Identification of Genes that Contribute to Drought Tolerance in *Populus*

by

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Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY

Spring 2014

Approval

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Date Defended: January 15, 2014

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Abstract

Populus species and derived hybrids are valued for their fast growth and are cultivated all over the Northern hemisphere. They are grown primarily for pulp, paper and oriented strand board production. Fast growing poplar also has potential to be used for carbon sequestration as well as a feedstock for the carbon-neutral production of energy. Many of the commonly used species and hybrids are, however, regarded as drought sensitive, which poses a problem for large-scale cultivation, particularly in light of climate change-induced drought spells in areas of poplar growth including the Canadian prairies. To evaluate the extent of drought tolerance variation in commercially important Canadian poplar hybrids, we tested their ability to withstand drought and ranked them based on a series of physiological and morphological responses. Gene expression analysis of the response to drought in the least and most tolerant clones revealed differences in abscisic acid-mediated signaling, in particular a putative negative and a putative positive regulator of this pathway. Thereafter, we tested the functional importance of these two genes by transformation experiments. Overexpression of the putative negative regulator led to reduced drought tolerance in transgenic *Arabidopsis thaliana*, whereas overexpression of the putative positive regulator led to improved drought tolerance in transgenic *Arabidopsis thaliana* and transgenic poplars. Taken together, we have generated a better understanding of drought tolerance in available fast-growing poplar hybrids, functionally characterized two poplar genes, and identified strong candidate genes for targeted improvement of drought tolerance in poplar hybrids.

Keywords: *Populus* hybrids; *Arabidopsis;* gene expression; drought tolerance; global-warming; physiology; abscisic acid

"And say: My Lord! Increase me in knowledge."
(The Holy Quran)

I dedicate all my work and achievements to my parents who always inspired me and encouraged me throughout my studies.

In addition, I would also like to dedicate my work to rest of my family, friends and colleagues from all over the world.
The work in this thesis would not have been possible without their support.

Acknowledgements

First of all, I would like to thank my senior supervisor Dr. Jim Mattsson for his generous support and obtaining the funding for this work as well as to attend the numerous conferences. I also want to thank him for his invaluable suggestions about experimental work and editorial review throughout my degree. Next, I would like to thank Dr. Aine Plant for accepting me as a PhD student in her lab. Also, I thank her for providing me reference letters and editorial advice whenever I needed. I am thankful for her support and advice at many stages during my PhD. Thanks also go to Dr. Sherryl Bisgrove for happily allowing me to work in her lab when needed. I also give my thanks to the members of my examination committee Dr. Barbara Hawkins, Dr. Julian Christians and Dr. Allison Kermode.

Thanks to Aktari Begum for her assistance in tissue culture. I also obtained valuable help from postdoctoral fellows during my work. I would like to give my thanks to Kamal Biswas for his help in conducting trials in the greenhouse and to Oliver Corea for his support.

The support of the present and past members of the Mattsson lab and other grad students in the department is greatly appreciated. Special thanks to Adam Foster for his assistance in the lab and also for being a good friend. Also thanks to Dua'a Riyal, Tammy Baylis and Fatemeh Abedi for all their support. I was lucky enough to obtain valuable assistance from undergraduate students. Thanks to Lina Jang, Sarah Calbick and Ryan Thomas for their help in the lab and greenhouse work.

The support of Departmental staff particularly Marlene Nguyen and Amelia Shu, is greatly valued. I truly appreciate their regular reminders and alerts. Also, thanks to Alex Fraser, Debbie Sandher and Barbara Sherman for their help.

I thank all who have financially contributed to my graduate studies. Thanks to the Natural Sciences and Engineering Research Council of Canada, Higher Education Commission of Pakistan and Mutual Fire Insurance Company of BC for funding my research project, and Simon Fraser University for granting me scholarships and Teaching Assistantships.

I would also like to thank to every member of my large family for all the moral support they have provided me throughout my academic career.

Admittedly, this work would likely have not been possible without all of your support and to you all, I give my cordial thanks.

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Muhammad Arshad analyzed the physiological data and performed quantitative PCR.

Chapters 3, 4 and 5: Muhammad Arshad performed all experimental work and analyzed the data.

Chapter 1.

Introduction

1.1. Drought stress in plants

The abiotic stresses, drought and saline soils are responsible for substantial plant growth reduction and yield losses worldwide with sometimes devastating economic effects (Wang et al. 2003). Drought causes 6-8 billion ($US) loss annually worldwide, generally affecting more people than any other kind of natural disaster (Wilhite 2000) and is considered the major cause for crop loss around the world (Ding et al. 2011). In recent times, severe and more frequent drought episodes have been observed all over the world (Le Comte, 1994, 1995). According to the United Nation's Intergovernmental Panel on Climate Change, drought frequencies are expected to increase over the next few decades at many regions of the world. In addition, climate models have predicted more frequent and intense drought episodes worldwide in the future due to global warming (Cook et al. 2007; Hogg and Bernier 2005; Salinger 2005) IPCC, 2013.

Most of Canada experiences sporadic drought, however, Canadian prairies are more susceptible to drought due to irregular spatial and temporal precipitation (Bonsal and Regier 2007). Global warming has raised the temperature of Western Canadian regions by nearly 2 ^{0}C since 1940 (Hogg and Bernier 2005). This continuous warming of the regions may lead to drier conditions in the future, which may ultimately reduce forest productivity in the region. Furthermore, drier conditions tend to make forests vulnerable to diseases and insect attacks (Hogg and Bernier 2005). More recently, severe drought caused high mortality and die back (35%) of aspen in Western Canada (Hogg et al. 2008; Michaelian et al. 2011), suggesting an urgent need to develop drought tolerant plant varieties to cope with changing environments and increased drought periods in the region.

1.1.1. Plant growth and water relations

Drought is characterized by a temporary dry period when precipitation is below-normal (Dai 2011). In case of water deficit, transpiration exceeds water uptake and it could be a part of different stresses such as drought and salinity (Bray 1997). Drought adversely affects plant performance by causing cellular dehydration. Drought also weakens plants defenses, increasing their susceptibility to other stresses such as insect, herbivores and diseases (Englishloeb 1990; Vinocur and Altman 2005). Drought can lead to pre-mature leaf senescence, wilting, desiccation, and finally death of the plant (Neumann 2008).

Drought induces adaptive and acclimative responses in plants that help them tolerate adverse environmental conditions. Adaptation to changes in the environment includes genetic modification over many generations during the process of natural selection (Taiz and Zeiger 2010). For example, *Populus euphratica* is adapted to dry and semi-arid conditions and can grow in deserts characterized by drought, saline soils and low moisture. On the other hand, *P. trichocarpa* is a riparian tree and adapted to a wet and cool climate (Gries et al. 2003; Pearce et al. 2006). Acclimation is characterized by non-permanent changes in plant morphology or physiology without any genetic modification. These changes help plants to cope with environmental conditions and are also reversible upon relieving the stress (Taiz and Zeiger 2010). For example, plants close stomata during water deficit to reduce water loss whereas stomata re-open upon re-watering (Bartels and Sunkar 2005). Similarly, plant growth is inhibited and leaf senescence is stimulated during drought, which reduces the leaf area for transpiration, and renewed access to water stimulates formation of new leaves (Munne-Bosch and Alegre 2004).

Water uptake is essential for plant growth. Plant growth is the result of cell division and, to a larger extent, cell expansion. Cell expansion is driven by a combination of cell turgor gained by water uptake and cell wall loosening (Neumann 1995). Water content in plants depends on water uptake by roots and water loss by transpiration primarily in leaves. Since maintenance of a high water content and cellular turgor is essential for plant growth, plants can reduce water deficit by maximizing the absorption of water from the soil or minimizing the water loss by transpiration. Guard cells in a plant leaf control the stomatal aperture, and thereby much of the transpiration of water from plants (Chaves et

al. 2003). Drought reduces leaf area, and overall plant growth, which is an acclimation response to decrease shoot area for transpiration through stomata (Sanchez-Blanco et al. 2009; Tardieu 2005).

Drought-induced cavitation is common in woody plants and may be harmful to growth because it blocks the conduction of water through the xylem, which can lead to leaf desiccation and even branch die back (Rood et al. 2000). Resistance to cavitation is positively correlated to drought tolerance in some woody plants whereas in other species cavitation helps to prevent water loss and enhance drought tolerance (Bucci et al. 2013; Pineda-Garcia et al. 2013; Tyree et al. 2003).

1.1.2. Photosynthesis

Photosynthesis in higher plants is impaired as leaf water potential and relative water content decrease (Lawlor and Cornic 2002). Reduced CO_2 diffusion into carboxylation sites of Rubisco due to stomatal closure is considered a major cause of photosynthesis inhibition during mild water stress (Pinheiro and Chaves 2011). The amount of CO_2 molecules entering the mesophyll cell may also be reduced due to changes in intercellular spaces caused by leaf shrinkage during water deficit stress (Lawlor and Cornic 2002). Severe drought also impairs ribulose bisphosphate regeneration, ATP synthesis and Rubisco activity (Maroco et al. 2002). In addition, when stomatal conductance is markedly decreased, reactive oxygen species are generated in chloroplasts, which damage ATP synthase and cause overall impairment of photosynthesis (Lawlor and Tezara 2009). There is also evidence that reduced cellular water content affects photosynthesis by impairing metabolism (Reddy et al. 2004).

1.1.3. Osmotic adjustment

Plant cells respond to water deficit by synthesizing solutes, which in turn increase water uptake by osmosis – a process known as osmotic adjustment (Ashraf 2010). During osmotic adjustment, cell water potential drops due to accumulation of solutes. Thus the major function of solute accumulation is to create a water potential gradient for water uptake to maintain cell turgor (Wang et al. 2003).

When the water potential is lower than that of the surroundings, water will flow via osmosis down its concentration gradient into the cells. Solute accumulation protects proteins and membranes from dehydration (Hincha and Hagemann 2004). Both organic solutes and inorganic ions play vital roles in osmotic adjustment during drought, and their type varies among different plant species and varieties (Chen and Jiang 2010). They not only contribute to osmotic adjustment but also detoxify reactive oxygen species, protect membrane integrity as well as stabilize enzymes and other proteins (Ashraf and Foolad 2007). The solutes include sucrose, hexoses, sugar alcohols, sorbitol, mannitol, galactitol, proline, glycine betaine, myo-inositol, ononitol and pinnitol (Ashraf and Foolad 2007; Chen et al. 2007; Kurz 2008).

1.2. Molecular responses of plants to drought stress

Plant must sense the drought conditions in order to generate a response. How plants perceive drought signals is still largely unknown, however, multiple theories exist. For example, receptor-like kinases are localized on the plasma membrane and are thought to be involved in perceiving external environmental signals and activation of downstream signaling cascades during drought (Osakabe et al. 2013). The *A. thaliana* histidine kinase AtHK1 was identified as a putative osmosensor, which acts as a non-hormonal receptor. AtHK1 detects reduction in plant turgor and conveys a stress signal downstream to mitogen-activated protein kinase (MAPK). Subsequently, a MAPK signaling cascade induces the expression of several drought responsive genes (Hamanishi and Campbell 2011; Osakabe et al. 2013; Urao et al. 1999). Two HKT1 homologs have also been identified and characterized in *Eucalyptus*, which altered the sodium and potassium flow indicating that they can detect changes in solute concentration and play a role in osmosensing and osmoregulation (Liu et al. 2001). Similarly, transgenic *A. thaliana* overexpressing AtHK1 exhibited enhanced drought tolerance whereas the *athk1* mutant plants were hypersensitive to osmotic stress (Tran et al. 2007; Wohlbach et al. 2008). Another model suggests that in the absence of water flow in a drying rhizosphere, plant-derived ABA accumulates, which is perceived as a water deficit signal by plant roots, triggering downstream responses to drought (Hamanishi and Campbell 2011; Hartung et al. 1996).

Drought perception leads to two main signaling pathways; one that depends on the production of the plant hormone ABA, and one that does not (Huang et al. 2012).

1.2.1. The ABA-dependent pathway

ABA is a hormone involved in many developmental processes including the regulation of seed maturation and dormancy. It also regulates plant responses to various environmental stresses. ABA acts both at short and long distance. There is evidence that ABA triggers a response already in the cell in which it is produced (Seo and Koshiba 2002). ABA produced in roots in response to water stress is transported in xylem via the transpiration stream to shoot organs, where it acts as a messenger to elicit a response (Nambara and Marion-Poll 2005).

ABA Biosynthesis

As shown in Fig. 1.1, ABA is synthesized from carotenoids that in turn are built from isopentenyl diphosphate (IPP), with the majority of enzymatic conversions occurring in plastids. IPP is the substrate for geranylgeranyl diphosphate (GGPP) biosynthesis. GGPP is then converted to phytoene by phytoene synthase. Phytoene desaturase catalyzes the conversion of phytoene to carotene, which is then converted to lycopene and β-carotene. β-carotene is further converted to zeaxanthin, which undergoes an epoxidation catalyzed by zeaxanthin epoxidase to form all-trans-violaxanthin. Oxidative cleavage of 9-cis-neoxanthin and 9-cis-violaxanthin to form xanthoxin is catalyzed by 9-cis epoxycarotenoid dioxygenase (NCED), a key regulatory step in ABA biosynthesis (Iuchi et al. 2001; Qin and Zeevaart 1999; Thompson et al. 2000). Conversion of xanthoxin to ABA takes place in the cytosol and three pathways have been proposed: (1) via abscisic aldehyde (2) via xanthoxic acid and (3) via abscisic alcohol, however, abscisic aldehyde is believed to be the major pathway for ABA synthesis; Fig. 1.1, (Seo and Koshiba 2002; Seo and Koshiba 2011).

ABA homeostasis in a particular plant organ or tissue depends not only on its biosynthesis but also removal and deactivation (Cutler and Krochko 1999). ABA catabolism restores the balance of ABA within plant tissues by reducing ABA content. In most plant tissues, the catabolism of ABA occurs primarily by hydroxylation of the 8'-carbon atom forming 8'-hydroxy ABA (8'-OH ABA), a reaction that is catalyzed by a

cytochrome P450 monooxygenase (Kitahata et al. 2005; Kushiro et al. 2004). 8'-OH ABA is further converted to phaseic acid, which is then reduced to dihydrophaseic acid (Zhou et al. 2004), compounds that lack ABA-like activity. ABA may also be inactivated by conjugation to form, for example, ABA glucose ester (Qin and Zeevaart 1999).

Overexpression of enzymes involved in ABA biosynthesis enhances drought tolerance in plants. For example, overexpression of zeaxanthin epoxidase (ZEP) increased ABA levels and drought tolerance in *A. thaliana* (Park et al. 2008). Similarly, higher levels of ABA were measured in *A. thaliana* and tobacco transgenic plants overexpressing NCED3 and subsequent experiments revealed an improved drought tolerance in transgenic plants (Iuchi et al. 2001; Qin and Zeevaart 1999).

ABA-induced stomatal closure

ABA stimulates stomatal closure by triggering signal cascades in guard cells. Ion channels are found on the vacuolar and plasma membranes and include inward and outward transporting K^+ channels, anion channels and Ca^{2+} channels. ABA causes ion channels in the vacuolar and plasma membranes to open, releasing ions from the cell. As ionic concentration in the cell decreases, water flows out of the cell by osmosis resulting in shrinkage of the cell volume and stomatal closure (Taiz and Zeiger 2010).

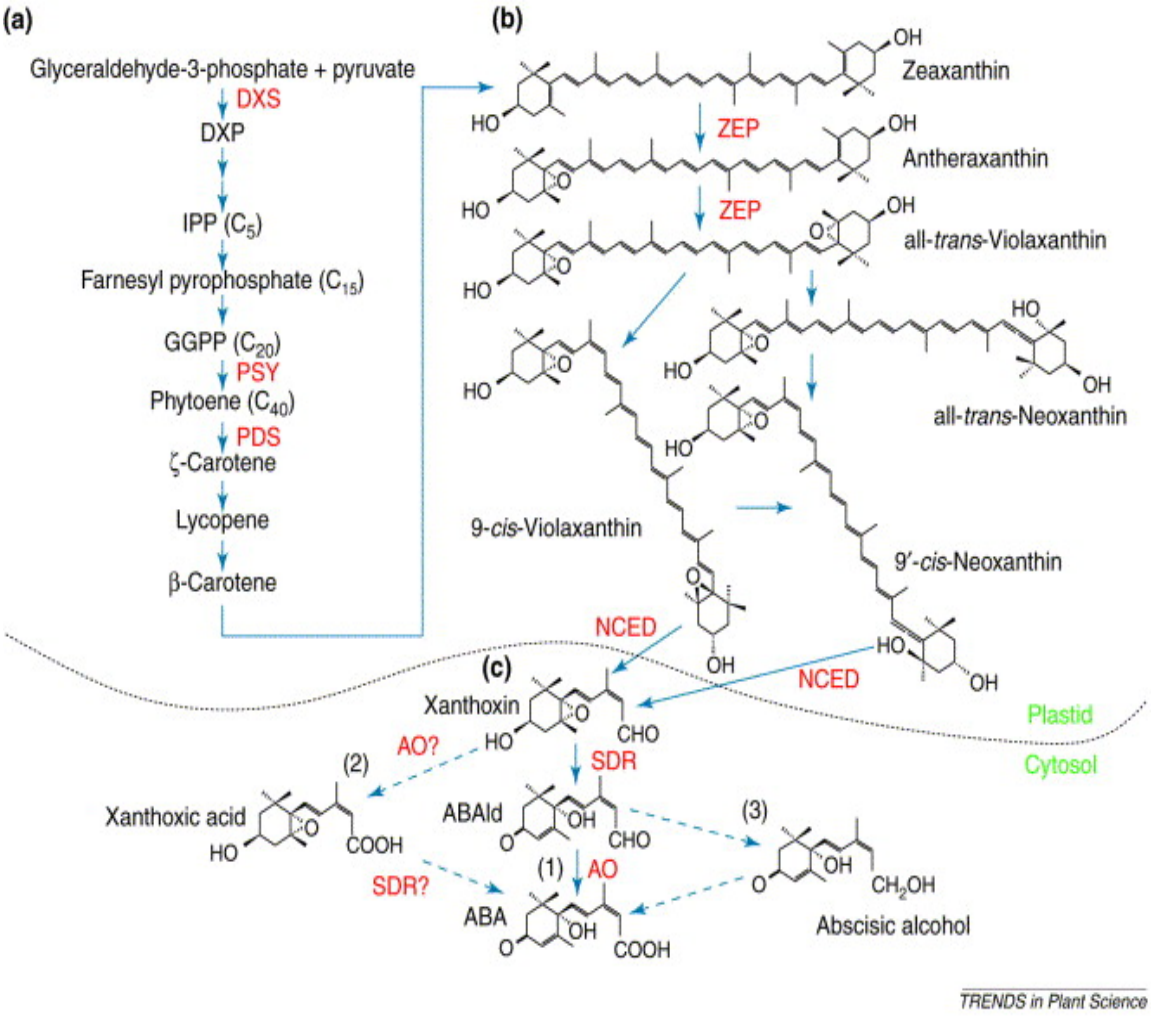

Figure 1.1. ABA biosynthesis pathway: Compound names are shown in black and enzyme names in red. A solid blue arrow indicates major whereas a dotted blue indicates a presumed pathway. Abbreviations: ABA, abscisic acid; ABAld, abscisic aldehyde; AO, aldehyde oxidase; DXP, 1-deoxy-D-xylulose-5-phosphate; DXS, DXP synthase; GGPP, geranylgeranyl pyrophosphate; IPP, isopentenyl pyrophosphate; NCED, 9-cisepoxycarotenoid dioxygenase; PDS, phytoene desaturase; PSY, phytoene synthase; SDR, short-chain dehydrogenase/reductase, ZEP; zeaxanthin epoxidase (Seo and Koshiba 2002). Reprinted with permission from Elsevier Limited.

ABA-induced signaling

Protein kinases and phosphatases are involved in ABA signal transduction. Open stomata 1 (OST1) is an ABA-activated protein kinase and the *ost1* mutant lacks the ability to limit transpiration during drought stress (Mustilli et al. 2002). Similarly, stress-induced kinase 1 (SIK1) is a receptor-like protein kinase and transgenic *Arabidopsis*

plants overexpressing it exhibit enhanced drought tolerance whereas knockout mutants show hypersensitivity to drought stress (Ouyang et al. 2010).

Overexpression of protein phosphatases 2C (PP2C) reduced tolerance to drought stress of *Arabidopsis* by increasing water loss (Liu et al. 2009). As shown in Fig. 1.2, ABA binding to the RCAR/PYR/PP2C/SnRK2 complex results in a conformation change that prevents PP2C dephosphorylation of SnRK2 (Umezawa et al. 2009; Vlad et al. 2009). Subsequent phosphorylation of SnRK2 triggers gene expression and other SnRK2-dependent events (Fig. 1.2) that ultimately lead to ABA-dependent acclimation to water deficit (Bartels and Sunkar 2005). Functional characterization of several PP2C genes in different plant species indicates that several PP2Cs are negative regulators of ABA signaling and drought tolerance (Kuhn et al. 2006; Merlot et al. 2001; Nishimura et al. 2007; Rubio et al. 2009; Saez et al. 2004; Saez et al. 2006; Zhang and Gan 2012).

Plant responses to ABA vary among different organs and depend on water status as well as ABA concentration. Under well-watered conditions, ABA promotes shoot growth by supressing ethylene levels in *Arabidopsis* and tomato (LeNoble et al., 2004; Sharp et al., 2000). However, external application of ABA inhibits root growth in the absence of stress conditions. Additionally, supranormal concentration of ABA reduces root growth but normal endogenous concentration of ABA helps to maintain root growth by supressing ethylene production (Sharp and LeNoble 2002).

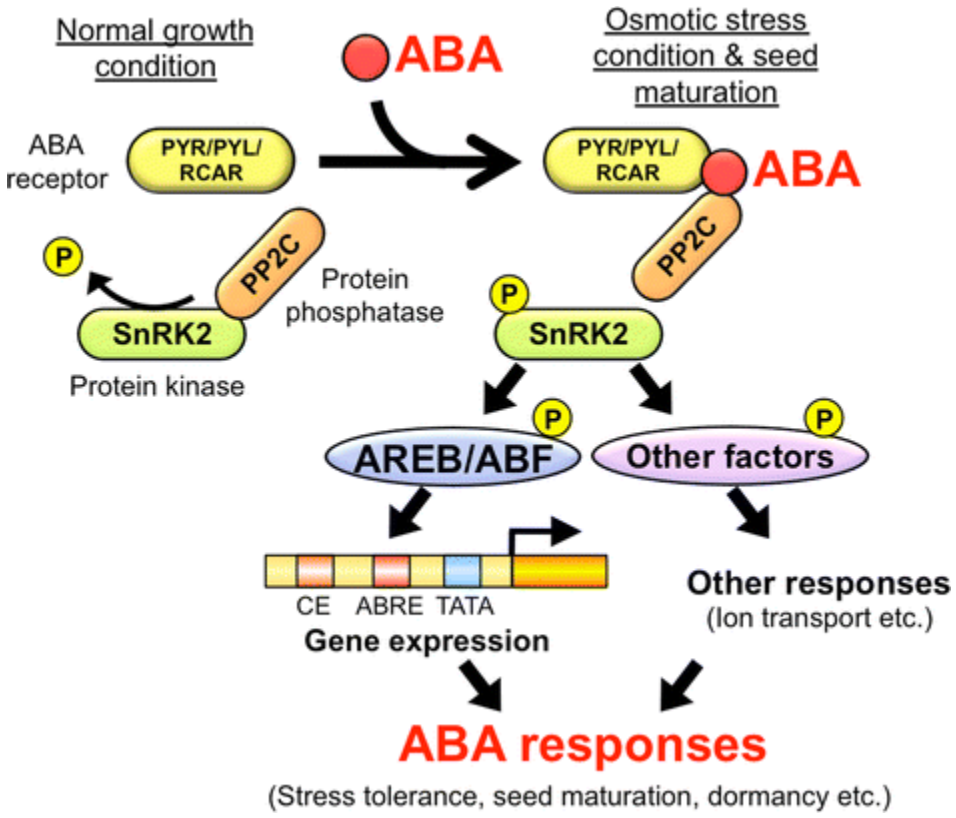

Figure 1.2. A model for ABA signaling pathway. Under normal growth conditions, PP2C dephosphorylates SnRK2 and inactivates the pathway. ABA prevents SnRK2 dephosphorylation by PP2C and a downstream signaling pathway is activated. Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; AREB, ABRE-binding protein; ABF, ABRE binding Factor; PYR, pyrabactin resistance; PYL, PYR1-like; SnRK2, SNF1-related protein kinase 2; PP2C, 2C-type protein phosphatase. Reprinted from, (Nakashima and Yamaguchi-Shinozaki 2013) with permission from Springer Verlag.

ABA-induced gene expression

Expression of stress-inducible genes such as RD29A, RD22, COR15A, COR47, P5CS is significantly reduced or blocked in ABA deficient mutants, providing evidence for the ABA-dependent pathway (Xiong and Zhu 2003). As shown in Fig. 1.3, the ABA-responsive element (ABRE) is an essential cis-acting element in the ABA-dependent gene expression pathway. Basic domain/leucine zipper *(bZIP)* transcription factors consist of ABRE-binding protein (AREB) and ABRE-binding factors (ABF). AREB1/ABF2

binds to ABRE motif in the promoter region of ABA-inducible genes and activates their expression (Huang et al. 2012). Overexpression of AREB1 in *A. thaliana* results in enhanced drought tolerance and ABA sensitivity whereas loss of function mutants exhibit reduced drought tolerance and ABA insensitivity indicating that AREB1 plays an essential role in ABA signaling and drought tolerance (Fujita et al. 2005).

Treatment of *A. thaliana* plants with ABA results in accumulation of the transcription factors MYB2 and MYC2, suggesting that they act in the ABA-dependent pathway (Fig. 1.3; Shinozaki and Yamaguchi-Shinozaki, 2007). Evidence in that direction comes from overexpression of MYB2 and MYC2, which results in *A. thaliana* plants that have enhanced drought tolerance. In addition, the overexpression of MYB2 and MYC2 results in elevated expression of many genes, revealing putative downstream targets of these transcription factors (Huang et al. 2012). Similarly, expression of the RD26 NAC transcription factor is also induced by drought and ABA, suggesting a third transcription system in the ABA-dependent pathway (Fig. 1.3). In support of that notion, transgenic plants overexpressing RD26 exhibit an ABA hypersensitive phenotype (Shinozaki and Yamaguchi-Shinozaki 2007).

1.2.2. ABA-independent pathway

The *A. thaliana* RD29A/COR78/LT178 genes are cold, drought and ABA inducible, however, these genes are also induced in *aba* or *abi* mutants when exposed to drought or cold stress, indicating that these genes are activated by both ABA-dependent and ABA-independent pathways; Fig. 1.3, (Shinozaki and Yamaguchi-Shinozaki 2007). The promoter of all three genes contains two cis-acting elements known as ABREs (see above) and drought responsive elements (DRE) responsible for ABA-dependent and ABA-independent responses respectively (Shinozaki et al. 2003). Completely ABA-independent induction of transcription factors is seen in the dehydration responsive element binding (DREB) transcription factors. DREB1 and DREB2 bind to DRE and regulate the expression of cold and drought inducible genes respectively (Yamaguchi-Shinozaki and Shinozaki 2006).

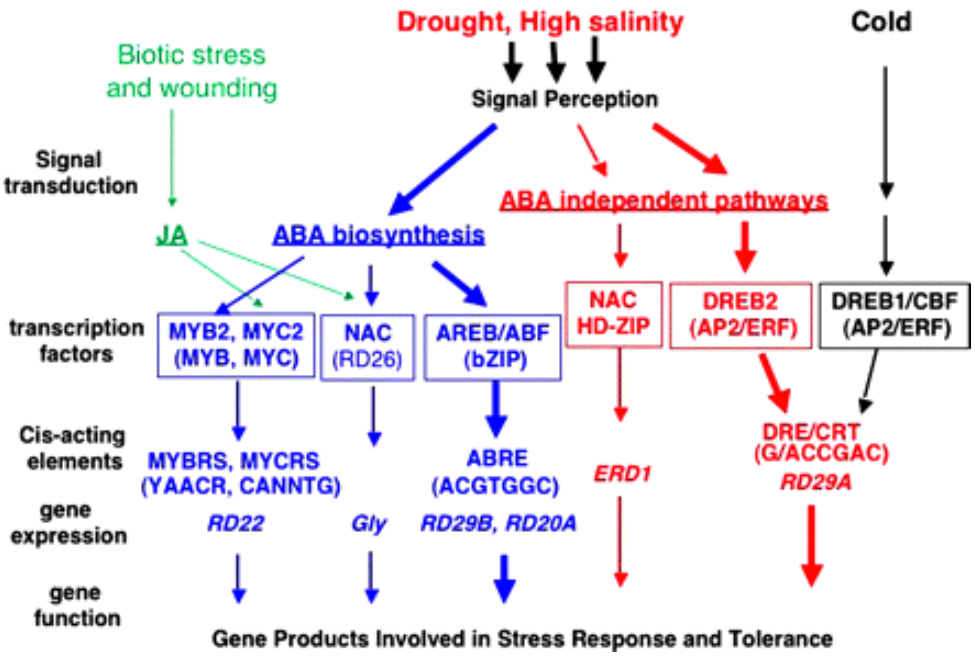

Figure 1.3. A biotic stress signaling pathways and downstream gene expression. Six different signaling pathways exist in drought, cold and salinity responses and only three are ABA dependent. Red color indicates ABA-independent, blue color indicates ABA-dependent pathway whereas green color indicates biotic stress and wounding pathway. Thick arrow indicates a major and important signaling pathway. Reprinted from, Shinozaki and Yamaguchi-Shinozaki, 2007 with permission by Oxford University Press.

Eight DREB2 homologues are present in *A. thaliana*. DREB2A and DREB2B appear to be the two major transcription factors mediating gene expression in the ABA-independent pathway especially as plants overexpressing them exhibit enhanced drought tolerance (Sakuma et al. 2006). Several mutants have also been produced in different studies that show enhanced drought tolerance, for example, DREB2A interacting protein 1 (DRIP1) and DRIP2 appear to be negative regulators of DREB2A, as a more stable and enhanced expression of drought responsive genes was observed in *drip1* and *drip2* double mutants than in wild type *A. thaliana* plants (Qin et al. 2008).

1.2.3. Identification of drought-induced effector genes

Drought stress-induced signaling leads eventually to activation of genes that participate in mounting an acclimation response to the perceived stress. These genes are known as effecter genes, and produce effecter proteins. Microarray analysis has been used to great effect to identify many hundreds of drought and ABA induced genes in many plant species (Bogeat-Triboulot et al. 2007; Hamanishi et al. 2010; Rabbani et al. 2003; Seki et al. 2001; Seki et al. 2002; Shinozaki et al. 2003; Street et al. 2006). Stress-induced genes are not only involved in protecting cellular functions during stress but they also play a crucial role in regulating signal transduction, metabolism and osmoprotection as well as acting as chaperones and ROS scavengers. Similarly, several genes are involved in synthesis of compatible solutes such as proline, glycine-betaine, galactinol and mannitol, which are accumulated during drought and help plants to not only conserve cellular water but also stabilize protein and cell structure under stress conditions (Umezawa et al. 2006). Overexpression of a glycine-betaine synthesis gene enhances glycine-betaine levels and drought tolerance in transgenic *A. thaliana* indicating its role in stress modulation (Chen and Murata 2002). Late embryogenesis abundant proteins (LEA) act as chaperons to prevent protein aggregation, and overexpression of LEA genes confers drought tolerance (Bartels and Sunkar 2005).

Drought stress can cause oxidative stress by inducing the production of ROS that have a role in signaling i.e. activation of the defense system to scavenge the ROS. ROS are also involved in imposing significant damage in any organelle undergoing oxidative reactions (Cruz de Carvalho 2008; Sanchez-Diaz et al. 2007). Plants, however, produce antioxidants to scavenge ROS and protect cellular functions (Cruz de Carvalho 2008). Overexpression of genes involved in the production of antioxidants improves drought tolerance in various plant species, for example, superoxide dismutase (SOD) overexpression increase drought tolerance in rice, alfalfa and potato (McKersie et al. 1996; Perl et al. 1993; Wang et al. 2005). Similarly, *A. thaliana* ascorbate peroxidase overexpression also improves drought tolerance in plants (Badawi et al. 2004).

Carbohydrate metabolism is strongly affected by drought stress. Drought enhances carbohydrate accumulation by up-regulating the expression of genes involved in carbohydrate synthesis (Regier et al. 2009). Studies have also shown a positive

correlation between carbohydrate accumulation and drought tolerance (Bartels and Sunkar 2005). Some proteins are involved in water transport during drought for example; aquaporins that mediate water transport in many plant species and their expression is altered in response to drought stress (Aroca et al. 2006). Aquaporin proteins are membrane-bound water channels and play a crucial role in cellular water homeostasis. Down-regulation of aquaporin has been observed in *A. thaliana* during drought stress indicating that this is a way to minimize water loss and uphold turgor in the leaves (Aharon et al. 2003; Alexandersson et al. 2010; Hachez et al. 2006).

1.3. Poplar cultivation and responses to drought

Species in the *Populus* genus have adapted to a wide range of environments with respect to access to water. For example, *P. euphratica* is well adapted to arid regions of North Africa, the Middle east and China (Hukin et al. 2005). It can even grow on saline soils, and can tolerate brackish water. At the other end of the spectrum, *P. trichocarpa* is found primarily in riparian ecosystems, i.e. along rivers and creeks, in the wet and moist north west of North America, and can tolerate a water-logged root system, but is sensitive to drought (Chen et al. 1997; Marron et al. 2006). The related species, *P. deltoides* and *P. balsamifera* are more able to grow in the inlands of North America, with warm and dry summers. Already these few examples illustrate a considerable adaptive genetic variation with respect to drought tolerance in *Populus* species. Similar to other plants, *Populus* species respond to drought with physiological changes that include stomatal closure, leaf area reduction and osmotic adjustment, which together increase the availability of water and decrease water loss (Marron et al. 2002). In addition, the root/shoot ratio is increased, which further helps them to absorb water from the soil (Yin et al., 2005).

1.3.1. Utilization of hybrid poplar

Poplar hybrid clones have been selected primarily based on rapid growth and are grown all over the northern hemisphere for forestry and reclamation purposes where they represent an important commercial resource. Poplar trees are primarily harvested for pulp and paper production and composite lumber and board products (Dickmann 2001).

In addition, poplars are also planted to provide shelterbelts and windbreaks on the prairies to reduce water and wind erosion, in phytoremediation and as a riparian buffer to prevent contaminants such as fertilizer from going into streams (Dickmann 2001). Poplars have the potential to be used as a prominent component of programs to optimize carbon sequestration and so aid Canada in meeting its international commitments to reduce greenhouse gas emissions (McKenney et al. 2004; Yemshanov and McKenney 2008). Furthermore, poplar chips are used as feedstock to heat the houses in many towns and villages of Sweden (Christersson 2008). More recently, fast growing hybrid poplars have gained attention as a potential source of feedstock to meet the growing demand for non-fossil based transportation fuel (Gonzalez-Garcia et al. 2010; Sannigrahi et al. 2010; Yemshanov and McKenney 2008).

Commonly used and highly productive hybrids are generally considered drought sensitive (Marron et al. 2003; Monclus et al. 2006) and the patterns of global episodic drought over the last decade have pinpointed the need to develop drought tolerant poplar genotypes for use as a tool to achieve sustained forest productivity (Rood et al. 2003). While water use efficiency -the amount of water used for production of a given amount of biomass- of poplars is comparable to or better than some annual crops (Blake et al. 1984; Borrell et al. 1997; Sivamani et al. 2000) and even some conifers (Blake et al. 1984; Zhang et al. 1996), they cannot escape drought by setting seeds as annual plants do, and their large leaves do not prevent water loss and excess heating as conifer needles do (Mohammadian et al., 2007). In addition, there are predictions that global warming will increase the frequency and severity of summer droughts in poplar growing areas of Canada, USA, Europe and China (Hogg and Bernier 2005; Schindler and Donahue 2006) IPCC, 2013). Taken together, these observations suggest that expanded, and in some areas even sustained plantation of poplars depend upon the development of drought tolerant poplar genotypes (Rood et al. 2003).

1.3.2. Drought tolerance variation in hybrid poplar

Depending on the traits of parents, and the particular combination of traits in the selected offspring, there is also considerable variation in drought tolerance of poplar hybrids (Bradshaw et al. 2000; Chen et al. 1997; Marron and Ceulemans 2006; Tschaplinski et al. 1994). Two assessments of the effect of drought on productivity of

segregants after hybridizations show that the most productive clones tend to be the least drought tolerant (Marron et al. 2006; Monclus et al. 2005), indicating that there is typically a trade-off between these traits (Darychuk et al., 2012). There are exceptions though (Tschaplinski et al. 1998), implying that this is not a strict interdependence and that there are loci and alleles that can uncouple these two traits to some extent. As indicated above, drought tolerance is not usually the most important trait in the selection of a clone or clones to be deployed at a particular site. In addition, smaller site pre-trials that inform the choice of clones may not be evaluated well enough to warrant publication. As a consequence there are few systematic studies available comparing the performance of poplar hybrids with respect to drought tolerance (Monclus et al. 2006).

1.3.3. Poplar as a genetic model tree

The first tree and poplar species to have its genome sequenced was *P. trichocarpa*, which has a 485-megabasepair haploid genome, with a predicted 45,555 protein-encoding genes (Tuskan et al. 2006). The *P. trichocarpa* genome size is much smaller than other tree species with sequenced genomes, for example, Norway spruce with a 20-gigabase genome (Nystedt et al. 2013) and Eucalyptus with a 1.13-gigabase genome (Neale and Kremer 2011), making poplar a suitable model system to study biological functions in trees (Bradshaw et al. 2000; Jansson and Douglas 2007; Strauss and Martin 2004; Wullschleger et al. 2002). Vital molecular biology techniques such as quantitative trait loci analysis, expressed sequenced tags database and microarrays analysis are also available in poplar (Nanjo et al. 2004; Street et al. 2006). Furthermore, proteomic and metabolite profiling studies have also been performed in poplar (Brosche et al. 2005; He et al. 2008; Morreel et al. 2006; Plomion et al. 2006). Access to the full genomic sequence and gene transformation protocols makes poplar suitable for molecular and biochemical studies of tree growth and development (Tuskan et al. 2006). These tools are basic to understand plant responses to environmental conditions and to facilitate the identification and functional characterization of genes for traits of interest in trees, for example, stress tolerance with a major aim to develop strategies to improve tolerance to global warming-induced drought periods.

One major drawback of using poplar as a model organism is its dioecious nature with individual trees bearing either male (pollen-bearing) or female (seed-producing) flowers

(Pineda-Garcia et al. 2013). Poplar also has a long generation time that varies from 7 to 15 years before flowering and therefore, poplar breeding takes a long time to obtain progeny with desired traits. Poplar, on the other hand, can be transformed with DNA constructs, which provides overexpression of genes of interest that contains dominant alleles for overexpression or knockdown transformations, bypassing the need for sexual reproduction (Flachowsky et al. 2009).

1.3.4. Molecular genetic analysis of drought responses in poplar species

Genomic and proteomic studies have identified several hundreds of drought responsive genes in poplar but they are not functionally characterized, hence their involvement in drought tolerance is not known (Hamanishi et al. 2010; Plomion et al. 2006; Raj et al. 2011; Street et al. 2006; Wilkins et al. 2009). Only a few studies have been done to shed some light on the actual function of genes in drought tolerance. Transgenic *A. thaliana* overexpressing two poplar calcineurin B-like (CBL) genes, PeCBL6 and PeCBL10, exhibit enhanced drought tolerance as compared to non-transgenic plants (Li et al. 2013). Overexpression of drought-induced poplar scarecrow-like 7 (PeSCL7) also confers enhanced drought tolerance in transgenic *A. thaliana* plants (Ma et al. 2010). Another study demonstrates functional characterization of a poplar aquaporin and results show that aquaporins act as water transporters (Almeida-Rodriguez et al. 2010). Similarly, overexpression of poplar nuclear factor Y subunit B7 (NF-YB7) increases drought tolerance in transgenic *A. thaliana* (Han et al. 2013). Thus, taken together, there is still much to learn about poplar gene function in drought tolerance.

1.4. Objectives

To date there is no systematic study available that assesses the available variation with respect to drought tolerance in commonly used Canadian poplar hybrids. Growers have of course observed clonal differences in drought tolerance in the field over the years, but those differences are influenced by many other abiotic and biotic factors, and observations have, to our knowledge, not been quantified in a systematic manner. Therefore, we thought that the prudent first step was to induce drought under

standardized greenhouse conditions where the only obvious variable is the comparison of different hybrid clones. It stands to reason that if large differences in drought responses are found, they should trace back to adaptive and acclimative gene expression differences. Thus we hypothesize that a comparison of the expression of genes that represent different components of a drought response can be used to identify processes, and even individual genes that differ between drought sensitive and resistant clones. Such analyses may yield correlations between gene expression and drought tolerance, but do not provide evidence whether or not that change in expression actually has an impact on drought tolerance.

We hypothesize that if the identified genes have key functions in conveying a stress signal or mounting a stress response, altering the expression of those genes will have an impact on drought tolerance. As indicated above, poplars can be genetically transformed, providing an avenue for testing of gene function via altered levels of gene expression in transgenic poplar plants. While poplar transformation is fast and easy relative to other tree species, it is still a time consuming process. *A. thaliana* transformation is faster and a large number of individuals can be generated in a short time, allowing systematic analysis of drought responses. Despite the obvious morphological differences, *A. thaliana* is also a surprisingly close relative to poplars (Taylor 2002), suggesting that functional results obtained in *A. thaliana* are likely to reflect closely functions in poplar. For this reason, we thought it prudent to carry out a first functional analysis in *A. thaliana*, while pursuing poplar transformation and regeneration as a secondary objective. Here we describe the four main objectives of this thesis project.

- To compare physiological and growth responses of commonly used Canadian poplar hybrids to drought stress under standardized greenhouse conditions, and use the results to generate a ranking with respect to drought stress tolerance.

- To compare gene expression responses to drought in the least and the most tolerant clones identified above to pinpoint potential differences in the activity of processes and genes that may contribute to differences in drought tolerance.

- To test if identified genes play a role in drought tolerance by over expressing them in transgenic *A. thaliana* and assess transformants for altered responses to osmotic and drought stress.

- To carry out poplar transformation in which the expression of candidate gene is altered in a manner that may result in improved drought tolerance in regenerated poplar plants.

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Chapter 2.

Ranking of drought tolerance in nine poplar hybrids and identification of candidate genes for drought tolerance breeding

Abstract

Poplar hybrids are cultivated in North America as a fiber source for the pulp and paper industry primarily because of their fast growth, and they have, for the same reason, the potential to be used for carbon sequestration as well as a feedstock for the carbon-neutral production of energy. They are generally regarded as drought sensitive, which poses a problem for large-scale cultivation, particularly in light of predicted droughts caused by global warming. To approach this problem, we tested nine commonly used North-American poplar hybrids with respect to a series of physiological responses to drought, resulting in a ranking of drought tolerance. The difference between the least and the most tolerant variety was surprisingly large, and we used this to look for differences in the expression of genes involved in various aspects of a drought response. A set of genes displayed significant differences, including orthologs of genes in other species with known functions in drought tolerance, pinpointing potential aspects of drought tolerance in poplar.

2.1. Introduction

Poplars have been planted for centuries in agricultural areas of North America to provide wind and snow breaks, shade, and in more recent times, to prevent soil erosion and fertilizer run-off into waterways (Balatincz and Kretschman, 2001). More recently, fast-growing hybrid poplars have been planted to provide feedstock for pulp and paper production (Dickmann, 2001) and they are also being considered as a carbon-neutral alternative to fossil fuels for large-scale heating (Yuan et al. 2008), for carbon sequestration (McKenney et al. 2004) and as a feedstock for ethanol production (Gonzalez-Garcia et al. 2010; Karp and Shield 2008; Yemshanov and McKenney 2008). Life cycle assessments considering the energy used for cultivation, fertilizer production, harvest and conversion into biofuel show that hybrid poplars compare favorably to alternative annual crops (Butnar et al. 2010) and this holds true when ecological perspectives are considered (Robertson et al. 2000).

Poplar plantation forestry is not without problems, however, as the planted hybrids may not be fully adapted to local biotic and abiotic stressors. Commonly used hybrids are generally considered drought sensitive (Marron et al. 2003; Monclus et al. 2006). In addition, there are predictions that global warming will increase the frequency and severity of summer droughts in poplar growing areas of North America (IPCC, 2013). In particular, large parts of the Canadian prairies are frequently affected by low levels of precipitation coupled with high temperatures during summer, conditions that have worsened in the past century and are predicted to continue to do so (Hogg and Bernier 2005; Schindler 2009). These effects on water availability are compounded by human activities that have caused 20-84% reductions in summer water flow over the past 90 years in the major rivers of the Canadian prairie provinces (Schindler and Donahue, 2006). Taken together, these observations suggest that expanded, and in some areas the sustained plantation of poplars depends upon the development of drought tolerant poplar genotypes (Rood et al. 2003). From this perspective, it is important to characterize drought response and tolerance in hybrids currently deployed in the Canadian prairies to identify clones better able to cope with drought.

Poplars respond to water deficit in much the same way as many other plants. The response includes closing the stomata to reduce transpiration (Blake et al. 1984;

Hamanishi et al. 2010) which in turn reduces gas exchange and photosynthesis (Larcheveque et al. 2011; Silim et al., 2009). The reduced access to water and photosynthate in turn reduces the rate of leaf formation, stem and root growth (Bogeat-Triboulot et al., 2007; Liu and Dickmann, 1992; (Harvey and vanden Driessche 1997; Larcheveque et al. 2011; Yin et al. 2004; Yin et al. 2009). Poplars acclimate by reducing leaf area (Larcheveque et al. 2011), the density of stomata on leaves (Hamanishi et al., 2010), the shoot to root biomass ratio (Liu and Dickmann, 1992; Tschaplinski et al., 1998), and the width of tracheary elements, which results in improved water uptake and retention and avoids vascular embolism (Cai and Tyree 2010; Hacke et al. 2010). In extreme drought, leaves abscise and branch embolisms stop water transport (Rood et al. 2000) both of which reduce water loss. Many of these responses and the resulting ability to cope with drought differ considerably between species, within species, and between different hybrid clones, providing evidence for genetically based differences in adaptation (Bray 2004; Mazzoleni and Dickmann 1988; Monclus et al. 2005; Monclus et al. 2006; Xiao et al. 2008). Genotype comparisons also reveal strategies to cope with drought stress such as better stomatal control of water loss and higher root to shoot biomass growth (Tschaplinski et al. 1998), but also opposing alternatives, such as keeping stomata open followed by early leaf abscission, coupled with good recovery after re-watering (Larcheveque et al. 2011). Two assessments of the effect of drought on the productivity of segregants after hybridizations showed that the most productive clones tend to be the least drought tolerant (Monclus et al., 2005; Monclus et al., 2006), indicating that there is typically a trade-off between these traits. There are exceptions though (Tschaplinski et al., 1998), implying that this is not a strict interdependence and that there are loci and alleles that can uncouple these two traits to some extent.

At the molecular level poplars respond to drought by altering the expression of many hundreds of genes (Hamanishi et al. 2010; Street et al. 2006; Wilkins et al. 2009). Genes likely to be involved in photosynthesis and various biosynthesis processes are generally down-regulated, whereas genes with sequence similarity to stress-related genes are up-regulated (Street et al., 2006). The sets of up-regulated genes include candidates for ABA synthesis, signaling and ABA-induced transcription factors (Chen et al. 2013; Hamanishi et al. 2010; Wilkins et al. 2009). Many genes encode putative effecter proteins, including enzymes involved in the synthesis of compatible sugars, sugar

alcohols and amino acids that maintain cellular turgor, and protective proteins such as late embryogenesis-abundant and heat shock proteins that presumably act to stabilize proteins and membranes, as well as enzymes that inactivate reactive oxygen species (Cohen et al. 2010; Plomion et al. 2006; Street et al. 2006; Xiao et al. 2009). Quantitative trait loci mapping has identified several genomic domains that contain loci involved in regulation of drought tolerance (Street at al., 2006). Street et al. (2006) also showed that offspring that differed in their response to drought also differed with respect to the levels of expression of many drought-induced genes. Another positive correlation has been found for superoxide dismutase activity and drought tolerance, presumably due to better capacity to handle drought-induced oxidative stress (Regier et al. 2009). Taken together though, the body of evidence linking any specific gene of the drought induced genes in poplars to drought tolerance is currently weak to absent.

Here we sought to rank a set of hybrid poplar clones grown on the Canadian prairies with respect to drought tolerance and thereafter use the most sensitive and tolerant clones to identify potential differences between them with respect to the expression of poplar orthologs of genes implicated in various aspects of drought responses in other plants. This comparison yielded a small set of genes whose expression correlated with drought tolerance. The identified genes includes putative orthologs of genes in other species for which there is functional evidence for a role in drought tolerance, implying that our approach has identified relevant genes also in poplar trees.

2.2. Materials and Methods

2.2.1. Poplar hybrids

Hardwood cuttings of nine hybrids were obtained from Barb Thomas, Alberta Pacific Forest Industries, and Bill Schroeder, Agriculture & Agri Food Canada, Indian Head, Saskatchewan. Poplar hybrids included Green Giant (GG), Assiniboine (Schroeder and Lindquist 1989), AP36, Canam, Katepwa (Kate), Hill, Walker (Lindquist et al. 1977), WP69 (also known as Okanese; Schroeder et al., 2013) and WP86. Hill, AP36, and Walker are hybrids of *P. deltoides* x *P. petrowskyana* where *P. petrowskyana* is a hybrid of *P. nigra* x *P. laurifolia* (Silim et al., 2009). Walker is a parent of WP-69 and WP-86,

which are therefore half siblings. In addition, *P. petrowskyana* is the second parent of WP69 (Schroeder et al., 2013). Walker is also a parent of Katepwa and Assiniboine whereas the second parent of these hybrids is unknown. Similarly, the Green Giant clone is a hybrid of *P. deltoides* x *P. petrowskyana* (Silim et al., 2009). Hill, Katepwa, WP69, Walker and Assiniboine originate from Indian Head, Saskatchewan whereas Green Giant and AP36 originate from Brooks (Silim et al. 2009).

2.2.2. Drought stress trials

Cuttings were placed in 2.7 L nursery pots containing a soil mixture of two parts Promix BX peat (Westgro, Delta, BC) and one part sand. When the cuttings established roots, a 20-20-20 (NPK) fertilizer solution (66.7 g/L, Plants Products Co. LTD, Brampton, Ontario) was provided once every week. The plants were grown under 16/8 hour light/dark periods with ~50-70% relative humidity at ~25 ^{0}C in a greenhouse. Trees were flood-irrigated once every two days with water containing 0.26 g/L fertilizer. Trees were inspected regularly for signs of insect pests, which were controlled using biological control methods. Two weeks before the start of a drought stress trial, trees were transferred to 5.7 L pots in the same soil mixture. Up to 18-24 trees per clone were maintained in the irrigation troughs whereas the same number of trees was placed outside the irrigation trough so that water was withheld.

2.2.3. Physiological data collection

A plastochron index was established at the start of the trial for all trees by tying string to a partially opened leaf (~0.5cm), which was designated as leaf 0. The leaves above leaf 0 were designated -1, -2, -3, -4 and so on in order of emergence whereas the leaves below leaf 0 were designated +1, +2, +3, +4 and so on. The 3 uppermost young leaves were collected for RNA extraction, the leaf below these was collected for water potential (ψ_w) measurements and the leaf below the ψ_w leaf was collected for relative water content (RWC).

Physiological data were collected on (1) Day zero -the day on which the trial started- (2) when the trees attained a mild stress level, indicated with the same time point at day 4 (3) and when the trees attained a more severe stress level i.e. all leaves wilted. Tree

height growth rate, number of newly emerged leaves, ψ_w and RWC were measured on each data collection day. Height growth rate was calculated as increase in height (cm/day) whereas leaf formation as number of new leaves emerged per day. Leaf ψ_w was measured using a pressure bomb (PMS instrument Co). RWC was determined by removing ten discs from the leaf into a pre-weighed vial and sealed. The fresh, full turgid, and the dry weights of leaf discs were obtained and RWC was calculated as: RWC = [FW-DW/FTW-DW] x 100.

2.2.4. Quantitative real time PCR (RT-qPCR)

RNA was extracted as described in (Change et al., 1993). RNA was treated with DNase1 (1 unit; Fermentas) before being used for cDNA synthesis. Approximately 1 μg RNA was used to prepare cDNA using the iScript cDNA synthesis kit (BIO-RAD Laboratories, Mississauga, ON). The qPCR was set up according to the manufacturer's protocol (BIO-RAD) and SYBR Green master mix (BIO-RAD) in a volume of 20 μL. Target genes were amplified along with reference genes (Ubiquitin & Elongation factor 1-α) in triplicate in a DNA Engine Opticon 2 system (BIO-RAD). The target and reference genes were quantified using the delta Ct and delta-delta Ct methods (Livak and Schmittgen 2001).

2.2.5. Quantification of ABA

Freeze dried samples from control and drought stressed GG and Walker genotypes were sent to the Plant Biotechnology Institute, National Research Council Canada for analysis. Analysis was performed on a UPLC/ESI-MS/MS utilizing a Waters ACQUITY UPLC system.

2.2.6. Statistical analysis

Statistical analyses were performed with JMP8 software (SAS institute, NC, USA). The values are presented as mean from three to twenty-four replications per experiment. One-way analysis of variance (ANOVA) along with Tukey's honest significant difference (HSD) method was used to test whether or not the means of several groups were equal (Figs. 2.2 and 2.3). Means with letters A, B, C, D, E and F indicate that they are

statistically significant from each other, two means with same letter indicate an insignificant difference between them. Student's t test was used in Fig. 2.4 to 2.8 and supplementary figures.

2.3. Results

The major objectives of this study were to identify potential differences in drought tolerance among popular Canadian poplar clones and to use the obtained differences as a tool to identify gene expression markers correlating with drought tolerance.

2.3.1. Ranking of poplar hybrid clones with respect to drought tolerance

We tested nine clones; Green Giant (GG), Assiniboine, AP-36, Canam, Katepwa, Hill, Walker, WP69 and WP86. The trials were carried out in a greenhouse to minimize the impact of environmental variation on the outcome. In each trial, 18-24 trees per clone were subjected to drought, while a similar number of trees were kept as well-watered controls. We observed that clones differed considerably in the time it took to display severe drought stress symptoms as indicated by flaccid, somewhat shriveled leaves and leaf water potentials (ψ_w) between -1.4 and -2.4 MPa. For example, GG attained a severe stress state after 7.5 days, Walker after 11 days, WP69 and Katepwa took 11.5 days (Fig. 2.1a). The time to show severe drought symptoms were intermediate for the remaining five clones. These data indicated large differences in the development of drought symptoms between the selected clones and also implied that the characterization of responses at specific time points may be misleading as clones will be at different stages of drought symptom progression. To accommodate for these differences, we chose to compare physiological and molecular responses at a specific time point as well as when plants displayed comparable external physiological symptoms, regardless of the time taken to develop those symptoms. To this end, plants were characterized four days after the withdrawal of water resulting in mild stress (MS), and then again when plants displayed obvious wilting of leaves and shedding of lower leaves indicative of severe stress (SS; Fig.2.1b). We recognize that the definitions of the mild and severe stress symptoms are imprecise, but this method provided us with plants

with a more comparable physiological status thereby facilitating sampling. As will be seen, the two different comparisons also produced similar rankings.

Ranking of clones at mild stress

Clones displaying uniform mild drought stress symptoms (some leaf wilting) did not show similar leaf ψ_w and RWC values. For example, GG leaves had both ψ_w and RWC values that were significantly lower than the other clones (Figure 2.2 A, B). At the other end of the spectrum, Walker leaves displayed ψ_w and RWC values that were significantly higher than the other clones. The remaining seven clones were intermediate between GG and Walker with respect to these physiological traits. This trend was maintained upon assessment of growth parameters. GG height growth was reduced by 55% and leaf formation by 63% (Figure 2.2 C,D). At the other extreme, both Canam and Walker showed significantly higher levels of height growth compared to the other clones. To our surprise, the height gain of mildly stressed Canam and Walker plants was actually higher than the corresponding well-watered controls (Figure 2.2 C). Water to field capacity can inhibit poplar growth (Dickmann et al., 2001) and this is supported by these data showing that when Canam and Walker were watered to field capacity they displayed reduced growth relative to that observed when water was initially withdrawn. Similarly, the frequency of leaf formation was significantly higher in Walker compared to the other clones (Figure 2.2 D). The mildly stressed Walker plants gained 38% more leaves than the well-watered controls, providing additional evidence that the withdrawal of water (leading to a mild water deficit stress) increased overall growth. The mildly stressed trees of two other clones, WP69 and Katepwa, also had leaf formation rates above 100% compared to well-watered controls providing additional data that supports the growth promotion observed during a mild stress. Taken together, the analysis of these four parameters in poplar clones displaying mild stress symptoms identified GG as highly sensitive to mild stress and Walker as tolerant to mild stress, with the other clones falling between these two extremes.

Ranking of clones at severe stress

More so than at mild stress, we predicted that plants displaying severe stress symptoms would lose approximately equal amounts of water because the symptoms displayed were similar across clones. Instead severe stress exacerbated the differences between

clones (Figure 2.3 A). The ψ_w of GG was less than 30% of the corresponding control. AP36 ψ_w were significantly higher than GG. In turn, ψ_w in Assiniboine, WP86, Katepwa and Walker was significantly higher than AP36. Finally, ψ_w in Walker was significantly higher than all other clones with only a 30% loss after 11 days as opposed to a 70% loss in GG after 7.5 days. The differences in water potential across clone indicate clone differences in the underlying osmotic capacity of cells. Differences in RWC were not as large as those for ψ_w. GG and Canam nevertheless had more than 20% lower RWC than Walker. The largest impact of severe stress was seen when height growth was recorded (Figure 2.3C). This also resulted in a series of group-wise differences of significance levels. Height growth of GG was reduced to a mere 5% of control after 7.5 days, while Walker retained 57% of the control after 11 days of stress. In total, six clones had height growth reduced to less than 30% of controls, significantly lower than the three top clones, with reductions of between 45 and 60% of controls. A larger range of differences was seen when leaf formation rates were compared, ranging from a rate of 5% in GG to 75% in Walker compared to controls, with a series of group-wise significance levels in between. In summary, the characterization of responses to severe stress produced more marked differences between clones than at mild stress and maintained GG as the least tolerant and Walker as the most tolerant clone. Significant differences between the other clones were also observed, but the ranking order varied with the applied criteria.

2.3.2. Comparison of GG and Walker at the molecular level

The characterization of responses to drought stress provided a ranking of clones. In particular, it showed that GG was the least tolerant and Walker was the most tolerant clone. We reasoned that a comparison of these two distinctly different clones might shed some light on potential molecular differences pertaining to tolerance, and therefore pursued further analysis with these two clones. In Figure 2.4B, we show a direct comparison of leaf ψ_w in GG and Walker. ψ_w is comparable in well-watered Walker and GG plants. GG responds to severe stress by an almost four-fold decrease in ψ_w, while Walker responds with a marginal, although significant, decrease in ψ_w. ABA is produced at higher levels in response to drought, and acts as a signaling molecule at both the long distance and local levels to elicit an acclimation response. We therefore compared the foliar content of ABA in GG and Walker to assess whether ABA levels reflected the ψ_w

levels. In the absence of stress, there were no significant differences in ABA levels between these clones (Figure 2.4A). GG leaves responded to severe stress with a 20-fold increase in ABA levels, while Walker leaves responded with a 16-fold increase. In absolute terms the differences are larger, with 39 μg /g dry weight ABA in GG and 18 μg /g dry weight ABA in Walker. With respect to absolute levels, the accumulation of ABA appears to reflect the ψ_w levels in severely stressed leaves. It could be argued though that Walker mounts an almost equally strong response as GG in terms of fold-induction of ABA, and a considerably stronger response than GG if ABA accumulation is considered in context with the absolute and fold change in ψ_w between control and stressed leaves (see discussion).

Gene expression markers of drought stress

Since it is well known that a large number of genes respond to stress by changes in RNA levels, we identified 26 poplar genes that are known to be expressed at elevated levels in response to drought stress, including a set with high homology to genes in other species that have been implicated in stress response and tolerance. We obtained these candidates from genomic and proteomic studies, which were conducted on several poplar genotypes (Hamanishi et al. 2010; Plomion et al. 2006; Street et al. 2006). Only genes that were common to the three publications and belonged to different but important functional categories for drought tolerance were selected. This includes proteins involved in ABA synthesis and signaling, potential stress response effectors such as an aquaporin and a LEA protein, and also those involved in metabolism and protein processing (Table 2.1). The primers used to amplify these genes are given in the table 2.2. While this is a small set of genes, we reasoned that they may serve as markers to identify processes whose regulation may differ significantly between the GG and Walker clones.

Gene expression in respons to mild stress

In line with published results (Plomion et al. 2006; Street et al. 2006), we found that many of the stress-induced genes we examined were expressed at elevated levels in leaves of mildly stressed GG and Walker plants (Figure 2.5). In GG, 13 out of 26 genes showed average steady-state levels of transcripts between 10 and 1,000-fold higher in stressed leaves than in control leaves (Figure 2.5A), although only five of these had

transcript levels that were significantly higher than that of well-watered control plants due to biological variation. In Walker, the transcript levels of none of the 26 genes were 10-fold higher in stressed leaves, and none of the 26 genes were up-regulated at statistically significant levels (Figure 2.5B). In addition, a subset of the genes had average steady state transcript levels that were lower in stressed compared to control leaves, although again not at statistically significant levels.

Gene expression in response to severe stress

At the molecular level the response of GG to severe stress was marginally different from its response to mild stress with 14 instead of 13 genes showing a 10-fold or higher average transcript levels, and the same number of genes with significantly higher levels of expression relative to that of control leaves (Figure 2.6A). Walker, on the other hand, showed a considerably stronger response to severe stress compared to mild stress. Now 10 instead of 0 genes had an average 10-fold or higher transcript levels, and 4 rather than 0 genes had significantly higher transcript levels. There were also five genes in Walker that responded to severe stress with a significant reduction of transcript levels (Figure 2.6B).

Clonal differences in response to mild stress

Below we directly compare the expression of individual genes in GG and Walker. Of the 26 tested genes, eight genes showed significantly different transcript levels in GG compared to Walker, with all of them showing higher levels of expression in GG (Figure 2.7). These genes encode putative orthologs of a protein phosphatase 2C (PP2C), a cytochrome P450 (CYPA), ring finger protein (RF), drought responsive family protein (DRFP), DC1 domain-containing protein (DC1) a putative ABA responsive element binding protein (ABF2) transcription factor, a galactinol synthase (Gal) and starch branching enzyme (SBE), with 10, 4, 22, 9, 27, 53, 31, and 22 times higher levels of expression in GG, respectively. The remaining 18 genes showed non-significant differences in gene expression. We note though that there is a clear tendency towards higher expression in GG compared to Walker (Supplemental fig. 2.1). In well-watered control plants, seven of the eight genes in fig. 2.7 showed no significant differences in expression between GG and Walker, indicating that differences in expression were the result of stress (Supplemental fig. 2.3). Among the eight genes, the putative PP2C

stands out as different from the others as orthologs from other species are negative regulators of ABA signaling (Gonzalez-Garcia et al. 2003; Kuhn et al. 2006; Rubio et al. 2009a; Saez et al. 2004). It is therefore interesting that PP2C is expressed at significantly higher levels in GG than in Walker (Figure 2.7), as this implies that PP2C may be inhibiting ABA-mediated stress responses more in GG than in Walker (see discussion).

Clonal differences in response to severe stress

We found that eight genes were expressed at significantly different levels between GG and Walker, including four genes with significantly different levels of expression at mild stress (Fig. 2.7, 2.8). The four genes were PP2C, DC1, ABF2 and SBE with 82, 50, 22, and 78 times higher average expression in GG respectively. Three genes showed significantly higher expression in GG compared to Walker only at severe stress. These genes encode a putative late embryogenesis abundant protein (LEA), an aquaporin (aqua), and a CBL-interacting protein kinase (CIPK) and they had 10, 20, and 20 times higher expression in GG respectively. One gene, encoding a putative nine-cis expoxy carotenoid dioxygenase-like 3 (NCED3), defied the trend in that it was expressed at significantly higher levels in Walker rather than in GG (Fig. 2.8). No significant differences in the expression of these genes were found in well-watered GG and Walker plants (Supplemental fig. 2.4), indicating that differences in expression were stress induced. The expression of the remaining 18 genes did not differ significantly between GG and Walker (Supplemental fig. 2.2).

In summary, we identified a set of genes whose responses to stress differed significantly in the two assessed varieties. Three of the genes are potential orthologs of genes encoding putative functions in ABA synthesis or signaling, indicating that this aspect of drought responses may be part of the explanation as to why Walker is more drought tolerant than GG.

2.4. Discussion

With the poor prospects for access to sufficient water in mind, we evaluated the response to controlled drought of a set of poplar hybrid clones grown in the Canadian

prairies. This produced a ranking based on physiological and growth responses. We are aware that this ranking does not replace a long-term field trial, which by its nature will capture the effect of many more factors that interact with drought stress, e.g. biotic stressors. On the other hand, field trials can be hard to interpret. Our standardized greenhouse-conditions allow us to state with confidence that the observed differences between clones reflect different responses to the same withdrawal of water and not to other environmental variables. While there have been other studies assessing the drought responses of hybrid poplars, the majority of them compare and contrast the physiological response of no more than two to three clones (DesRochers et al. 2007; Dickmann et al. 1992; Lei et al. 2006; Xiao et al. 2009; Yin et al. 2004; Yin et al. 2005).

Here we compared and ranked nine hybrid poplar clones that are in use on the Canadian prairies. The differences in response to drought stress were remarkably large considering that many of the tested hybrid clones are closely related. For example, the rate of leaf formation in GG during mild stress was ~ 20 % that of Walker (Fig. 2.2D). At severe stress, the rate of leaf formation in GG was a mere 6% of that of Walker (Fig 2.3D). Similarly, the water potential of GG leaves was significantly lower than that of Walker leaves at mild stress (Fig. 2.2A). While it is clear that Walker ranks as the overall top clone and GG as the overall bottom clone with respect to drought tolerance, the ranking of the intermediate seven clones varied for the different criteria. To assess if any overall tendency could be seen, we averaged the ranking in figures 2.2 and 2.3 (Supplemental fig. 2.5). This ranking showed rather small differences between the seven clones with WP69 and Katepwa emerging as distant second and third ranked drought tolerant clones after Walker. This averaging attached the same weight to all assessed criteria. WP69 and Katepwa again joined Walker as the top three clones with respect to rate of leaf formation, but not with respect to height growth. From a growers' point of view, field performance in terms of leaf formation and shoot growth in response to stress would probably be more relevant than maintenance of water potential and relative water content. The standardized conditions used in this study revealed differences among the clones that in the end may be attributed to underlying genetic differences in multiple loci controlling responses to drought stress. It also raises the promise that recurrent selection from offspring after crosses between the most drought resistant clones

identified here can be used to increase the overall frequency of beneficial alleles to generate novel, more drought tolerant varieties.

Most of the tested genes were up-regulated in response to drought stress in the GG and Walker clones. GG responded to mild stress with an overall stronger up-regulation of genes compared to Walker. This observation is consistent with the stronger physiological responses of GG relative to Walker described above. The marginally stronger response in gene expression of tested markers in GG to severe stress indicate that, at least with respect to this set of genes, the response of GG had reached a maximum already at mild stress.

Walker mounted a stronger marker gene expression response at severe compared to mild stress, but the overall response was still weaker than that of GG. This is consistent with the physiological data (Figure 2.3) as well as the lower ABA content in Walker leaves compared to GG after severe stress (Figure 2.4). Curiously, Walker but not GG had a set of genes that were down- rather than up-regulated in response to both mild and severe stress. Thus, even in this limited set of genes, we found striking differences in the expression of stress-induced gene expression between these two clones. Speaking against this option though is the rather limited physiological and growth reduction symptoms indicating limited stress. Several of the significantly down-regulated genes were also down-regulated to a limited extent in mildly stressed Walker plants (Figure 2.5B). It appears that Walker responds to stress by down-regulation of these genes instead of the up-regulation seen in GG as well as in previous publications reporting on the expression of these genes (Plomion et al. 2006; Street et al. 2006) and that down-regulation of these genes may be part of the response that contributes to the relative drought tolerance in Walker plants. In support of this hypothesis, one of the genes that is up-regulated in GG but down-regulated in Walker encodes a potential ortholog of a protein phospatase, known as PP2C, which acts as a negative regulator of ABA signaling and drought responses in *A. thaliana* (Kuhn et al. 2006; Rubio et al. 2009b; Saez et al. 2006). Lower expression in Walker imply reduced inhibition of ABA-mediated stress responses.

Another gene that is significantly down-regulated in Walker is a potential ortholog of an *A. thaliana* aquaporin-encoding gene. Members of aquaporin-encoding genes have been

observed to be both up- and down-regulated in response to drought stress (Bartels and Sunkar 2005). Down-regulation of aquaporin genes may enhance drought tolerance by preserving cellular water in tobacco and citrus (Rodriguez-Gamir et al. 2011; Smart et al. 2001), and it appears plausible that leaf cells could limit water loss by having fewer aquaporin proteins in their plasma membranes. While the largest differences in gene expression between GG and Walker can be traced to lower or unchanged expression in Walker and up-regulation in GG (Figure 2.8), there is one gene that is expressed at significantly higher levels in Walker than in GG in response to severe stress. This gene encodes a potential ortholog of the *A. thaliana* nine-cis-epoxycarotenoid dioxygenase 3 (NCED3), an enzyme that catalyzes an important step in ABA biosynthesis (Seo and Koshiba 2002; Thompson et al. 2000). While the elevated expression is consistent with the ability of Walker to withstand severe stress better than GG, Walker leaves actually have lower ABA content than GG at severe stress (Fig. 2.4). If ABA levels in GG and Walker leaves are compared by fold-induction between well-watered and stressed plants, the response of Walker is comparable to that of GG. If compared in terms of fold-increase of ABA per fold decrease in ψ_w, Walker actually mounts a much stronger ABA response than GG, which is in line with the significantly higher levels of NCED3 transcripts compared to that of GG leaves. NCED 3 as an enzyme in ABA biosynthesis could mediate stomatal closure and gene expression changes that promote drought tolerance (Bartels and Sunkar, 2005).

We acknowledge that there may be many factors contributing to the differences in drought tolerance between GG and Walker. For example, there may be differences in the number of fine roots and root hairs and hence ability to take up water. Structural differences, however, cannot explain the differences observed in the expression of individual genes in the two clones responding to a similar stress. That the identified differentially expressed genes may indeed be involved in conferring drought tolerance is supported by the altered expression of their potential *A. thaliana* orthologs in transgenic plants. Overexpression of AtNCED3 increases drought tolerance in *A. thaliana* (Iuchi et al. 2001) demonstrating its role in providing this trait. Gain of function mutants expressing *A. thaliana PP2CA* have reduced drought tolerance, whereas loss of function mutants of the same gene have enhanced drought tolerance (Kuhn et al. 2006; Rubio et al. 2009b; Saez et al. 2004; Saez et al. 2006), demonstrating its role as a negative

regulator of drought tolerance. Likewise, overexpression of a *Zea mays* PP2C (ZmPP2C) ortholog in transgenic *A. thaliana* plants also result in plants with reduced drought tolerance (Liu et al. 2009).

In conclusion, we have generated a first ranking of the response of nine Canadian poplar hybrids to drought as well as identified gene markers whose presumed activities correlate with drought tolerance. We predict that identified drought-tolerant clones may be further improved either by transgene technology altering the expression of these genes, or by further hybridization of identified tolerant clones, using these expression markers for early selection of offspring with improved drought tolerance.

Table 1. Genes assessed by RT-qPCR. Functional category (in bold heading) and annotation based on similarity to genes in other species. Abbreviated gene names used here, poplar transcript name and ortholog genes in separate columns.

	Poplar transcript name	Ortholog gene	Functional Annotation
1			Signal /hormone transduction
	Potri.018G119200.1	At1g30270	CBL-interacting protein kinase (CIPK)
	Potri.010G123400.1	At1g68300	Ethylene-responsive protein (ERP)
	Potri.008G011300.1	At2g27050	Ethylene –insensitive 3 (EIN3)
	Potri.008G059200.1	At3g11410	Protein phosphatase 2C, putative (PP2C)
	Potri.001G393800.1	At3g14440	Nine-cis-epoxycarotenoid dioxygenase 3 (NCED3)
	Potri.014G028200.1	At1g45249	ABA-responsive element binding protein 1 (AREB1)
	Potri.014g028200.1	At1g45249	ABA responsive element-binding protein 2 (ABF2)
2			Stress effectors
	Potri.006G092700.1	CAC18558	Universal stress protein (USP)
	Potri.005G020900.1	At1g56280	Drought responsive family protein (DRFP)
	Potri.004G174100.1	At4g35985	Senescence/dehydration-associated protein (ERD7)
	Potri.002G165000.1	At2g46140	Late embryogenesis abundant protein (LEA)
	Potri.008G039600.1	BAD14371	Aquaporin, putative PIP2.1-like (Aqua)
	Potri.015G078900.1	At5g63030	Glutaredoxin, putative (Gludox)
3			Metabolism
	Potri.008G149200.1	At1g54870	Short chain dehydrogenase/reductase (SDR)
	Potri.002G189900.1	At1g23800	Aldehyde dehydrogenase (AD)
	Potri.013G005900.1	At1g09350	Putative galactinol synthase (Gal)
	Potri.006G033300.1	At2g40890	Cytochrome P450, putative (CYPA)
	Potri.019G100000.1	At5g02380	Metallothionin protein, putative (MT2A)
	Potri.005G251000.1	At5g03650	Starch branching enzyme (SBE)
4			Protein processing or binding
	Potri.006G141700.1	At5g60360	Cysteine protease (CP)
	Potri.004G133800.1	At5g26360	Chaperonin, putative (Chap)
	Potri.014G170400.1	At2g04240	RING-H2 finger protein (RF)
	Potri.014G071600.1	At1g01940	Peptidyl-prolyl cis-trans isomerase (PPCI)
5			Unknown
	Potri.005G244700.1	At1g60420	DC1 domain-containing protein (DC1)
	Potri.005G002100.1	At3g05880	Hydrophobic protein (HP)
	Potri.015G091900.1	At3g48510	Unknown protein (UP)

Table 2. Name and sequence of primers used for RT-qPCR experiments. Primer names correspond to the gene abbreviations used in table 1.

Primer name	Primer sequence
SDAP	F: AAG AGT TCT GCC GCG TAT TG R: CCT CCT TTT GAT CCT CCT CA
SBE	F: CTC AGG ATG CCC AAA TTC AT R: TGT GTT GCT TAT GCC GAG AG
CIPK	F: CAT CAG ACT TGC GAA CCT CA R: GCT TGT TAA ACG GGA AAC GA
PPCI	F: TGT CAC GCG ATT GAT CCT TA R: AGA GCA TAA TGC GAG GGG TA
AREB1	F: CCC CAG CTC TCA ACA AAA AC R: AGA CAC AGA CCA TGG CAA CA
Gludox	F: CCA CCA ATG TGT TTC CCT TC R: AAA CAG GCT GCG CTT AAA AA
MT2A	F: GGCTGTAGCTGTGGCTCTGA R: GGATCGCAGGTGCAGTTTG
DRFP	F: TGA TTC ATG GAG TGC TCG AC R: GAA CAG AAA GGG CAT GGA AA
USP	F: GGG AGG AGA TGC CAG AGA GA R: CCA TGC CCA GAT GAT TGC T
ERP	F: GGT GGG TGA TCC TCA AAC AG R: ACT ACC AGA ACG GGG CAC TT
LEA	F: TGC CAA GGT GTC TGT CAA AA R: CAA CGT CCT TCA CCA AGC TC
DC1	F: AGT CCA GGC TTT CCC GTT TA R: TTG CCT ACC AAT GAG GCA AC
ABF2	F: GGT GGA ATC AGA CCC ACT CA R: TTC CAA CAA CCG ATC GTC TC
Gal	F: CCA TGG AGG TTC ACT GGA AA R: GGA TGG GGC GGT AAC ATA GT
CP	F: GGA AGG AGT TTT CAC CAC CA R: ATG CAC AGG TTG CAA TAC CA
PP2C	F: GTG CTT TGT CGA AAC GGT GT R: CGG TAT GAC GTA GGG CTT CA
NCED3	F: ATC CGG GGA GAA TCT GAA GT R: TGT TCG TGT ACT GCC CTC TG
EIN3	F: AAC CGA GCT GAC TCT GGT GT R: CAC ATC CTC CTC TCC AGC TC
CYPA	F: AAG ACG ACG ACG ACG AAG AC R: CGA GCG AGG CTA TCA ATC TC
AD	F: ACC AAT CGG TGT AGC AGG TC R: CAT GAA ATA GCT TGG CAG CA
Chap	F: CAC GCT CCA GTT CTC GTT CT R: ACT CAC GGA GGA TTG CAT TG
HP	F: TTG CCT CCT CTT GGT GTC TT R: TCA CTT GGT GAT GGC GTA GA
RF	F: GAC TGC TCG GTT TGC TTG AC R: GCT CAC CAA AAG CAA GAT GC
UP	F: TGC AAA ACA ACC ACT GTC CA R: GAA AGT GCC GAT GAA GGT CA
SDR	F: GTT TCC TTC ACC AGG GGA TT R: AGG CTG TCC TGC TCT TTT CA
Aqua	F: CGC TGG TAT CTC TGG TGG AC R: ATC TCA GCA CCC AAA GCT G
UBQ	F: GTT GAT TTT TGC TGG GAA GC R: GAT CTT GGC CTT CAC GTT GT
EF1a	F: GAC GGT ATT TTA GCT ATG GAA TTG R: CTG ATA ACA CAA GTT CCC TGC

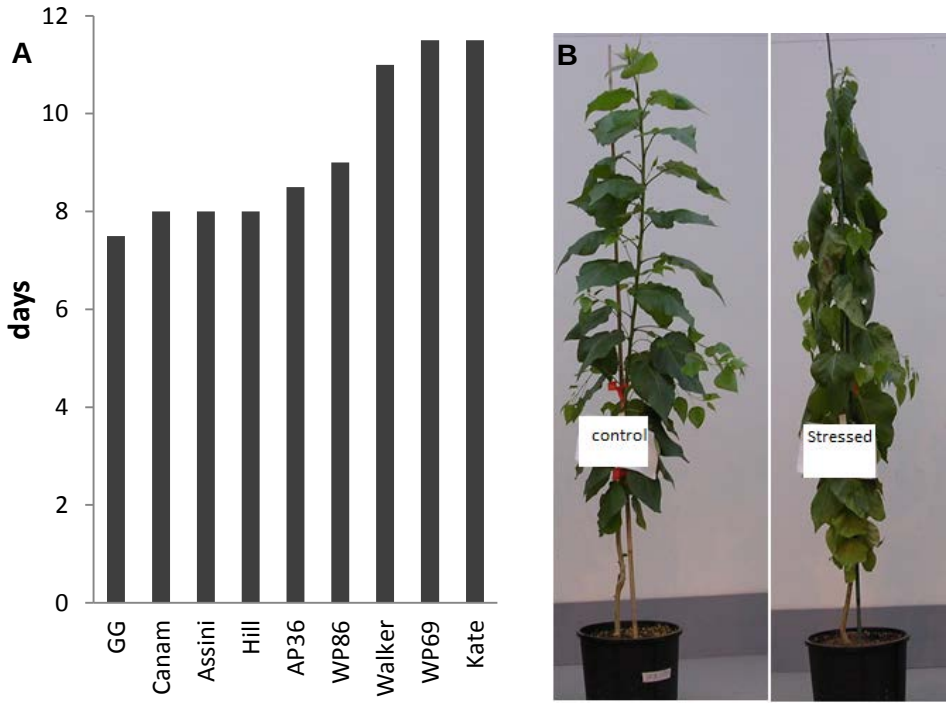


Figure 2.1. Differences among clones in the time taken to display severe drought stress symptoms. A, number of days each clone took to exhibit severe drought stress symptoms after withholding water. The values are an average of two independent experiments (n = 16-24 per experiment). B, example of a well-watered control plant (left) and a severely stressed plant with wilted leaves (right).

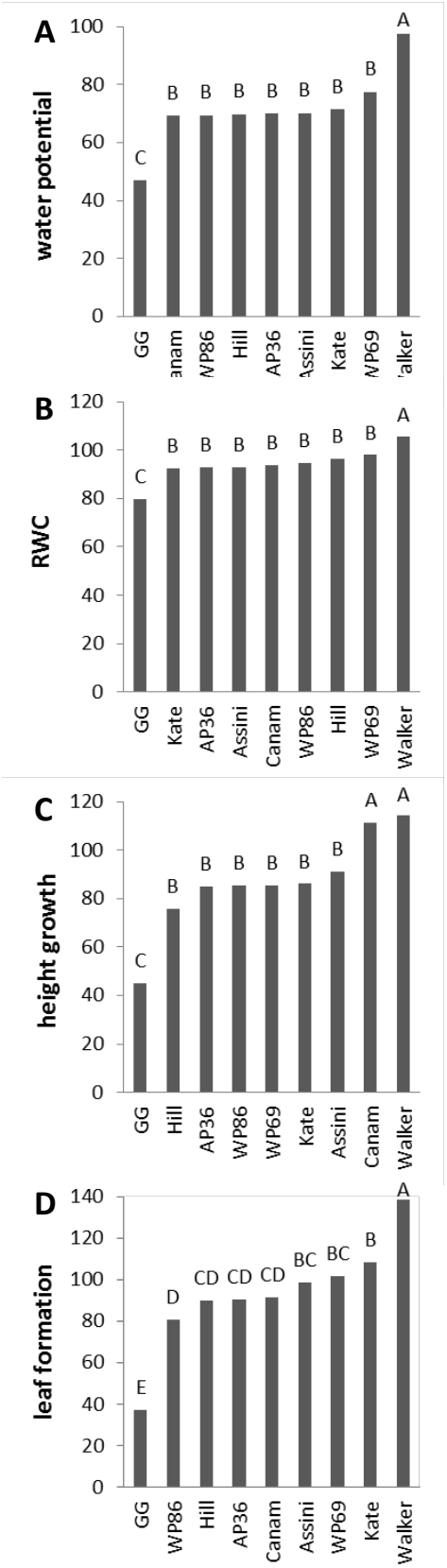


Figure 2.2. Physiological response of poplar hybrids to mild drought stress. A, water potential; B, relative water content; C, height growth rate (cm/day) and D, leaf formation (newly emerged leaves per day). Values are the mean presented as a percentage of well-watered control plants. Different letters indicate that means are statistically significant from each other at $P < 0.05$ (Analysis of variance/Tukey HSD). For water potential and RWC, n=3-6; for Height growth and leaf formation, n=15-24.

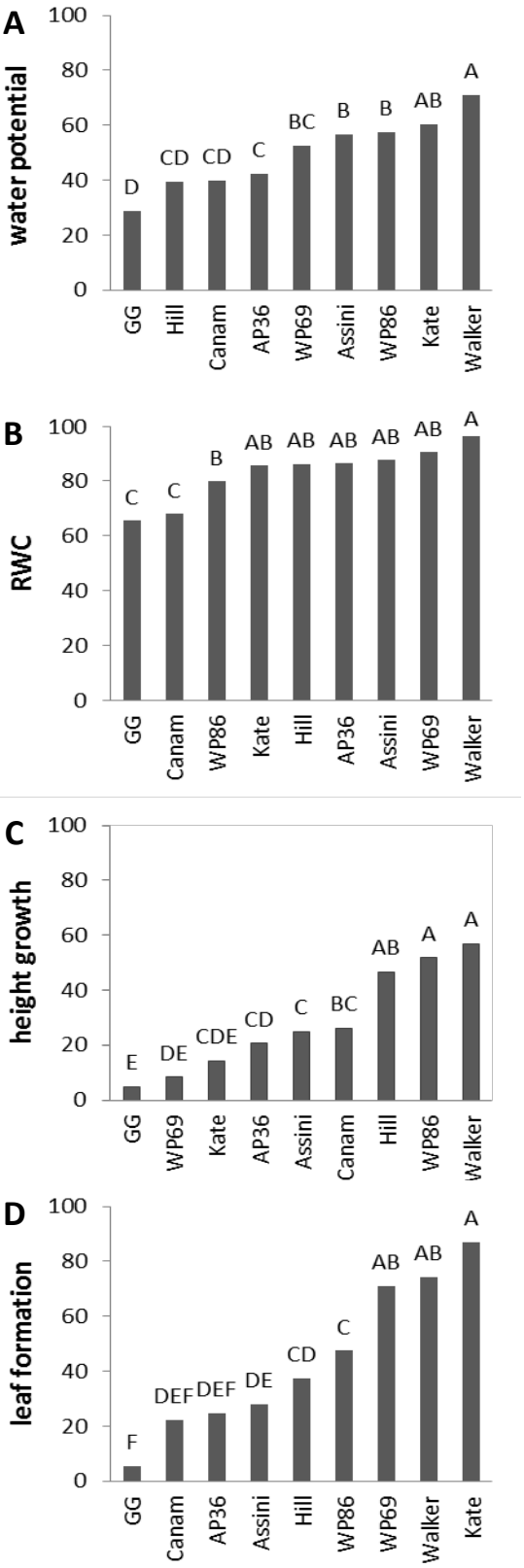


Figure 2.3. Physiological response of poplar hybrids to severe drought stress. A, water potential; B, relative water content; C, height growth rate (cm/day) and D, leaf formation (newly emerged leaves per day). Values are the mean presented as a percentage of well-watered control plants. Different letters indicate that means are statistically significant from each other at $P < 0.05$ (Analysis of variance/Tukey HSD). For water potential and RWC, n=3-6; for height growth and leaf formation, n=10-18.

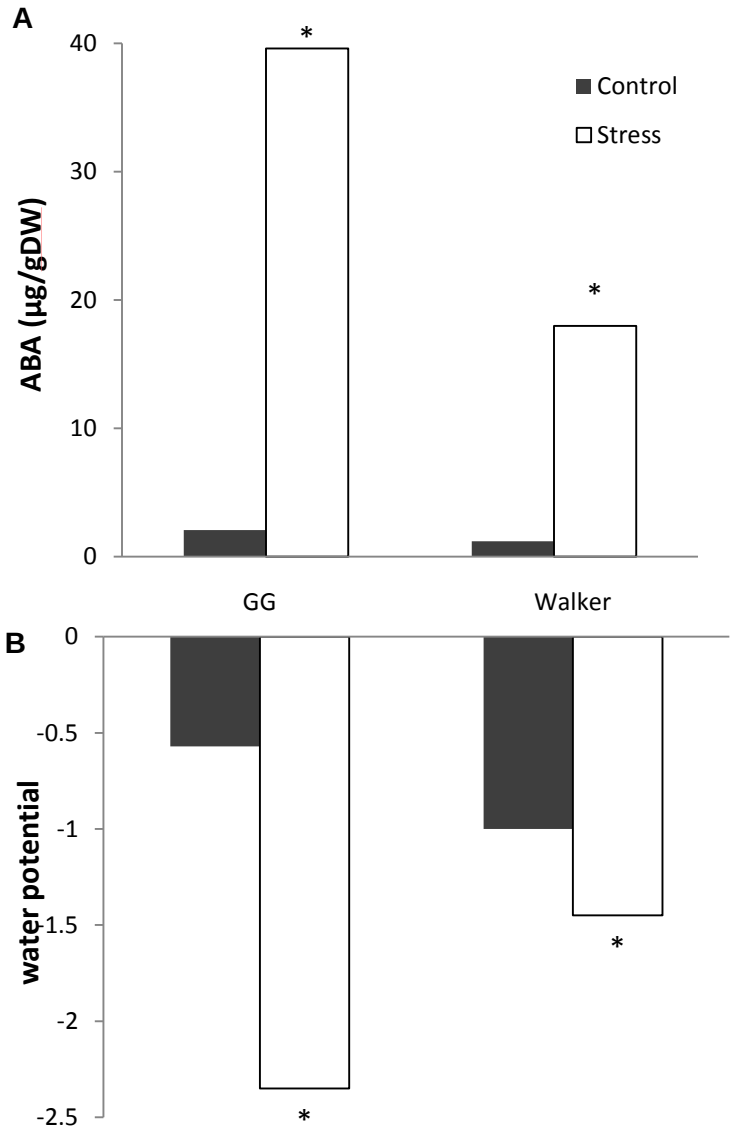


Figure 2.4. A correlation between ABA and ψ_w levels of severely stressed GG and Walker leaves. A, ABA content in well watered controls and severely stressed leaves of GG and Walker (n=3). B, water potential of well watered and severely stressed leaves of GG and Walker (n=3). The * indicates that means are statistically significant from each other at $P < 0.01$; Student's t-test.

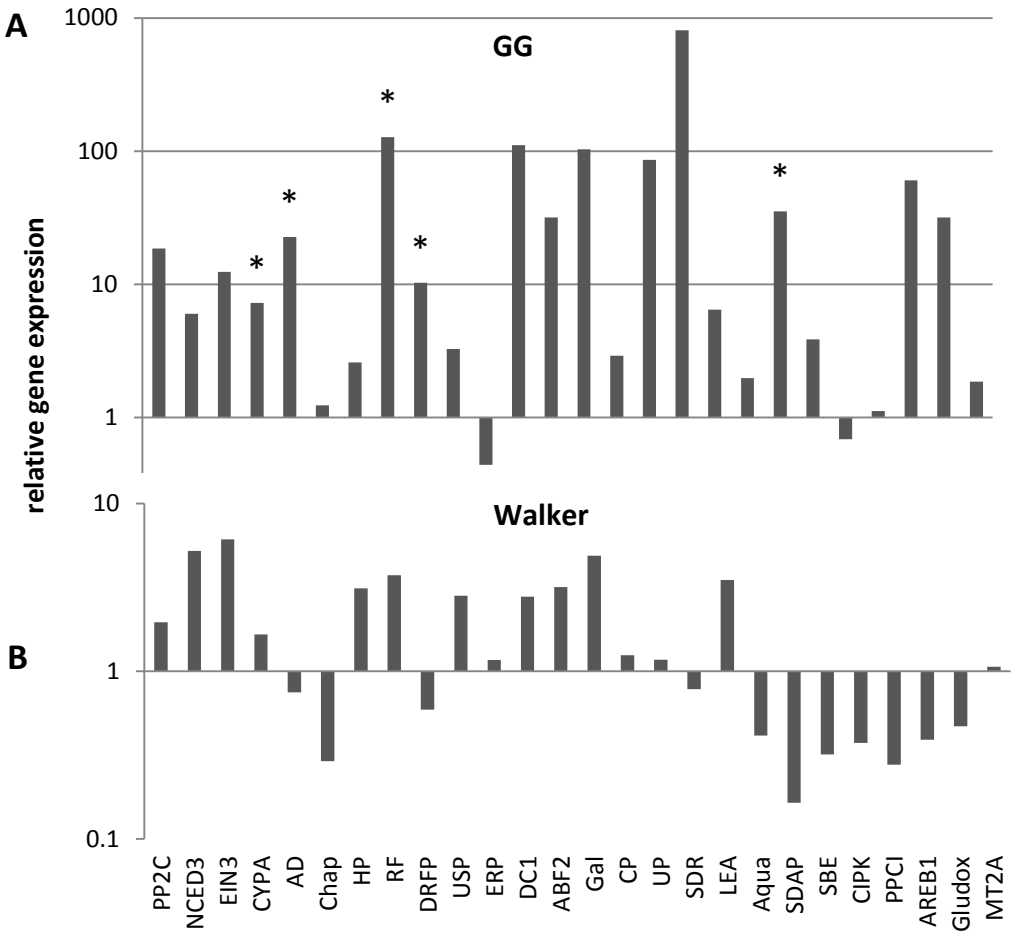


Figure 2.5. Drought induced gene expression at mild drought stress. A, expression of 26 genes in stressed GG plants. B, expression of 26 genes in stressed Walker plants. Gene expression values are relative to gene expression in non-stressed control plants and expression of house-keeping genes. Asterisks (*) show a significant difference in expression between mildly stressed and well-watered control plants at $P < 0.05$ (student's t test). The mean values were obtained from three biological and three technical replications using RT-qPCR.

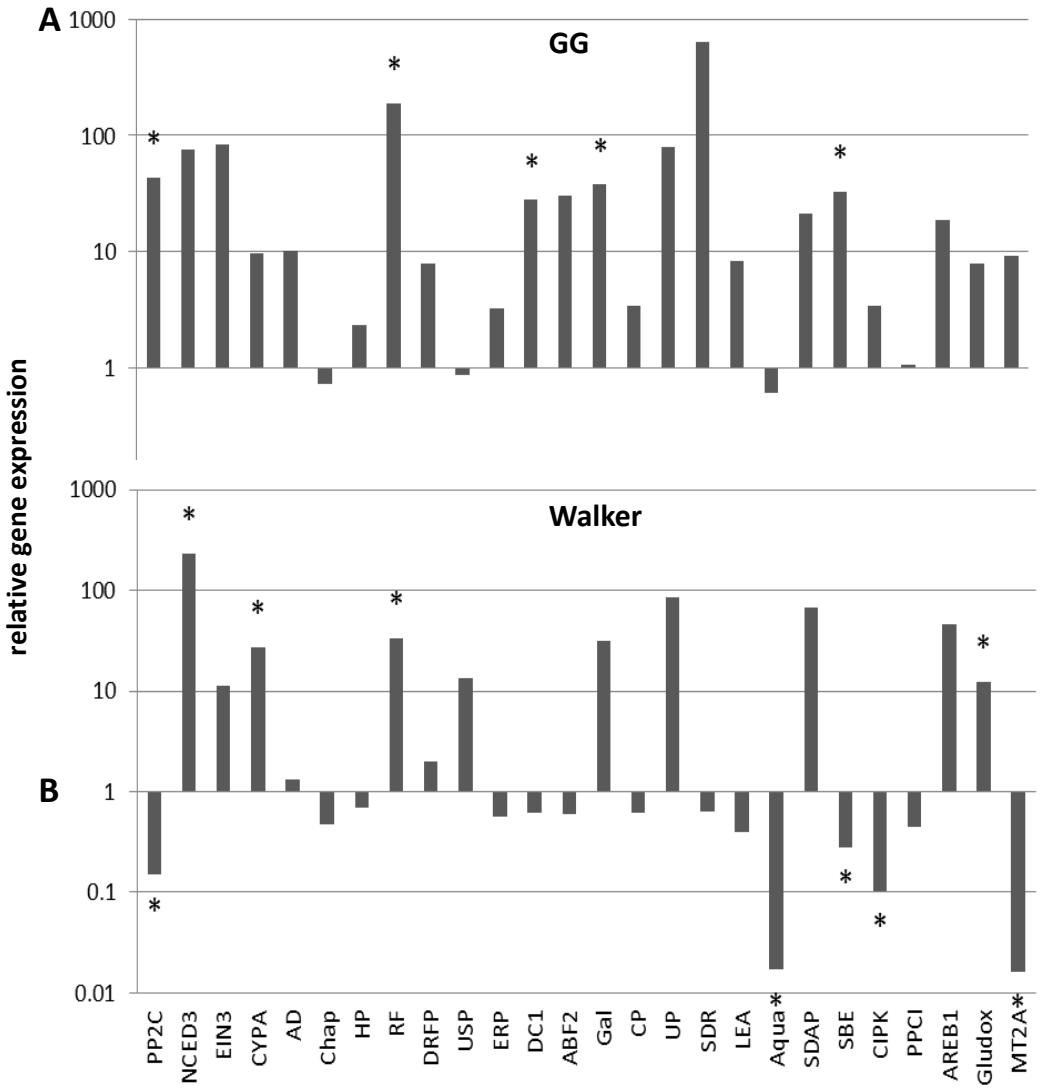


Figure 2.6. Drought induced gene expression at severe drought stress. A, expression of 26 genes in stressed plants of GG. B, expression of 26 genes in stressed plants of Walker. Gene expression values are relative to gene expression in non-stressed control plants and expression of house-keeping genes. Asterisks (*) show a significant difference in expression between severely stressed and well-watered control plants at $P < 0.05$ (student's t test). The mean values were obtained from three biological and three technical replications using RT-qPCR.

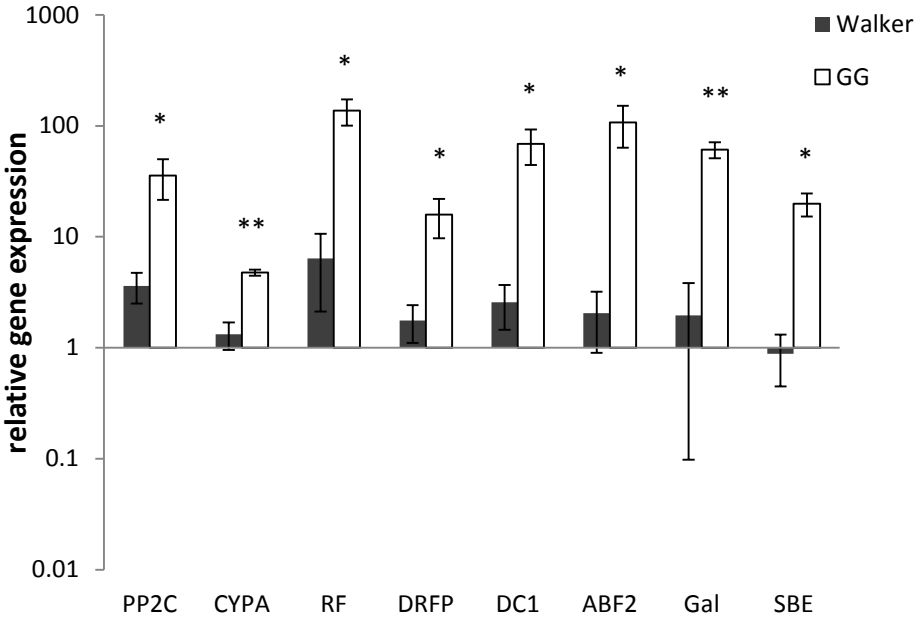


Figure 2.7. Differential gene expression between GG and Walker at mild drought stress. Gene expression values are relative to gene expression in non-stressed control plants and expression of house-keeping genes. Asterisk (*) shows that expression differences between GG and Walker are statistically significant at $P < 0.05$ whereas (**) indicate significance at $P < 0.01$ (student's t test). Only genes with significant differential expression are shown. The mean values were obtained from three biological and three technical replications using RT-qPCR.

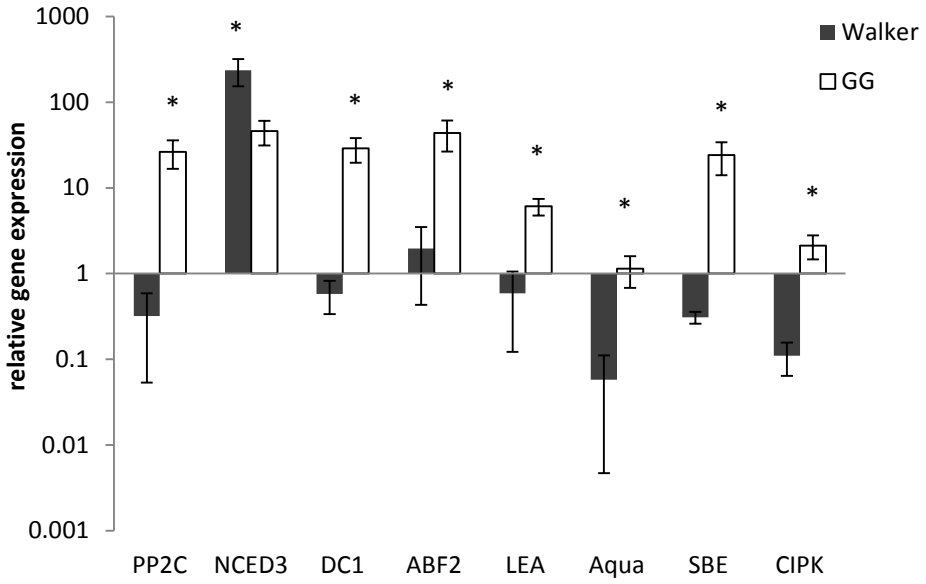
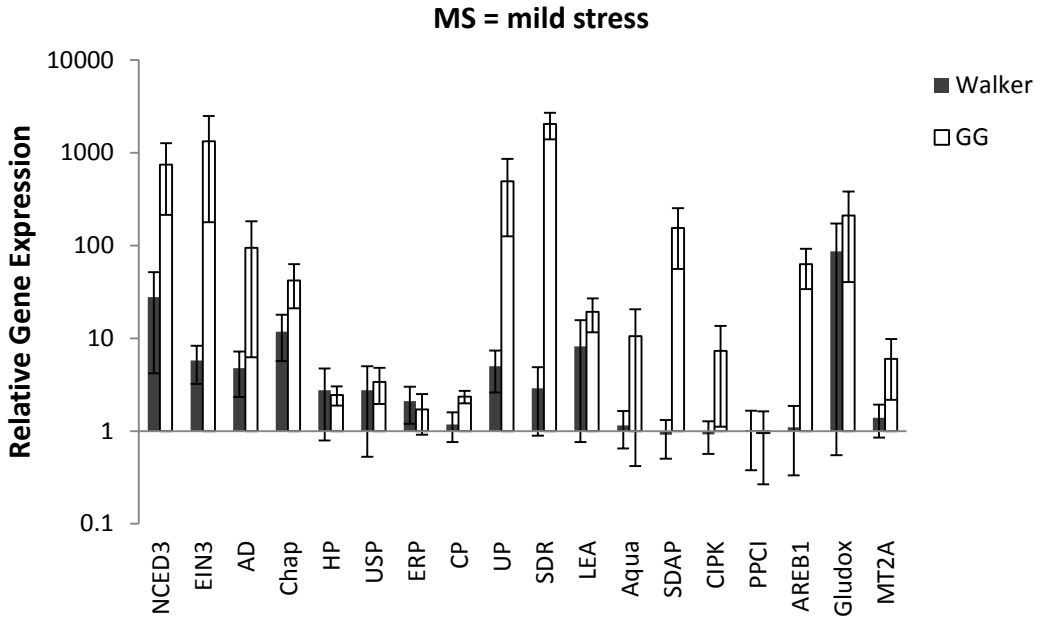
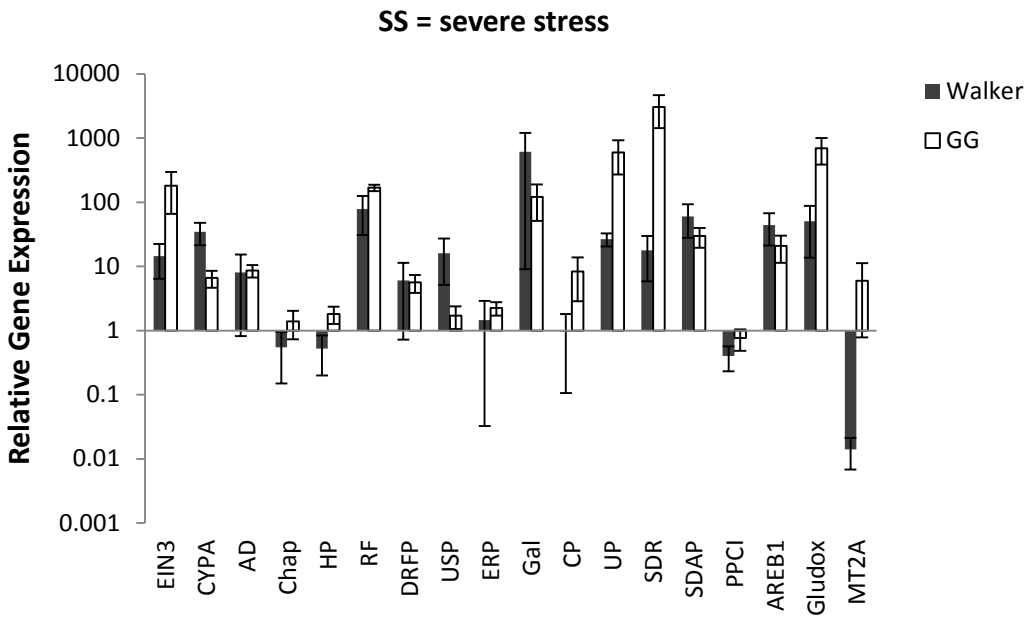


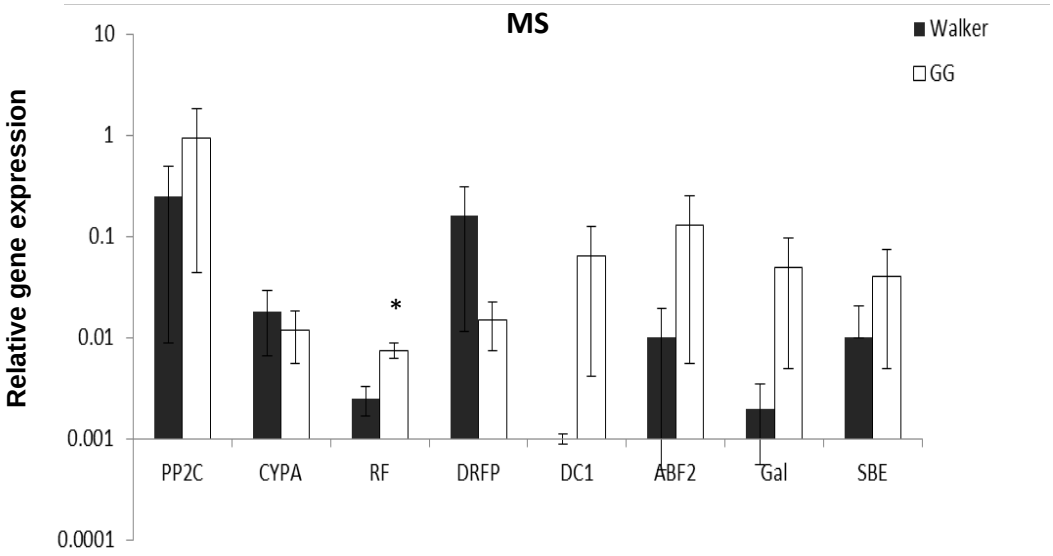
Figure 2.8. Differential gene expression between GG and Walker at severe drought stress. Gene expression values are relative to gene expression in non-stressed control plants and expression of house-keeping genes. Asterisks (*) show that expression differences between GG and Walker are statistically significant at $P < 0.05$ (student's t test). Only genes with significant differential expression are shown. The mean values were obtained from three biological and three technical replications using RT-qPCR.



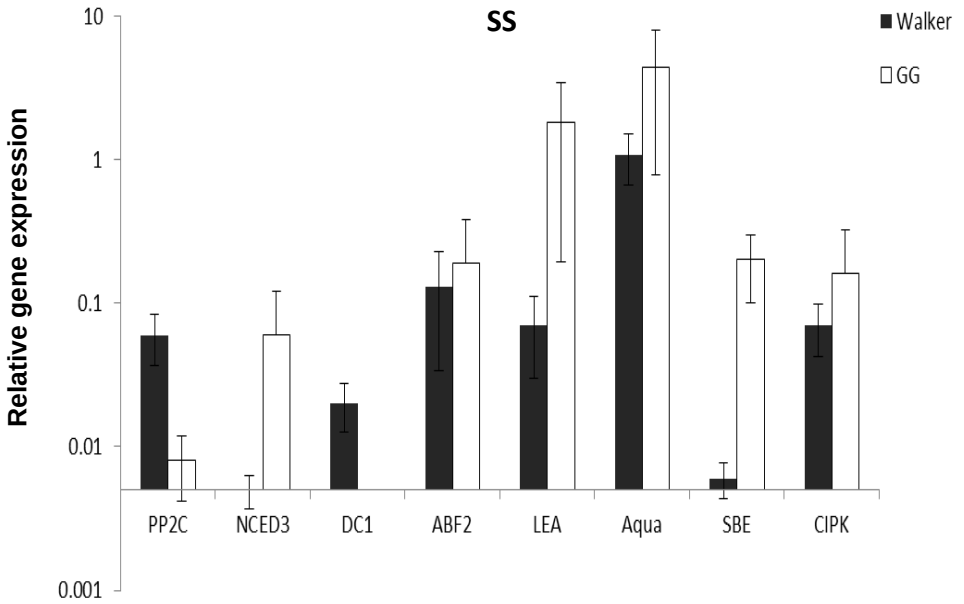
Supplementary Fig. 2.1: Genes with relative expression differences that were not statistically significant between Walker and GG at MS. The expression is relative to well-watered control plants and two housekeeping genes, n=3, with three technical replications per biological sample.



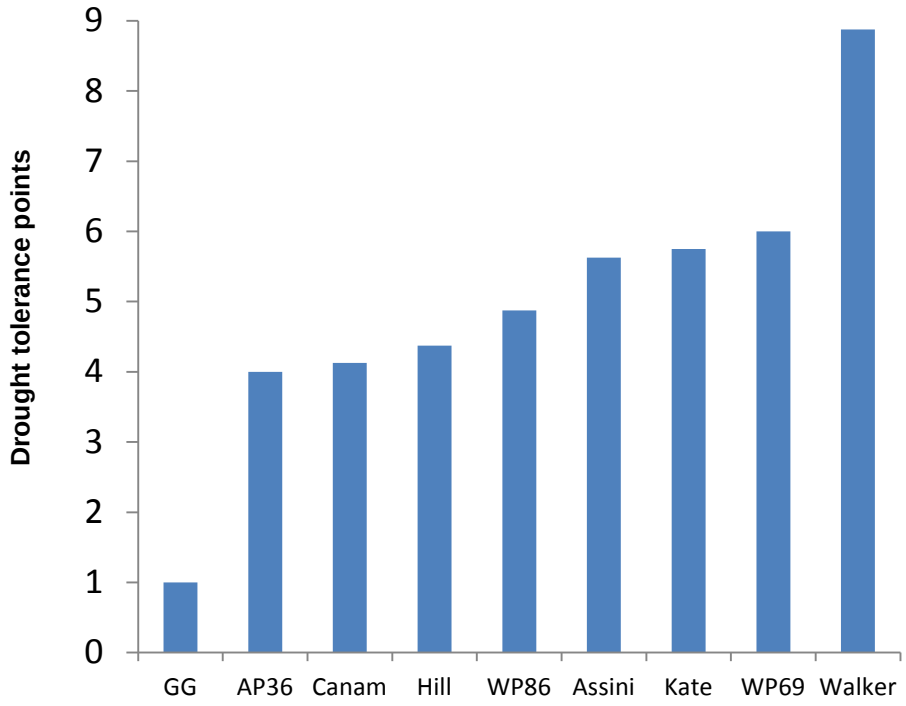
Supplementary Fig. 2.2: Genes with relative expression differences that were not statistically significant between Walker and GG at SS. The expression is relative to well-watered control plants and two housekeeping genes, n=3, with three technical replications per biological sample.



Supplementary Fig. 2.3: Constitutive gene expression in well-watered control plants of Walker and GG. The expression is relative two housekeeping genes, n=3, with three technical replications per biological sample. Asterisk (*) shows statistical significance at $p < 0.05$.



Supplementary Fig. 2.4: Constitutive gene expression in well-watered control plants of Walker and GG. The expression is relative two housekeeping genes, n=3, with three technical replications per biological sample. Asterisk (*) shows statistical significance at $p < 0.05$.



Supplementary Fig. 2.5: Ranking based on averages of ranking orders from assessments of water potential, relative water content, rate of height and leaf formation in the nine assessed clones shown in Figures 2 and 3. Higher value indicate higher ranking, showing that Walker had the highest drought tolerance points (8.9), whereas GG had the lowest (1).

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Chapter 3.

A putative poplar PP2C-encoding gene negatively regulates drought and abscisic acid responses in transgenic *Arabidopsis thaliana*

Abstract

Populus species are valued for their fast growth and are cultivated all over the Northern hemisphere. Many of the commonly used species and hybrids are, however, regarded as drought sensitive, which poses a problem for large-scale cultivation particularly in light of climate change-induced drought spells in areas of poplar growth. While many hundreds of drought-induced genes have been identified in *Populus spp*., very little is known about the genes and the signaling process that leads to a drought response in these species. Based on sequence similarity, the poplar *G059200* gene is a potential ortholog of *AtPP2CA*, an inhibitor of drought and ABA responses in *Arabidopsis thaliana*. To test if *G059200* has a similar function, we generated transgenic *A. thaliana* plants overexpressing this gene. These transgenic lines exhibited reduced responses to exogenous ABA and reduced tolerance of osmotic stress. Finally, drought tolerance of plants was also significantly reduced. Taken together, these data provide evidences that *G059200* acts as a negative regulator of ABA responses. The ability to negatively regulate drought stress responses suggests that *G059200* may be targeted for drought tolerance breeding, for example by identification of individuals harboring natural or induced loss-of-function alleles, or by RNA interference technology, to generate poplar plants with reduced activity of *G059200*.

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; PYR, Pyrabactin resistance; SnRK2, SNF1-related protein kinase 2; PP2C, protein phosphatase 2C

3.1. Introduction

Fast-growing *Populus* hybrids are cultivated all the over northern hemisphere and are used in the production of paper, pulp, plywood, veneer and other composite products (Balatinecz et al., 2001). Furthermore, fast growing poplars are, and may increasingly be, used for biofuel production (Karp and Shield, 2008; Yemshanov and McKenney, 2008). While there are poplar species growing in arid climates (Gries et al., 2003), the fast-growing hybrids currently deployed are generally considered drought sensitive (Liang et al., 2006). Climate modeling predicts an increased frequency of drought episodes in many regions of the world, including sites in North-America, Europe and Asia where poplar clones are currently planted (Salinger et al., 2005). For example, large parts of the Canadian prairies, harbouring poplars for shelter belts, pulp and paper production, have seen a drastic decrease in available water, and is predicted to receive even less water (Hogg and Hurdle, 1995; Hogg and Bernier, 2005).

Responses to drought in poplar species and hybrids have been studied in some detail, revealing physiological and morphological traits that correlate with drought tolerance. This includes better stomatal control of water loss (Blake et al., 1984; Liu and Dickmann, 1996; Silim et al., 2009; Tschaplinski et al., 1998), increased sensitivity to abscisic acid (Chen et al., 1997; Li et al., 2004; Yin et al., 2004) increased accumulation of osmolytes, photoprotective compounds, and antioxidant enzymes (Gebre et al., 1994; Marron et al., 2002; Regier et al., 2009; Xiao et al., 2008; Zhang et al., 2012), increased extent of leaf area reduction (Monclus et al., 2006) increased root to shoot ratios and increased carbon storage in roots (Marron et al., 2003; Tschaplinski et al., 1998), and improved recovery upon rewatering (Marron et al., 2003). Two assessments of the effect of drought on the productivity of segregants after hybridizations showed that the most productive clones tend to be the least drought tolerant (Monclus et al., 2005; Monclus et al., 2006), indicating that there is typically a trade-off between these traits. There are exceptions though (Tschaplinski et al., 1998), implying that this is not a strict interdependence. QTL mapping has identified several genomic regions that contribute to drought tolerance (Street et al., 2006) but not yet individual genes. To date, many hundreds of drought-induced genes have been identified in poplar species (Bogeat-Triboulot et al., 2007; Caruso et al., 2008; Hamanishi et al., 2010; Plomion et al., 2006;

Street et al., 2006; Wilkins et al., 2009). Altered expression of drought-induced genes in transgenic plants has provided evidence in support of a role in regulation of drought responses and tolerance. Overexpression of two putative poplar transcription factors in *A. thaliana* resulted in plants with improved drought tolerance (Chen et al., 2011; Ma et al., 2010). Similarly, overexpression of poplar calcineurin B-like proteins, presumably involved in calcium-dependent signaling, led to improved drought tolerance in transgenic *A. thaliana* (Li et al., 2013a) and poplar (Li et al., 2012) plants.

The plant hormone abscisic acid (ABA) plays a key role in the signaling after perception of water deficit and related salt stress, cold, and wounding (Leon et al., 2001; Raghavendra et al., 2010; Xiong et al., 2002; Zhang et al., 2006). Water deficit perceived by roots leads to ABA production and transport via the transpiration stream to leaves and other organs, where it triggers alteration of gene expression, stomata closure and osmotic adjustments (Nambara and Marion-Poll, 2005; Szabados and Savoure, 2010). ABA is also produced locally in leaves in response to water deficit (Seo and Koshiba, 2002). The molecular basis of ABA signaling is increasingly well understood, particularly from work in *A. thaliana*. ABA binding to the RCAR/PYR/PP2C/SnRK2 complex results in a conformation change that prevents PP2C dephosphorylation of SnRK2 (Umezawa et al., 2009; Vlad et al., 2009). Subsequent phosphorylation of SnRK2 triggers gene expression and other SnRK2-dependent events that ultimately lead to ABA-dependent acclimation to water deficit (Bartels and Sunkar, 2005). Thus, ABA triggers a release of signaling suppression by PP2C/Protein Phosphatase 2C. The PP2C-family of genes can therefore be viewed as modulators or negative regulators of ABA signaling (Fuchs et al., 2013; Gonzalez-Garcia et al., 2003; Kuhn et al., 2006; Tougane et al., 2010). Clade A PP2C (PP2CA)-encoding genes may for several reasons be suitable for targeted breeding of drought tolerance. First, drought tolerance can be manipulated in *A. thaliana* by overexpression, resulting in enhanced water loss, and down regulation, resulting in reduced water loss (Rubio et al., 2009; Saez et al., 2004; Saez et al., 2006). Second, overexpression of potential PP2CA orthologs from a dicot tree, a monocot grass and liverwort in transgenic *A. thaliana* results in reduced ABA signaling and drought tolerance (Gonzalez-Garcia et al., 2003; Liu et al., 2009; Tougane et al., 2010), indicating that PP2CA function is well preserved across the plant kingdom.

Identification of PP2CA-like genes with a function in the regulation of drought responses is, however, not without problems. PP2CA-like genes occur as families of genes in plant genomes. When known, it appears that they may have different, albeit partially overlapping functions, acting in various processes and in different organs (Gosti et al., 1999; Li et al., 2013b; Miyazaki et al., 1999; Reyes et al., 2006; Schweighofer et al., 2004). Functional characterization of seven *A. thaliana* PP2CA paralogs (PP2CA, ABI1, ABI2, HAB1, HAB2, AHG1, SAG113) has shown that they are all negative regulators of ABA signaling, however, loss-of-function mutation only in PP2CA, ABI1, ABI2, HAB1 and SAG113 caused a reduction of water loss (Kuhn et al., 2006; Merlot et al., 2001; Nishimura et al., 2007; Rubio et al., 2009; Saez et al., 2004; Saez et al., 2006; Zhang and Gan, 2012). There is also evidence for PP2CA-like genes acting in gibberellin and jasmonic acid signaling (Reyes et al., 2006; Schweighofer et al., 2007). Finally, there is experimental support for a PP2CA-like gene acting as a positive rather than negative regulator of ABA signaling (Reyes et al., 2006).

PP2CA-like genes have to date not been characterized in poplar species. Here we sought to identify a potential poplar ortholog of the *A. thaliana* PP2CA gene (hereafter referred to as *AtPP2CA*). Since the *Populus trichocarpa* genome has been sequenced, we used it to identify the genes with the highest similarity to PP2C-like genes in *A. thaliana* and *Medicago truncatula*, and thereafter used phylogenetic analysis, to identify *Potri.008G059200* (hereafter referred to as G059200) as the most likely candidate. We proceeded to generate transgenic *A. thaliana* lines overexpressing G059200 and to test whether this gene has the ability to act as a negative regulator of ABA signaling as well as osmotic and drought stress responses.

3.2. Materials and Methods

3.2.1. Bioinformatics

The BLASTX algorithm (Altschul et al., 1990) was used to identify highly similar protein sequences in the *Populus trichocarpa* and *Medicago truncatula* genomes using the Phytozome v9.1: *Populus trichocarpa* v3.0 and KEGG databases. The phylogenetic relationships among the identified protein sequences were thereafter analyzed and

visualized using the MEGA5.2 software (Tamura et al., 2011). The peptide alignment in Fig. 3.1b was made by the use of the EMBL-EBI Clustal Omega software. The sequences were also scanned for potential sequence motifs using the *PlantP* software (http://plantsp.genomics.purdue.edu/). PCR primers were designed by the use of the Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/).

3.2.2. Plant material and growth conditions

Stem cuttings of the poplar hybrid Green Giant were grown in 2.7 L plastic pots containing a mixture of two parts Promix BX peat (Westgro, Delta, BC) and one part sand under 16/8 hour light/dark periods with 50-70% relative humidity at approximately 25^0C in a greenhouse. When the cuttings established roots, a 20-20-20 (NPK) fertilizer solution (66.7 g/L, Plants Products Co. LTD, Brampton, Ontario) was provided once every week until a height of 1-1.5m. *A. thaliana* seeds (Col-0, stock # CS28166) were sown on plates containing Murashige and Skoog modified basal medium (Invitrogen, prod # M404) 1% sucrose and 0.8% agar (PhytoTechnologies Labs, Kansas city, USA). Seeds were stratified at 4 ^{0}C for 3 days and transferred to the growth chamber at 22 ^{0}C, 60-80% humidity with 16 hour light (80-100 µmol m^{-2} s^{-1})/8 hour dark periods. Seedlings were transferred to plastic pots filled with PRO-MIX soil and kept at conditions above until plants were ready for floral dip or phenotypic analysis.

3.2.3. Vector construction and plant transformation

The G059200 gene containing introns and exons was PCR amplified from DNA from a *P. trichocarpa x P. deltoides* hybrid with number K9, obtained from Michael Carlsson, British Columbia Ministry of Forests Kalamalka research station. 5`- TCTGAACTAATGGCTGGGATTT - 3` and 5`- TTCTCAAATCCACAACAACAACA - 3` (see supplementary fig 3.1). The amplicon was recombined first into pDONR/Zeo gateway vector using the B/P reaction kit (Invitrogen), then after confirmatory Sanger sequencing, recombined into the pK2GW7,0 vector (Karimi et al., 2007) using the LR reaction kit, (Invitrogen). Subsequently, pK2GW7,0 was transformed into the GV3101 strain of *Agrobacterium tumefaciens* by electroporation. *A. thaliana* plants (Col-0) were transformed with *A. tumefaciens* containing the G059200 gene by the floral dip method (Bent, 2006). Plants were confirmed for insertion of a single T-DNA element by

observing 3:1 resistance-sensitive ratio to kanamycin. Thereafter, transgenic plants were tested for homozygosity in the T3 generation. Only populations showing 100% resistance to kanamycin were considered homozygous and used for further phenotypic experiments. Presence of T-DNA was confirmed by PCR of the NPTII gene conferring kanamycin resistance.

3.2.4. Germination and root growth assays

To measure the response to osmotic stress or ABA, wildtype and transgenic seeds were plated on solid media described above, supplemented with mannitol (0, 0.1, 0.2, 0.3 or 0.4M) or ABA (0, 0.5, 1, 5 or 10µM). Seeds were stratified at 4 ^{0}C for 3 days before transferring to the growth chamber. Resistance to treatments was scored at day 7 based on seedlings with green (tolerant) or yellow (sensitive) cotyledons as described in (Saez et al., 2006). For root growth measurements and fresh weight measurements, five days old seedlings grown on MS media were transferred to a new MS media with or without indicated concentration of mannitol or ABA and data were collected at 12 d as described (Rubio et al., 2009; Saez et al., 2006). Each independent experiment contained three replications.

3.2.5. Quantitative real time PCR (qRT-PCR)

RNA was extracted using Plant RNA Purification reagent (Invitrogen). One µg RNA was used to synthesize cDNA using superscript III (Invitrogen). qRT-PCR amplifications were performed using an Opticon 1 thermocycler (BioRad) with three technical and three biological replications. G059200 expression was analyzed using the primers 5`GTGCTT TGTCGAAACGGTGT3` and 5`CGGTATGACGTAGGGCTTCA3` and its expression was normalized against the APT1 house-keeping gene using the primers 5`TGGAAGGTTATTCGGAGGAG3` and 5`AGGATCAAATCCCACGCAAA3`. Relative gene expression was calculated using the ΔCT method for Fig. 3.2a and $\Delta\Delta$CT method for Fig. 3.2b as described by (Livak and Schmittgen, 2001).

3.2.6. Drought stress and water loss assays

At the start of the drought stress of poplar plants, 3 plants were kept in the irrigation troughs and watered every second day, while 3 plants was placed outside the trough, withholding water. After 7 days, the 3 uppermost young leaves were collected for RNA extraction from drought stressed as well as well-watered plants. In *A. thaliana* plants, two different assays were performed to measure the water loss. A rapid water loss assay was performed as described (Dai et al., 2007) with two independent experiments, n=3 per experiment. The rosettes of 25 day-old plants were decapitated and fresh weight (FW) was measured at different time points. Percent water loss was calculated by decrease in FW at specific time point/FW after decapitation multiplied by 100. Long-term water loss assay was performed as described (Rubio et al., 2009; Saez et al., 2006). Two independent experiments were performed in which 7 leaves were collected per plant from 10 different plants of each line per experiment. Wildtype and transgenic plants were grown on soil in the growth chamber under normal watering conditions for 3 weeks before drought was imposed by withholding water completely. Leaves were removed at the indicated time points and weighed. Afterwards, leaves were incubated in demineralized water for 3 hours, blotted dry, and weighed again. The difference in weight was considered as water loss.

3.2.7. Statistical analysis

Statistical analyses were performed with JMP9 software (SAS institute, NC, USA). The values are presented as mean ± SE from three to ten replications per experiment. Analysis of variance (ANOVA) along with Tukey's honest significant difference (HSD) method was used to test whether or not the means of several groups were equal (Figs. 3.2b, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9). Means with letters a, b, c and d indicate that they are statistically significant from each other, two means with same letter indicate an insignificant difference between them. Means without any letter indicate an insignificant difference. Student's t test was used in Fig. 3.2a.

3.3. Results

3.3.1. Identification of a putative *Populus* PP2CA ortholog

We used the predicted *AtPP2CA* (accession # At3g11410) protein sequence as a template to search for PP2CA-like proteins among the predicted open reading frames (ORFs) in the *P. trichocarpa* and *M. truncatula* genomes using the BLASTX algorithm selecting only sequences with high similarity based on an e-value ≤ 1.2e-26. The phylogenetic relationship between the identified protein sequences, together with those of the PP2CA-like proteins was thereafter analyzed and visualized (Fig. 3.1a). *M. truncatula* sequences were included to reduce the risk for arbitrary associations. The most parsimonious tree, generating the topology with the fewest number of mutations, provided a sub-clade in which *AtPP2CA* clustered with the proteins of the *M. truncatula* gene MTR_5g080680 and the *P. trichocarpa* gene G059200 (Fig. 3.1a). These three proteins also shared a putative glutamine amidotransferases class-II active site motif (indicated in the peptide alignments in Fig. 3.1b), which was not present in any of the other predicted peptides including the closely related poplar peptide Potri.010G199600. The overall sequence similarity between AtPP2CA and G059200 is 72.5%, which is high relative to for example the similarity between *A. thaliana and Citrus* PP2CA orthologs (Romero et al., 2012). Based on these results, we concluded that the most likely *P. trichocarpa* ortholog of *AtPP2CA* is G059200. The *G059200* gene is expressed in leaves, and the steady-state level of transcripts is significantly enhanced in response to drought stress (Fig. 3.2a), consistent with other PP2CA-like genes (Miyazaki et al., 1999). We were unable to detect any expression in roots, with or without drought stress (Fig. 3.2a).

3.3.2. Responses of plants overexpressing G059200 to ABA

If G059200 encodes a PP2C ortholog that acts as a negative regulator of ABA responses, we expect that overexpression results in plants with reduced responses to ABA, osmotic and drought stress. To test this hypothesis, we generated *A. thaliana* transgenic lines constitutively over-expressing the G059200 transcript under the control of the 35S CaMV promoter (see materials and methods). Since there is no native G059200 gene in *A. thaliana*, we used an artificial CT value of 35 as a reference to get

an indication of the degree of upregulation in comparison to a silent gene, and a comparison of expression between independent lines. The L2, L6 and L7 lines showed an average of 110, 129 and 384 fold upregulation respectively compared to a CT value of 35 (Fig. 3.2b), which was higher than several other identified lines (not shown). We therefore chose these lines for assessment of potential phenotypes induced by over expression of G059200. Here we will compare their responses to exogenous ABA, osmotic and drought stress relative to untransformed *A. thaliana* plants.

ABA and germination

ABA and ABA signaling suppress premature seed germination in many plant species, including *A. thaliana*. If *G059200* is a negative regulator of ABA signaling, then we expect that seeds overexpressing *G059200* may be partially insensitive to ABA and therefore able to germinate in the presence of exogenous ABA. We sowed wildtype and transgenic *A. thaliana* seeds on MS media with increasing concentrations of ABA. As predicted, ABA imposed significantly less ($P < 0.01$) inhibitory effect on germination of transgenic lines compared to wildtype (Fig. 3.3). For example, at 1 µM ABA, 30% of wildtype seeds germinated whereas 67% (L2), 78% (L6) and 90% (L7) of transgenic line seeds germinated. At 5 µM ABA, no wildtype seeds germinated while approximately 10% (L2 and L6) and 57% (L7) germination was still observed in transgenic lines (Fig. 3.3). In addition to the enhanced germination in all transgenic lines, we also observed significantly higher germination in the L7 line compared to the L2 and L6 lines at 1, 5 and 10 µM ABA, indicating that the dosage of G059200 expression makes a difference in the response to exogenous ABA.

ABA and root growth

While low concentration of ABA can stimulate root growth, higher concentrations can be supra optimal, resulting in reduced root growth (Liu et al., 2009). To test if roots of transgenic lines showed reduced sensitivity to exogenous ABA, we transferred 5 day old seedlings to solid MS media supplemented with 0, 5 or 10 µM ABA and incubated plates in a vertical position in the growth chamber. In the presence of 5 and 10 µM ABA, we observed a significant reduction of root growth in wildtype plants compared to transgenic lines (Fig. 3.4 a,b), providing evidence that G059200 overexpression reduces the sensitivity also of the root to ABA.

ABA and fresh weight

A. thaliana plants also respond to ABA by a reduced rate of fresh weight accumulation (Rubio et al., 2009). Again, if ABA signaling is reduced, we would expect a reduced water loss in the transgenic lines. Wildtype plants responded to 1 µM and 5 µM ABA by a 50 and 80% loss in water content respectively. The fresh weight of transgenic lines did not change significantly after exposure to 1 µM ABA. Fresh weight was dramatically reduced in response to 5 µM ABA, but was still significantly higher than that of wildtype plants (Fig. 3.5), consistent with reduced ABA responses in the *G059200* over-expressing plants.

3.3.3. Response of plants overexpressing G059200 to osmotic stress

Since ABA signaling is required for a proper response to osmotic stress, we expect that if G059200 acts as a negative regulator of ABA responses in transgenic *A. thaliana*, its overexpression should result in reduced resistance or ability to cope with osmotic stress. Similar to ABA treatment, we scored the effect of osmotic stress on seed germination, root growth and fresh weight.

Osmotic stress and germination

We observed a significant difference between wildtype and *G059200*-overexpressing plants when seeds were grown on the same medium supplemented with different levels of mannitol or NaCl (Fig. 3.6 a,b). Data showed that 0.1M and 0.2M mannitol had almost no effect on germination of wildtype plants, whereas 0.1M mannitol reduced germination by approximately 10% and 0.2M mannitol reduced it by 35-40% in the transgenic lines, revealing osmotic stress-sensitive behavior of transgenic lines. Although a reduction in germination was noticed in wildtype when mannitol concentration was further increased to 0.3M, germination of transgenic lines was still significantly lower than wildtype at this concentration (Fig. 3.6a). A similar trend of germination inhibition was observed when seeds were germinated at different concentrations of NaCl. A concentration of 100 mM NaCl was enough to significantly reduce the germination of transgenic lines by up to 60%, while only 7% reduction was observed in wildtype seed germination (Fig. 3.6b). Likewise, 150 mM NaCl further inhibited the germination significantly in transgenic lines

and only 25% germination was observed, whereas about 85% germination was still recorded in wildtype seeds (Fig. 3.6b). These results revealed that transgenic *A. thaliana* overexpressing G059200 are hypersensitive to osmotic stress during seed germination.

Osmotic stress and root growth

Roots absorb water from the surrounding soil and are therefore usually the first plant organ to sense a change in water availably. To assess the effect of *G059200* overexpression on root growth in the presence of osmotic stress, we transferred 5 days old seedlings to MS media plates containing 0, 0.2, and 0.3M mannitol. Seedlings were grown vertically on plates in the growth chamber and root elongation was measured at 12 d after transfer in two independent experiments. As expected, an impaired root growth was observed in transgenic lines when grown on different concentration of mannitol (Fig. 3.7 a,b). About 30% reduction of root growth was measured in transgenic lines of *A. thaliana* when grown on MS supplemented with 0.2M mannitol. By increasing the mannitol concentration to 0.3M, we observed similar root growth in wildtype and L2 transgenic lines whereas L6 and L7 exhibited a 19% and 29% reduction of root growth respectively (Fig. 3.7 a,b).

Osmotic stress and fresh weight

We also determined the gain in fresh weight by wildtype and transgenic lines grown at different levels of mannitol treatment. Under normal growth conditions, fresh weight gained by transgenic lines was not significantly different than wildtype plants (Fig. 3.8). However, when grown on media supplemented with 0.2M mannitol, transgenic lines accumulated 35-50% less fresh weight compared to wildtype (Fig. 3.8), indicating that G059200 overexpression inhibits biomass accumulation in *A. thaliana* during osmotic stress. We observed no significant difference in fresh weight accumulation of wildtype and transgenic lines at 0.3M mannitol as this concentration resulted in a sharp reduction of fresh weight accumulation in wildtype on increasing mannitol concentration (Fig. 3.8). These results revealed that *A. thaliana* transgenic lines overexpressing *G059200* displayed an osmotic stress hypersensitive phenotype, in line with reduced signaling and response to osmotic stress.

3.3.4. Overexpression of G059200 reduces drought tolerance by enhancing water loss

Due to the ABA-insensitive phenotype of transgenic plants described above, we expected an impaired stomatal closure in transgenic lines in response to drought stress. To test this hypothesis, we performed two previously described assays (Dai et al., 2007; Rubio et al., 2009; Saez et al., 2006). The first assay, measuring rapid response to water loss, was performed by severing rosettes of wildtype and transgenic lines from their roots followed by weighing rosettes to measure rate of water loss. As expected, transgenic lines lost significantly more water (48-61%) as compared to wildtype plants, which transpired only 38% water after 3 hours (Fig. 3.9a). The second assay measured long term water loss. While plants were watered to field capacity, no significant difference between wildtype and transgenic lines were observed. When drought was imposed by withholding water, transgenic lines lost 19-33% more water than wildtype in 16 days (Fig. 3.9b). At 18 d, transgenic plants turned brown, wilted and died unlike wildtype plants that were still green and alive (Fig. 3.9c). Taken together, two assays revealed that *G059200* over-expression in transgenic *A. thaliana* enhanced water loss with detrimental effects on plant survival.

3.4. Discussion

Here we show that overexpression of the poplar gene *G059200* results in transgenic *A. thaliana* plants that have reduced sensitivity to exogenous ABA and enhanced sensitivity to osmotic and drought stress treatments. It may seem as a contradiction that ABA and stress treatments result in opposite responses considering that these stressors are known to trigger production of ABA and induction of ABA signaling, and therefore are part of the same signal transduction pathway. It is important to keep in mind, however, that the ABA treatments involved hyper-physiological concentrations of ABA resulting in strong inhibition of germination and root growth (Kuhn et al., 2006), whereas the stressors presumably evoke production of physiological levels of ABA, in turn resulting in activation rather than inhibition of downstream stress responses. In the end, the results from the three treatments are consistent with G059200 acting as a negative regulator of ABA signaling in drought stress responses, as predicted based on its high similarity to

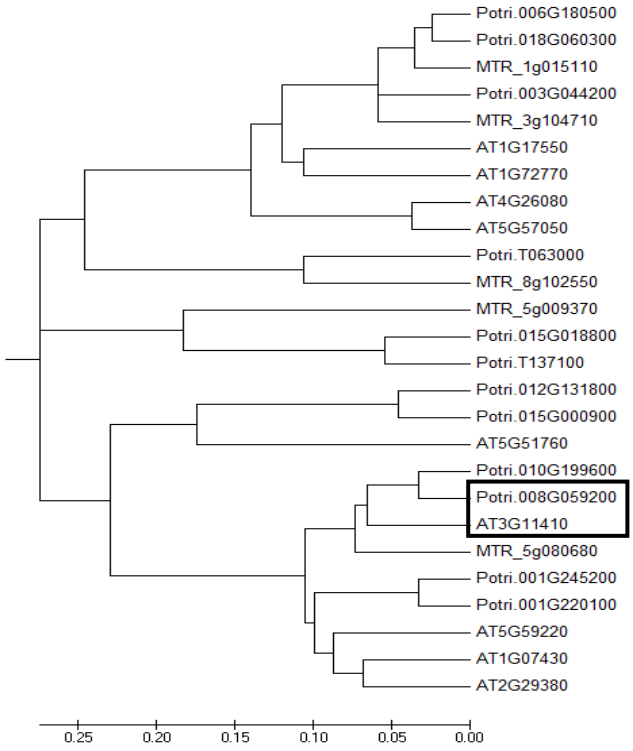
the *A. thaliana PP2CA* gene. First, overexpression of *G059200* reduces the negative effects of ABA on germination and root growth, providing evidence that G059200 acts as a negative regulator of ABA signaling. Second, overexpression of *G059200* reduces the positive effects that ABA signaling normally has on mounting appropriate responses to osmotic and drought stress, providing additional evidence in support for G059200 being a negative regulator of ABA signaling.

While our phylogenetic analysis indicates that *G059200* is an ortholog of AtPP2CA, there is no functional evidence to that effect in the literature. A mutant overexpressing AtPP2CA has been identified in a screen of a population generated by inserting cDNAs into a binary plasmid for over-expression followed by large-scale transformation of *A. thaliana* plants (Kuhn et al., 2006). The overexpression of the *AtPP2CA* gene resulted in seeds with reduced sensitivity to ABA, allowing them to germinate in the presence of ABA. This phenotype is identical to what we describe here for seeds overexpressing the poplar *G059200* gene. However, roots of *G059200* overexpressing plants have reduced sensitivity to ABA whereas the *AtPP2CA* overexpressing line does not (Kuhn et al., 2006). This discrepancy may be due to differences in the level of overexpression though as the roots of loss-of-function *Atpp2ca* mutants showed an enhanced sensitivity to ABA, a phenotype opposite to the gain-of-function over-expression of *G059200*. The similarities extend also to effects on water loss; over expression of both *AtPP2CA* and G059200 results in enhanced water loss from detached rosette leaves. Loss-of-function *Atpp2ca* mutants showed an opposite phenotype, i.e. a reduced water loss, thereby providing evidence that a large spectrum of *PP2CA* levels affect this trait. Since overexpression of *G059200* and *AtPP2CA* resulted in the same set of phenotypic drought and osmotic stress responses in multiple organs it appears likely that they fit into the same molecular context, i.e. that both proteins partake in an RCAR/PYR/PP2C/SnRK2 complex and that ABA binding results in a conformation change that prevents both of them from dephosphorylating SnRK2, triggering downstream signaling. Since at least *G059200* is not normally expressed in roots, but ectopic expression of it in this organ results in a predicted resistance to osmotic stress, it appears that the components that it needs to interact with are present also in the root, whether or not those components are the same as those in shoot tissues. While other comparisons can be made between *G059200* and *AtPP2CA*, the intent of this study was

to use *AtPP2CA* as an entry point to identify a poplar gene that acted as a negative regulator of ABA signaling and drought responses, and the obtained phenotypes indicates that the *G059200* gene indeed has that function.

It remains to be seen if *G059200* over expression in transgenic poplar results in a similar drought sensitive phenotype, and, more importantly, if a loss of function poplar mutant have improved drought tolerance. It is possible that extensive functional redundancy among poplar PP2CA genes prevents the elucidation of a phenotype from loss of function in a single PP2CA-like gene in poplar. On the other hand, as part of the ABA receptor complex, *A. thaliana* PP2CA proteins appear essential for ABA-dependent signaling in response to drought stress; (reviewed in Bartels and Sunkar, 2005; Raghavendra et al., 2010). The phylogenetic analysis in Fig. 3.1 reveals nine *A. thaliana* and twelve *P. trichocarpa* proteins that are closely related. Based on these numbers, functional redundancy is not likely to be much more widespread among PP2CA-like genes in *P. trichocarpa* than in *A. thaliana*. It is also possible to target more than one gene for loss of function mutation. Expression of RNA interference and artificial microRNAs in transgenic plants are methods that can be adapted to target single as well as multiple genes for reduced expression (McGinnis, 2010; Schwab et al., 2006). Although untested in poplar, the advent of a recent genome editing technology that allows targeted deletions of parts of genes in animal as well as plant cells is of particular interest, as it allows the production of bi-allelic, non-transgenic mutant plants (reviewed in Belhaj et al., 2013).

a



b

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AT3G11410           MAGICCGVVGETEPAA-PVDSTSRASLRRRLDLLPSIKIVADSAVAPPLENCRK--RQKR
Potri.008G059200    MAGICCGVVAENEAAA-AVEARSRASRRRRLELRLVSDVSV------PPSTILDVAPAKR
MTR_5g080680        MAEICCGVVGEEETLTLPVEPSSRPSGRRSLDLLPLKYMAD------PPENSRKR--QKL
                    ** ******.* *  :  *:  ** * ** *:*     :        * ..  .    *

AT3G11410           ETVVLSTLPGNLDLDSNVRSENKKARSA-VTNSN-SVTEAESFFSDVPKIGTTSVCGRRR
Potri.008G059200    KKLELFPIPISRDCGNAVENCKRIEENKNNSISDSSK-PESVKLKEALKFGMTSVCGRRR
MTR_5g080680        DPSVSPPPPPARECENAVDSCESSTDEESKENEVVLENKVVVVEECSPKYGITSVCGRRR
                    .       *   :  . * . :    .     .           .   * * ********

AT3G11410           DMEDAVSIHPSFLQRNSENHHFYGVFDGHGCSHVAEKCRERLHDIVKKEVEVMA---SDE
Potri.008G059200    DMEDAVSIHTSFTT---KNTSYFGVFDGHGCSHVAMKCRDRLHEIVKQEVEGFKEEESVE
MTR_5g080680        EMEDAVSVHPSFCRE--KQDHFFGVYDGHGCSHVATMCKERLHEIVEEEVE----KEKVD
                    :******:* **     ::  ::**:*********  *::***:**::***      . :

AT3G11410           WTETMVKSFQKMDKEVSQRECNLVVNGATRSMKNSCRCELQSPQCDAVGSTAVVSVVTPE
Potri.008G059200    WKETMERSFVEMDKEVGNWCV-------EGENCSTCRCGLQTPQGDAVGSTAVVAVVTPE
MTR_5g080680        WKSTMEKSFIRMDEEVLNSSK--------TKQSFSCKCELQTPHCDAVGSTAVVAVVTPE
                    *..** :** .**:** :           .   :*:* **:*: *********:*****

AT3G11410           KIIVSNCGDSRAVLCRNGVAIPLSVDHKPDRPDELIRIQQAGGRVIYWDGARVLGVLAMS
Potri.008G059200    KIIVSNCGDSRAVLCRNGVAIPLSSDHKPDRPDELLRIQEAGGRVIYWDGPRVLGVLAMS
MTR_5g080680        KIIVSNCGDSRAVLCRNGVAIPLSSDHKPDRPDELDRINKAGGRVIYWDGARVLGVLAMS
                    ************************ ********** **::********** *********

AT3G11410           RAIGDNYLKPYVIPDPEVTVTDRTDEDECLILASDGLWDVVPNETACGVARMCLRGAGAG
Potri.008G059200    RAIGDNYLKPYVIPEPEVTVTERMEEDECLILASDGLWDVVSNDTACGVVRMCLRAQKPP
MTR_5g080680        RAIGDNYLKPYVISEPEVTITERSDEDECLILASDGLWDVVQNDTACKVVQMCLKANKTV
                    ************* :****:*:* :**************** *:*** *.:***:.

AT3G11410           ---------DDSDAAHNACSDAALLLTKLALARQSSDNVSVVVVDLRKRRNNQASS
Potri.008G059200    SPPGSNG---ALGSSDKACSDASVLLTKLALARHSTDNISVVVVDLRRNQH-----
MTR_5g080680        TPPGSPGLEVAGNGSDKACSDASILLTKLALARNSSDNVSVVVVDLRRDQRQQ---
                             .:.:*****::*********.*:**:********: :.
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Figure 3.1. Characterization of the G059200 peptide sequence. A, phylogenetic representation of *Populus trichocarpa, A. thaliana and Medicago truncatula* PP2CA class of proteins by maximum likelihood analysis. The G059200 protein was the phylogenetically closest *P. trichocarpa* protein to *AtPP2CA* (AT3G11410). B, Alignment of *AtPP2CA* with poplar *G059200* and *M. truncatula* MTR_5g080680. Identical amino acids are marked with asterisk, functionally similar amino acids are marked with colons and related ones are marked with dots. A dark solid bar indicates presence of a putative glutamine amidotransferase class-II active site motif.

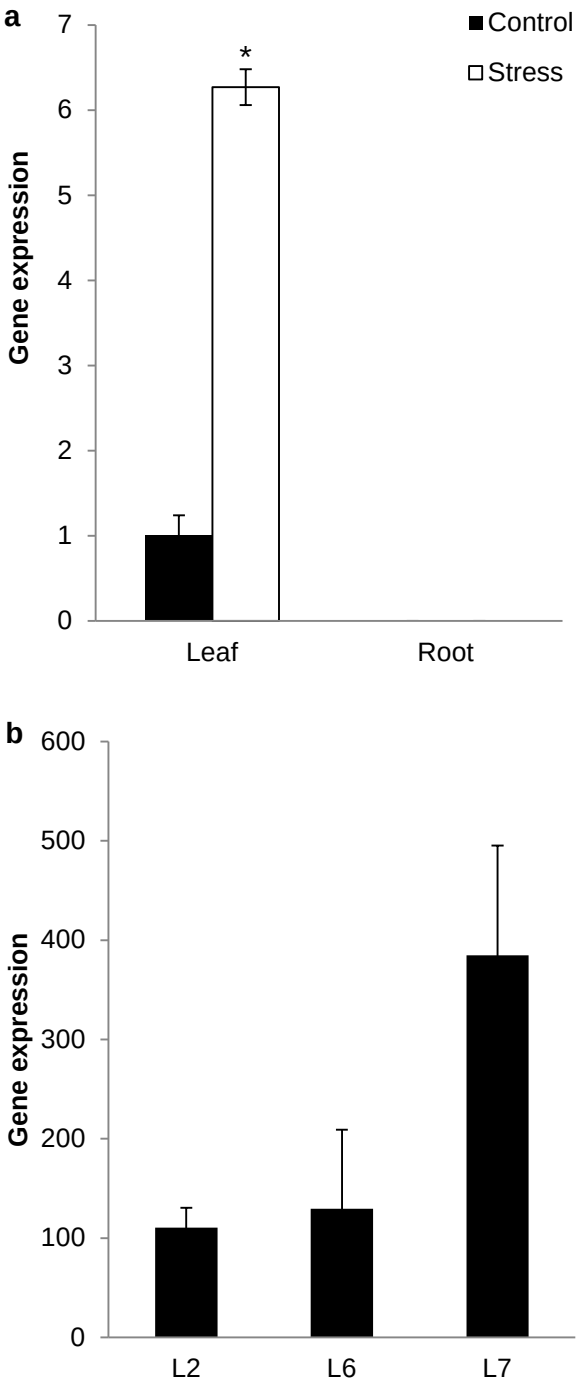


Figure 3.2. Quantitative real time PCR analysis of G059200.
A, Drought-induced expression of *59200* in poplar leaves. Each bar represents a mean from three plants.
Bars = ±SE, * Student's t-test, significance at P < 0.01. B, Expression of *59200* in three transgenic *thaliana* lines relative to each other. Since there is no equivalent gene in *A. thaliana*, the obtained CT values for transgenic plants is related to a cycle in which we did not detect any product in control plants (CT=35). The obtained values were also normalized against the expression of the native APT1 gene. Each bar represents a mean of three independent experiments.
Bars = ±SE, Analysis of variance.

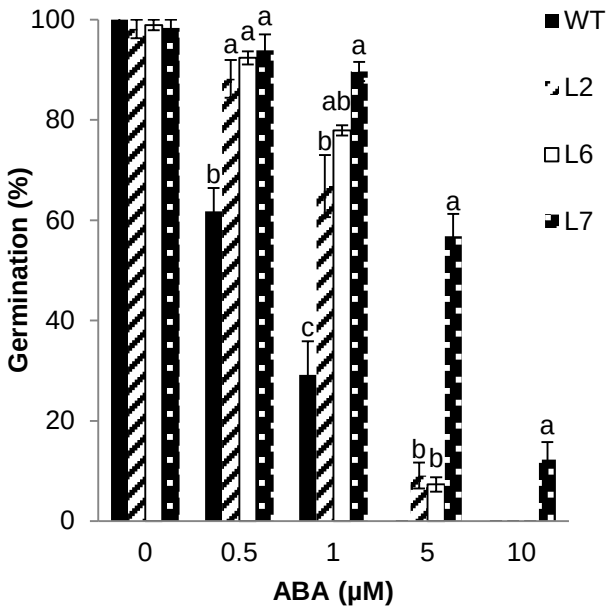


Figure 3.3. Percentage germination of wildtype and transgenic line seeds on ABA-containing media. More than 150 seeds of each line were plated in three replications and values are averages ± SE of single experiment. Different letters indicate that means are statistically significant from each other at $P < 0.01$ (Analysis of variance/Tukey HSD).

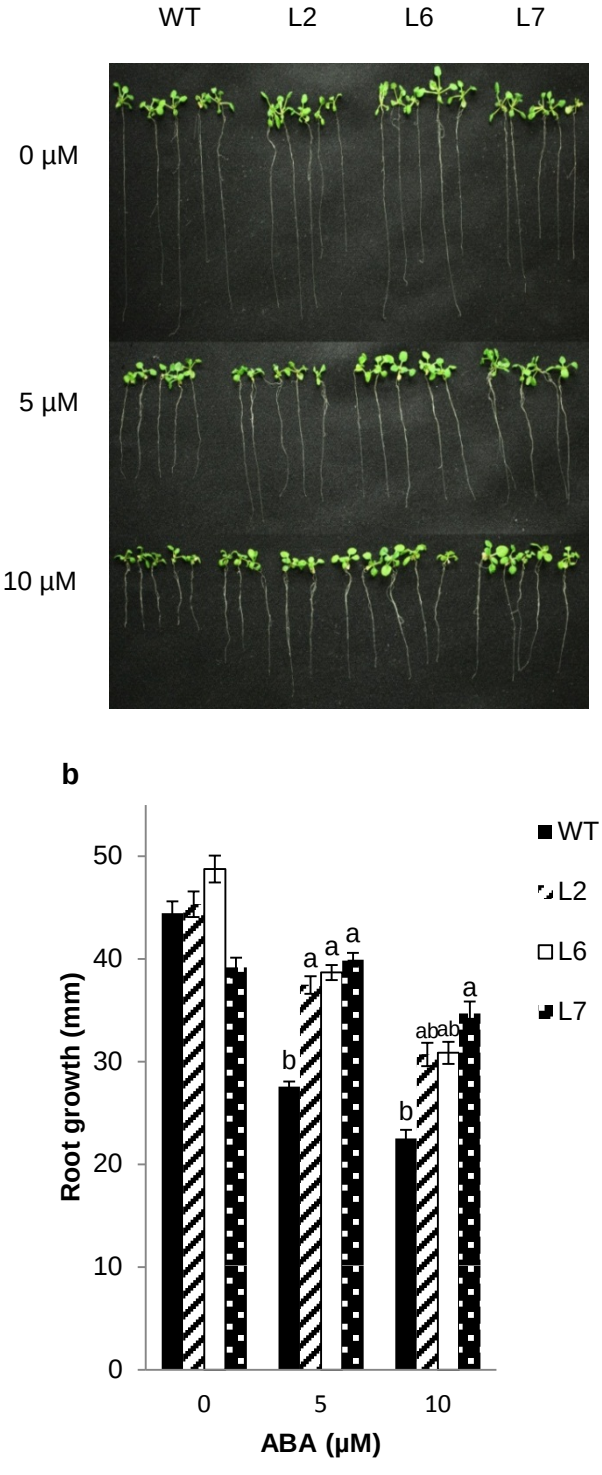


Figure 3.4. Transgenic *A. thaliana* lines show ABA insensitive root growth. A, photograph showing root growth of wildtype and transgenic lines was taken at 12 d. B, Root growth data are mean ± SE of two independent experiments (n=15 in three replications per experiment). Different letters indicate that means are statistically significant from each other at $P < 0.05$ (Analysis of variance/Tukey HSD).

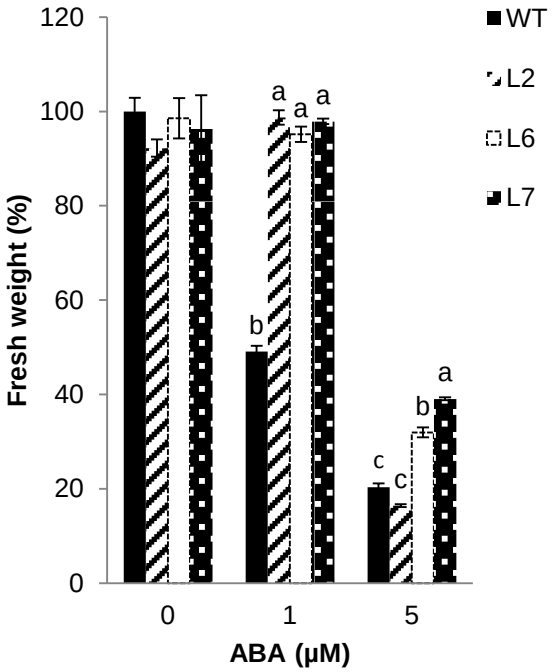


Figure 3.5. Fresh weight accumulation in wildtype and transgenic lines supplemented with or without indicated concentration of ABA. Percent fresh weight accumulation in wildtype and transgenic lines was calculated with respect to fresh weight of wildtype grown in MS media without ABA. Values are mean ± SE of single experiments (n=30 in three replications). Different letters indicate that means are statistically significant from each other at $P < 0.01$ (Analysis of variance/Tukey HSD).

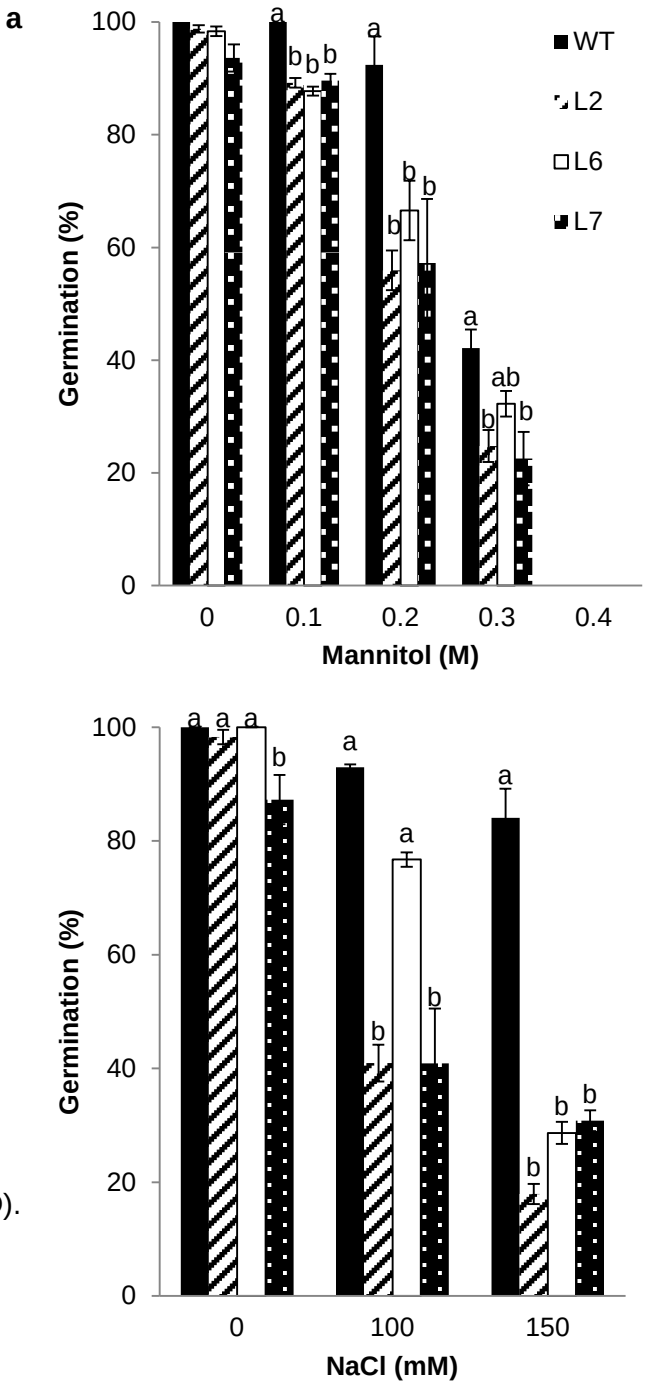
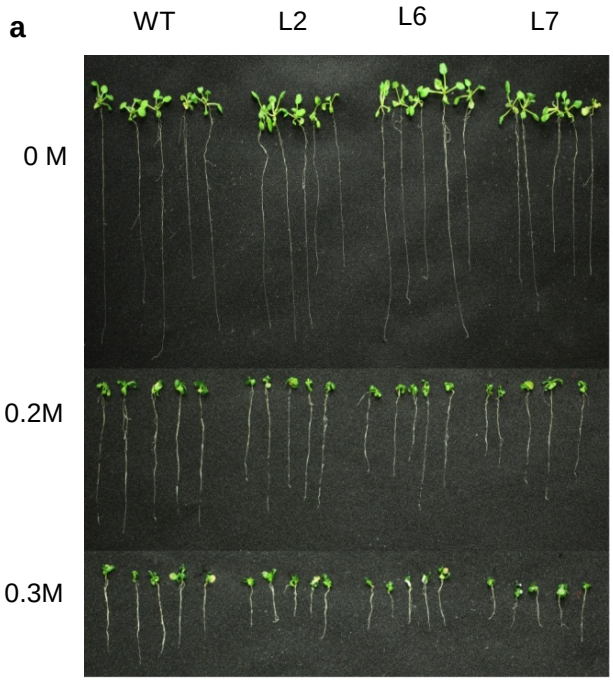


Figure 3.6. Percentage of germinated seeds on MS agar plates supplemented with or without indicated concentration of mannitol (A) or NaCl (B). About 100-150 seeds of each line were plated in three replications for each experiment; two independent experiments (A), single experiment (B). Data of seed germination are mean ± SE. Different letters indicate that means are statistically significant from each other at $P < 0.01$ (Analysis of variance/Tukey HSD).



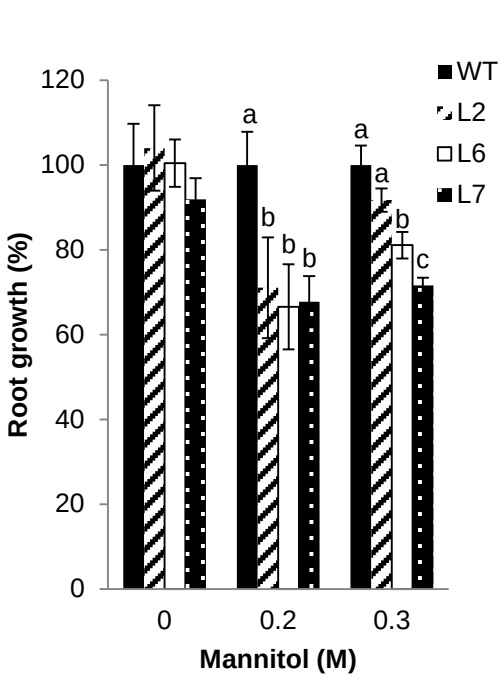


Figure 3.7. Transgenic A. thaliana lines show hypersensitive root growth during osmotic stress. A, photograph showing root growth of wildtype and transgenic lines taken 12 days after germination. B, Percent root growth of transgenic lines was calculated with respect to root growth of wildtype grown in the same medium. Values are mean + SE of two independent experiments (n=15 in three replications per experiment). Root growth of wildtype grown in MS media supplemented with 0.2M and 0.3M mannitol was 80% and 42% respectively of the wildtype grown in MS media without mannitol. Wildtype values were set to 100% to aid visualization of differences between wildtype and transgenic lines. Different letters indicate that means are statistically significant from each other at $P < 0.01$ (Analysis of variance/Tukey HSD).

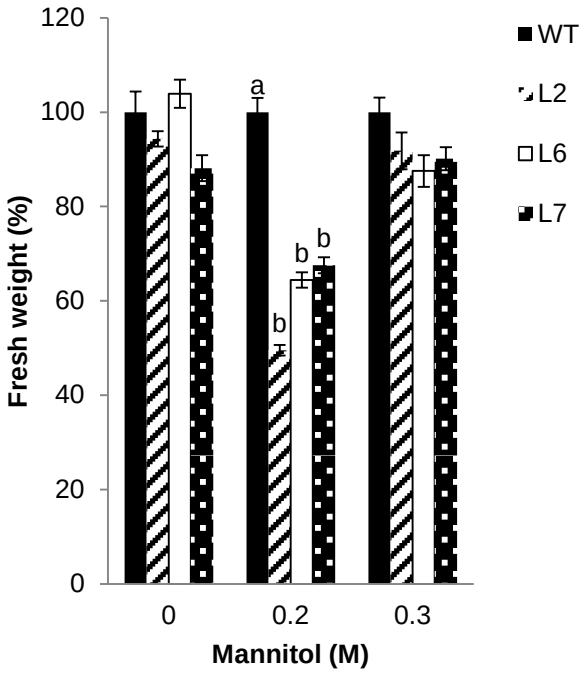
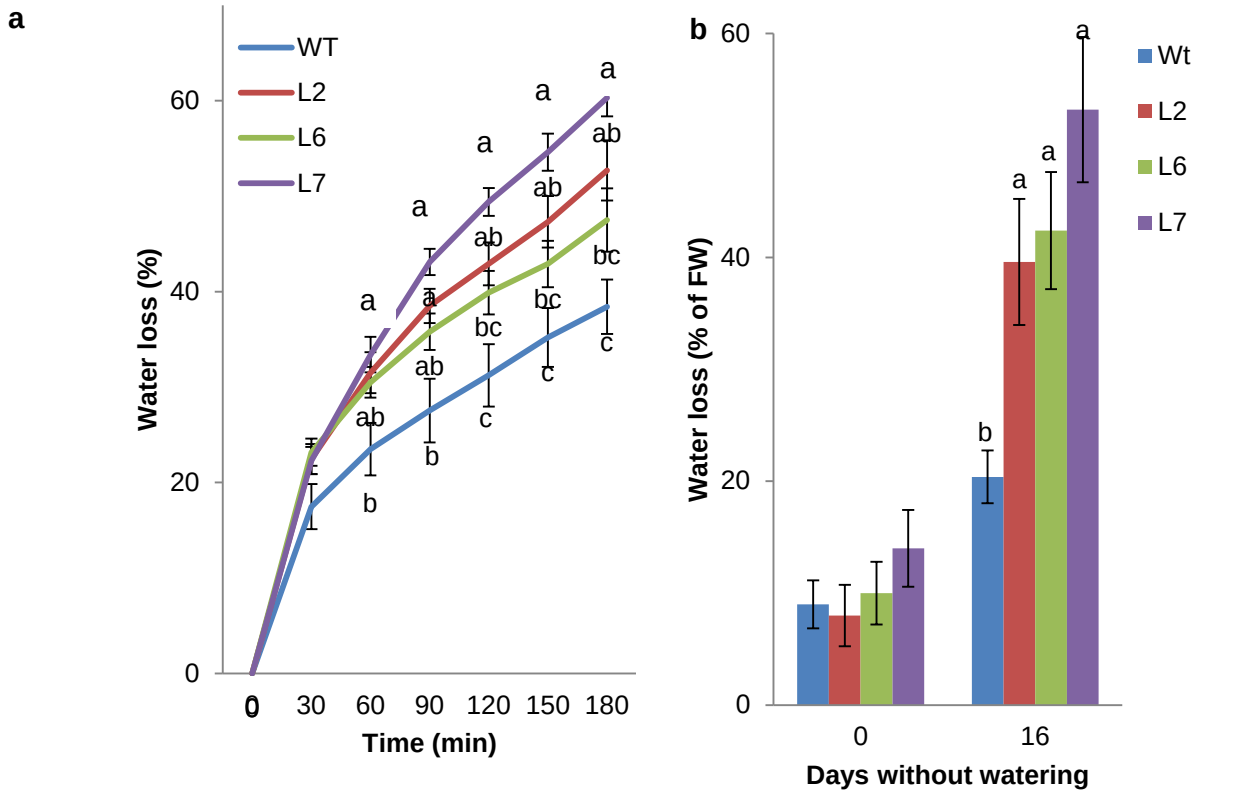


Figure 3.8. Fresh weight accumulation in wildtype and transgenic lines. Percent fresh weight data were calculated with respect to fresh weight of wildtype grown in the same media. Values are mean ± SE of two independent experiments (n=30 in three replications per experiment). Fresh weight of wildtype grown in MS media supplemented with 0.2M and 0.3M mannitol was 73 and 44% respectively of the wildtype grown in MS media without mannitol. Wildtype values were set to 100% to aid visualization of differences between wildtype and transgenic lines. Different letters indicate that means are statistically significant from each other at $P < 0.01$ (Analysis of variance).


a
WT
L2
L6
L7
Water loss (%)
Time (min)
0
30
60
90
120
150
180
b
Wt
Water loss (% of FW)
Days without watering
16


c
WT
L2
L6
L7
Control
Stressed

Figure 3.9. Enhanced water loss and reduced drought tolerance of transgenic lines overexpressing poplar G059200 gene. A, water loss was calculated from decrease in FW compared with fresh weight at time zero. Percent values are mean ± SE of two independent experiments (n=3 per experiment). B, Quantification of water loss in plants grown on soil for indicated number of days without watering. Water loss data are mean of 7 leaves per plant, collected from 10 different plants of each line per experiment (two independent experiments). Different letters and * indicate that means are statistically significant from each other at $P < 0.01$ (Analysis of variance). C, Reduced drought tolerance in transgenic *A. thaliana* lines overexpressing poplar *G059200* gene compared to wildtype. Photograph was taken 18 days after withholding water. Inflorescences were removed to better show the effect of drought on rosette leaves.

TCTGAACTAATGGCTGGGATTTGCTGCGGAGTTGTTGCTGAGAATGAAGCAGCTGCAGCAG
TTGAGGCAAGGTCACGAGCATCAAGACGCAGGAGATTAGAACTCCGGTTGGTTTCCGACGT
GTCTGTTCCACCGTCGACGATTTTGGATGTCGCTCCGGCGAAAAGGAAGAAACTCGAGTTG
TTTCCCATTCCCATCTCACGTGATTGTGGTAACGCAGTAGAAAACTGCAAAAGAATTGAAGAA
AATAAAAACAATAGCATCTCGGATTCAAGTAAACCAGAATCTGTGAAATTGAAAGAAGCTCTT
AAATTCGGCATGACTTCTGTTTGTGGTAGAAGAAGAGATATGGAAGACGCCGTTTCGATACA
CACTTCTTTTACTACAAAAAACACCTCGTATTTTGGTGTTTTCGACGGTCATGGTTGCTCACA
TGTAAGAGCGAACTTCCTCCCTTAAATTGCTTTCCAGTTTCTTTTTTTTAATCATGTTATTTTAT
GGTGCTGATTTTGGTGTGTTATTTTTAATTTAGGTGGCGATGAAGTGTAGAGATCGGTTGCAT
GAGATAGTAAAACAAGAAGTTGAAGGTTTTAAAGAAGAAGAAAGTGTAGAATGGAAGGAAAC
TATGGAGAGAAGTTTTGTAGAGATGGATAAAGAAGTGGGTAATTGGTGCGTTGAAGGAGAAA
ACTGTTCTACTTGTAGGTGTGGGTTACAGACGCCACAAGGCGACGCCGTTGGATCTACTGC
TGTGGTTGCTGTGGTGACGCCTGAGAAGATTATTGTCTCCAACTGTGGCGATTCTCGTGCCG
TGCTTTGTCGAAACGGTGTCGCTATTCCTCTCTCCTCTGATCACAAGGTGAGGTTAATCATTT
CCTTTAAATTGATCCATGCTTTTAGTTTTTGTTTGGAGTGATTATTTATGAACCATGTTAAAGA
ACAAGTCGAAATGTTGTTTCCACTAAAAGAATTAGGCGTGCGGATGTGGGTCAAGGTTTGAG
CTTTAGCTTTGTTGATTGTTGATAGCTAAGGAACTTTTTAGGAATCGGCGTGTGCCGTGCACA
TTTTTAATTCTTAGGACCTAATTATAATATTATTGTTAATCATGAT**G/A**TAGATTTTATTTCGATT
TTATTTT**G/T**CTGACTTCTGTTTATA**T/C**TTGTAATTACAGCCTGATCGACCAGATGAATTGCTT
CGAATCCAAGAAGCTGGTGGGCGTGTAATTTATTGGGATGGCCCAAGAGTCCTTGGTGTTTT
GGCCATGTCTAGAGCCATAGGTAATTAACATTGCAC**C**TTGATTTATTACACCCCCATCAAATT
GTGGCTGGTTTCTGCTTGATTCATGTACTCATGTTTTTTTGGCTTGTCATCAGGTGACAATTA
CTTGAAGCCCTACGTCATACCGGAACCGGAAGTGACTGTGACGGAACGGATGGAGGAGGAT
GAGTGTTTGATATTGGCAAGTGATGGACTGTGGGATGTGGTGTCAAATGACACTGCTTGTGG
GGTTGTGCGAATGTGCCTCCGTGCCCAAAAGCCACCTTCACCACCAGGTTCTAATGGCGCC
CTTGGGAGCTCTGATAAGGCCTGCTCAGATGCGTCAGTTCTG**T/C**TGAC**T/C**AAGTTGGCCT
TGGCTAG**A/GC/A**ATAGCACGGACAATATTAGTGTTGTTGTTGTGGATTTGAGAA

Supplementary Fig 3.1: DNA sequence of the G059200 variant cloned from the *P. trichocarpa* X *P. deltoides* K9 hybrid. A comparison of this sequence with that of G059200 (*P. trichocarpa* v3.0 genome) reveal 7 SNPs (bold) with nucleotide before slash indicating described gene variant, nucleotide after slash indicating template genome nucleotide. The described variant also had a single nucleotide insertion (C) in the last intron. Exons as defined in the *P. trichocarpa* v3.0 genome are underlined.

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Chapter 4.

Overexpression of a putative poplar NCED3 enhances drought tolerance in transgenic *Arabidopsis thaliana*

Abstract

Fast growing poplar hybrids are valued for their fast growth and are cultivated for multiple purposes all over the northern hemisphere. In many environments, sensitivity to drought is a growing concern, especially in light of human-induced changes in water availability. We have previously shown that the poplar G393800 gene in response to severe drought stress is expressed at higher levels in a relatively drought tolerant hybrid than in a highly sensitive hybrid. This gene encodes a potential ortholog of the *Arabidopsis thaliana* gene *AtNCED3*, whose gene product carries out a key step in ABA biosynthesis, thus acting in ABA-dependent responses to drought. To test if G393800 has a similar function, we generated transgenic *A. thaliana* plants overexpressing this gene. Transgenic plants had elevated levels of ABA and ABA catabolites. Seeds showed reduced germination during osmotic stress. In addition, two assays testing both rapid and slow response to water deficit in plants demonstrated that G393800 over expression resulted in enhanced drought tolerance. Taken together, these data provide evidence that G393800 can act as a positive regulator of drought tolerance.

4.1. Introduction

Poplar hybrids have been selected for fast growth and their biomass is used primarily for the production of pulp and paper, plywood and oriented strand boards (Dickmann, 2001). Their high rate of biomass accumulation makes them suitable for carbon sequestration and renewable biofuel production (Karp and Shield, 2008; Yemshanov and McKenney, 2008). Commonly used hybrid poplar clones have been selected for rapid growth and often regionally important disease resistance but not for drought tolerance (Liang et al., 2006). Human activities are rapidly reducing water availability in important poplar growing regions of Canada, Europe and China (Huo et al., 2008; Schindler, 2001; Schindler and Donahue, 2006; Vorosmarty et al., 2010). Climate change, past and predicted (Dai, 2011; Hogg and Bernier, 2005; Salinger et al., 2005) may also have a negative impact on poplar cultivation.

Among the most commonly studied *Populus* species and hybrids there are some traits that generally correlate with relatively high drought tolerance. These include stringent regulation of stomatal closure (Blake et al., 1984; Yin et al., 2004), ability to accumulate osmoprotectants and proteins and compounds that protect against free radical, membrane and photo damage (Gebre et al., 1998). Structural acclimation responses include ability to reduce leaf area, shoot to root ratio and xylem vessel diameter (Harvey and van den Driessche, 1999; Munne-Bosch and Alegre, 2004; Yin et al., 2004).

The genetic basis of drought tolerance in *Populus* species is largely unknown although there are some encouraging developments. QTL mapping has identified several genomic regions for which markers co-segregate with higher drought tolerance (Street et al., 2006; Tschaplinski et al., 2006), providing opportunities for marker-based early selection of segregating individuals harbouring a larger set of desired loci. The identification of drought-induced poplar genes has provided another entry point. Hundreds of drought responsive genes have been identified in *Populus* species and hybrids (Hamanishi et al., 2010; Street et al., 2006; Wilkins et al., 2009). These sets of genes encode proteins with potential roles in the responses described above, but also signaling components, both abscisic acid (ABA)-dependent and independent, transcription factors and proteins for which no annotations can be made. Altered expression of a few of these genes in transgenic plants has yielded interesting results.

Overexpression of two putative poplar transcription factors in *A. thaliana* resulted in plants with improved drought tolerance (Chen et al., 2011; Ma et al., 2010). Similarly, overexpression of poplar calcineurin B-like proteins, presumably involved in calcium-dependent signaling, led to improved drought tolerance in transgenic *A. thaliana* (Li et al., 2013b) and poplar (Li et al., 2012) plants.

We have previously compared the drought responses of a drought sensitive and a drought tolerant clone and found several genes differentially expressed between two clones (Arshad et al. in manuscript). Among these genes, a potential poplar ortholog of the *A. thaliana* NCED3 gene was of a particular interest due to its potential involvement in ABA biosynthesis. The AtNCED3 gene encodes an enzyme with 9-cis epoxycarotenoid dioxygenase (NCED) activity (Shinozaki and Yamaguchi-Shinozaki, 2007). There are five members of the NCED family in A. thaliana, and NCED3 is believed to be the most important of them (Seki et al., 2007). Zeaxanthin, a precursor of ABA, is converted to 9-cis-neoxanthin and 9-cis-violaxanthin in plastids (Seo and Koshiba, 2002). Subsequently, NCED3 catalyzes the conversion of these compounds into xanthoxin, which is transported to the cytosol, where it is converted into ABA (Seo and Koshiba, 2002). Taken together, the pathways of ABA biosynthesis and breakdown are well defined in *A. thaliana* (Nambara and Marion-Poll, 2005), providing an opportunity to map similar functions onto similar genes in the poplar genome.

ABA can be transported over long distances. Upon binding to cellular receptor complexes, ABA triggers signaling leading to stomatal closure and activation of downstream genes leading to other drought responses (Shinozaki and Yamaguchi-Shinozaki, 2007). *A. thaliana* nced3 knockout mutants exhibit impaired stomatal closure and drought tolerance (Seki et al., 2007). Plants overexpressing AtNCED3 and putative orthologs in other species show enhanced ABA levels and improved tolerance (Hwang et al., 2010; Iuchi et al., 2001; Li et al., 2013a; Tan et al., 2003; Wan and Li, 2006; Zhang et al., 2009). *AtNCED3* expression is regulated by the A. thaliana activating factor 1 (ATAF1), a NAC-type transcription factor (Lu et al., 2007). Plants overexpressing ATAF1 exhibit increased transcripts of NCED3 and ABA levels (Jansson and Douglas, 2007), presumably via binding to an identified cis element in the *AtNCED3* promoter region (Behnam et al., 2013).

Here, we assess the function of the poplar G393800 gene, a putative ortholog of AtNCED3, by overexpression in transgenic *A. thaliana* plants. Our results show that this gene can act as a positive regulator of drought tolerance in *A. thaliana*.

4.2. Materials and methods

4.2.1. Bioinformatics

We used the BLASTX algorithm (Altschul et al., 1990) to search for highly similar protein sequences in the *Populus trichocarpa* and *Glycine max* genomes using the Phytozome v9.1: *Populus trichocarpa* v3.0 and KEGG databases. The phylogenetic relationship was generated between *A. thaliana* NCEDs, identified poplar and soybean proteins and analyzed using EMBL-EBI Clustal Omega software. PCR primers were designed by the use of the Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/).

4.2.2. Plant material and growth conditions

We sowed *A. thaliana* seeds on plates that contained Murashige and Skoog modified basal medium (Invitrogen, #M404), 1% sucrose and 0.8% agar (PhytoTechnologies Labs. Kansas city, USA). Seeds were then stratified at 4 ^{0}C for 3 days and transferred to the growth chamber at 22 ^{0}C, 60-80% humidity with 8 hour light (80-100 µmol m^{-2} s^{-1})/16 hour dark periods. About three-week old seedlings were transferred to plastic pots filled with PRO-MIX soil (Premier Horticulture Inc. Philadelphia, USA) and kept at the same conditions mentioned above until plants were ready for phenotypic analysis.

4.2.3. Vector construction and plant transformation

A genomic fragment of the poplar G393800 gene containing the entire open reading frame plus introns was amplified by PCR from DNA from a *P. trichocarpa x P. deltoides* hybrid with number K9, obtained from British Columbia Ministry of Forests Kalamalka research station using following primers: 5`- AGCTCAAACAACTTTGCCAAA - 3` and 5`- ACCAGGCTATACCATCCCAG- 3`. AttB1 and 2 sites were attached to the amplicon ends during PCR, followed by recombination into pDONR/Zeo gateway plasmid using

the B/P reaction kit (Life Technologies). Identity of the cloned fragment was confirmed by Sanger sequencing, followed by recombination into the pK2GW7,0 vector (Karimi et al., 2007) using the L/R reaction kit, (Life Technologies). Subsequently, we transformed pK2GW7.0 into the GV3101 strain of *Agrobacterium tumefaciens* by electroporation. Finally, *A. thaliana* plants (Col-0) were transformed with *A. tumefaciens* containing the G393800 gene by the floral dip method (Bent, 2006). *A. thaliana* plants were confirmed for a single T-DNA insertion by observing 3:1 resistance-sensitive ratio of transgenic seeds to kanamycin in the T2 generation. Transgenic plants were also tested for homozygosity in the T3 generation and only plants with 100% resistance to kanamycin were considered homozygous and used for further phenotypic analysis. The T-DNA presence was confirmed by PCR of the NPTII gene conferring kanamycin resistance.

4.2.4. ABA quantification

Transgenic and wildtype seeds were grown on the MS media for 3 weeks in 5 replications. A total of 25 seedlings per line were collected, pooled and freeze-dried for each line. The samples were sent to the Plant Biotechnology Institute, National Research Council, Saskatoon, SK for ABA quantification.

4.2.5. Germination assays

Wildtype and transgenic seeds were plated on solid media described above, supplemented with 0, 100 or 200mM mannitol. Seed germination percentage was recorded at day 7 based on seedlings with green (tolerant) or yellow (sensitive) cotyledons as described in (Saez et al., 2006).

4.2.6. Drought stress and water loss assays

We performed two different water loss assays to analyze the transpiration rate in wildtype and transgenic plants. To measure a rapid water loss, an assay was performed as described by (Dai et al., 2007) where the rosettes of 4 weeks old plants were decapitated and kept on the lab bench. Fresh weight (FW) was measured from decapitated rosettes at different time points. We calculated water loss percentage by recording the decrease in FW at specific time point/FW at time zero multiplied by 100.

Long-term water loss assay was performed as described by (Rubio et al., 2009; Saez et al., 2006). Wildtype and transgenic plants grown in soil in pots kept in the growth chamber with normal watering regimes before drought was imposed on four weeks old plants by withholding water completely. Subsequently, leaves were removed at the indicated days after withholding water, and weighed. Detached leaves were then soaked in demineralized water for 3 hours followed by drying on blotting paper, and weighing again. The difference in weight was considered as water loss.

4.3. Results

While the poplar *Potri.001G393800* (hereafter referred to as *G393800*) gene share sequence similarity to the *A. thaliana* gene *AtNCED3*, encoding a 9-cis-epoxycaratenoid dioxygenase, there is no phylogenetic analysis available assessing how it and other related poplar genes relate to *AtNCED3*. We therefore used the predicted protein sequence of *AtNCED3* (accession # *At3g14440*) as a probe to search for highly similar predicted open-reading frames (ORFs) in the *P. trichocarpa* genome. Thereafter, we generated and analyzed the phylogenetic relationship between the identified poplar ORFS with those of *A. thaliana* NCED proteins, including also NCED3-like protein sequences from soybean to reduce the risk of arbitrary associations between sequences. (Fig. 4.1). The generated phylogenetic tree provided a sub-clade in which *AtNCED3* (At3g14440) clustered with *P. trichocarpa* gene G393800 (Fig. 4.1). We found a 72 % sequence similarity between the predicted AtNCED3 and G393800 peptide sequences. Based on these results we conclude that G393800 is the most likely *P. trichocarpa* ortholog of *AtNCED3*.

We hypothesized that if the poplar G393800 protein acts as a 9-cis-epoxycaratenoid dioxygenase, then over expression of it in transgenic *A. thaliana* plants may result in responses similar to that of plants overproducing ABA. To that effect, we generated three transgenic lines that were monogenic homozygous for a construct that carries a CaMV 35S promoter fused to a genomic fragment encompassing the complete predicted ORF of the G393800 gene (see materials and methods). Here we will describe the response of these lines relative to that of untransformed control plants, first regarding ABA levels, thereafter during seed germination and drought stress.

4.3.1. ABA and ABA catabolites

We hypothesized that if G393800 is involved in ABA biosynthesis then we will observe enhanced levels of ABA in transgenic lines. To test this hypothesis, we quantified ABA in wildtype and transgenic lines. The results demonstrated that L1 and L3 transgenic lines have 42% and 25% more ABA respectively compared to wildtype whereas L5 has ABA levels similar to wildtype (Fig 4.2a). Upon further analyzing ABA quantification data, we found that plants from the three transgenic lines contained much higher levels of the ABA catabolites phaseic acid (PA) and dihydrophaseic acid (DPA) than wildtype plants. In addition, transgenic lines also contained higher levels of ABA glucosyl ester (ABAGE), an inactive conjugate of ABA (Fig 4.2b).

4.3.2. Germination

Since ABA induces seed dormancy (Koornneef et al., 2002), we hypothesized that we will see a reduced germination in transgenic seeds compared to wild type due to higher levels of ABA in transgenic lines. As predicted, we found significant difference in germination between wildtype and transgenic plants overexpressing G393800 when seeds were grown on the same medium supplemented with different levels of mannitol (Fig. 4.2). Data showed that under control conditions without addition of mannitol, the germination of transgenic seeds and wildtype was not significant, except for the L5 line, which has a 13% reduction in germination. Furthermore, 100 mM and 200 mM mannitol had almost no effect on germination of wildtype plants, whereas 100 mM mannitol concentration reduced germination of L5 line by approximately 30% (Fig. 4.2). Similarly, 200 mM mannitol caused a germination reduction by 25-70% in the transgenic *A. thaliana* lines overexpressing poplar G393800 (Fig. 4.2) revealing osmotic stress sensitive behavior of transgenic lines. Based on these results, we conclude that transgenic *A. thaliana* seeds overexpressing poplar G393800 gene are hypersensitive to osmotic stress during germination.

4.3.3. Water loss

Based on the seed germination results, we predicted a reduced water loss in transgenic lines due to higher ABA levels. To test this hypothesis, we performed two previously

described water loss assays (Dai et al., 2007; Rubio et al., 2009; Saez et al., 2006). The first assay was performed by measuring a quick response to water loss. Plants were decapitated and rosettes were weighed after every half an hour to measure rate of water loss in wildtype and transgenic lines. As predicted, wildtype plants lost significantly more water in three hours (75%) as compared to two transgenic lines (L3, L5), which lost approximately 55% water during this time. Water loss in the L1 line was, however, not significantly different from the wildtype (Fig 4.3a). Similarly, a second assay was carried out to measure water loss for a longer duration. We withheld water from wild type and transgenic plants growing on soil for 20 days. At day zero, water loss was not significantly different between wild type and transgenic lines whereas after depriving plants with water for 20 days, we recorded 8-12% of water loss in transgenic lines, which was significantly lower than wildtype plants that lost about 56% of water during this time (Fig. 4.3b). In conclusion, these assays revealed that poplar G393800 gene over-expression in transgenic *A. thaliana* reduces water loss and enhances drought stress tolerance. As shown in the Fig. 4.3c, drought caused lethal effects on the wild type plants that mostly turned brownish after 45 days of withholding water whereas transgenic plants were still greenish except the L1 line.

4.4. Discussion

Since ABA signaling has a central role in the perception of drought stress, its manipulation has the potential of delivering a complete package of responses. Also within the pathway, manipulation of early steps is likely to have a stronger effect than manipulation of later steps as it may interfere with the pathway before it ramifies extensively. Members of the poplar gene family encoding putative NCED proteins, or for that matter, other putative poplar components of ABA signaling, have not been evaluated functionally before. Here we tested if the overexpression of a putative poplar NCED-encoding gene, potentially involved in the biosynthesis of ABA, and thereby an early step in this pathway, could have that effect in transgenic plants. We chose to express this gene in *A. thaliana* both because of the rapid generation of transgenic plants relative to poplar, and because the production of large number of monogenic homozygous seeds allowed large number of seedlings or biological replicates to be tested under highly standardized conditions.

Our results show that one of the tested transgenic *A. thaliana* lines overexpressing the poplar G393800 gene exhibit reduced germination (Fig. 4.2). Germination in the presence of osmolytes showed that also the other two tested lines had reduced germination frequency compared to wildtype seeds. Given that ABA induces seed dormancy (Koornneef et al., 2002), we speculate that the observed reduction of seed germination in transgenic plants is a result of excess ABA biosynthesis due to 9-cis epoxycarotenoid dioxygenase (NCED) activity of the G393800 protein. ABA quantification provided direct evidence that G393800 may be involved in ABA biosynthesis (Fig. 4.2). Higher levels of ABA catabolites (Fig. 4.2), however, suggest that transgenic lines synthesized much more ABA than quantified, which was degraded or inactivated by ABA hydroxylase and ABA glucosyl transferase enzymes that are induced by ABA (Kushiro et al., 2004). Subsequent water loss experiments, however, provide additional indirect support for this conclusion. The observed effect of NCED3 overexpression was especially strong after a longer drought trial on adult plants, in which the transgenic lines lost 45% less water than the wildtype control plants (Fig. 4.3b). This is a strong phenotype for overexpression of a single gene, pointing towards the central role of ABA biosynthesis and signaling in providing a strong response to drought stress.

In theory, constitutive overexpression of an NCED3 enzyme may lead to constantly elevated ABA levels and signaling. ABA-induced stomatal closure in turn would reduce gas exchange, photosynthesis and growth. Seriously reduced growth would argue against this approach in targeted breeding for drought resistance. A review of tested plant lines suggests, however, that mild overexpression of G393800 had no obvious effect on plant size (Fig 4.3c). This aspect needs to be quantified properly though before any conclusions can be made, which in turn can inform if a similar approach can be taken to generate transgenic poplar plants with improved drought tolerance.

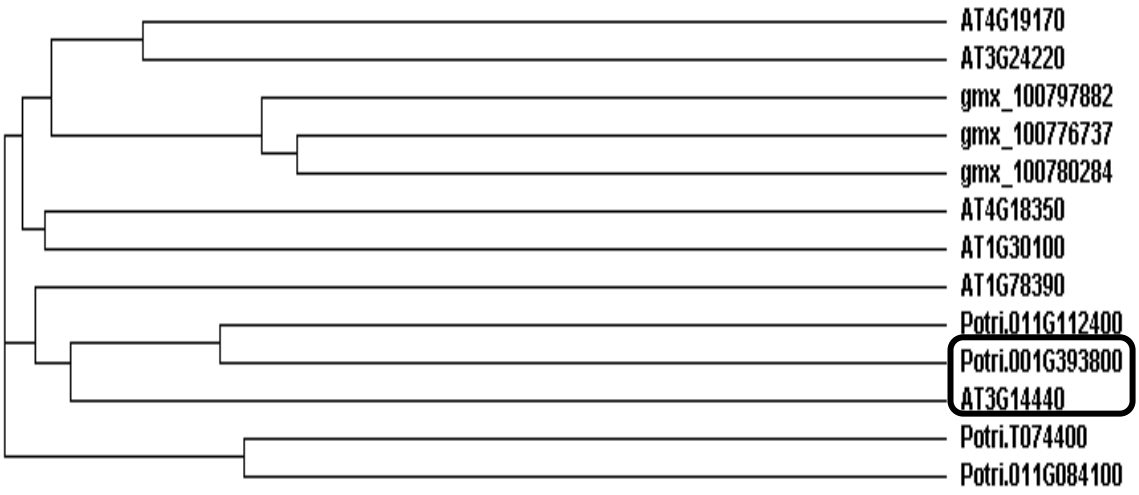

Figure 4.1. Maximum likelihood phylogeny of *Populus trichocarpa, A. thaliana and Glycine max* NCED class of proteins. The G393800 protein was phylogenetically the closest *P. trichocarpa* protein to *A. thaliana* NCED3 (AT3G14440).

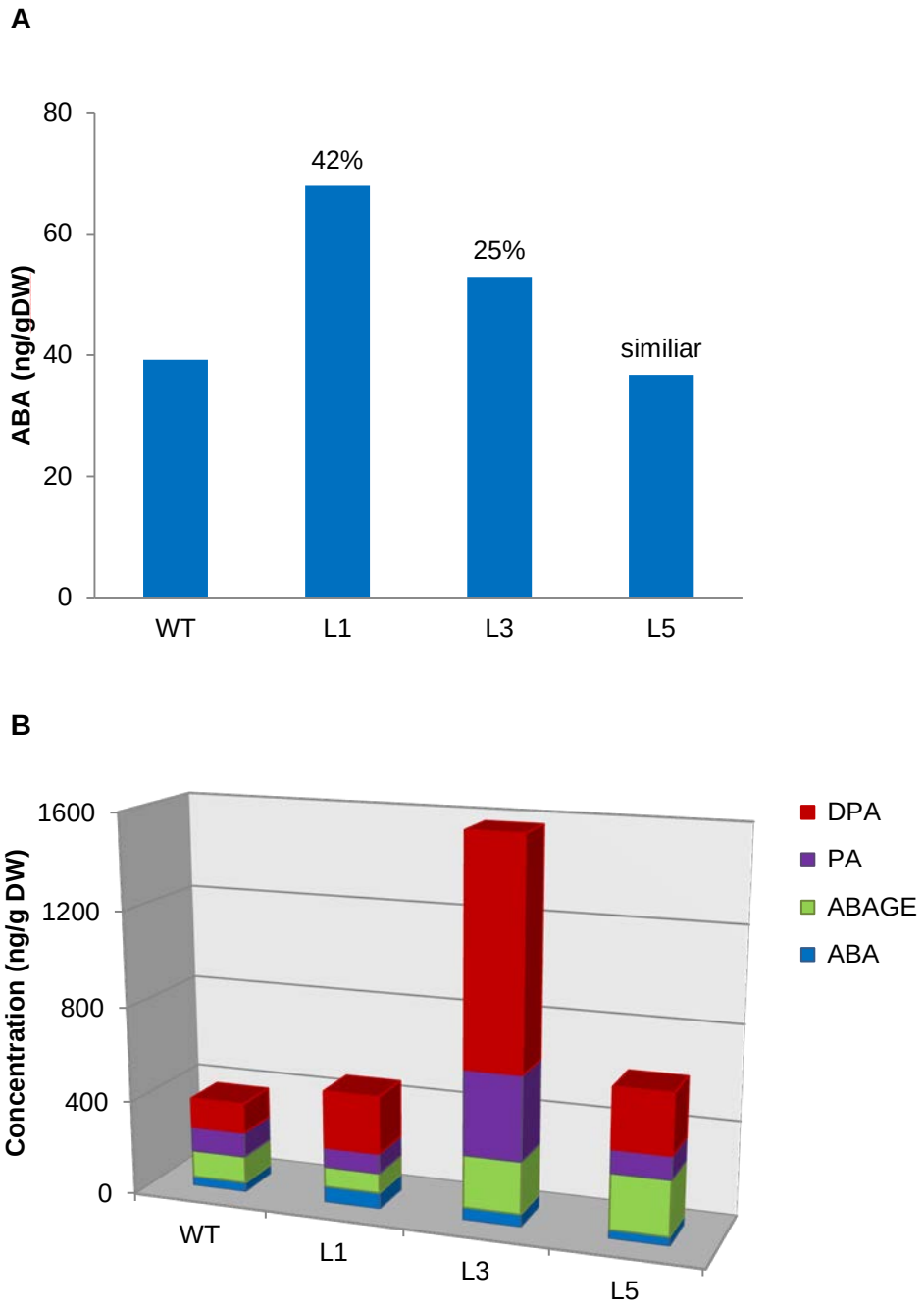

Figure 4.2. ABA quantification from wildtype and transgenic lines of *A. thaliana* overexpressing putative poplar NCED3. A, ABA levels in wildtype and transgenic plants. Enhanced ABA levels in transgenic plants were calculated as percent of those in wildtype, and are indicated on top of each bar. B, ABA and ABA catabolites accumulation in wildtype and transgenic plants. The values are mean of 25 plants per line in 5 replications.

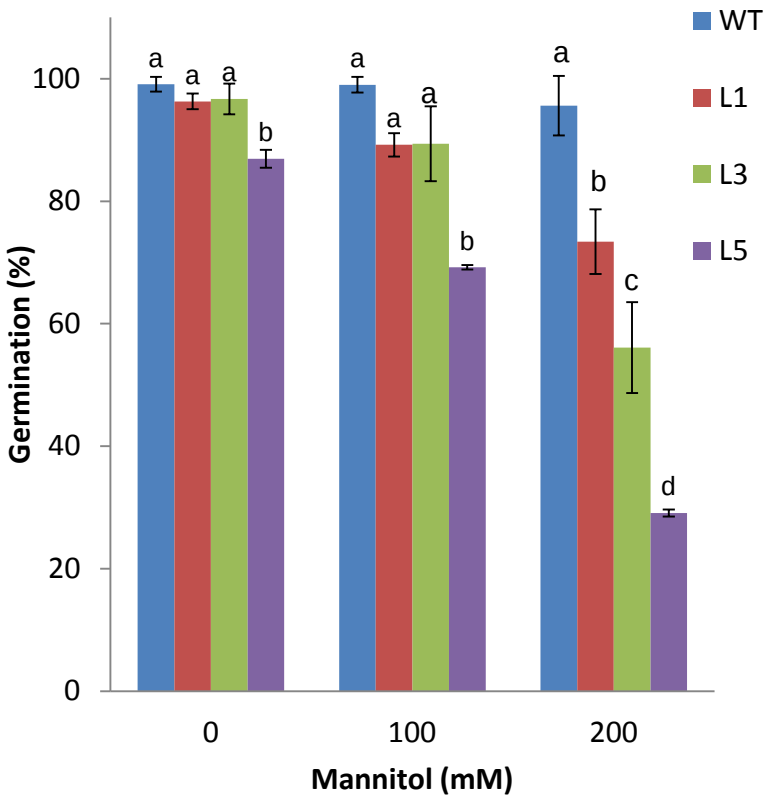

Figure 4.3. Germination percentage of wildtype and transgenic seeds on media containing different concentration of mannitol. Approximately 100 seeds of each line were sowed on plates. Values are averages ± SE of three independent experiments; three replications per experiment. Different letters indicate that means are statistically significant from each other at $P < 0.01$ (Analysis of variance/Tukey HSD).

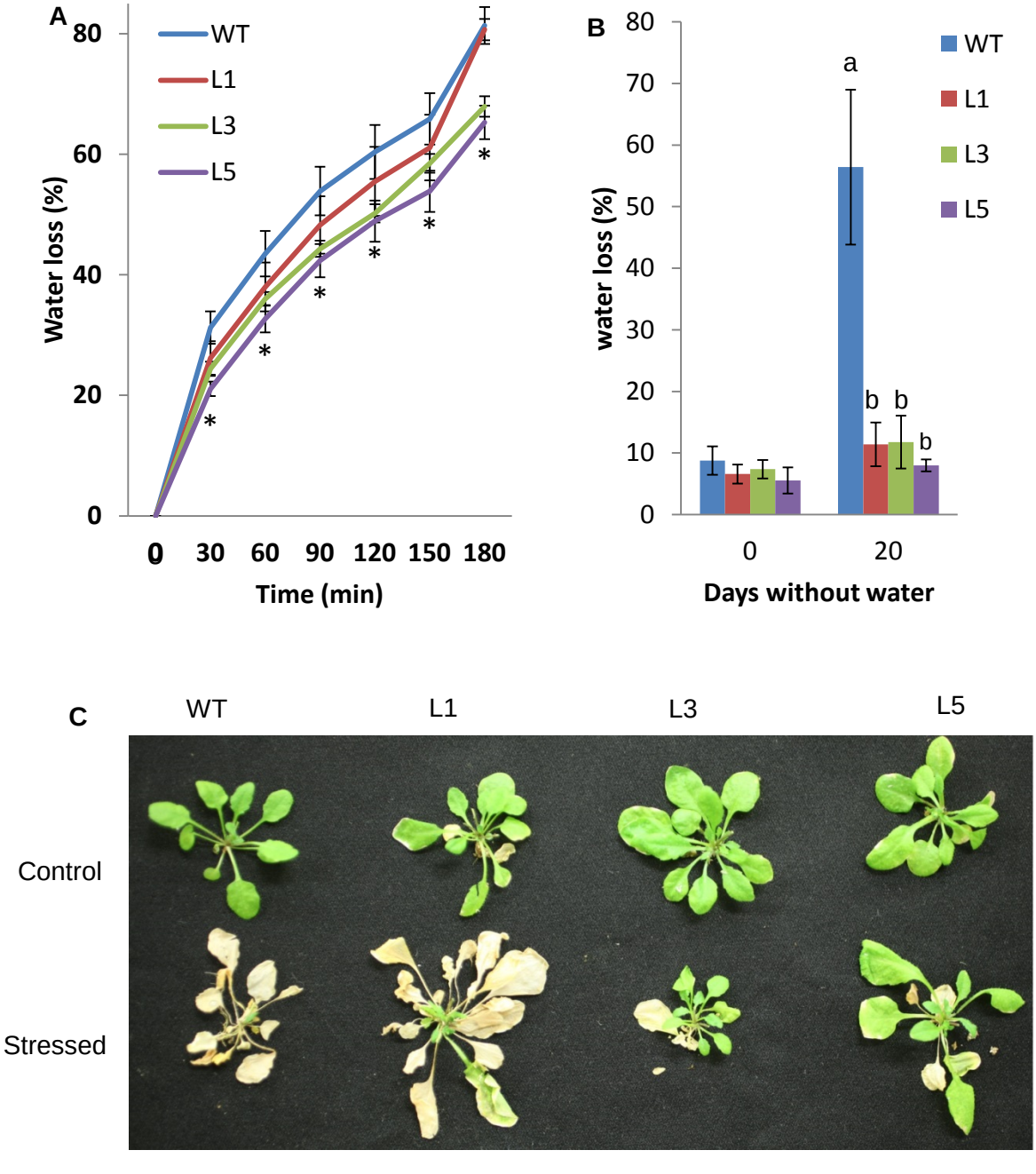
A
Water loss (%)
80
60
40
20
0
WT
L1
L3
L5
0
30
60
90
120
150
180
Time (min)
B
water loss (%)
80
70
60
50
40
30
20
10
0
a
b
b
b
WT
L1
L3
L5
0
20
Days without water
C
WT
L1
L3
L5
Control
Stressed

Figure 4.4. Reduced water loss and enhanced drought tolerance of transgenic lines overexpressing poplar G393800 gene. A, water loss measurements from plant rosettes incubated at the lab bench. Percent values are averages ± SE of two independent experiments (n=5 per experiment). B, water loss quantification from plants grown on soil and exposed to drought stress for indicated number of days. The data are average of 5-7 leaves per plant; 10 plants per line in a single experiment. Different letters and * indicate that means are statistically significant from each other at $P < 0.01$ (Analysis of variance/Tukey HSD). C, plants grown on soil were exposed to drought by withholding water for 45 days till lethal effects were observed on wildtype plants. The inflorescence was cut before taking the pictures to clearly show the effect of drought on rosettes.

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Chapter 5.

Overexpression of a putative poplar NCED3-encoding gene results in enhanced drought tolerance in transgenic poplar plants

Abstract

We have previously identified the poplar *G393800* gene as a potential ortholog of the *Arabidopsis thaliana* gene *NCED3*, encoding an enzyme in ABA biosynthesis. Overexpression of this gene in transgenic *A. thaliana* results in plants with increased tolerance to drought. To assess whether overexpression of the same gene in transgenic poplar plants results in a similar phenotype, we tested three *Populus alba* lines carrying the *G393800* under control of the 35S CaMV promoter in a water loss assay from excised leaves. This assay showed that leaves from the transgenic lines lost water at a slower rate than leaves from wildtype plants. Our result indicate that overexpression of poplar *G393800* reduces water loss also in transgenic poplar plants.

5.1. Introduction

Populus species are found across the northern hemisphere in a large range of ecological niches. For example, *P. trichocarpa* is found primarily in riparian environments in the north west of North America. *P. balsamifera* also grows in North America, but is better adapted to growth in drier areas than *P. trichocarpa*, away from water streams. *P. euphratica* in turn is adapted to warmer and much drier regions and is found from North Africa to the Taklimakan desert in China, which is characterized by high temperature, saline soils and frequent drought stress (Gries et al., 2003). The traditional human uses of these and other species range from firewood and lumber, to windbreak in open landscapes. Many *Populus* species can hybridize, and have been used to generate offspring with considerable hybrid vigor as manifested by high growth rates and biomass accumulation (Dickmann, 2001). The identified individuals are then propagated by stem cuttings, generating clonal populations with uniform rapid growth. These traits have made *Populus* hybrids a popular choice as a ligno-cellulosic feedstock for pulp and paper production, but also plywood, oriented strand production and to a limited extent, lumber production (Dickmann, 2001).

Populus hybrids and hybrids of related *Salix* species are also used as feedstock for communal heating and are also being considered as a potential source for fermentation to generate ethanol biofuel (Karp and Shield, 2008). Generally, selected *Populus* hybrids are sensitive to drought stress and they need plenty of water to maintain their fast growth. Given that climate change models have predicted increased drought frequencies in regions where *Populus* hybrids are commonly planted, including Canadian prairies, central and northern United States, and north-west China (IPCC, 2013), there is a need to rapidly generate hybrid clones that are better adapted to a drier environment (Rood et al., 2003). Preferred clones may quite possibly have a compromised growth rate, but survive drought when sensitive clones do not, thereby providing better long-term productivity. To gain a better understanding of what regulates drought tolerance in *Populus* species, the aforementioned and unusually drought-tolerant species *P. euphratica* is under particular scrutiny (Tang et al., 2013). This species harbours morphological adaptations such as small and hairy leaves with a thick, waxy cuticle and sunken stomata that reduce transpiration. It also has improved

acclimation responses such as extensive accumulation of osmolytes that facilitate water uptake also from soils with low water potential (Ottow et al., 2005). There is also genetic capacity among the species and their derived hybrids that can be used to improve drought tolerance.

Previously, we have identified 12 poplar candidate genes differentially expressed in drought sensitive and tolerant poplar genotypes (see chapter 2). Subsequently, we tested the function of two poplar genes in *A. thaliana*; putative PP2C; a potential negative regulator, and putative NCED3; a potential positive regulator of drought tolerance. We transformed these two poplar genes into *A. thaliana* first for preliminary assessment because poplar transformation is tedious and it takes long time to get transgenic plants for analysis. Poplar PP2C overexpression caused drought sensitive phenotype in transgenic *A. thaliana* plants (see chapter 3), which is consistent with PP2C being a negative regulator of ABA and drought tolerance. Poplar *NCED3*-overexpressing transgenic *A. thaliana* showed higher level of ABA and an improved drought tolerance (see chapter 4), which is also consistent with NCED3 being a positive regulator of drought and ABA biosynthesis.

The goal of this study is to assess the function of the identified putative NCED3 and PP2C in transgenic poplar plants. For that purpose, we generated transgenic poplars carrying a construct that should result in overexpression of NCED3 and another construct that should result in RNA interference-mediated down-regulation of the endogenous PP2C-like gene. Here we will describe our results to date.

5.2. Materials and Methods

5.2.1. Vector construction, plant transformation and growth conditions

The predicted open reading frame of the G393800 gene containing introns and exons was PCR amplified from DNA extracted from a *P. trichocarpa x P. deltoides* hybrid with number K9, obtained from British Columbia Ministry of Forests Kalamalka research station. The vector was constructed as described in Chapter 4, and *P. alba, clone 717* leaf discs were transferred to plates containing Murashige and Skoog modified basal

medium (Invitrogen, prod # M404) for transformation as described by (Ma et al., 2004) with some modifications, i.e. CIM2 contained 250mg/L and 500mg/L of cefotaxime and carbenicillin, respectively. In addition, for plant selection 100 mg/L kanamycin was used in CIM and SIM media.

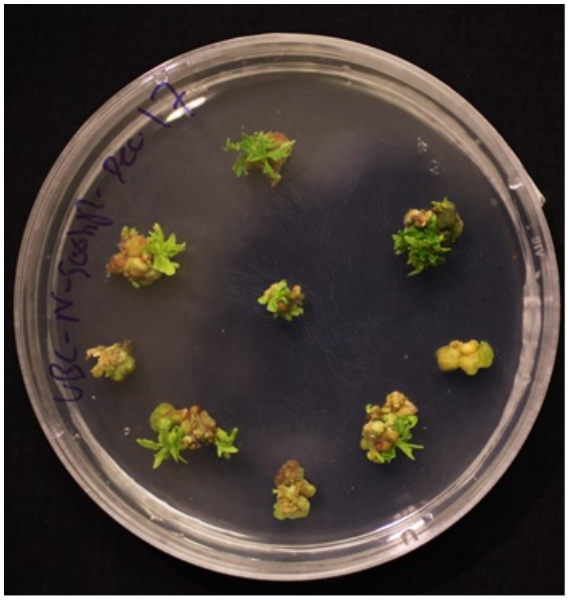


Figure 5.1. Calli with shoots growing on medium with kanamycin and hormones for shoot induction.

Plates were transferred to the growth chamber at 22 ^{0}C, 60-80% humidity with 8 hour light (40-80 µmol m^{-2} s^{-1})/16 hour dark periods. The plates containing callus or shoots were covered with Kimwipes (Kimberly-Clark) to reduce the intensity of light. Transgenic poplars with roots were transferred to plastic pots filled with PRO-MIX soil (Premier Horticulture Inc. Philadelphia, USA) and acclimatized under the lab conditions at first and were subsequently transferred into the greenhouse.

5.2.2. Confirmation of transformation by PCR

To confirm the transgenic plants, we used Phire plant direct PCR kit (Thermo Scientific) and amplified the neomycin phospotransferase NPT III gene using the following primers: 5` - TTATGCCTCTTCCGACCATC – 3` and 5` - ATTCCGACTCGTCCAACATC – 3`. The PCR conditions were set and the gene was amplified according to manufacturer's protocol. The PCR product was analyzed by gel electrophoresis.

5.2.3. Water loss assay

For water loss assay, we cut the leaves from wildtype and transgenic poplar plants and wrapped the leaf petiole in parafilm to avoid water loss from wounded part. The leaves were then placed on the greenhouse bench containing aluminum foil (Fig. 5.4A). Fresh weight of leaves was recorded after every hour for 5.5 hours. The water loss was then calculated by decrease in fresh weight at the indicated time point (Fig. 5.4B).

5.3. Results:

5.3.1. Verification of transgenic poplar

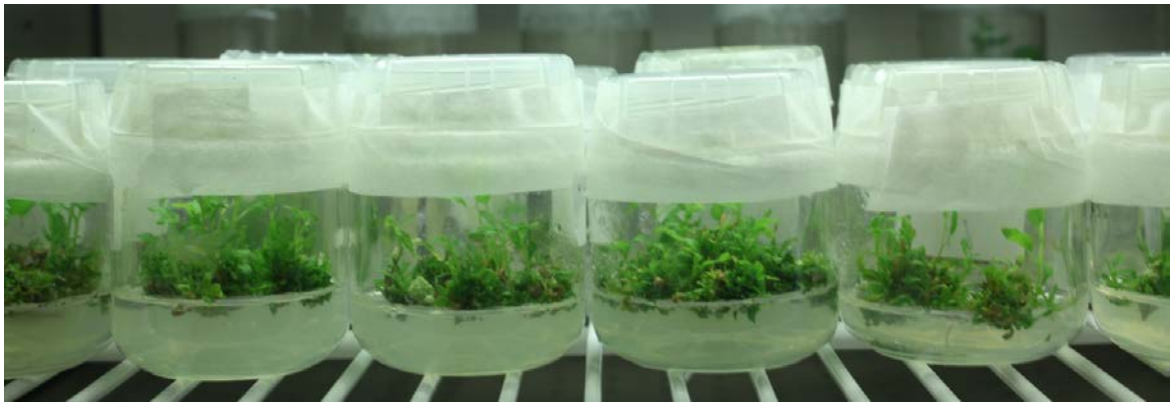
Since the transgenic plants should harbor the neomycin phospotransferase (NPTII) gene providing kanamycin resistance, we tested for the presence of this gene in putative transgenic plants. As predicted, we were unable to detect the presence of this foreign gene in wildtype plants. In putative transformants we were able to PCR amplify a fragment of the NPTII gene from all tested independent lines (Fig. 5.2b). We also used transgenic *A. thaliana* plants transformed with the same vector as positive controls. To date, we have generated transgenic poplar overexpressing G393800 and currently they are growing the greenhouse (Fig. 5.3).

5.3.2. Water loss

To test if overexpression of G393800 reduces water loss in poplar, we performed an assay (see materials and methods) measuring water loss in excised leaves of wildtype

and transgenic poplar plants. The results showed that transgenic poplar plants overexpressing G393800

A.



B.

Figure 5.2. Confirmation of genetic transformation of poplar plants. A; putative transgenic poplar with elongating shoots. B; PCR verification for presence of NPTII gene in putative transformants. The minus sign indicates results from attempted amplification using DNA from an untransformed poplar plant. The plus sign indicates results from amplification using DNA from previously identified kanamycin resistant *A. thaliana* plants. The five lanes in between are PCR products from DNA extracted from five independent putative poplar transformants. The lane to the far right is a DNA ladder, indicating that the expected length of the PCR product was obtained.

poplar gene lost 5% less water than the wildtype, which was statistically significant (Fig. 5.4). This experiment provides evidence that overexpression of G393800 reduces water loss also in poplars.

Figure 5.3. Transgenic poplars in the greenhouse

A

B

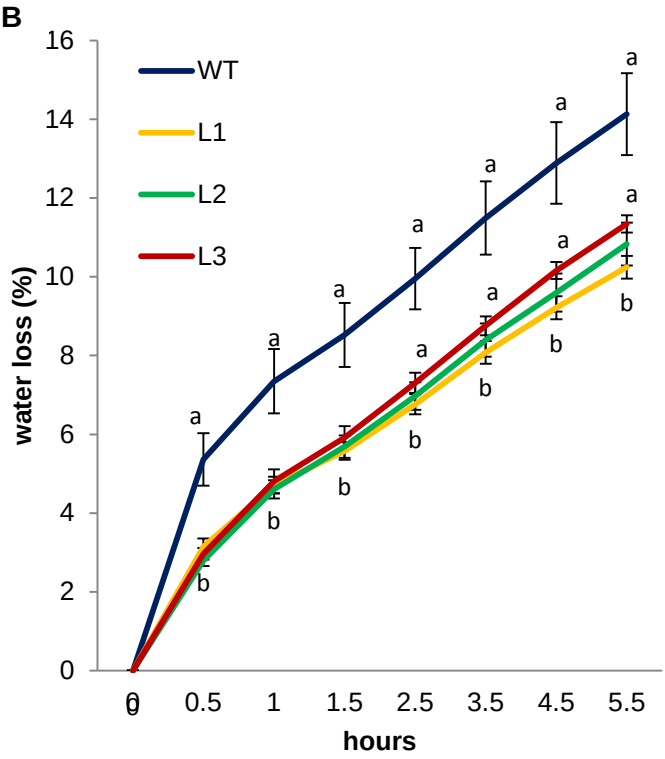


Figure 5.4. Reduced water loss in transgenic poplar lines overexpressing poplar G393800 gene. A, excised poplar leaves on the table with petiole wrapped in parafilm. B, water loss measurements from wildtype and transgenic poplar leaves incubated at the greenhouse bench. Percent values are averages ± SE of single experiments (n=5). Different letters indicate that means are statistically significant from each other at P < 0.01 (Analysis of variance/Tukey HSD).

WT **35S-NCED3**

Figure 5.5. Comparison of wildtype and transgenic poplars in the greenhouse. Three poplar plants on the left are wildtype whereas three poplar plants on the right are transgenic constitutively overexpressing putative poplar NCED3 (G393800).

5.4. Discussion

While transformation of *A. thaliana* is straight forward, transformation of poplar involves much more time and effort. It is, in our experience, not always successful. After initial attempts, we obtained expert advice from the laboratory of Dr. Peter Constabel, University of Victoria that allowed us to obtain calli growing at the margin of *Agrobacterium*-infected leaf discs. We now have some 30 plants growing on soil (Fig. 5.3), some of which have already been confirmed as transformants (Fig. 5.2). Additionally, we performed a water loss assay, which provided evidence that

overexpression of putative poplar NCED3 gene reduces water loss in poplars (Fig. 5.4) suggesting that this gene is a positive regulator of drought tolerance of poplar.

For future work, our intention is to carry out additional genotyping to confirm the presence of T-DNA and the NPTII gene. Once plants have reached about 1 m in height, we will collect stem sections for rooting to generate clones of all independent lines, allowing the use of biological replicates and the sacrifice of plants in severe drought stress experiments. Here we outline the key planned experiments.

We hypothesize that plants overexpressing putative poplar NCED3 will have smaller stomatal aperture compared to wildtype because our results show that transgenic plants lost less water than wildtype (Fig. 5.4). An experiment will be conducted to measure the stomatal aperture of wildtype and transgenic lines.

Similarly, we hypothesize that transgenic plants will have better growth and higher survival rate during drought compared to wildtype. If transgenic plants have similar growth and survival rate as wildtype, the hypothesis is rejected. If, however, transgenic plants are able to maintain growth and survival better than wildtype, the hypothesis is supported. To test this hypothesis following two experiments will be conducted; 1) growth measurements and 2) survival rate.

If the putative poplar NCED3 gene is overexpressed constitutively and strongly, transgenic poplar plants may produce higher levels of ABA, which may result into reduced growth as compared to wildtype under well water conditions. Hence, we hypothesize that under well watered conditions, transgenic line with medium level of NCED3 expression will grow better than the lines with higher levels of NCED3. We expect that transgenic lines with medium levels of NCED3 expressions will show moderate tolerance to drought and normal growth during well watered conditions whereas transgenic lines with higher levels of NCED3 will show higher tolerance to drought but stunted growth in well watered conditions. We, however, did not observe the huge morphological differences between transgenic and wildtype poplars growing under well watered conditions in the greenhouse (Fig. 5.5).

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Chapter 6.

Summary, Significance and Future Perspectives

Here we present a summary of progress to date, and a brief reflection on the significance of the obtained results (see also chapters 2, 3, 4, 5). In addition, we also provide a perspective on how the work may be expanded to have an impact on breeding for drought tolerance in poplar hybrids.

6.1. Summary of the completed work

Typically, an agricultural breeder would first take an inventory of available varieties with respect to a trait of interest, and then go on to use the best variety for further improvement. We took such an inventory by evaluating drought tolerance in nine hybrid poplar clones. Available variation among poplar species and quite possibly among hybrid clones developed outside Canada may be considerably large, but we focused on hybrids with recognized performance in the Canadian prairies. The generated ranking provides useful information to forest tree growers to select the hybrid of choice for field trials and deployment (Fig. 6.1A). The identified differences in drought tolerance are ultimately caused by differences at the gene and gene expression level. Therefore, we compared the expression of 26 genes, marking different processes involved in drought response, in the least and the most tolerant hybrids i.e. Green Giant and Walker (Fig. 6.1B).

There is considerable variation in expression of these genes in cloned replicates, so we focused our attention only on the genes that were determined to be expressed at significantly different levels in the two extreme hybrids. This helped us to narrow down the list to 12 genes (*PP2C, NCED3, CYPA, RF, SBE, Gal, DRFP, DC1, ABF2, LEA, Aqua, CIPK*). This set of genes also includes putative *PP2C*, *NCED3* and *P450* genes,

which may be involved in ABA signaling, biosynthesis and catabolism respectively (Fig. 6.1C), implying that differences in ABA-mediated signaling may be part of the explanation why Walker is more drought tolerant than Green Giant. In addition, the identified genes are the potential orthologs of *A. thaliana* genes believed to be involved in drought responses and ABA signaling, which provides additional evidence that these gene may serve as suitable target for drought improvement in poplars.

We went on to functionally characterize two genes with putative functions in ABA biosynthesis (NCED3) and signaling (PP2C), by cloning genomic fragments containing their open reading frames into binary plasmids providing constitutive expression and transforming them into *A. thaliana*, and later in poplar. We obtained multiple lines of evidence that the putative PP2C-encoding gene acts as a negative regulator whereas the putative NCED3-encoding gene acts as a positive regulator of drought tolerance (Fig. 6.1D). The implication of these findings is that overexpression of NCED3 and reduced expression of PP2C may contribute to the development of drought tolerant poplars (Fig. 6.1E; work in progress). There is evidence from other similar studies that constitutive overexpression of components in ABA signaling may reduce growth. Therefore, generated transgenic poplars should be evaluated for growth performance along with their ability to withstand drought. If promising results are obtained, transgenic poplar plants may be gauged for field performance in collaboration with potential commercial partners (Fig. 6.1F).

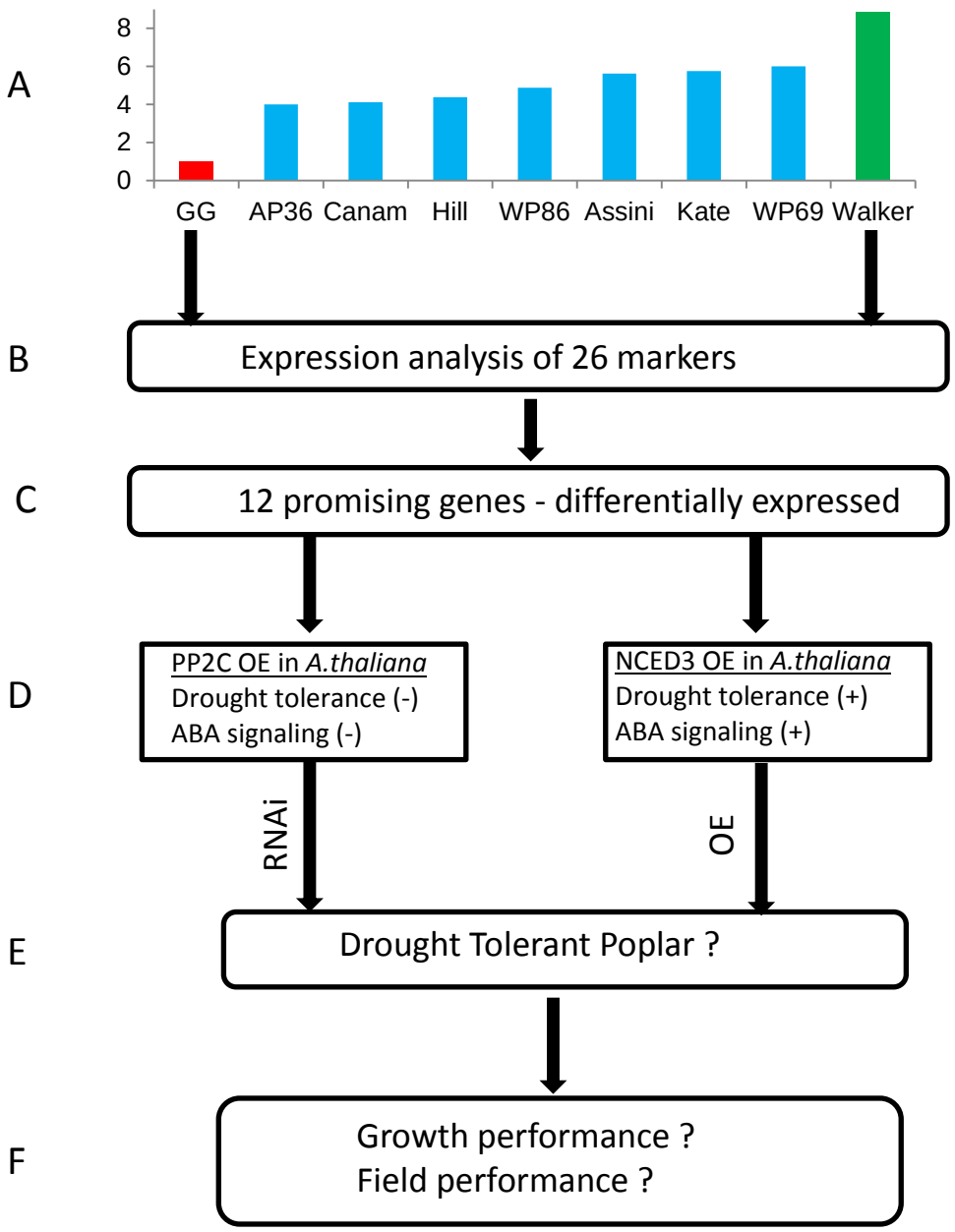

Figure 6.1. A flow chart of the completed work; A, ranking of nine poplar hybrids grown on the Canadian prairies with respect to relative drought tolerance. A higher value indicates more tolerance. The color (green) is for the most tolerant and red for the least tolerant hybrid, whereas blue colour indicates moderate drought tolerant hybrids, based on the different physiological traits; B, candidate drought responsive poplar genes used in this study; C, genes with differential and significant expression between two extreme hybrids during mild and severe stress; D, functional characterization of two poplar genes by transformation into *A. thaliana*; E, proposed alteration of expression to increased drought tolerance in transgenic *Populus*. OE refers to over expression, RNAi refers to overexpression of sense and antisense RNA transcripts of target genes, resulting in RNA interference with the translation of the endogenous gene's transcript; F, suggested work to test the performance of poplars with altered gene expression.

6.2. Significance of the obtained results

These Canadian hybrids have been cultivated on the prairies for some time but they have not been systematically compared with respect to their ability to tolerate drought. We provided a ranking of nine Canadian commercially important hybrids based on the performance of different various physiological traits during drought stress. As mentioned before, the generated ranking may therefore be used by tree growers to select the hybrid of choice for afforestation purposes. This information may become even more important as predicted climate change-induced drought episodes affect the Canadian prairies.

Although, microarray studies have shown that the 26 genes, used in this study are drought responsive, their actual involvement in drought tolerance is unknown. In addition, most of the 26 genes are members of gene families in which individual genes may have functions ranging from similar and overlapping, to vastly different. This is the case also for the PP2C and NCED3-like genes that we chose to focus on for functional analyses. The obtained results demonstrate that the two genes have the capacity to strongly influence the response to drought and osmotic stress. To my knowledge, this is the first assessment of any ABA-related genes in poplar. The results therefore provide valuable insights into ABA synthesis and signaling in *Populus*, a genus of commercial and ecological importance across the northern hemisphere. The results also indicate that mapping of *A. thaliana* gene functions in ABA synthesis and signaling onto the poplar genome followed by functional assessments is a valid, time and cost effective way of gaining insights into these processes. Our findings can also be used to expedite the selection of tolerant poplars using both conventional and unconventional methods (see below).

6.3. Future perspectives

Although there are presently no plans to expand this work beyond the analysis of obtained transgenic poplar plants, formulation of strategies to apply obtained results towards breeding for drought tolerance in poplar may facilitate discussions with potential end users regarding research collaborations. Here we have formulated such strategies

based on both conventional and unconventional methods, providing alternatives for consideration by end users.

6.3.1. Conventional approach to drought tolerance

To date, poplar breeding has been done primarily by hybridization of so-called plus trees based on size and also traits such as disease tolerance, followed by screening for offspring with desired traits. The most promising individuals are then vegetatively propagated via stem cuttings, and clones compared in field trials to make statistically valid conclusions about which of the individuals have the best traits. This is a resource intensive procedure in terms of space and the time it takes to identify the few final individuals. Here we will provide a similar scenario based on our results. First, the generated ranking can be used to choose suitable parents based on drought tolerance. Walker, identified as the most drought tolerant clone, is a female. It can therefore be crossed with WP69, the clone with the second highest ranking, as it is a male (Fig. 6.2). Similarly, Walker can also be crossed with any other male poplar species carrying a trait of interest but characterized by low drought tolerance. The offspring population can then be screened for the plants that show relatively low expression of PP2C and high expression of NCED3 genes in response to non-fatal drought stress. This pre-selection can reduce both the time and space that it typically takes to obtain a limited number of individuals for futher testing. The idea is that the combination of genomes from two relatively drought tolerant clones can be combined to enhance the frequency of desired gene variants or alleles that contribute to drought tolerance. Selection based on the expression of putative NCED3 and PP2C genes may allow early identification of a smaller population having expression profiles that are equal to or even better than the original parents.

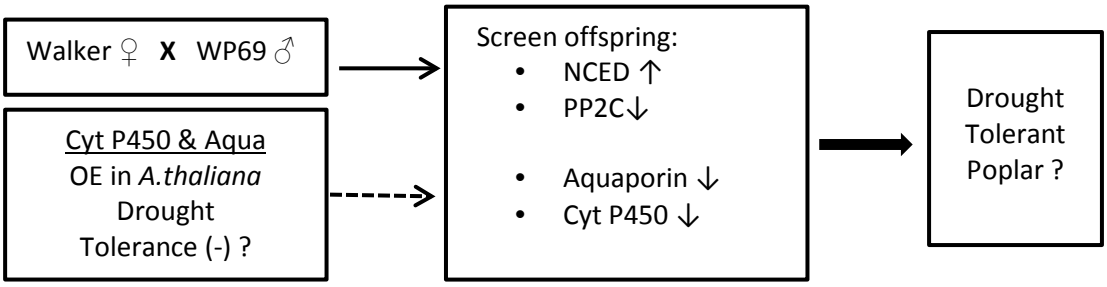

Figure 6.2. Drought tolerance breeding in poplar. Figure shows how expressions of candidate genes can help in breeding programs to improve drought tolerance. A solid arrow indicates a pathway supported by our results in transgenic *A. thaliana* whereas a dotted arrow shows a proposed pathway for drought tolerance in poplar.

Furthermore, we hypothesize based on expression profiles and predicted gene functions that the identified poplar aquaporin-like and cytochrome P450-like genes may act as negative regulators of drought tolerance. To test this hypothesis, these poplar genes can first be transformed and overexpressed in *A. thaliana*. If overexpression of these genes makes plants more sensitive to drought, these genes could also be included in poplar breeding programs as potential negative regulators of drought tolerance just like the PP2C-like gene (Fig. 6.2). After crossing Walker and WP69, the offspring populations can be screened for lower expression of aquaporin and cytochrome P450 along with PP2C and NCED3 genes. We hypothesize that the poplar plants with lower expression of PP2C, aquaporin, Cytochrome P450 but higher expression of NCED3 may have improved drought tolerance (Fig. 6.2).

6.3.2. Unconventional approach to drought tolerance

Based on the results from overexpression of the putative PP2C and putative NCED3 poplar genes in *A. thaliana*, it may be possible to obtain improved drought tolerance in

poplar by altering the expression of these genes. To alter the expression of candidate genes, either transgenic or non-transgenic approaches can be used. For a transgenic approach, the expression of poplar PP2C and NCED3 can be manipulated, for example, reducing the expression of PP2C and overexpressing NCED3 may improve tolerance in poplars (Fig. 6.3A).

As discussed before (chapter 4), there is a distinct possibility that constitutive ABA synthesis or signaling may affect growth negatively. For example, constitutive overexpression of *A. thaliana* DREB1A resulted in drought tolerance, however, it also reduced overall growth rendering plants poorly suited for production (Kasuga et al., 1999). To address this problem, we have generated a construct that may provide Enhanced Endogenous Expression (EEE) instead of constitutive overexpression of the gene (Fig. 6.3A). EEE occurs when multiple CaMV 35S enhancers are used to increase the expression of the gene from a nearby promoter. Multiple CaMV 35S enhancers act in a different way than complete CaMV 35S promoters and cause enhancement of endogenous gene expression instead of constitutive expression (Weigel et al., 2000; Zheng et al., 2007). Thus, there is a possibility that EEE may be a suitable approach to enhance tolerance without growth retardation, usually caused by constitutive overexpression. We have generated transgenic poplars harbouring a tandem repeat of four 35S enhancers in front of the identified poplar NCED3-like gene to test the validity of the EEE approach, but generated plants have not yet been evaluated.

While genetically modified plants can be most useful in agriculture, the presence of foreign genes is an ethical concern for many consumers and non-governmental organizations. These plants also have to be extensively evaluated for any side effects in terms of ecological risks - procedures that can prove prohibitively expensive compared to varieties produced by conventional methods.

The year 2013 saw the emergence of a method that allows targeted modification of genes in both animal and plant genomes – the CRISPR/Cas system – without the introduction of foreign genes (Feng et al., 2013; Jiang et al., 2013; Mao et al., 2013; Miao et al., 2013; Nekrasov et al., 2013; Shan et al., 2013; Xie and Yang, 2013). While other methods exist for modification of genes of interest, they are either inefficient in plants (homologous recombination) or extremely resource intensive (TILLING) or require

extensive cloning (ZFN and TALENs) preventing wider adoption within the breeding community (Belhaj et al., 2013).

The CRISPR/Cas system, on the other hand, requires only transient expression of two genes to trigger high frequency modification of a targeted locus (up to 90%) of the genome of targeted cells (Feng et al., 2013; Jiang et al., 2013; Mao et al., 2013; Miao et al., 2013; Nekrasov et al., 2013; Shan et al., 2013; Xie and Yang, 2013). One gene encodes a bacterial endonuclease that cuts chromosomal DNA, and the other gene encodes an RNA that (i) interacts with the nuclease, and (ii) has a gene-specific sequence that guides the nuclease to a specific gene. The resulting gene-specific cut triggers either of two endogenous repair mechanisms, both of which result in small deletions, and a loss of the targeted gene's function. Stable transgenic and non-chimeric *A. thaliana* and rice plants have been generated with this method (Belhaj et al., 2013) with more to come. Bi-allelic mutant cells, i.e. having both copies of targeted genes altered, have also been recovered, which is particularly important for a dioecious tree with long generation time like poplar, as it excludes the need to wait for reproductive age to generate homozygous mutant individuals. The absence of transgenes in the organism also limits the need to pay royalties to inventors. The method is currently limited to breeding by loss of gene function.

In this context, we have obtained the evidence that the PP2C-like poplar gene act as a negative regulator of drought responses and tolerance. CRISPR-mediated inactivation of this gene may therefore result in improved drought tolerance (Fig. 6.3B). Based on putative protein function and expression profiles, it is also possible that the putative cytochrome P450 and the aquaporin-encoding genes may also be negative regulators of drought tolerance and may therefore be suitable targets for CRISPR/Cas9 in activation (Fig. 6.3B).

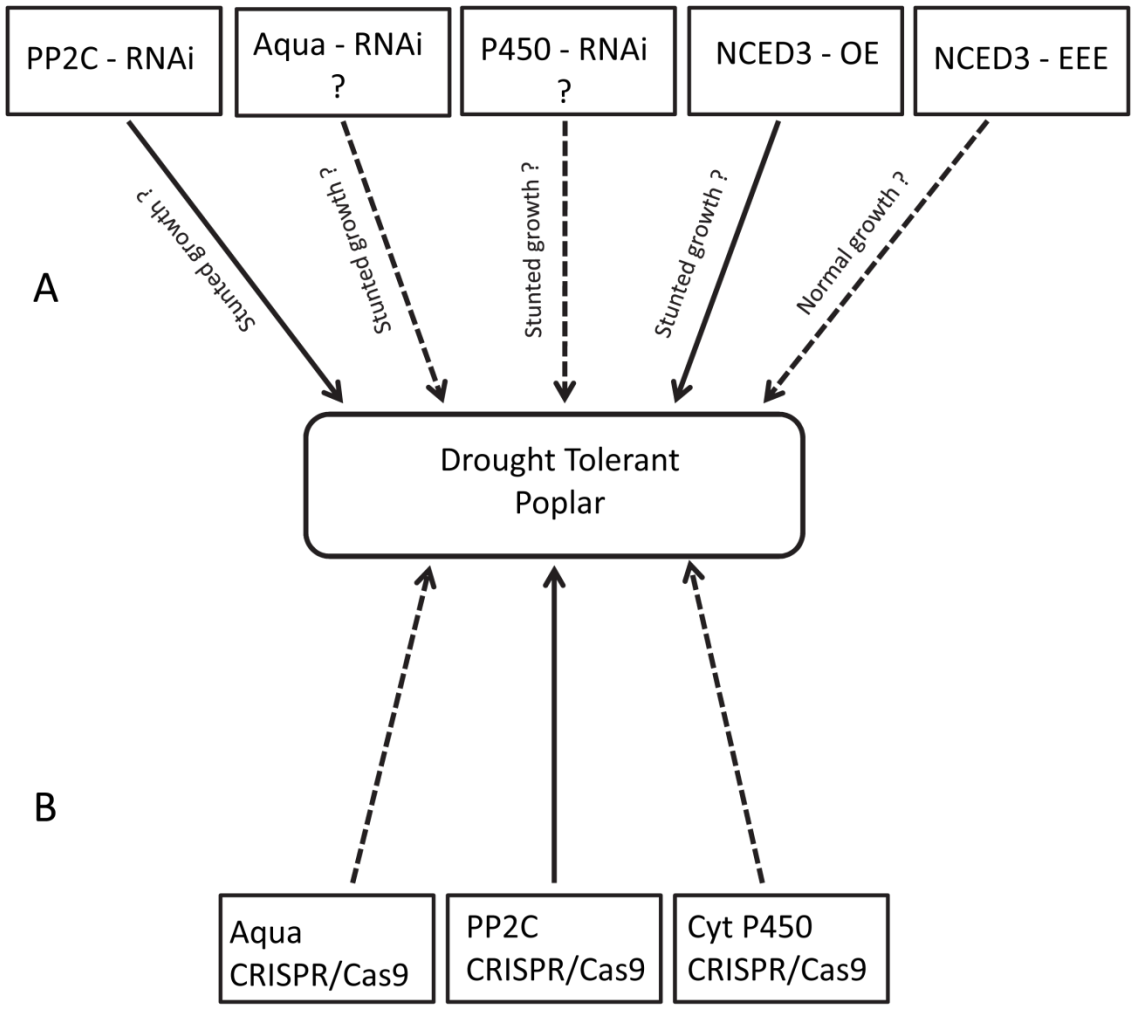

Figure 6.3. Unconventional and novel approaches to improve drought tolerance in poplars. A, transgenic approach to overexpress NCED3-like and reduce the expression of PP2C-like poplar genes. These constitutively overexpressing genes may cause growth retardation, which may be avoided by adopting the EEE approach. B, non-transgenic, the latest and innovative technology. CRISPR/Cas9 inactivates the negative regulator of drought tolerance. A solid arrow indicates a pathway supported by our results in transgenic *A. thaliana* whereas a dotted arrow shows a proposed pathway for drought tolerance in poplar.

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