought, so that the gene would be overexpressed under stress conditions (T.J. Tschaplinski, personal communication, April 2010).

The third was a knockdown construct, in which the gene had reduced expression. Reduced expression was achieved through post-transcriptional gene silencing, also known as RNA interference (RNAi). This strategy uses a double stranded RNA trigger, two inverted copies of the target gene segment, behind a strong promoter to achieve sequence-specific gene silencing (Early *et al.* 2006; Hannon 2002).

Table 3.1 One line represented each transgenic construct used in the drought study.

<i>Gene and expression type</i>	<i>Line</i>
AREB1/ABF2 const. OE	534454
AREB1/ABF2 ind. OE	533001
AtMYB ind. OE	534338
AtMYB knockdown	534220
DREB2A const. OE	532675
DREB2A ind. OE	534831
DREB2A knockdown	534516
DREB2B const. OE	532967
DREB2B ind. OE	533557
DREB2B knockdown	534528
MYB const. OE	534354
MYB ind. OE	534476
NAC const. OE	534504
NAC ind. OE	533552
Vector Control	533584

The study also included two controls, a Vector control and an untransformed Wildtype *P. deltoides* clone. The Vector control was the same *P. deltoides* clone transformed with a vector lacking a target gene. It accounted for the effects that transformation itself may have had on the phenotype. The untransformed Wildtype is a proprietary clone of the Mead Westvaco Corporation and was the original background *P. deltoides* clone that was used in transformation. It provided a benchmark against which the Vector control could be compared. Individuals with basal stem diameters below 5 mm were excluded, because it would take too long for their pots to dry down to the point of drought stress. Due to availability and plant condition, some genes did not have adequate replicate numbers for all three constructs to be fully represented in this study.

All constructs and Wildtype clone were grown from tissue culture. After their arrival at Oak Ridge National Laboratory, they were transplanted into tall one-gallon pots in 2 parts Fafard 3B potting mix to 1 part perlite. The trees were approximately 8 months old by the beginning of the drought experiment and some were nearly 2 m tall.

Experimental design

The drought experiment was a completely randomized design (CRD) with factorial treatments and took place in the greenhouse at Oak Ridge National Laboratory. Differences in light exposure along the bench were initially believed to be a concern so a blocking factor was used to control light variation, but was later determined not to be a factor. Temperatures ranged between 63 and 74° F with 73 to 99% relative humidity.

The blocking was done by splitting the bench lengthwise to form two blocks with the most difference in terms of light levels. One hundred forty-five trees were assigned

to the two blocks, A and B. Replicate plants of each construct were randomly assigned to a block and to a treatment, either water or drought in such a way as to ensure there were equal treatment replicate numbers in each block. For example, construct 305 had 3 individuals assigned to drought and 3 assigned to water in block A. This construct had 2 replicate plants assigned to drought and 2 assigned to water in block B. Each construct had the same number of replicates assigned to drought as to water and as close to the same number as possible in each block. Extra plants from constructs with uneven numbers were placed in either block A or B, making sure to maintain an equal number of plants in each block. Due to the small number of replicates within each construct several constructs only had one treatment x block combination.

The plants were irrigated by spaghetti drip tubes inserted in each pot. Plants assigned to the watered treatment had these tubes inserted in the container substrate while drought plants had them removed. Plants assigned to the well-watered treatment were irrigated 3 times each day for 40 minutes at a time.

A t-test by treatment was used (SAS 1999) to determine whether there was a block effect. For droughted plants there was no difference in growth ($p=.972$) or leaf loss ($p=.399$) between blocks A and B. Watered plants also showed no difference between growth ($p=.881$) and leaf loss ($p=.081$) between blocks. Due to this absence of differences between blocks A and B, as well as the small number of replicates per construct the experiment was analyzed as a CRD.

Trees were taken through 4 drought cycles, each one ending when the droughted plants were shown to be under water stress, which was determined by using a Scholander-type pressure chamber (as described later in the chapter). At the end of each

drought cycle, all plants were irrigated with municipal water and received a liquid fertilizer solution made from 42 g of Jack's Multipurpose 20-10-20. Due to the evidence from the literature that trees become acclimated to drought, each successive cycle was one day longer than the previous one. Stomata respond more quickly to low water conditions in acclimated plants and it takes longer for them to become drought stressed again. The first cycle started July 30, 2009 and lasted 5 days. Plants were irrigated for two consecutive days following the end of a drought cycle. The next cycle began immediately afterwards. The leaf water potential (LWP) of all plants was measured at the end of the fourth drought cycle.

Leaf material collection

After the fourth drought cycle, trees were rewatered and 1 to 2 unexpanded apical leaves were collected mid-day for DNA expression and possible metabolite analysis. The plants were then taken through a fifth drought cycle and leaves were collected pre-dawn for osmotic potential analysis on constructs with significant differences in LWP between treatments, as determined using a t-test (Figure 3.1). Leaves were frozen on dry ice to stop physiological function of cells (Praba *et al.* 2009) and then stored in a -80°C freezer until they were analyzed.

Relative growth rate

Measuring biomass production

Productivity is an essential characteristic of a desirable feedstock. A low-yielding clone would be of little use as a plantation tree, regardless of how drought tolerant it



Figure 3.1 Collecting leaf samples. Leaves were collected before dawn for measuring osmotic potential and metabolite composition

proved to be. Growth rate was determined to show which constructs produced greater biomass under both watered and stress conditions. Two consecutive diameter measurements were taken at 5 cm above the soil surface at the beginning of the experiment and every two weeks thereafter to determine stem diameter relative growth rate (Merriam *et al.* 1995). Relative Growth Rate (RGR) is calculated as:

$$\text{RGR} = (\ln D_2 - \ln D_1) / (t_2 - t_1)$$

D_1 and D_2 are plant diameters at times t_1 and t_2 , and \ln is the natural logarithm.

Effect of tree size on RGR

RGR has been employed by forest researchers to determine growth differences resulting from various experimental treatments, allowing comparisons to be made between plants with unequal starting sizes. It has been used to measure the effect of a wide range of variables, including fertilization, soil moisture, CO₂, sulfur dioxide, ozone, and genotype (South 1995). RGR assumes that tree growth follows the compound interest law, that is, as a constant percentage of initial size. Difficulties may arise in situations where growth actually follows the variable interest law, which is when the percentage increase in biomass changes as tree sizes increase. RGR can be strongly correlated with tree size and many organisms show a declining RGR over time (South 1995). To minimize the effect of tree size on RGR, initial diameter was used as a covariate during the statistical analyses.

Leaf abscission

Eastern cottonwood (*Populus deltoides*) naturally occur in riparian habitats or other moist environments. For this reason it is not surprising that even mild water deficit can substantially reduce leaf growth and photosynthesis (Pallardy and Rhoads 1997). Poplar drought responses fall into the ‘avoidance’ category, meaning these trees prevent drought stress by increasing water uptake or by reducing transpiration. Water uptake is increased by favoring root growth over shoot growth and transpiration is minimized by closing stomata and shedding leaves.

The number of leaves (with a laminar length greater than 2 cm) on the main stem was assessed at the beginning of the study and every two weeks thereafter to determine the extent of leaf abscission. The number of leaves removed (for analyses, insect control, accidental damage etc...) was subtracted from the initial leaf number, before calculating the percentage of leaves that were lost. A construct with increased drought tolerance should have a lower degree of leaf loss in response to drought.

Leaf water potential

PMS pressure chamber

Drought stress was determined by using a Model 610 plant moisture stress (PMS) pressure chamber (PMS Instruments, Corvallis, OR) to measure the predawn leaf water potential (LWP) of fully expanded leaves. Leaves were collected before dawn and immediately put into the pressure chamber so that the petiole was protruding through the chamber lid (Figure 3.2). The cut end of the petiole was observed with the aid of a magnifying glass as pressure was gradually increased. The pressure at which water was



Figure 3.2 A hand lens was used to help identify the appearance of sap on the cut surface of the petiole protruding through the lid (From PMS Instrument Company)

first seen on the petiole surface was recorded. This equals the tension the water column was under when the leaf was removed (Cleary *et al.*, n.d.). Plants requiring high pressure levels for liquid to appear on petiole were under a greater degree of moisture stress, indicating they had low water potential. LWPs are at their highest before sunrise.

Drought tolerant plants maintain high LWP

Predawn LWP is considered indicative of the entire plant's water status and varies according to genotype (Praba *et al.* 2009). Comparisons between the responses of two *Eucalyptus* clones to water stress showed that the drought resistant CN5 clone maintained higher LWP (Costa *et al.* 2004). This CN5 clone had 25% more leaf expansion than the susceptible ST51 clone under moderate stress. The drought resistant clone also experienced less of a reduction in leaf growth under severe stress, 44% vs. 53% (Costa *et al.* 2004). Similar observations were made in experiments involving rice and wheat, with drought tolerant cultivars maintaining higher LWPs under stress.

Populus leaf abscission steadily increases as predawn LWP falls from 0 to -3 MPa, with near-total leaf abscission occurring at levels below -3 MPa (Pallardy and Rhoads 1997). Mild water stress begins with a predawn LWP around -0.5 MPa and becomes severe below -1.0 MPa. The proportion of plants experiencing complete leaf loss and suppression of new growth rises as predawn LWPs approach -2 MPa.

Determining length of drought cycle

Each drought cycle ended when plants were experiencing water stress. When droughted plants reached a predawn LWP of -2 MPa or began wilting, whichever came

first, they were considered stressed and were rewatered, marking the end of that drought cycle. All plants were on the same drought cycle, so if a single droughted plant was experiencing stress, the cycle ended for all plants. At the end of the fourth cycle the predawn LWP of all plants was measured and samples for osmotic potential and metabolite profiles were collected, as described below. Each construct was then compared to the vector control to see if they responded differently to drought. The vector control was compared to the Wildtype, nontransformed control.

Osmotic potential

Osmotic potential of greenhouse-grown plants

Tree species from xeric ecosystems have low leaf osmotic potentials even when they have access to plenty of water (Gebre *et al.* 1998). Comparing the osmotic potential under normal conditions to that of the lines when they are under drought stress will enable an assessment of how each transgene affects drought tolerance. Lower osmotic potential indicates increased drought tolerance. Since these genes were selected based on osmotic potential QTL, their expression level will likely affect osmotic potential. Under watered conditions, constitutively overexpressed (OE) constructs are likely to have lower osmotic potential than knockdown and inducibly overexpressed (OE) constructs. Under drought conditions the constitutively and inducibly overexpressed constructs will likely be similar to each other, but lower than the knockdown construct. The caveat is that these greenhouse-grown plants must be able to achieve osmotic potentials that are evident under field conditions with high solar radiation. This has not been the case thus far, where the greenhouse-grown plants typically have had much

higher osmotic potentials than field-grown plants (T.J. Tschaplinski, personal communication, June 2009).

LWP determines which constructs to measure

At the end of the fourth dry-down cycle, the seventh fully expanded leaf from the shoot apex was collected from each plant before dawn. They were placed on dry ice to prevent changes in osmotic potential or cell damage and then put into labeled Ziploc bags and stored in a -80 C freezer until measured. Due to the narrow range of osmotic potentials observed in most greenhouse grown plants, only constructs varying in the differential between watered and droughted predawn LWP were analyzed. Osmotic potential was determined using the osmolality, solute concentration, measured by the VAPRO vapor pressure osmometer (Model 5520, Wescor Inc., Logan, UT).

VAPRO Protocol

Leaves were removed from the freezer and thawed on ice, one at a time. Leaf tissue, excluding the midrib, was rolled and inserted into a leaf press. Approximately 10 microliters of liquid were expressed onto a solute-free paper disc and inserted into the vapor pressure osmometer. The wire thermocouple above the sample in the chamber estimates osmolality in mmol/kg based on the dew point temperature, the temperature at which condensation no longer occurs (Wescor, n.d.). Osmolality (mmol/kg) was converted to osmotic potential (MPa) using the van't Hoff relationship at 25°C:

$$\text{osmotic potential} = -(8.314 * 10^{-6}) * 298 * \text{osmolality}$$

The machine was calibrated prior to measuring leaves that day, as explained in the user's manual (Wescor 2004). The metal surface, onto which the paper discs were placed, was wiped down with 70% ethanol between samples to remove sap residue.

Metabolite analysis

Osmotic adjustment

Osmotic adjustment helps plants survive dry spells, it occurs when plants lower their osmotic potential by accumulating solutes (Gebre *et al.* 1998). It is calculated as the difference between a construct's osmotic potential under dry and wet conditions (Tschaplinski *et al.* 2006). There is a positive correlation between osmotic adjustment and grain yield under greenhouse and field conditions. The observed increase in solute concentration maintained turgor and processes necessary for growth in wheat exposed to moderate and severe drought stress (Praba *et al.* 2009). Osmotic adjustment is considered a possible selection criterion in breeding drought tolerant crops.

If any of the constructs had indicated a large degree of osmotic adjustment between droughted and control plants, metabolites would have been analyzed to determine which metabolites were being accumulated to account for the difference. In addition to those used for LWP measurement, leaves were collected predawn for metabolite analysis. Plant tissue was put on dry ice immediately following harvest to prevent post harvest changes in metabolite levels (Robinson *et al.* 2005). Compounds present in leaves would be identified and quantified using an Agilent 5972A gas-chromatograph/mass-spectrometer (GC/MS).

Separating molecules and identifying composition

GC reliably separates a sample into its components, but is unable to identify specific substances. Mass spectrometry (MS), on the other hand, provides a spectral output of its constitution. This output is compared to mass spectra of known compounds to identify the substance (Douglas, n.d.). A GC/MS readily detects compounds, such as organic acids, fatty acids, soluble carbohydrates, amino acids, and phenolic compounds in a quantitative manner, allowing the determination of their concentrations in plant tissue.

Metabolic profiling can help determine gene function by revealing how metabolite levels vary between overexpressed and down-regulated constructs of the same transgene. Plants containing an up-regulated construct of a putative drought tolerant gene are thought to have greater metabolite concentrations, and hence increased drought tolerance. The compounds that accumulate under stress versus well-watered conditions can show which metabolic pathways are involved in the drought response. In plants shown to be drought tolerant, it would clarify the mechanisms by which tolerance is achieved in *Populus*.

Gene expression

Reverse transcription PCR

Validation is important in studies involving differential gene expression. It's done by quantifying the level of a gene transcript of interest (Jawdy 2006). These steady-state mRNA levels can be quantified using reverse transcription polymerase chain reaction (RT-PCR) (Freeman *et al.* 1999). After isolation, RNA is reverse transcribed

into a complementary DNA copy (cDNA) that can be multiplied exponentially in a PCR reaction. These amplification products are detected and quantified during the last step of in RT-PCR (Freeman *et al.* 1999).

Real-time RT- PCR

Real-time RT-PCR is unique because it measures the amount of amplified PCR product and each cycle of the reaction, rather than just at the end (Gachon *et al.* 2004). This is possible because a fluorescent, DNA-binding dye (SYBR Green) is added to the solution. This dye fluoresces when bound, giving visible estimates of how much amplified DNA is present at the end of each cycle (Bustin 2000).

Plants respond to various stresses, such as drought, by altering gene expression and quantifying this expression is an important part of defining gene function (Bustin 2000). RT-PCR is used to evaluate differences in gene expression between transgenic crops and the wildtype. It was used to determine differences in transgene copy number from multiple lines of transgenic maize (Rudenko *et al.* 2004).

Chapter 4

Results and Discussion

Vector control plants

Large variability resulting from low sample size is believed to have prevented significant differences from being detected. Small sample size increases variability, making it difficult to detect differences that truly exist. To increase this experiment's power, gene expression results of already analyzed constructs were assessed to identify any constructs that could be pooled with the Vector control. The Vector control was a clone transformed with an empty vector to account for the effects that transformation itself may have had on the phenotype. All constructs were compared to the Vector control to determine the effect of each on productivity and drought tolerance.

Gene expression analysis had been carried out on half of the transgenic constructs. DREB2A ind. OE, DREB2A knockdown, and AtMYB ind. OE did not show the intended altered expression, but only AtMYB ind. OE was pooled with the Vector control plants. In addition to similar gene expression, AtMYB ind. OE closely resembled Vector control plants in terms of RGR, leaf loss, and LWP. The response of the other two constructs differed quite a bit from that of the Vector control, making it possible that their expression had been altered just enough to affect downstream genes but not enough to be statistically different. A small increase in expression may be enough to trigger a cascade of downstream responses (T.J. Tschaplinski, personal communication, April 2010).

Growth

Growth rate considerations

Relative growth rate is used here to minimize any size-related growth differences. It operates on the assumption that tree growth is a constant percentage of initial size, similar to the compound interest law (South 1995). In many cases however, a plant's percentage of biomass increase changes according to overall plant size, generally declining as the plant becomes larger. In these cases, RGR is strongly correlated with tree size and no longer follows the compound interest law (South 1995).

Relationship between initial diameter and RGR

RGR was highest in the smallest plants, decreasing in trees with a larger stature (Figure 4.1). These were often knockdown constructs that in a previous study had appeared to have considerably lower growth rates than other expression types (Figure 4.2). Due to low biomass production in a previous growth trial, the three knockdown constructs, DREB2B, DREB2A and AtMYB began the drought experiment with considerably smaller statures (less than 8mm diameter). DREB2A const. OE was the only overexpression construct that began the experiment with a diameter less than 8mm.

RGR in this previous growth trial was calculated slightly differently. It used each trees' volume index, commonly used to estimate biomass, which is the product of the stem diameter squared and height of each tree (Scarascia-Mugnozza *et al.* 2000).

Although RGR was calculated slightly differently in the previous growth trial,

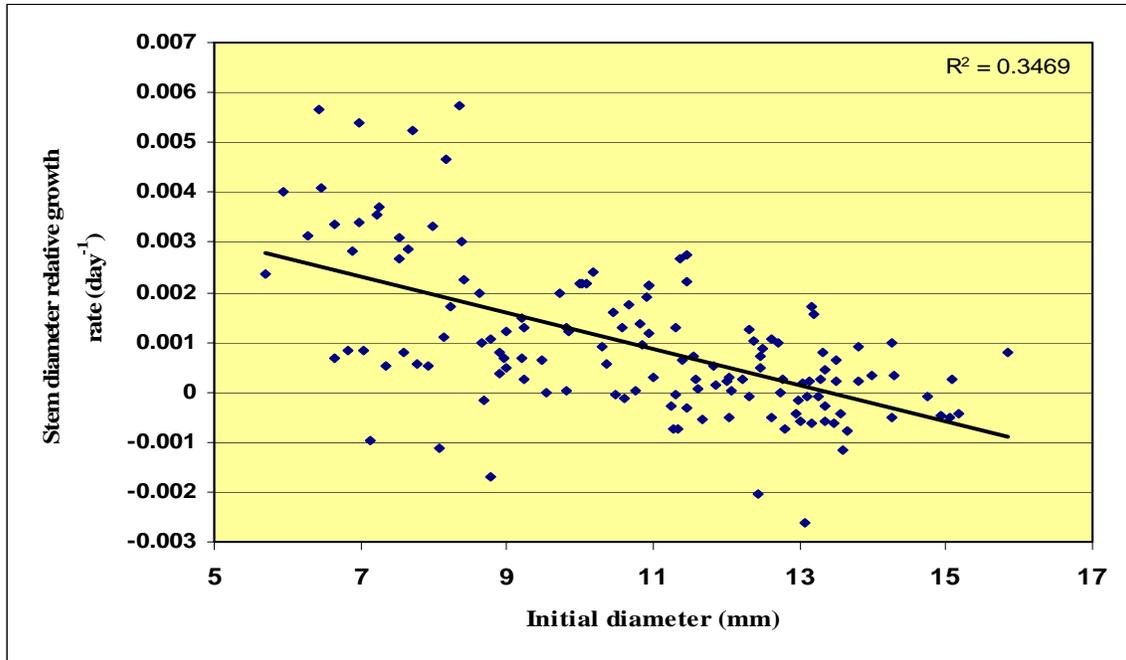


Figure 4.1 Smaller plants had the highest RGR, with the largest plants growing very little or in some cases not at all

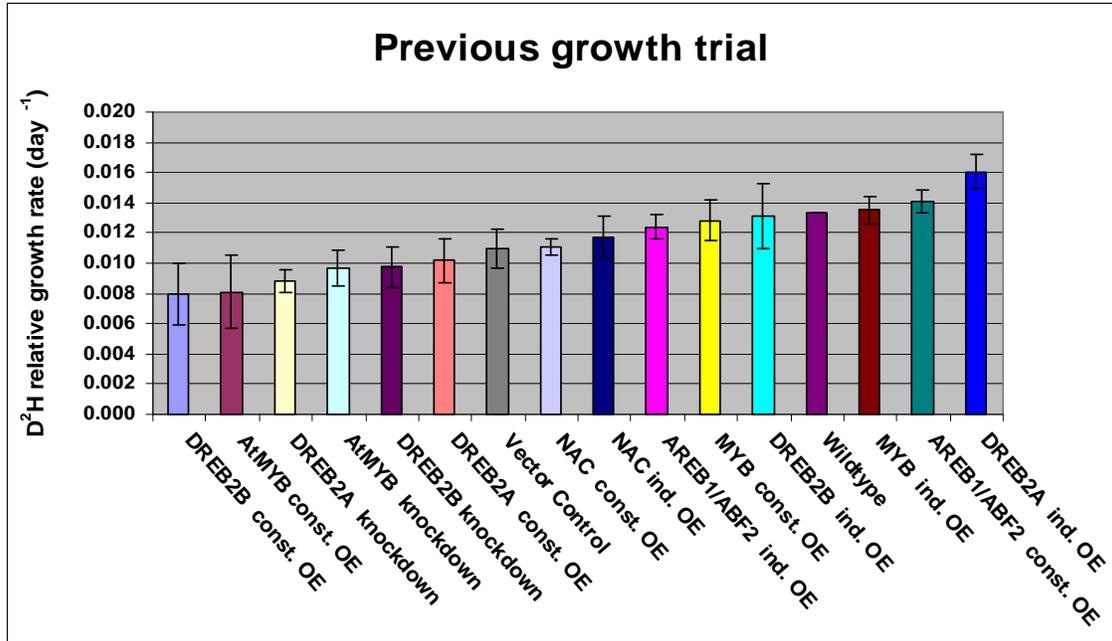


Figure 4.2 *D²H* relative growth rate of each construct averaged across lines during a previous growth trial.

comparisons can still be made with RGRs from the drought experiment, because a single growth rate calculation was used within each study.

Based on RGR alone, it would have seemed that DREB2B, DREB2A, and AtMYB knockdown and DREB2A const. OE were the most productive constructs. Under irrigated conditions these four smallest constructs had among the highest growth rates (Figure 4.3). Under drought conditions the DREB2A and DREB2B knockdown constructs had the highest growth rate, whereas AtMYB knockdown and DREB2A const. OE showed median RGRs (Figure 4.4). Similarly, constructs with large mean starting diameters, such as DREB2B ind. OE grew very little. A few constructs are shown having negative growth rates. Growth was so minimal in these constructs, close to zero, that this negative growth rate is within the standard deviation of the mean. Some constructs with negative RGR under drought may have actually contracted their tissues in response to the lack of water.

Statistical analysis

A correlation was run (JMP 8) and a strong negative relationship was shown to exist between initial diameter and RGR ($r = -0.606$). Initial diameter was used as a covariate in the growth analysis to account for differences in plant size. NAC ind. OE and DREB2B knockdown were dropped from the analysis because of very low sample sizes. These constructs had 3 and 4 total observations respectively, with only one drought replicate each. Lack of replication makes it impossible to effectively study the effect of drought on these constructs. Low sample size was due to lack of plant material at the start of the experiment, mortality, and data omission due to failure of some drought

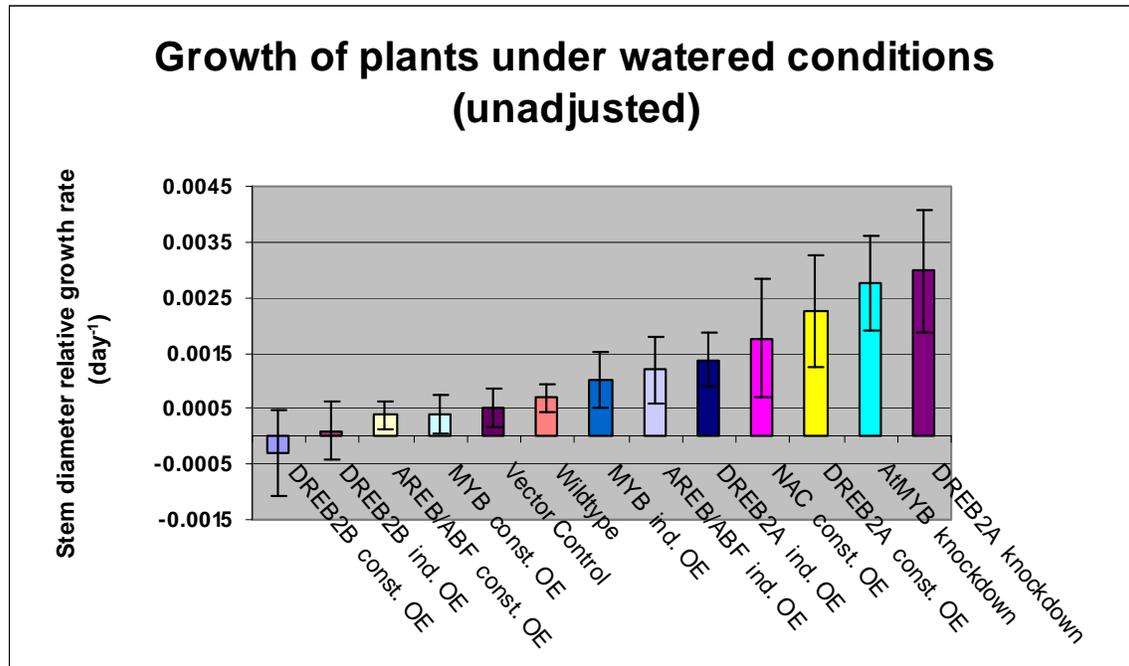


Figure 4.3 Stem diameter relative growth rate of Wildtype and 11 transgenic constructs of well-watered eastern cottonwood plants over a 34 day period. These growth rates are not adjusted to account for the effect of initial diameter on growth.

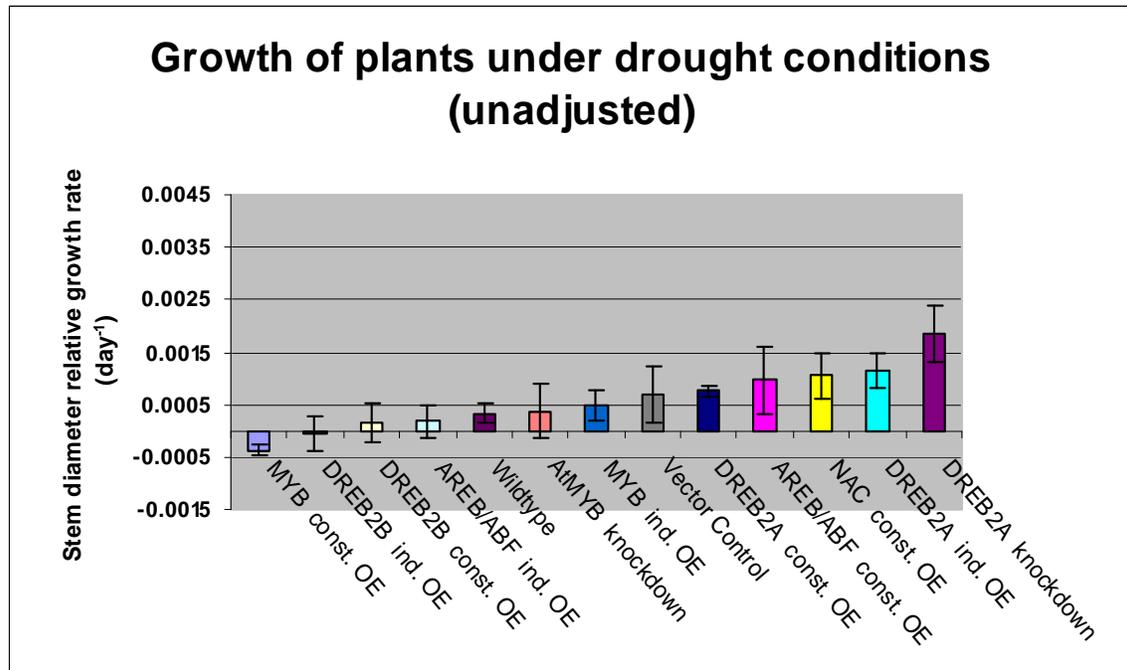


Figure 4.4 Stem diameter relative growth rate of Wildtype and 11 transgenic constructs of eastern cottonwood plants taken through 4 drought cycles over a 34 day period. These growth rates are not adjusted to account for the effect of initial diameter on growth.

plants to dry out. After removing constructs with excessively small sample sizes each construct ranged between 7-10 observations.

RGR was analyzed as a CRD with a covariate and factorial treatments using mixed models (SAS 1999). There was a significant interaction between initial diameter and treatment, indicating that for growth, the slope of the covariate differed between watered and drought conditions. The covariate of each treatment must have parallel slopes in order to be valid. To address this issue, the RGR of drought and watered plants were analyzed separately, as a CRD with a covariate, using mixed models (SAS 1999).

Growth under watered conditions

Initial diameter had a significant effect on RGR under watered conditions ($p < 0.0001$), but RGR did not significantly differ among constructs ($p = 0.254$). It is believed that the failure to detect significant differences was due to low sample size of transgenic plants, which resulted in low power. Small sample size increases variability, increasing the standard error and increasing the likelihood of making a type II error, which is failure to detect differences that truly exist. A sign of this test's low power is that mean separation shows standard errors that are very large compared to the means. Although the standard errors had a fairly narrow range most of them were greater than 50% of the mean. The most extreme case was DREB2A const. OE which had a standard error that was 90% of the mean.

Adjusting for the covariate minimized the growth differences between constructs. The highest RGRs were of DREB2A ind. OE (0.00164), MYB ind. OE (0.00161), and Wildtype (0.00149) (Figure 4.5). In a previous growth trial DREB2A ind. OE had the

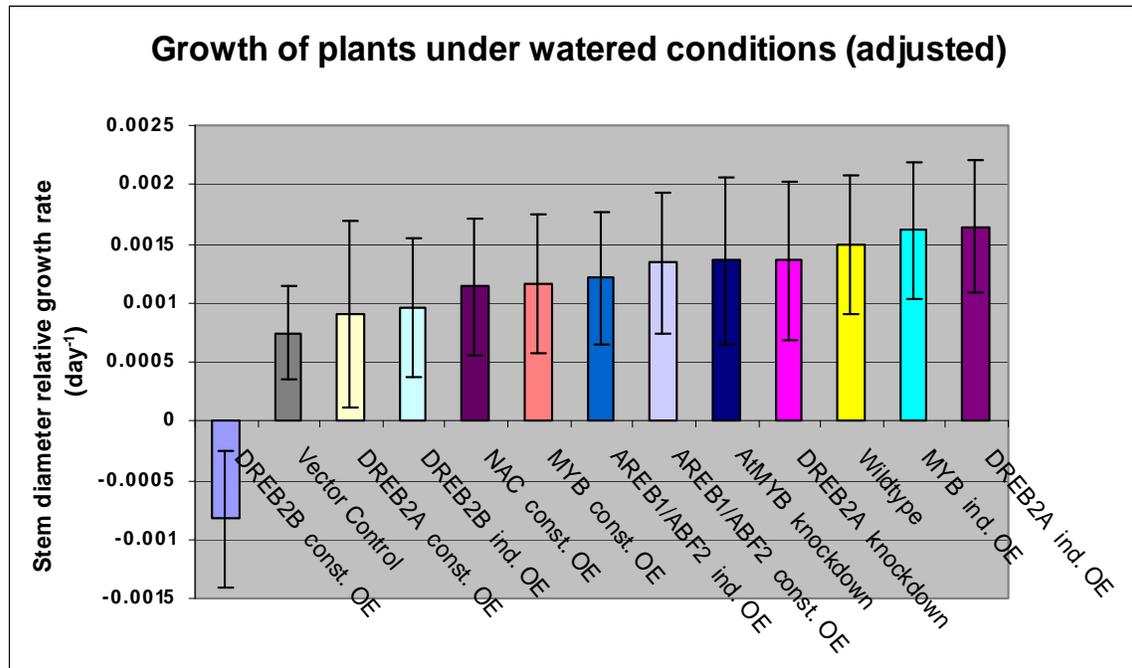


Figure 4.5 Stem diameter relative growth rate of well-watered Wildtype and 11 transgenic constructs of eastern cottonwood plants over a 34 day period. These growth rates are adjusted to account for the effect of initial diameter on growth. Growth did not differ significantly between constructs.

highest RGR averaged across lines (Figure 4.2). MYB ind. OE and Wildtype also had among the highest growth rates in the previous growth trial.

Surprisingly, the next highest RGRs belonged to the two knockdown constructs, DREB2A (0.00135) and AtMYB (0.00134). These constructs began the experiments with the smallest diameters due to their low productivity in the previous growth trial. It is possible that using initial diameter as a covariate doesn't completely address the decreased growth rate of larger trees. Another possibility is that they are not actually experiencing reduced gene expression. Since these drought tolerance transcription factors are likely involved in many physiological functions, reducing their expression should have a negative impact on growth and development. The DREB2A and AtMYB knockdown constructs, chosen for the drought experiment, had RGRs approximately 50% larger than the other lines in their respective constructs. RT-PCR analysis showed that the DREB2A knockdown line used actually was not experiencing reduced expression (Table 4.1). It is not known whether or not the other lines of this construct had reduced DREB2A expression. Expression analysis has not yet been completed on the AtMYB knockdown construct.

Only one overexpression construct, DREB2B const. OE, had lower RGR than the control. In the previous growth trial DREB2B const. OE averaged across lines also had the lowest growth rate of all constructs. This may be partly due the negative impact constitutive overexpression often has on growth (Hsieh et al 2002). It is not clear why DREB2B would have more of an impact on growth, when constitutively overexpressed, than the other genes, but it may be related to gene function.

Table 4.1 Results of RT-PCR expression analysis were available for some constructs included in the drought experiment.

<i>Construct</i>	<i>Target gene expression (in relation to Vector control)</i>
AREB1/ABF2 const. OE	overexpressed
AREB1/ABF2 ind. OE	induced under drought
DREB2A const. OE	overexpressed
DREB2A ind. OE	not induced
DREB2A knockdown	not reduced
DREB2B knockdown	not reduced
AtMYB ind. OE	not induced (pooled with Vector control)
NAC const. OE	overexpressed

Growth under drought conditions

A square root transformation with a transvalue of 0.00262 was used in the drought analysis to correct unequal variance. Means and standard errors for growth under drought conditions listed here are back-transformed and have been adjusted for differences in initial plant size. Initial diameter had a significant effect on RGR under drought ($p < .0001$). Although construct also had a significant effect on RGR of droughted plants ($p = 0.023$), only constructs differing from the control were of interest. The Least Significant Difference (LSD) mean separation table shows that three constructs differed from the vector control at the 0.05 significance level (Table 4.2).

The Vector control had a RGR of 0.0005. AREB1/ABF2 const. OE and DREB2A ind. OE had significantly higher growth rates than the control under drought with 0.00170 and 0.00162, respectively. With a RGR of -0.0007, AtMYB knockdown was less productive than the vector control. Under drought, MYB ind. OE and Wildtype still had among the highest RGRs of all constructs, although these differences were not statistically significant (Figure 4.6).

Mean growth of MYB ind. OE under drought was 25% less than growth when watered, .0016 vs. .0012, but it had the third highest RGR. Wildtype had the fourth highest growth rate, regardless of treatment, 0.00149 and 0.00116, under irrigated and drought conditions, respectively. Vector control's RGR was low relative to other constructs under irrigated conditions and median under drought treatment. Its low productivity relative to the Wildtype could indicate that transformation itself had a negative effect on growth.

. Table 4.2 Mean separation table for RGR under drought conditions indicates some of the constructs differ from the Vector control. Constructs followed by the same letter are not significantly different from one another.

<i>Gene and expression type</i>	<i>Adjusted RGR (x1000)</i>	<i>Letter group</i>
AREB1/ABF2 const. OE	1.70 (+/- 0.52)	A
DREB2A ind. OE	1.62 (+/- 0.45)	AB
MYB ind. OE	1.20 (+/- 0.44)	ABC
Wildtype	1.16 (+/- 0.44)	ABC
DREB2B ind. OE	0.98 (+/- 0.50)	ABCD
DREB2A knockdown	0.53 (+/- 0.43)	ABCD
Vector Control	0.50 (+/- 0.27)	CD
MYB const. OE	0.40 (+/- 0.44)	BCDE
AREB1/ABF2 ind. OE	0.37 (+/- 0.37)	CDE
NAC const. OE	-0.02 (+/- 0.38)	CDE
DREB2B const. OE	-0.24 (+/- 0.37)	DE
DREB2A const. OE	-0.42 (+/- 0.40)	DE
AtMYB knockdown	-0.71 (+/- 0.37)	E

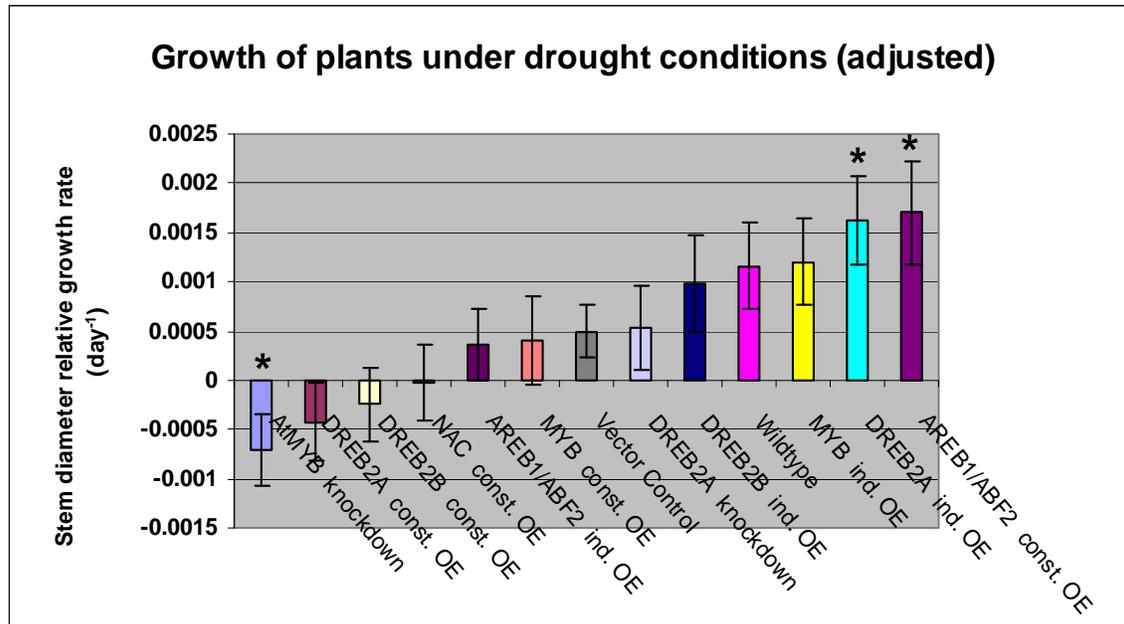


Figure 4.6 Stem diameter relative growth rate of Wildtype and 11 transgenic constructs of eastern cottonwood plants taken through 4 drought cycles over a 34 day period. These growth rates are adjusted to account for the effect of initial diameter on growth. RGR of constructs that differed from the Vector control are designated with an asterisk. *AREB1/ABF2 const. OE*, *DREB2A ind. OE* grew more than the Vector control while *AtMYB knockdown* grew less.

AREB1/ABF2 const. OE had the highest RGR of all constructs under drought. Its RGR was actually 22% higher under drought, but since the differences between drought and watered RGR were less than the standard errors, they are statistically equal. DREB2B const. OE, DREB2B ind. OE, AtMYB ind. OE and Wildtype were other constructs appearing to have higher growth rates under drought. These differences were also not significant, meaning that statistically these constructs grew the same under both treatments.

Small sample size was a concern under watered conditions and is believed to have contributed to the low power of this analysis as well, leading to few significant differences being detected. Small sample size increases variability which inflates the standard error and increases the likelihood of making a type II error. Most constructs had back-transformed standard errors over 50% of the means. The standard errors of DREB2B const. OE, NAC const. OE, and MYB const. OE were actually greater than their means. AREB1/ABF2 const. OE and DREB2A ind. OE had the lowest standard errors relative to their means, 31 and 28% respectively. Having standard errors that are large compared to the means is a sign of low power.

Leaf Abscission

Initial diameter influenced leaf loss

Overall size appeared to influence how drought-stressed plants became. This was observed by the pots of smaller drought plants not drying out completely and was thought to affect the amount of leaves lost overall. More leaf loss was expected in larger trees. A correlation was run (JMP 8) and a moderate positive relationship was found between

initial diameter and leaf loss ($r=.218$). Initial diameter was used as a covariate in the leaf abscission analysis to account for differences in plant size and differences in drought stress severity. It is assumed that larger plants experienced more severe drought stress than smaller plants, because of their greater demand for water.

Statistical analysis

NAC ind. OE and DREB2B knockdown were not included in this analysis, because their low sample sizes made it impossible to effectively study the effect drought has on these constructs (explained above). Leaf abscission was initially analyzed as a CRD with initial diameter as a covariate and drought and water as factorial treatments, using mixed models (SAS 1999). Similar to the initial RGR analysis there was a significant interaction between initial diameter and treatment, resulting in unequal covariate slopes. This indicates that initial diameter affected leaf loss differently according to what treatment was applied. Each treatment must have parallel covariate slopes in order for this statistical model to be valid. To address this leaf abscission of drought and watered plants was analyzed separately, as a CRD with a covariate, using mixed models (SAS 1999).

Leaf abscission under drought

A power transformation was used with a transvalue of 2 to correct unequal variance. All means and standard deviations for percent leaf loss listed here have been back-transformed. Initial diameter had a significant effect on leaf abscission ($p<.0001$). Construct also had significant effect on leaf abscission ($p=0.003$), meaning that not all

constructs experienced the same rate of leaf loss under drought. Standard errors for percent leaf abscission were quite variable, but most were relatively small. They averaged 10 to 20% of the mean. AREB1/ABF2 const. OE was the exception, with a standard error 162% of the mean.

The LSD mean separation table shows that two constructs differed from the vector control at the 0.05 significance level. The DREB2A knockdown construct experienced significantly higher rates of leaf loss than the control, 90% compared to 68% (Figure 4.7). The other knockdown construct, AtMYB, also had a larger mean leaf abscission than the Vector control, but this difference was not statistically significant. It is not clear why DREB2A knockdown had a high rate of leaf abscission, given the fact that it didn't actually have reduced expression (Table 4.1). The tolerance mechanisms activated by the DREB2A transcription factor under drought should still have been functioning in these plants.

Wildtype and NAC const. OE had the second and third highest rates of leaf loss, 87%.and 76% respectively. The NAC const. OE construct's lack of growth under drought is likely due to the fact that it lost such a large proportion of its leaves. Fewer leaves results in less biomass produced.

The other construct that differed from the Vector control was AREB1/ABF2 const. OE. Its 20% leaf loss was the lowest rate of leaf abscission under drought (Figure 4.7). Since percent leaf loss was being used to assess each construct's drought tolerance, it appeared that this construct had a greater drought tolerance than the Vector control. DREB2A ind OE had the next lowest mean percentage leaf loss, but this was not significantly different from the control.

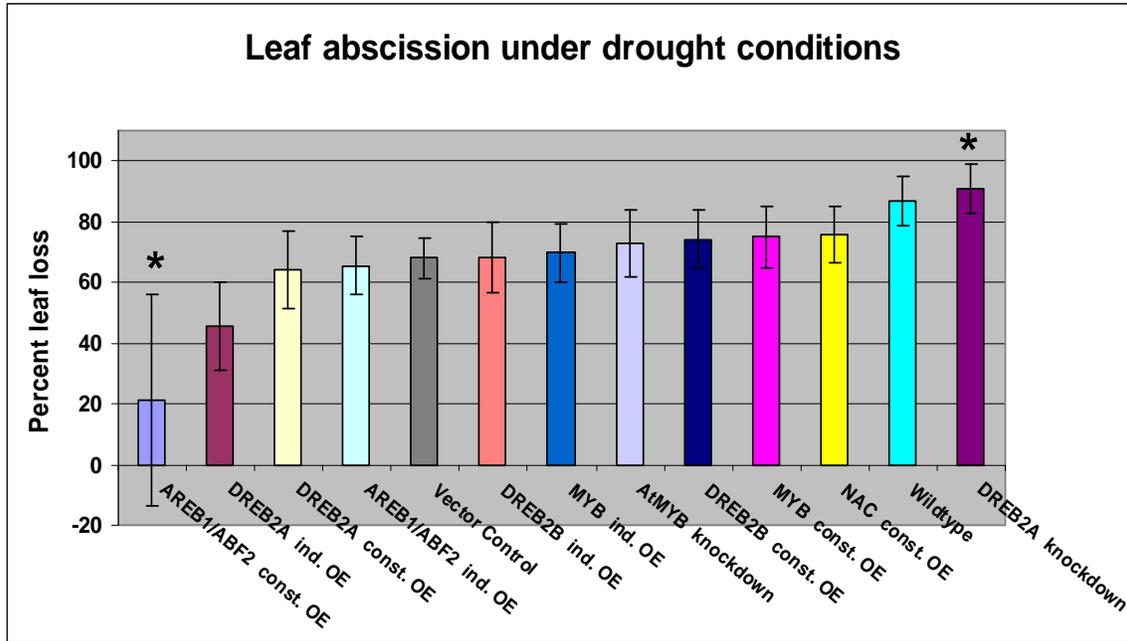


Figure 4.7 Percent leaf loss of Wildtype and 11 transgenic constructs of eastern cottonwood plants taken through 4 drought cycles over a 34 day period. These leaf loss percentages are adjusted to account for the effect of initial diameter on leaf loss. Percent leaf abscission of constructs designated with an asterisk differed significantly from the Vector control. AREB1/ABF2 const. OE, DREB2A ind. OE lost fewer leaves than the Vector control while DREB2A knockdown lost more.

Percent difference leaf loss

To get a better idea of how each construct was affected by drought, the percent difference was calculated (Street *et al.* 2006). This is beneficial because it takes into account that some constructs had high levels of leaf abscission, even when fully watered. Irrigated replicates of DREB2A ind. OE and AtMYB ind. OE lost over 20% of their leaves, but the overall mean leaf number of the AtMYB knockdown actually increased over the course of the experiment. The percentage increase in leaf abscission under drought is the difference between leaf loss of droughted plants and mean leaf loss of watered plants expressed as a percentage of mean leaf loss when watered. It was calculated using:

$$\% \text{ change} = (\text{drtLL} - \text{wtrLL}) / \text{wtrLL} \times 100$$

where drtLL denotes leaf loss under drought and wtrLL stands for mean leaf loss under watered conditions.

Leaf loss percentage differences were analyzed with mixed models using initial diameter as a covariate. A rank transformation was used to correct for unequal variance and non-normal data. Percentage change in leaf loss was shown to vary according to construct ($p=0.009$), but the LSD mean separation table indicated that Wildtype and MYB const. OE were the only constructs that differed significantly from the Vector control. Leaf abscission under drought for Wildtype and MYB const. OE were 49 and 9 times greater, respectively, than leaf abscission of irrigated replicates in each construct.

The Vector control only experienced a 3-fold increase in leaf abscission under drought (Figure 4.8). Although not statistically significant, AtMYB knockdown had the second highest increase, with a 20-fold increase in drought abscission. Mean increases in drought leaf abscission for AREB1/ABF2 const. OE (92%) and DREB2B ind. OE (167%) were smaller than for the Vector control (279%).

Possible xylem cavitation in MYB ind. OE

MYB ind. OE plants stood out after the first drought cycle, because they were the second largest construct in the study, but none were wilting or showing drought stress. By the time Wildtype plants of corresponding size had completely necrotic leaves, MYB ind. OE trees still looked fine (Figure 4.9). This construct's apparent drought tolerance was no longer visible in the second drought cycle. It is possible that the suddenness and intensity of the first drought cycle resulted in these large plants developing xylem cavitation. *Populus deltoides* are very susceptible to developing embolisms. Cavitation would have prevented these trees from taking up water when they were irrigated between drought cycles. Tolerance was not observed in these plants as the experiment progressed, indicating that serious damage may have resulted from the first drought cycle. MYB ind. OE plants remained one of the most productive constructs, but their medium rate of leaf abscission doesn't identify them as remarkably drought tolerant.

Xylem cavitation occurs when water in xylem vessels is under such great tension that dissolved air expands and forms an embolism. This can lead to branch dieback, which increases a tree's drought resistance by reducing the transpirational load

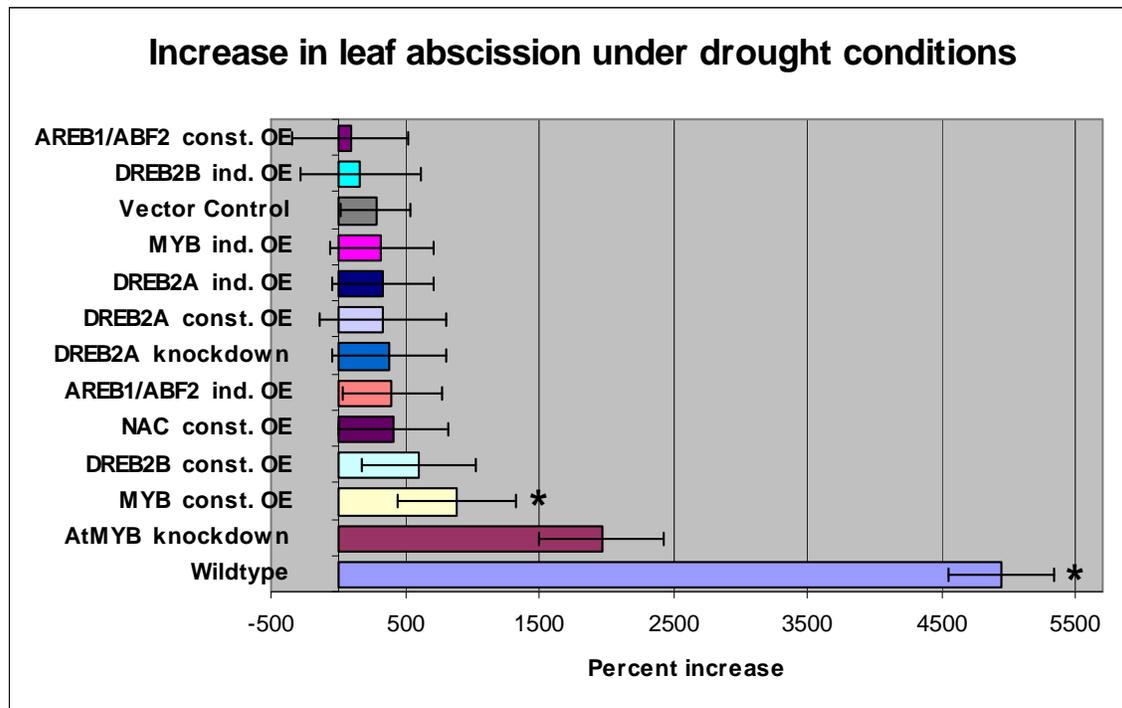


Figure 4.8 Mean percent difference of leaf loss of Wildtype and 11 transgenic constructs of eastern cottonwood plants under drought and watered conditions over a 34 day period consisting of 4 drought cycles. Constructs designated with an asterisk differed significantly from the Vector control. Wildtype and MYB ind. OE had a greater percent increase in leaf loss under drought than the Vector control..



Figure 4.9 At the end of drought cycle one MYB ind. OE (left) of similar stature to Wildtype (right) had not begun showing signs of drought stress.

(Rood *et al.* 2000). This mechanism for achieving drought resistance is undesirable for feedstocks because it reduces productivity.

Leaf Water Potential

Drought tolerant plants maintain high LWP

Predawn LWP is considered indicative of the entire plant's water status and varies according to genotype, with drought tolerant genotypes maintaining higher LWPs under stress (Praba *et al.* 2009). Pallardy and Rhoads (1997) stated that *Populus* leaf abscission increases as predawn LWP falls from 0 to -3 MPa, with near-total leaf abscission occurring at levels below -3 MPa. In this case, however, plants began wilting and lost the majority of their leaves earlier. Stress was believed to have begun around LWPs of -1 MPa, and trees became almost entirely necrotic at LWPs of -2 MPa.

Treatment differences

At the end of the fourth cycle the predawn LWP of all plants was measured. NAC ind. OE and DREB2B knockdown were not included in this analysis, because they lacked replication in the drought treatment, making it impossible to evaluate the effect drought had on these constructs (explained previously). An independent t-test was used to determine whether the LWP of each construct differed between treatments (Table 4.3).

Difference LWP

To better understand how each construct responded to drought, the change in

Table 4.3 LWP was measured at the end of drought cycle 4 to see whether droughted plants were experiencing stress. A significant difference ($P \leq 0.05$) in LWP between treatments indicates they were stressed.

gene & expression type	leaf water potential (MPa)		Did LWP differ by treatment? ($P \leq 0.05$)
	water	drought	
AREB1/ABF2 const. OE	-0.48	-0.52	no
AREB1/ABF2 ind. OE	-0.58	-0.95	no
AtMYB knockdown	-0.39	-0.57	yes
DREB2A const. OE	-0.38	-1.01	no
DREB2A ind. OE	-0.52	-0.98	yes
DREB2A knockdown	-0.40	-1.30	no
DREB2B const. OE	-0.43	-1.31	yes
DREB2B ind. OE	-0.53	-1.49	yes
MYB const. OE	-0.46	-1.63	yes
MYB ind. OE	-0.50	-1.10	no
NAC const. OE	-0.46	-0.98	no
Vector control	-0.51	-1.16	yes
Wildtype	-0.50	-2.10	yes

LWP was calculated by subtracting mean LWP under irrigated conditions from LWP under drought for each construct. It was calculated using:

$$\text{change} = \text{drtLWP} - \text{wtrLWP}$$

where drtLWP stands for leaf water potential under drought and wtrLWP stands for mean leaf water potential under watered conditions.

LWP differences were analyzed with a one-way Analysis of Variance. LWP changes did not vary according to construct ($p=0.07$), which means that all constructs experienced the same magnitude of change between their LWP under irrigated and drought conditions. Wildtype showed the most dramatic change with a 1.61 MPa decline under drought, with MYB const. OE having the next largest at 1.17 MPa (Figure 4.10). The 0.04 MPa decline observed in AREB1/ABF2 const. OE was lowest of all the constructs.

As in the previous analyses, low sample size increased variability and made it more difficult to detect differences between constructs. Some constructs, such as AtMYB knockdown, had significant treatment differences but little change. In contrast, DREB2A knockdown had one of the larger decreases but did not have treatment differences. This is most likely due to differences in each construct's standard error. T-tests to determine whether treatment differences existed, showed that AtMYB knockdown had very little variation, while DREB2A knockdown had the most variation

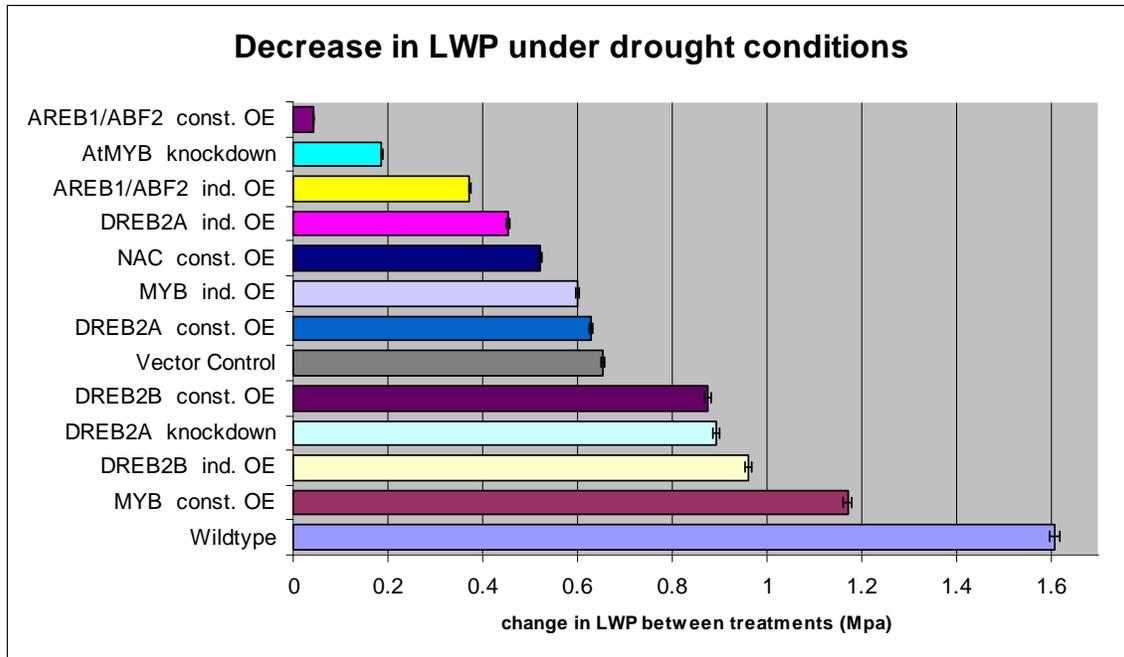


Figure 4.10 Change in LWP of Wildtype and 11 transgenic constructs of eastern cottonwood plants taken through 4 drought cycles over a 34 day period. Change in LWP did not differ significantly between constructs.

of all constructs. All constructs, except AREB1/ABF2 const. OE, whose LWPs didn't differ by treatment, also had high variation. It is less likely that treatment differences were detected in constructs with large standard errors relative to the mean, regardless of the magnitude their LWP changes under drought.

A small change in LWP is not the most important attribute of a drought resistant plant, but rather, it is the ability to maintain high LWPs (less negative) even under drought or maintaining turgor under low LWP. If a construct has little change but a low LWP under both drought and watered conditions, it is still not likely drought tolerant. AREB1/ABF2 const. OE, AREB1/ABF2 ind. OE, AtMYB knockdown, DREB2A const. OE, DREB2A ind. OE, NAC const. OE and Vector control maintained LWP for both treatments above -1 MPa. ABA accumulation under stress triggers stomatal closure. NAC const. OE, AREB1/ABF2 const. OE, and AREB1/ABF2 ind. OE constructs are overexpressing transcription factors that respond to ABA. This may be causing them to close their stomata more quickly under drought, which would help them maintain a high LWP.

Osmotic potential

Constructs with large LWP differentials selected for analysis

An Oregon study used a paired t-test to contrast field grown trees under drought and well-watered conditions. It found significant treatment differences in osmotic potential within each clone, but no treatment differences when all clones were grouped together. Mean osmotic potential of all clones under well-watered and dry conditions were -1.71 and -1.76 MPa, respectively (Tschaplinski *et al.* 2006).

Due to the narrow ranges of osmotic potentials typical of greenhouse-grown plants, only constructs showing differences between droughted and well-watered predawn LWP were analyzed. These constructs were DREB2B const OE, DREB2B ind OE DREB2A ind OE, MYB const OE, AtMYB knockdown and Wildtype. Samples of leaves that had already begun senescing were excluded from the analysis.

Paired t-tests did not indicate treatment differences within each construct or even when all constructs of each treatment were grouped together (Table 4.4). AREB1/ABF2 const. OE experienced the least amount of change in LWP. It fell from -0.48 to -0.52 MPa under drought. The largest change was experienced by the Wildtype, dropping from -0.50 to -2.10 MPa. Mean osmotic potentials of all clones under well-watered and dry conditions were -1.57 and -1.57 MPa, respectively.

Osmotic adjustment

Slow, progressive development of drought leads to greater increases in osmotic adjustment than a rapidly developing drought (Praba *et al.* 2009). In addition to the relatively high and narrow range of osmotic potential seen in greenhouse grown plants, the rapid onset of drought stress may be responsible for low osmotic adjustment values. Drought cycles were used to mimic a slow drought progression, but this may not have been the case for larger diameter plants. Given that these genes were selected from large-effect QTL for osmotic potential, it is expected that an osmotic potential differential between drought and well-watered plants become apparent in field trials. Even so, osmotic adjustment is only believed to become an important part of a plant's drought response once a critical stress level has been reached (Zhang *et al.* 1999).

Table 4.4 Mean osmotic potential (MPa) of constructs with significantly different LWP by treatment. No significant differences were found in osmotic potential.

<i>Gene and expression type</i>	<i>Treatment</i>	<i>Mean Osmotic potential (MPa)</i>
Control	Drought	-1.48 (± 0.24)
Control	Water	-1.56 (± 0.14)
Wildtype	Drought	-1.55 (± 0.08)
Wildtype	Water	-1.60 (± 0.11)
DREB2A inducible OE	Drought	-1.62 (± 0.18)
DREB2A inducible OE	Water	-1.52 (± 0.13)
DREB2B constitutive OE	Drought	-1.61 (± 0.17)
DREB2B constitutive OE	Water	-1.45 (± 0.19)
DREB2B inducible OE	Drought	-1.48 (± 0.02)
DREB2B inducible OE	Water	-1.69 (± 0.21)
MYB constitutive OE	Drought	-1.60 (± 0.04)
MYB constitutive OE	Water	-1.55 (± 0.12)
AtMYB knockdown	Drought	-1.59 (± 0.14)
AtMYB knockdown	Water	-1.66 (± 0.15)

Given that mean osmotic potentials for watered and stressed plants in each construct were not significantly different, osmotic adjustment was not observed. These plants growing under greenhouse conditions showed much higher osmotic potentials than what was observed for *P. deltoides* growing under field conditions (approximately -1.9 MPa) (Tschaplinski *et al.* 2006) . This difference is likely the result of lower light levels and milder temperatures, characteristic of greenhouse conditions, interacting with osmotic potential.

Metabolite analysis

Osmotic adjustment has previously been observed in greenhouse grown *Populus* (Gebre *et al.* 1998), but was not observed in this experiment. Due to the insignificant levels of osmotic adjustment (Table 4.4) observed between drought and well-watered treatments, leaf tissue metabolites were not analyzed. The absence of significant osmotic adjustment in these constructs may suggest that dehydration postponement strategies are playing a central role in conferring drought tolerance. Postponement strategies such as stomatal closure and leaf abscission prevent water loss via transpiration (Gebre *et al.* 1998).

Unlike this greenhouse experiment, field trials of *Populus* trees transformed with drought tolerance transcription factors will likely show differences in osmotic potential between treatments. Metabolite analysis would benefit these future studies by elucidating which molecules accumulate in plant tissues to increase dehydration tolerance. Osmotic adjustment may compete with productivity for substrate, but situations have been noted where trees accumulate high levels of solutes while maintaining a high growth rate

(Gebre *et al.* 1998). In the case of biomass feedstocks, and other agricultural crops, it is essential that yield not be negatively impacted by drought tolerance.

Chapter 5

Conclusions and Recommendations

Overview

Accelerated domestication would make it possible to design crop varieties that facilitate fuel production, either through growth, stress tolerance, or wood chemistry traits. Beneficial genes, such as those for height growth, response to competition, branching, cold tolerance, disease resistance, and cell wall chemistry have already been identified in poplar. Drought tolerance was chosen as a target trait to demonstrate the feasibility of this approach.

Genes that performed best

AREB1/ABF2 const. OE and DREB2A ind. OE performed better than the Vector control in terms of productivity and drought tolerance. They had high RGRs under drought and watered conditions, low to rates of leaf abscission, and maintained a high LWP relative to other constructs. MYB ind. OE also stood out as a potentially productive and drought tolerant construct, but neither its growth nor leaf abscission rates were statistically different from those of the Vector control.

AREB1/ABF2 const. OE

This construct had the second highest growth rate averaged across lines in a previous growth trial and had the highest RGR under drought in this experiment. The

statistical analysis indicated that growth of AREB1/ABF2 const. OE plants assigned to the drought treatment was significantly higher than the vector control. This construct also had significantly lower leaf abscission under drought than the vector control at the 0.05 significance level. AREB1/ABF2 const. OE maintained consistently high LWPs that did not differ according to treatment, while also having the smallest change between drought and irrigated LWP.

Results of RT-PCR analysis confirmed that AREB1/ABF2 is overexpressed in this construct (Table 4.1). Constitutive overexpression may be expected to negatively impact productivity, but that does not seem to be the case with this construct. This transcription factor is part of the ABA-dependent response pathway and is activated by ABA. Genes believed to be direct targets of this transcription factor encode for LEAs and regulatory proteins (Fujita *et al.* 2005).

DREB2A ind. OE

In a previous growth trial, DREB2A ind. OE had the highest RGR of all constructs averaged across lines, surpassing even the Wildtype. This construct showed similar productivity in the drought experiment, with an RGR significantly higher than that of the Vector control. Its rate of leaf abscission under drought was one of the lowest and it maintained a high LWP regardless of treatment.

Results of RT-PCR analysis showed that DREB2A expression in this construct was the same as in the Vector control (Table 4.1). Its significantly greater growth under drought, as well as visible differences in leaf abscission and LWP, prevented it from being pooled with the Vector control plants for analysis. It is possible that the DREB2A

transcription factor was being up-regulated enough to positively affect drought response. Even a minor increase in gene up-regulation could activate downstream genes, making the plants more drought tolerant. Future research should look at the drought response of constructs with more dramatic up-regulation.

MYB ind. OE

Although no statistical differences were found between this construct and the Vector control, MYB ind. OE had one of the highest RGRs in a preliminary growth trial, as well as during this drought experiment. Its median rate of leaf abscission may be partly due to xylem cavitation resulting from drought developing very suddenly, as opposed to the gradual development that occurs in nature. This construct had some of the largest trees and likely experienced stress more severely than other, smaller constructs. Expression analysis has not yet been done to confirm whether MYB is indeed up-regulated under drought.

Effect of transformation

No significant growth differences

Wildtype plants had a higher RGR than Vector control under both drought and irrigated conditions. Although they weren't statistically different, they may have been if a larger Wildtype sample size had been used. Another factor influencing the failure to detect differences can be attributed to the fact that the Vector control line chosen for the experiment wasn't representative of the construct. Line 533584 had an RGR 2 to 3 times larger than the other lines. A line with median productivity would have been more

representative of the construct and given a better idea of the effect transformation has on drought response.

Increased drought tolerance

Wildtype plants assigned to drought treatment lost more leaves overall than Vector control drought plants. This same difference was seen in the percent increase in leaf abscission under drought. Leaf abscission for Wildtype increased 49-fold under drought compared to irrigated replicates, while Vector control only increased 3-fold. Since percent leaf loss is being used to assess each construct's drought tolerance, it appears that transformation itself may have a positive effect on drought tolerance.

Field trials

DREB2A ind. OE, AREB1/ABF2 const. OE, and MYB ind. OE were the most productive constructs, as well as being likely to confer drought tolerance. Field trials would be the next step, providing a clearer picture of how these constructs would perform under long-term field conditions. Some constructs may be particularly susceptible to pests or diseases that weren't abundant in the greenhouse, influencing their suitability for plantations. Long-term field trials are a good way of evaluating performance when faced with multiple stress combinations.

Verify gene expression

Manipulating the expression of known genes is used to examine their contribution to phenotype (Carpenter and Sabatini 2004). The constructs in this study were transformed to have altered expression of target genes. Inducible OE constructs should have the same level of gene expression as the control under irrigated conditions, but greater expression under drought. Constitutive OE constructs should show greater expression of the target gene than the control under both drought and well-watered conditions. Knockdown constructs are expected to have reduced gene expression under drought and well-watered conditions compared to the control.

Expression of some constructs has been verified using Real-Time RT-PCR, but must still be done on the remaining constructs. New constructs could be made of genes not currently showing the intended altered expression and included in a new study to determine their effectiveness. Genes that failed to increase drought tolerance when overexpressed could be eliminated from the candidate gene list.

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Appendix

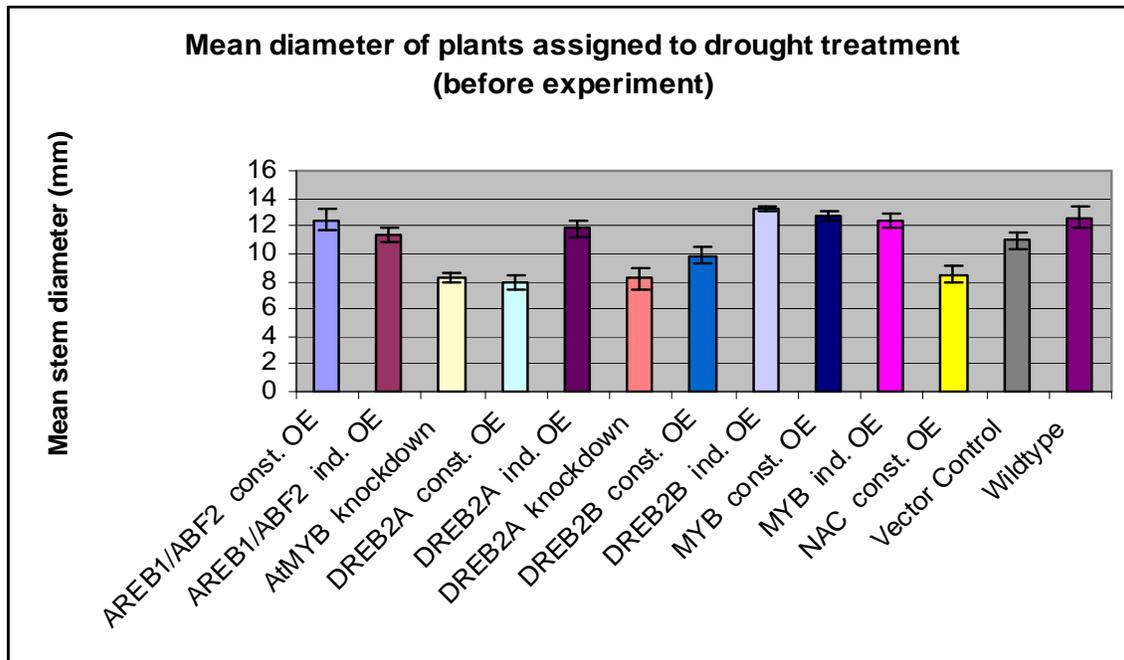


Figure A.1 Mean stem diameter of Wildtype and 11 transgenic constructs of eastern cottonwood plants assigned to the drought treatment, before the beginning of the experiment

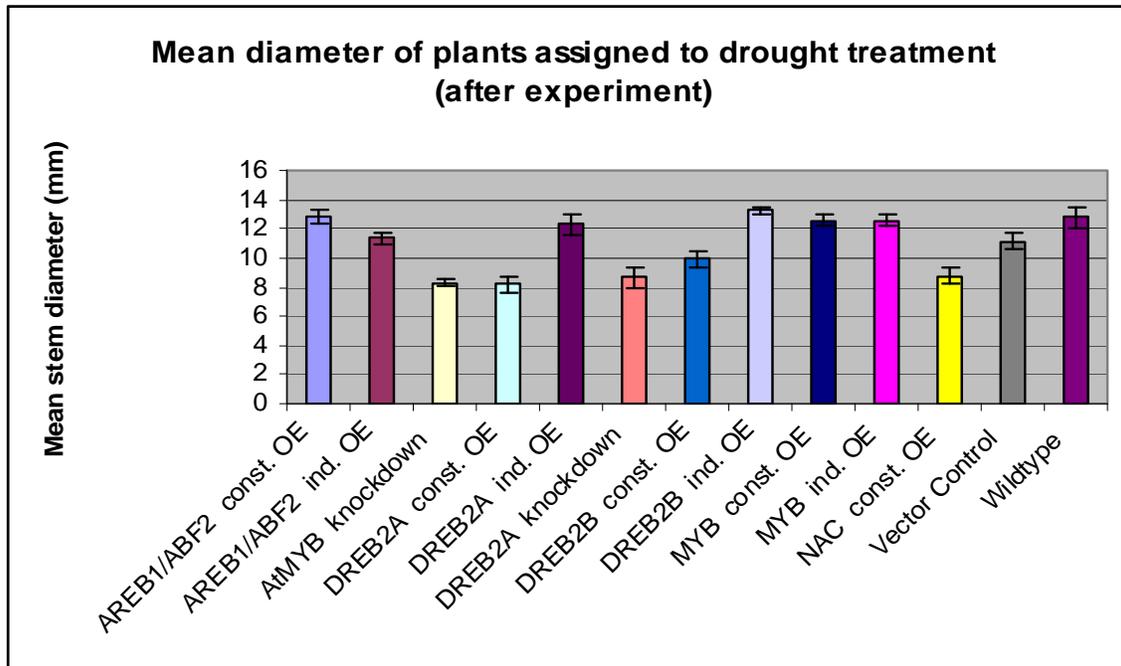


Figure A.2 Mean stem diameter of Wildtype and 11 transgenic constructs of eastern cottonwood plants assigned to the drought treatment, after they had gone through 4 drought cycles

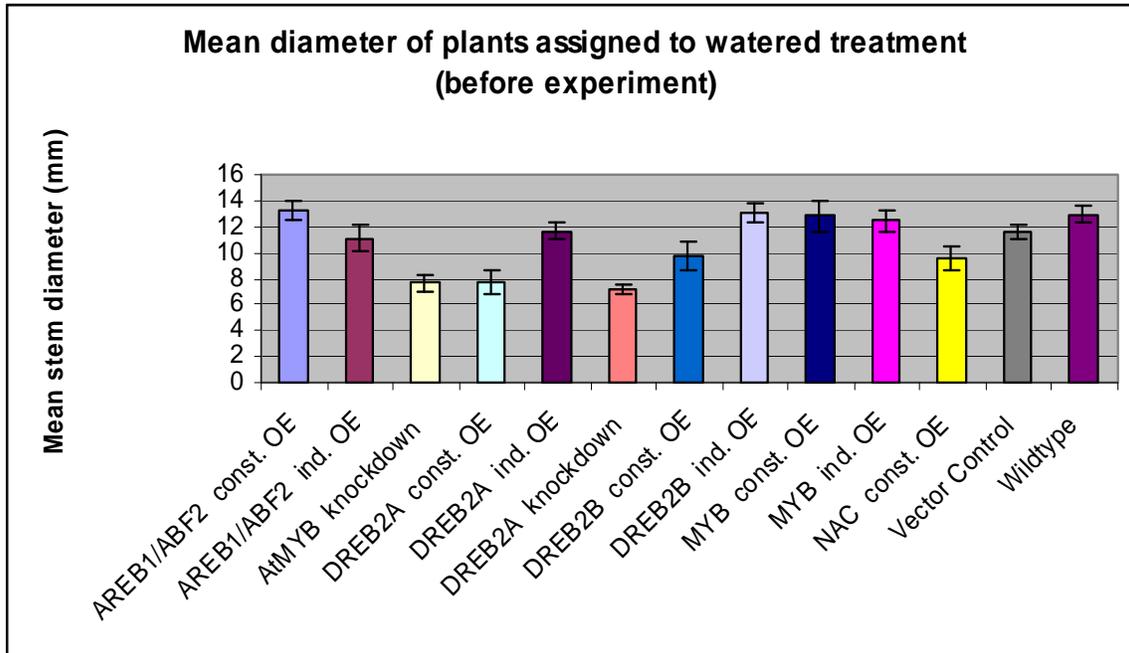


Figure A.3 Mean stem diameter of Wildtype and 11 transgenic constructs of eastern cottonwood plants assigned to the watered treatment, before the beginning of the experiment

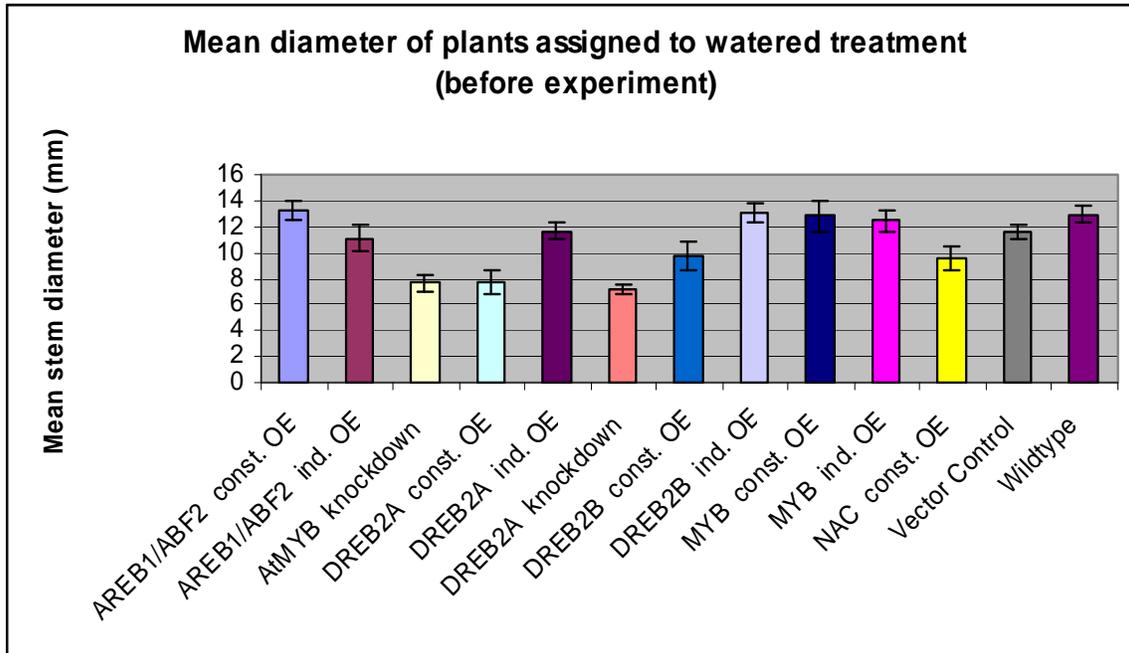


Figure A.4 Mean stem diameter of Wildtype and 11 transgenic constructs of eastern cottonwood plants assigned to the watered treatment, at the end of the experiment

Vita

Alina Campbell was born in Plantation, FL to Donald Fergus Campbell and Maria Estela Campbell Fernandez. She grew up in El Paso, TX and Sao Paulo, SP (Brazil).