Over-expression of *PIP2;5* aquaporin in a *Populus tremula*×*P.alba*clone and its effects on plant responses to low root temperature and osmotic stress

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Forest Biology and Management

Department of Renewable Resources

University of Alberta

ABSTRACT

This dissertation describes the studies that examined: 1. Production of transgenic poplar over-expressing the poplar aquaporin *PIP2;5* and 2. Comparison of the physiological properties of atransgenic poplar with the wild-type under different stress conditions including a) short-term low root temperature (LRT), b) prolonged LRT and c) polyethyleneglycol (PEG 6000)induced osmotic stress. Aquaporin *PIP2;5*was selected due to its water-transporting capacity and increased expression under water limitation. The coding sequence of *PtdPIP2;5* was cloned in the expression vector pCambia1305.2, under the control of maize ubiquitin promoter and the construct was introduced into *in vitro* grown *Populus tremula* × *P.alba* INRA Clone 717-1B4 by *Agrobacterium*-mediated transformation. Rooted plantlets of transformed poplar were transferred to the growth chamber and transformed lines were confirmed by PCR using primers against the hygromycin resistance gene and quantitative RT-PCR. Two lines exhibiting the highest expression of *PIP2;5* were selected and *ex vitro* plants were generated for experimentation. When the root temperature (RT) was lowered from 20°C to 5°C in solution culture, for 30 minutes, *PtdPIP2;5*-over-expressing plants had significantly higher net gas exchange rates and root hydraulic conductivities (L_p) compared with the wild-type plants. Recovery was rapid in transgenic lines when the RT was raised back to 20°C. In the transgenic lines, the transcript abundance of PIP2;5 was significantly higher at 20°C and remained highly significant at 5°C for 30 minutes, when compared with the wild-type plants. When the plants were exposed to LRT for 3 weeks in solution culture, transgenic lines showed improved gas exchanging properties and L_p values than the wild-type plants. The transcript abundance of PIP2;5 significantly increased in both transgenic lines at 20°C and PIP2;1 levels increased in one transgenic line, after 3 weeks at 5°C. Transgenic lines had significantly higher growth rates,

leaf size, gas exchange parameters and $L_p$than the wild-type poplars when subjected to PEG 6000 induced osmotic stress, at 50 g L^{-1}. The results suggest that transgenic linesmay be more resistant to LRT and osmotic stressesthan the wild-type plants. This study confirms the contribution of *PIP2;5*on plant water transport, gas exchange and growth.

Dedicated to

My Loving Parents, Wonderful Wife & Charming Daughters

ACKNOWLEDGEMENTS

I am greatly indebted to my research supervisor Professor.Janusz Zwiazek, first of all for giving me the opportunity to come to the University of Alberta, Canada, as a graduate student and secondly for his invaluable guidance, consistent encouragement, patience, understanding and kind hearted support throughout the course of this study. Thank you very much for what you have done for me, Sir. I am very grateful to my co-supervisor Professor.Janice Cooke, for all her excellent guidance, constructive criticism, support and encouragement to mould me throughout the project. I would like to thank specially to the research associate Dr.Walid El Kayal, for his excellent teaching of Molecular biological techniques and immense help in the production of the transgenic plants. I am thankful to my research committee member, Professor.Tarriq Siddiq who gave me advice and suggestions on my research and exams. I acknowledge the financial support provided by the National Sciences and Engineering Research Council to this project.

I would like to express my gratitude to a lot of present and past colleagues and post-doctoral fellows of Dr. Zwiazek and Dr. Cooke labs of the University of Alberta for their assistance and support for the various aspects of this project. My thanks to Adriana Almeida, Ale Equiza, Adriana Arango, Seonghee Lee, Navarro Alfonso, Calvo Polanco Monica, Eri Adams, Miranda Meents, Dominik Royko, Matt Bryman, Charles Copeland, Kimberly Lam, Jeremy Morris, Katherine Spencer, Tomas Major,Wenqing Zhang, Xu Feng, Hao Xu, Frances Leishman, Kelsey Ayton, Nnenna Onwuchekwa, Nathan Lauer, Evren Hisarli, Tan Xiangfeng, Mudeyu, Samantha Olivier and Shanjida Khan. I also extend my thanks to Amanda Brown, Mike Abley, Christie Nohos and Tammy Frunchak of Department of Renewable Resources and Troy Locke in the MBSU Facility ofthe Department of Biological Sciences for their timely help. I am very thankful to Chen Ding Klaus and Ahmed Najar of Renewable Resources, University of Alberta and Nagulan of NUS for their immense help. Specially, I would like to thank my friends, Nakeeran, Luxman, Aingaran, Prem, Jegatheesh and Pirasath Kirupa who frequently visited me with their families and gave me homely feeling in a foreign country.

I thank my loving parents, Ranganathan and Santhadevi and my loving brother Varathan, my parents in low Thangasivam and Vallinayaki, for their moral support, encouragement and

prayers that led to the successful completion of my degree. Most of all, I am forever indebted to the patience, support and sacrifice endured by my wonderful wife Suvarna and loving daughters Aarani and Biranavi throughout these long five years and four months of my race. My very sincere thanks from the bottom of the heart to everyone without whose help, successful completion of this journey would hardly have been possible.

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List of Abbreviations

ABA Abscisic acid

AQP Aquaporin

CaMV Cauliflower mosaic virus

CIM Callus induction medium

CT Cycle threshold

E Net transpiration rate

EDTA Ethylenediaminetetraacetic acid

EtOH Ethanol/Ethyl alcohol

gs Stomatal conductance

IBA Indole-3-butyric acid

2ip 6-(Y,Y-dimethylallyl-amino) purine

L_p Root hydraulic conductivity

LRT Low root temperature

NAA Alpha-naphthalene acetic acid

P_n Net Photosynthesis

PIP Plasma intrinsic protein

qRT-PCR quantitative reverse transcription polymerase chain reaction

PCR polymerase chain reaction

rpm revolutions per minute

RT room temperature

ROS Reactive oxygen species

SEM Shoot elongation medium

SIM Shoot induction medium

$T_{1/2}$ Halftime of water exchange

TDZ Thidiazuron (1 -phenyl-3-(1,2,3-thiadiazol-5-YL)-urea)

WT Wild-type

WUE Water use efficiency

Ψ Water potential

CHAPTER 1

1. Introduction and literature review

1.1 Poplar

Poplars are fast-growing, deciduous trees that grow in a variety of environmental conditions and are found commonly in the temperate climate of the northern hemisphere (Richardson et al. 2007). Genus *Populus*is categorised under the family Salicaceae, consists of 25–35 species of flowering plants including poplars,aspens andcottonwoods(Eckenwalder, 1996).

1.2 Biology and ecology of poplars

Poplars are dioecious trees that flower before leaf emergence in spring. The flowers are catkins and the number and density vary among species (Meikle, 1984). The leaves are alternate, stipulate, petiolate, simple and have glandular teeth along the margin. Thecapsule type fruitcontains numerous tiny brownseeds,surrounded by long, white spongy hairs, which is adapted for wind dispersal. Poplars have both preformed and neoformed leaves. Preformed leaves initiate at the end of one growing season and expand at the beginning of the next growing season, whereas neoformed leaves initiate and expand in one growing season. Species of *Populus* are widely distributed throughout the northern temperate regions with cooler temperatures including North America, Eurasia and northern Africa and some species are found in tropical regions of Africa and Asia but poplars are mostly wetland or ripariantrees (Meikle, 1984; Eckenwalder, 1996; Bradshaw et al. 2000). Aspen shows a broad east-west distribution from Atlantic coast to Pasific coast in the northern hemisphere (Richardson et al. 2007).

1.3 Economic importance of Poplars

Poplars are grown for diverse purposes such as wood products, stream bank protection, windbreaks and shelterbelts and biofuel production. In some scenarios, poplars have been considered a carbon sink to offset climate change. The trees are used for the pulp and paper industry, reforestation and phytoremediation of contaminated soils (Yadav et al. 2009;Huang and Dai, 2011).With the reduction of forested areas worldwide and an increasing human population, poplars are importantfor the wood products and to maintain theatmospheric carbon dioxide levelin nature (Stettler et al. 1996).

1.4 Poplar as a model treefor research

The genus *Populus*has advantages to be used as amodel for forest tree molecular biology. The advantages includea sequenced genome, high growth rate, short rotation age, ability to resprout and ease of vegetative propagation and wide range of economic importance (Bradshaw et al. 2000). The conserved chromosome number across the genus*Populus*(2n = 38), small sized genome (~$5x10^8$ bp), great variation in morphology, anatomy and physiology andhybrids attain sexual maturity early (Stettler et al. 1996) are more added advantages in *Populus*. Poplar was the first forest tree species to be genetically transformed (Fillatti et al.1987). The western balsam poplar (*Populus trichocarpa*) was the first tree to have its fullDNAcodesequenced (Tuskan et al. 2006) and this accelerated research on molecular biology. Fifty five full-length aquaporins have been analyzed fromthe *Populustrichocarpa*genome sequence data (Gupta and Sankararamakrishnan, 2009). Poplar is also chosen for research because of its prominent role in the Canadian landscape and in the Canadian forest products industry(Stettler et al. 1996).

1.5 Genetic modification in poplar

Conventional breeding of trees is a slow process and laborious due to long life cycles. There are a lot of complications in the poplar research area includinglong juvenile period(Jauhar, 2001). Genetic modification/ Genetic engineering isreferred to as a process of inserting new genetic material into existing cells in order to modify an organismfor changing its characteristics (Vert et al. 2012).In transformation, the inserted gene confers specific traits into selected genotypes without affecting their desirable genetic source (Pena and Seguin, 2001). Application of transformationin forest trees includes improving wood quality, enhancing resistance to pests and pathogens, developing resistance to herbicides, improving the productivity and developing stress resistance of the plants concerned (Vert et al. 2012).

1.5.1 Poplar transformation

In transformation, DNA is inserted into plant cells and it will incorporate into chromosomes and express in cells that can then be made to regenerate plants. Numerous transformation methods are practised for the introduction of exogenous genes into plant genomes and they are catogorized into two main groups.

1. Direct gene transfer - Physical and chemical methods involved in DNA introduction.

   Eg: electroporation, biolistics

Electroporation is a multi step process where electrical discharges are used to create reversible pores in the plasma membrane. The pores will function as conductive pathways for the movement of the foreign DNA into the plant cell (Chupeau et al.1994; Christou, 1995).

Gene gun / biolistic / microparticle bombardmentis a device that injects cells with genetic information into the target cells or tissues or whole plant. The foreign DNA containing the genes to be transferred is coated onto the surface of gold or tungsten particles and bombarded onto the target tissue or cells using a gene gun /microprojectile gun. Primary explants and the proliferating embryonic tissues are the two types of plant tissue commonly used in this technique (Neuhausand Spangenberg, 1990; McCown et al.1991).

2. Indirect gene transfer – A biological vector is used to introduce the exogenous DNA.

Eg: *Agrobacterium* mediated transformation

Among the techniques available to produce transgenic poplars, the*Agrobacterium* mediated transformationmethod is the one widely used for its higher efficiency (Yevtushenko and Santosh, 2010). A variety of explants are used in *in vitro* regeneration of poplar by thismethod and the explants used are leaves (Horsch et al. 1985; Cseke et al. 2007), petioles(Thakur et al. 2005), internodes (Ferreira et al. 2009, Yadav et al. 2009), stems(Gozukirmizi 1998), roots (Yadav et al. 2009)and shoot tips(Kang et al. 2009).Factors affecting transformation efficiency of poplar arethe genotype of poplar, type of *Agrobacterium tumefaciens* strain, concentration of bacteria,*in vitro* regeneration procedure used (Meilan and Ma, 2006) and the type of explants used (Han et al. 2000 and Ma et al. 2004). Different transformation methods have their own merits and demerits. In*Agrobacterium* mediated transformation, only thefragment of the transformation vector within the T-DNA borders of the Ti plasmid is transferred to the host cell. Only one or few copies of this fragment are integrated into the host genome. In direct DNA transfer, the whole transformation vector is transferred into the host cell and several copies of the transferred DNA will be integrated into the host genome (Charest et al.1997). In hybrid poplar*Populus davidiana*Dode×*Populus bollena* Lauche, transformation frequency was improved by

controlling the leaf age and by optimizing the initial concentrationof *Agrobacterium*($OD_{600=}$ 0.8–1.0) and using the optimized infection time (20–30 min) (Han et al. 2013). Nowadays for the selection of the transformed cell, hygromycinhas been widely used as a selectable marker. Hygromysin inhibits cytosolic proteins, therefore the chances of non tranformants getting killed are comparatively high. This might lead to relatively low number of escapes when hygromycin was used as a selectable agent (Cabanas et al. 1978; Cseke et al. 2007; Yang et al. 2013).When kanamycin is used as a selectable agent,chances of the non transformants not getting killed (escaping)are high because kanamycin inhibits protein synthesis of the plastid only. Therefore the cells that have no or less plastids might survive when kanamycin is used in the media. Sometimes it might take long time to show the complete effect (McGarvey et al. 2012).Unexpected changes in the expression levels of a foreign gene can occur after the transformation of the forest trees. Loss of a foreign gene, loss of expression of the foreign gene and different rearrangements caused by a foreign gene in the host genome are some reasons that may cause variability in the genome of the transgenic plants,after transformation (Ahuja and Fladung, 1996).

1.6 Water uptake and movement in plants

1.6.1 Driving forces and pathways in water uptake and transport

Water is important to plants for their routine metabolic functions, growth, solute transport, photosynthesis, seed germination, dispersal of fruits and seeds and for cooling of leaves.Fast growing trees such as poplarsare highly water demanding (Lambs et al. 2006). Soil-plant-atmosphere-continuum (SPAC) is the principle pathway for the movement of water fromsoil solution throughplantsto theatmosphere. Water moves passively according to the water potential

gradient (Knipfer and Fricke, 2011). When stomata are closed and there will beless or no transpiration, then ion uptake from soil will generate a force called root pressure that pulls the water from soil solution (Javot and Maurel, 2002). Therefore the main forces that regulate water uptake into theplant root are osmotic force generated by root pressure and hydrostatic forcegenerated by the transpiration pull (Steudle and Peterson,1998). The root will act as an organ for absorption of water from soil solution. When water is lost from the stomata and the aerial parts of the plant by evapo-transpiration, then there will be a negative pressure in the tracheary elements that draw water from the roots towards the aerial parts. The force for absorption of water is created at the leaf is the transpiration pull (Javot and Maurel, 2002).Transpiration pull is transmitted down to the tip of the root through the water column in the xylem. As transpiration proceeds, water absorption will also take placesimultaneously to compensate the water loss from the leaf. Most volume of water entering into the plants is by means of passive absorption, that is, no input of energy is needed for the water movement. Water will move freely from higher concentration to lower concentration.Sometimes absorption of water occurs with the help of energy that is released from the respiration of roots. Absorption takes place against a concentration gradient, even when the water potential of the cell sap is higher than that of soil solution.Once water is absorbed into the roots then water transport occurs through radial and axial transport. The axial transport consists of the water moving along the xylem vessels to aerial parts of the tall tree and it provides resistance of water transport through the whole plant because of the length of the tall tree (Doussan et al. 1998; Knipfer and Fricke, 2011). The axial transport faces the problem of resistance in the form of cavitation, in woody plants (Dalla-Salda et al. 2009). Radial water flow from the soil solution to the root xylem has comparatively higher resistance to water movement through roots mainly in the form of

casparian strips present in the endodermis and the cellular organization (Steudle and Peterson,1998).There are three pathways involved in the radial water movement in plants.

1.6.1.1 *Apoplastic Pathway*

The apoplastic pathwayrefers to the movement of water through the pores of the cell wall and through the intercellular spaces (Knipfer and Fricke, 2010). Hydrostatic and osmotic forces drive the movement of water and they are generated by the water potential differences and gradient between the soil, plant and atmosphere (Aharon et al. 2003). Casparian strips are water proof suberinised layers found on the cell wall of endodermis that control the water movement in the apoplastic pathway by inhibiting water advancement (Steudle and Peterson, 1998).

1.6.1.2 *Symplastic Pathway*

The symplastic pathwaycorresponds towater movement between cells through the cytoplasm and plasmodesmata (Steudle and Peterson, 1998). Plasmodesmata areextension of cytoplasm that interconnect the protoplasts of neighbouring plant cells through channels that traverse the cell wall space (Raven et al. 2005). Water movement in the symplastic pathway is controlled by the changes in the activity of the plasma membrane water channel proteins (Steudle and Peterson, 1998).

1.6.1.3 *Transmembrane (Transcellular)Pathway*

Transmembrane pathway refers to the movement of water across the plasma membrane and the tonoplast (Steudle and Peterson, 1998, Johansson et al. 2000). This path has higher resistance to the flow of water as the water has to move through the lipid bilayer membranes (Martre et al. 2002) and the movement of water is efficiently controlled by aquaporins, water channel proteins (Aharon et al. 2003).

The sum of symplastic and the trans-membrane pathways are considered as the cell-to-cell pathway(Steudle and Peterson, 1998). When plants show higher transpiration, the main route for radial water transport is the apoplastic pathway and when the transpiration is very low, the main water transporting route is the cell-to-cell pathway (Steudle and Peterson, 1998; Javot and Maurel, 2002). Water potential is the measure of the relative tendency ofwaterto move from one place to another and is represented by the Greek letter Ψ.If water is diluted with a solute, the water potential will become more negative (Mohr and Schoper, 1995). Water will always move from a region of higherwater potentialto the region of lower water potential, or down the concentration gradient. If solutes start to build-up across a biological membrane, water will move to the area where the solute concentration is high(Mohr and Schoper, 1995). Water potential (Ψ) is the sum of osmotic potential, turgor potential and gravitational potential (Taiz and Zeiger, 2010). During water absorption, root cell contents will be pressed to create a positive pressure in the cell because of the entry of water into the cell and the cell wall becomes more stretched (Boyer, 1995). The pressure build-up inside the cell is called turgour pressure. The osmotic gradient formed in the cell is called osmotic pressure. Higher plants adjust the turgour pressure, by exchanging solutes with other plant organs and by adjusting the cell wall elasticity(Tomos and Leigh,1999). Water can flow through the plant tissues by three different pathways, apoplastic, symplastic and transmembrane pathways. These path ways are driven by osmotic and turgour pressures (Steudle and Peterson, 1998).

1.7 Regulation of root water uptake and conductance under abiotic stress

1.7.1 Effect of low temperature stress

Chilling stress refers to temperatures between 0°C and 15°C and temperatures below 0°C are known as freezing stress. Leaf dehydration takes place by greater loss of water by leaf transpiration than water uptake by roots (Arocaet al. 2001;Vernieriet al.2001). The transpiration rate decreases under low temperatures due to reduced vapour pressure difference (VPD) between the leaf surface and the atmosphere (Arocaet al. 2003). When the temperature declines, the root water uptake will also decrease, because of the decrease in the VPD (Arocaet al. 2003) and the increase in the viscosity of water (Bloomet al. 2004). However, water viscosity increases cannot fully explain the decreased water uptake rate by roots (Wanet al. 2001;Bloomet al. 2004) and increase in the water flow by the apoplastic path (Leeet al.2008). When the roots were exposed to low temperature stress, hydraulic conductivity decreased faster than stomatal conductance (Wanet al. 2001). The reduction of hydraulic conductivity under low temperature is linked to the metabolism of the plant that lead to the inhibition of enzyme activity and aquaporin function (Wanet al. 2001;Murai-Hatanoet al. 2008).Low root temperature can happen even if there are relatively high air temperatures, with high transpirational demand and this can lead to water relations imbalance. Therefore, low temperature tolerance is correlated with drought resistance (Aroca et al. 2005). Reduced water flux at low temperatures occurs due to higher water viscosity (Muhsin and Zwiazek, 2002), inhibition of transmembrane water transport (Lee et al. 2005; Wan et al. 2001), reduced enzyme activities (Johansson et al. 1998) and reduced root respiration/metabolism (Kamaluddin and Zwiazek, 2001).In rice roots, when low temperature treatment was given, hydraulic conductivity started to decline in the first 5h before the amount of PIP (Plasma membrane intrinsic protein) and TIP (Tonoplast intrinsic protein) proteins started to change (Murai-Hatanoet al.2008). Aquaporins might be closed due to acidification of the cytosol during low temperature treatment and it will affect the plant water transport (Kawamura, 2008). Most putative mechanisms developed by plants to tolerate the low root temperature

stress, are schematically shown (Figure 1.1). The exact mechanism that suggestshow aquaporinregulates water flux through protein conformational change is affected by low temperature is not well understood (MuraiHatanoet al, 2008; Lee et al. 2012).

1.7.2 Effect of water deficit stress

Plants face water deficit (drought stress) when the water potential gradient between the plant and the atmosphere is affected by internal or environmental factors. When the soil water content is below the level in which the plants can absorb enough water to maintain their regular growth and metabolic activities then it will lead to water deficit stress. Consequently, transpiration and root water uptake of a plant starts to decrease (Chen et al. 2010).Plants detect waterdeficitconditions, which triggers molecular signaling that result in physiological changes such as change in growth, gas exchangereduction, osmoregulation and adjustments of water use efficiency (Bogeat-Triboulot et al.2007). When the drought event is exacerbated or is experienced for very long periods, embolisms in the xylemcould lead to plant death(Breda et al.2006).Plants that have a deeper root system can be more resistant to water stress than plants with a shallower root system (Alsina et al. 2011). When *Populus tremuloides*trees were subjected to mild drought stress, they showed an up-regulation of hydraulic conductivity(Siemens and Zwiazek, 2004).

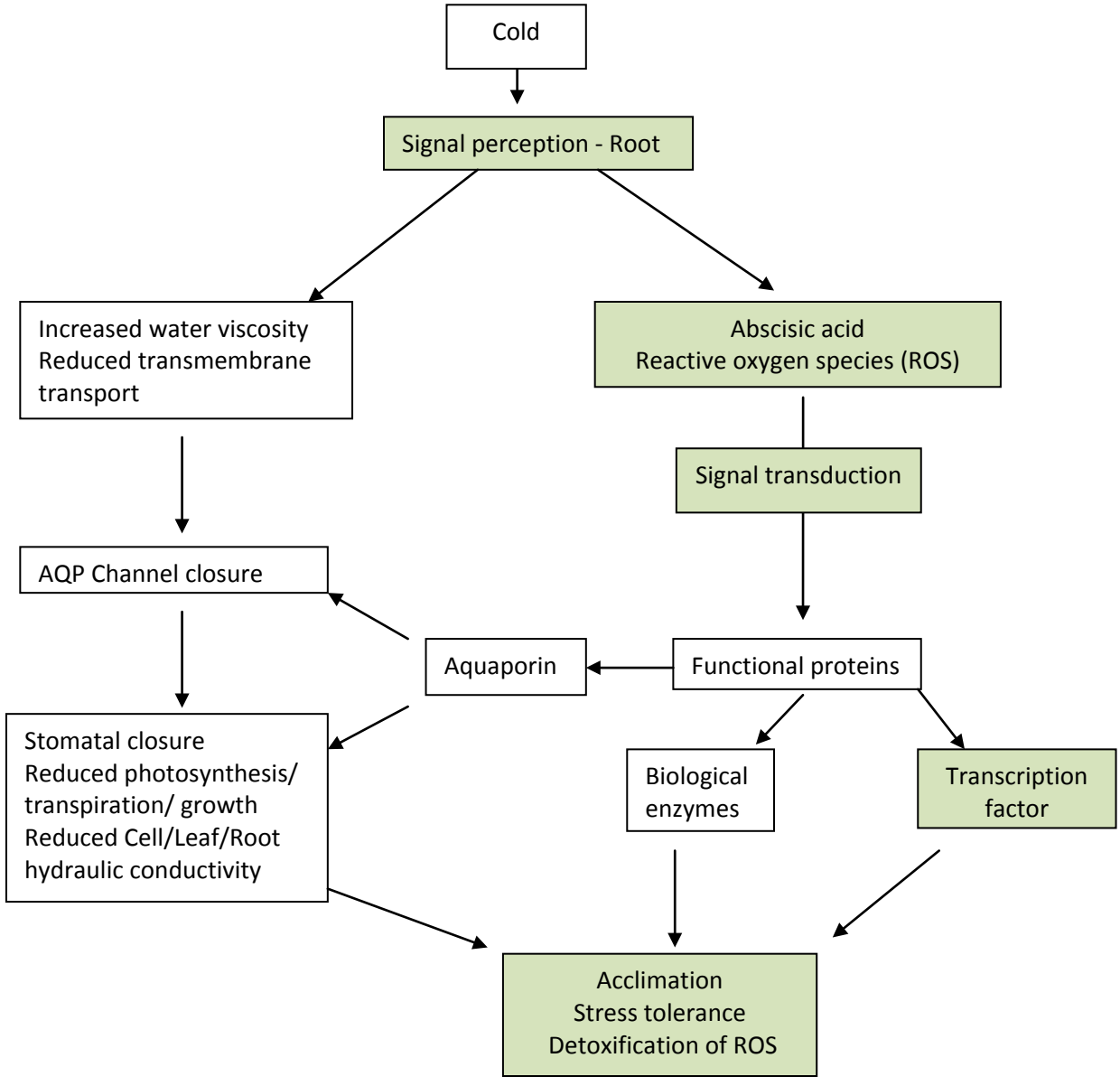

Figure 1.1: Putative response mechanism of plants to cold stress. Filled boxes are hypothesized processes.(afterAroca et al. 2005, Lee et al. 2012, Luu and Maurel,2005, Wan et al. 2001)

Depletion of soil moisture is first sensed by the root of the plant (Nilsen and Orcutt, 1996). Once the root sensed the water stress, it will initiate molecular signalsas abscisic acid (ABA) and reactive oxygen species (ROS) in leaves (Desikan et al.2004). ABA and ROSwill induce the calciuminflux into the cytoplasm, which will activate the ion channels and depolarize the plasma membrane. This depolarization will lead to the exclusion of potassium and other ions from the cytoplasm of the cell.Potassium exclusion will reduce the turgour pressure in the guard cells leading to the closure of stomata (Desikan et al.2004). When stomata are kept closed for long periods, carbon dioxide becomes depleted, resulting in 1.Induction of Rubisco (RuBP) in the leaf,2.Decrease inthe regeneration of RuBP,3.Increase in photorespiration(Flexas et al. 2004). These will lead to growth reduction in long term. Higher concentrations of ABA correlate with the severity of drought and the abundance of some AQPs (Johansson et al. 2000).

1.8 Aquaporins

Aquaporins (AQP) are transmembrane proteins that function as channels to facilitate and regulate the movement of water molecules across membranes (Tyerman et al. 1999; Maurel et al. 2008). The molecular weight of AQP family members ranges from 23 to 31 kDa (Gomes et al. 2009). Plant AQPs areclassified into five families depending on membrane localization and amino acid sequence (Hussain et al. 2011). They are plasma membrane intrinsic proteins (PIP), tonoplast intrinsic proteins (TIP), nodulin-26 like intrinsic proteins (NIP), small basic intrinsic proteins (SIP) and X intrinsic proteins / uncharacterized-intrinsic proteins (XIP) (Marjanovic et al. 2005; Tuskan et al. 2006; Gupta and Sankararamakrishnan, 2009). Plant species typically have a higher number of AQP genes than animals, ranging from 30 to 70 (Maurel et al. 2008; Gomes et al. 2009; Park et al. 2010). Poplar has 55 identified AQP genes (Gupta and

Sankararamakrishnan, 2009;Almeida-Rodriguez et al. 2010).The principle function of AQPs in plants is regulation of transmembrane water transport in situations where water flow needs to be adjusted when the flow is critically low (Luu and Maurel, 2005). In plants,AQPs are present in all organs including roots, leaves, stems, flowers, fruits and seeds (Maurel et al. 1995; Clarkson et al. 2000). AQPs are localized in the plasma membrane, tonoplasts, chloroplast membranes, endoplasmic reticulum and peribacteroid membranes of the nitrogen-fixing cells (Maurel et al. 2008; Rouge and Barre, 2008).

1.8.1 Structure of Aquaporins

AQP consists of six transmembrane helices with N and C termini facing the cytosol (Figure 1.2). There are five loops (A to E) connecting the transmembrane helices and among them B and Dare intracytoplasmic and A, C and E are extracytoplasmic (Murata et al. 2000; Chaumont et al. 2005). The N terminus of each protein is located on the cytoplasmic side of the membrane. Four AQP monomers assemble to form tetrameric holoproteins.Tetramers are stabilized by hydrogen bonds and interactions among the monomer loops (Murata et al. 2000). In AQP, hydrophobicNPAmotifs (Asparagine-Proline-Alanine) are located at the first intracellular loop (loop B) and the third extracellular loop (loop E)and form short helices. These helices fold back into the membrane from opposite directions (Murata et al. 2000). The appearance of AQP protein can be compared with an hourglass and the two hemipores facing each other in reverse within the membrane of AQP (Zardoya, 2005). Among the diverse cell membranes, the localization of each aquaporin protein inside the plant cells varies(Zelazny et al. 2007).

1.8.2 Function of Aquaporins

Aquaporin proteins are pores in the cellular membranes that allow therapid movement of water passively in either direction according to the water potential gradient (Maurel, 1997). SomeMIP family members can transport glycerol, CO_2, urea, ammonia, hydrogen peroxide, boron,silicon, arsenite, antimonite and lactic acid (Maurel et al. 2008) in addition to water. Change in the hydraulic conductivity of a plant could be partially controlled by the AQP activity, especially by PIPs (Javot et al. 2003; Postaire et al. 2010). AQPs can control the water flow across a cell membrane either by the change in the AQP abundance or by the change in the water flow rate (Bramley et al. 2007). The function of one AQPis influenced by its interaction with many other physiological and biochemical processes of the plant (Aharon et al. 2003).

1.8.3 The Plant Aquaporin Subfamilies

Different plant AQPshave different substrate specificity, localization, as well as transcriptional and posttranslational regulations (Danielson and Johanson, 2008; Maurel et al. 2008).

1.8.3.1 PIP subfamily

PIPs are plasma membrane MIPs with a molecular weight around 30 kDa and an isoelectric point of 9.0. PIPs are generally localized in organs characterized by large fluxes of water i.e. vascular tissues, guard cells and flowers. They have several basic amino acids at the C-terminal. Among the 35 full-length aquaporin genes of the *Arabidopsis* genome, 13 encode for PIPs. Based on the sequence similarities, PIPs are classified into two subgroups: PIP1 and PIP2. PIP1s and PIP2s structurally differ from one another in amino and carboxy termini length and amino acid substitutions.

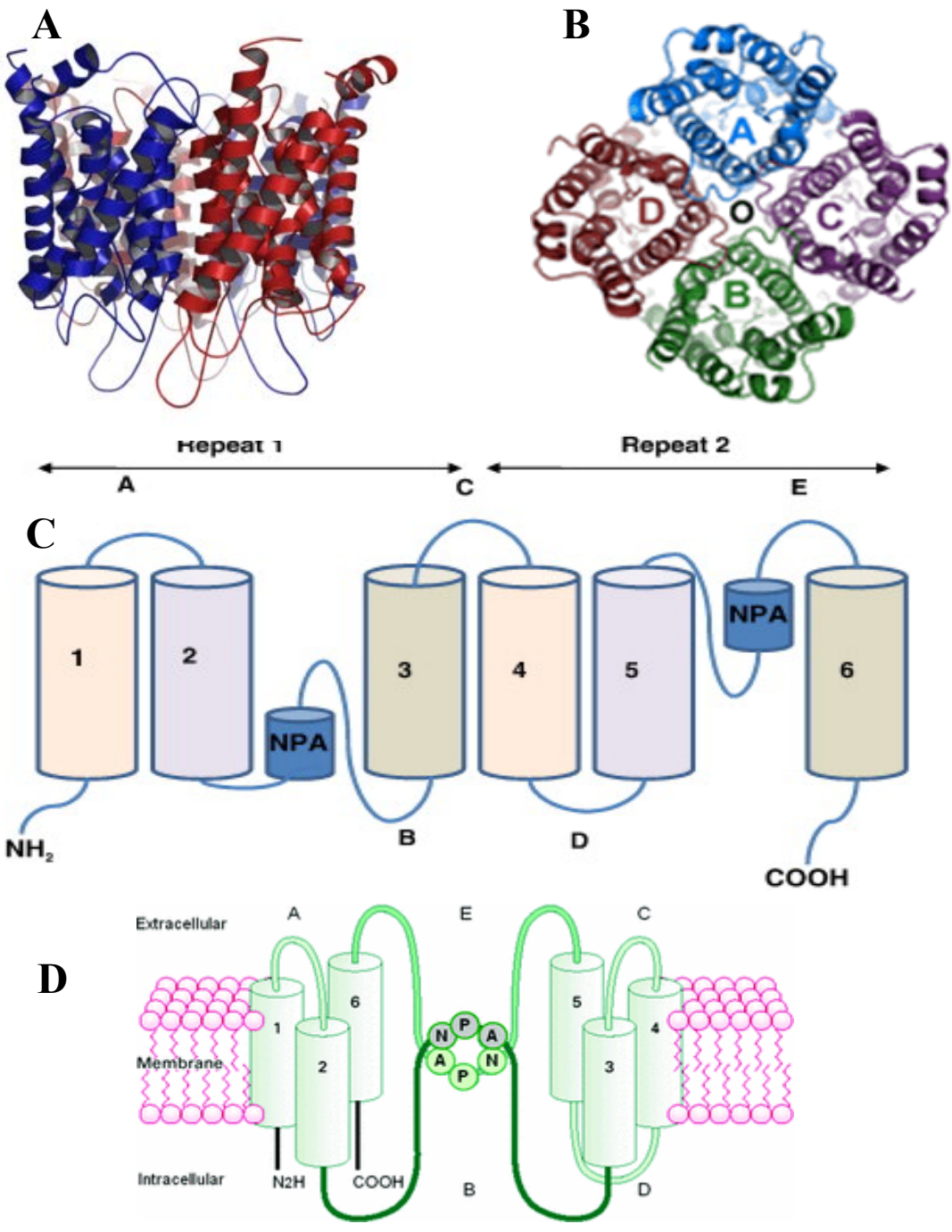

Figure 1.2: A) The basic structure of AQP, B) Top view of the AQP,C)Protein arrangementexplaining how the two regions of helical domains that interact to form the three dimensional structure of the protein. Pore of the AQP is composed of two halves called as hemipores. MIPs consist ofsixtransmembrane domains connected by five loops (A–E), with cytoplasmic N- and C-termini. Locations of NPA (Asn-Pro-Ala) motifs are at the loops B and E.D)Functional AQP formed by the interaction of the two hemipores (Gomes et al. 2009).

The PIP1 subgroup has five members (PIP1;1 to PIP1;5), whereas the PIP2 subgroup has eight isoforms (PIP2;1 to PIP2;8) (Maurel, 2007). PIP1 proteins have longer N-terminal but shorter C-terminal tails compared with PIP2(Chaumont et al. 2000). Based on the research and phylogenetic analysis of *Populus trichocarpa*, among the 55 full-length MIP protein sequences, there are 15 PIPs (five PIP1s and ten PIP2s), 17 TIPs, 11 NIPs, 6 SIPs and 6 XIPs (Tuscan et al. 2006; Gupta and Sankararamakrishnan, 2009).

1.8.3.2 TIP subfamily

Tonoplast Intrinsic Proteins (TIPs) are the most abundant AQPs in the vacuolar membrane (Maurel et al. 2008),butits role in other subcellular locations are not confirmed yet. The vacuole occupies 90% of most mature plant cells and tonoplast of the vacuole is involved in maintaining water and osmotic balance and functions as a barrier to intracellular transport. TIPs are involved in intracellular water movement and transportation of other small molecules(Nozaki et al. 2008). AQPs are the most abundant proteins of the tonoplast(Maeshima, 2001). Therefore water permeability of the tonoplast is higher than that of the plasma membrane.This leads to rapid osmotic adjustment of the cytoplasm and maintenance of cell turgour pressure. TIPs possess a molecular weight between 25 to 28kDa and an isoelectric point around 6.0(Delrot et al. 2001). In maize,*Arabidopsis*and rice, the TIP subfamily consists of five subgroups regarding their sequence homologies: TIP1, TIP2, TIP3, TIP4 and TIP5. TIPs are predominantly located at the tonoplast, but some TIP isoforms are found in protein storage vacuolesand small vacuoles (Fleurat-Lessard et al. 2005).

1.8.3.3 NIP subfamily

This sub family was named as NIP because of the sequence similarity they showed with the nodulin-26proteins, a protein that is expressed at the symbiosome membrane when root of leguminous plants were infected by rhizobacteria. In root nodules, communication about passage of water between the bacteria and the host plant is done by nodulin-26like proteins (NIPs) in an effective way. NIPs are different from other plant MIPs and located in plasma and intracellular membranes of both leguminous and non-leguminous plants(Maurel, 2007). Water permeability and expression of NIPsare very lowcompared to PIPs and TIPsbut they are permeable to small solutes (eg.glycerol, urea). Functions of TIPs are still poorly understood because they are usually associated with specialised structures (Maurel, 2007).

1.8.3.4 SIP subfamily

The SIP subfamily is not structurally and functionally very well characterized. In *Arabidopsis,*SIP members are suspected to involve in solute permeability and are localized in the endoplasmic reticulum (Ishikawa et al. 2005). In cotton, SIPs were classified phylogenetically in one SIP1 subgroup. Based on the NPA sequence SIP1 is subdivided into the SIP1;1 and the SIP1;2 (Park et al. 2010).

1.8.3.5 XIP sub family

TheseAQPs are a newly discovered phylogenetically distinct subfamily and was first characterized from upland cotton (Park et al. 2010). XIPs are widely found in moss, fungi and dicots. There are 19 XIP members that included 5*Populus*XIPs. Among the XIPs, 10 were from dicots other than*Populus*, three were from moss and one was from a protozoa. XIP homolog was absent in monocots (Danielson and Johanson, 2008). Expression analysis of*Populus*MIPs

indicates that*Populus*XIPs do notshow any tissue-specific transcript abundance.XIP subfamily is not functionally characterised in poplar(Gupta and Sankararamakrishnan, 2009).

1.8.4 Aquaporin gene expression

Gene expressionis a process by which information of ageneis converted into protein. Principle steps involved in the gene expression process aretranscription of mRNA, splicing,translation andpost-translational modificationof a protein.Quantitative real time PCR is used to determine the abundance of mRNA transcripts within a cell or tissue as a measure ofgene expression (Bustin et al. 2009). Expression of AQP may be induced by abiotic factors including drought, salinity, low temperatures and wounding. AQP expression is also affected by phytohormones such as ABA and GA. Since *PatPIP* from a *Populus tremula* x*P. alba*clone was strongly expressed in response to NaCl, *PatPIP* might be involved in the plant's response to water stress (Bae et al.2011).

1.8.5 Regulation / Control of aquaporin gene expression

Regulation of gene expression is an adaptation mechanism wherea gene is transcribed, mRNA is translated intoa protein and the protein is correctly targeted. MIP gene expression is regulated in a cell-specific manner via hormones and by environmental factors such as water stress, pathogens, low temperature and salinity. In plants, many signalling pathways controlMIP gene expression through complicated transcriptional, translational and posttranscriptional controls and it is very challenging to distinguish a standard expression pattern for each AQP gene (Maurel andChrispeels, 2001; Hachez et al. 2006). In plants, regulation of AQP expression has been used to evaluate the protein's activity on the osmotic water permeability and how the manipulation of the expression of the same genes acts. The low abundance of AQP proteinswillreduce the water

permeability and the high abundance (over-expression) will increase the hydraulic conductance of biological membranes (Martre et al. 2002).

1.8.5.1 Gating through phosphorylation and dephosphorylation

AQP gating is the opening and closing of the AQP water channel pore in the living cellular membranes. Gating factors involved in aquaporin regulation are phosphorylation and dephosphorylation. Gating can be regulated by protons (H^+) and divalent cations (Verdoucq, et al. 2008). The enzymes involved in gating are protein kinases and phosphatases. Phosphorylation enhances aquaporin activity (Maurelet al. 1995) by keeping the AQP pores in the open state (Tornroth-Horsefieldet al. 2006).

1.8.5.2 Tetramerization

AQPsare capable of forming homotetramers or heterotetramers in the biological membranes (Fetter et al. 2004).When AQPs form homo or heterotetramers, they are trafficked to cellular membranes and function as channel transporters. Some PIP2s, some TIPs and NIPs are functional when expressed alone in heterologous conditions and this indicates that they are active homotetramers. However aquaporins PIP1s alone, cannot form tetramers, but if PIP1s are co-expressed with other PIP2s, then thePIP1s will become active water channels and this proves that they are heterotetramers. Loop E of AQP controls tetramer formation in plants (Fetter et al. 2004). Among the PIP isoforms, generally PIP1 subgroup showed no or poor permeability to water, whereas PIP2 proteins showed comparatively increased water transport (Chaumont et al. 2000).The SoPIP1 from spinach (*Spinacia oleracea*) showed low water permeability in*Xenopus*oocyte whereas its PIP2 showed increased water permeability(Johansson et al. 1998; Tornroth-Horsefield et al. 2006). PIP2 proteins may interact with PIP1 members by forming

heterooligomer structures that can function as water channels (Fetter et al. 2004; Zelazny et al. 2007). Maize ZmPIP1;2is functional when it interacts with ZmPIP2s. ZmPIP1;2 increased membrane water permeability when co-expressed with the functional channel ZmPIP2 in*Xenopus*oocytes (Fetter et al. 2004; Chaumont et al. 2005). Some PIP1 members can also transport glycerol, boric acid, urea and CO_2 (Alexandersson et al. 2005). AtPIP2;1 is able to transport hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) involved in oxidative stress and also acts as a messenger in plant responses to wounding or pathogen attack. PIPs would facilitate cell to cell communication when they are infected by a pathogen (Dynowskiet al. 2008).

1.8.5.3 Hormones

Expression of some plant AQP is regulated by phytohormones such as gibberellins(Shapiguzov, 2004), abscisic acid (ABA) (Jang et al. 2004; Alexanderssonet al. 2005; Zhu et al. 2005), cytokinins and auxins(Lin et al.2007). Interaction between gibberellins and brassinosteroids have been shown to control aquaporin expression (Shapiguzov, 2004). ABA controls the transcription of many AQP isoforms in different parts of diverse plant species (Alexanderssonet al. 2005). ABA controls transcription of *PIP1* genes in guard cell of maize (Zhu et al. 2005). When ABA was applied to *Arabidopsis*, there was an increase in the abundance of AtAQPs (Jang et al. 2004). The expression of *OsAQP* gene encodes a tonoplast intrinsic protein in rice was increased in leaves and roots, by the application of gibberellic acid and abscisic acid (Liang et al. 2012). In ginseng(*Panax ginseng*)cells, *PgTIPI*was slightly more abundant when the cells were treated with exogenous cytokinins and low abundant whenexogenous auxins were supplied (Lin et al.2007).

1.8.5.4 pH and Cations

The pH change in the cytosol is sensed by the histidine (His-197) residue in the intercellular loop(Tournaire-Roux et al. 2003). Reversible reductions in AQP functionwas caused by high concentrations of H^+ (Kaldenhoff and Fisher, 2006). When the cytosolic pH dropped under flooding conditions,the histidine residue (His193 in SoPIP2.1) was protonated in spinach (Tornroth-Horsefield et al. 2006). A conserved H (His)-197 residue of the loop D was protonated by low pH, resulting in reduced water flow through the closure of the pore in*Arabidopsis*(Tournaire-Roux et al. 2003).Mono- and divalent-cations control AQP function under low pH. When *A. thaliana* protoplasts were treated with Ca^{2+}, there was a decline in the cell hydraulic conductivity. If divalent cations bind with AQPs directly, then the binding may cause changes in AQP configuration that trigger signal responses that may affect the water transport under someabiotic stressconditions (Gerbeau et al. 2002).

1.8.5.5. Reactive oxygen species

Reactive oxygen species (ROS) control signaling responses of plants under abiotic stress conditions. Reversible oxidative gating of AQPs might be caused by ROS (Henzler et al. 2004). Formation of ROS in parenchyma cells is induced by high light intensity and ROS might regulate AQP function (Kim and Steudle, 2008). There are two regulatory mechanisms suggested. 1. *OH may oxidize cysteine residues of the AQP pores and this would lead to conformational change of the protein and dephosphorylation will close the channel. 2. *OH will attack the triple bonds between the carbons ofthe plasma membrane through oxidation of lipids and the resulting lipid radicals will close the AQP pore (Henzler et al. 2004). The apoplast produces H_2O_2 in response to ABA and other adverse environmental conditions such as drought and salinity (Jubany-Marí et al. 2009). Theapoplastic ROS generation will lead to the ABA-

induced stomatal closure, during stress. Therefore the expression of AQPs willbe influenzed by the apoplastic ROS (Torres andDangl, 2005). Under stress conditions, it is suggested that ROS is excessively produced and causes signal transduction to activate the AQP and related enzymes to close the AQP channel (Figure 1.1, Luu and Maurel,2005).

1.8.5.6 Chemical inhibitors

Most of the AQPpores are blocked by mercurial compounds (Quigley et al. 2001). Mercurial compounds will bind to the SH-groups of cysteine residues attached to the NPA boxes in the central pore and block the pore and this will lead to a disturbance of water flow (Quigley et al. 2001). When sodium naphthenates were applied to *Populus tremuloides*, the hydraulic conductivity, root water transport and root respiration rate declined (Kamaluddin and Zwiazek, 2002). Sulphydryl reagents blocked AQPs through oxidation of cysteine residues.Inhibition by sulphydryl reagents could be reversed by reducing agents,eg: 2-mercaptoethanol (Tyerman et al. 1999).

1.8.5.7 Environmental stresses

Regulation of AQP expression under different abiotic stressvaries as a result of the plant's growth conditions, stages of development and tissue types (Alexandersson et al. 2005). Drought, cold, salinity and diurnal and circadian rhythms are some of the environmental factors that could inducethe mechanisms that in turn regulate transmembrane water movement (Javot and Maurel 2002; Jang et al. 2004; Alexandersson et al. 2005).AQPs play a key role in maintaining water homeostasis and balance under different environmental stress conditions (Luu and Maurel, 2005; Galmes et al. 2007). The abundance and activity of aquaporins in the plasma membrane and tonoplast may be regulated, hence plantscan control water fluxes within the cells

and in and out of the cells(Maurel, 2007). Salinity, drought and low root temperatures reduce water transport rates (Javot and Maurel 2002). Under different stress conditions, the AQP expression and abundance vary depending on the time duration and intensity of stress (Galmes et al. 2007), type of aquaporin examined (Jang et al. 2004),species, growing conditions and the plant tissue used for experimentation (Alexandersson et al. 2005). Not all the AQP transcripts are converted into proteins, because of the protein turn over and posttranslational modifications (PTM). PTM is a step in protein synthesis where polypeptides undergo structural and functional changes following initial synthesis of protein, e.g. phosphorylation. Turnover is the balance between synthesis and degradation of protein (Aroca et al. 2005; Yu et al. 2005).The exact role of AQPs in plant water status under different stress condition cannot be clearly explained, because differentAQP genes may be variously stimulated,reduced or remained unchanged under abiotic stress. This complex expression pattern clearly suggests that a reasonable water status is maintained by increased or reduced symplastic water transport via AQPs in cells under abiotic stress conditions (Shao et al. 2008).

1.8.6Responses of plants to AQP over-expression

Physiological responses of the plants to a variety of abiotic stress could be altered by the genetic manipulation of plants (Table 1.1). Increasing water use efficiency and the promotion of plant water retention in the cell are the important goals in the development of tolerance to abiotic stress. The mechanisms discussing the regulation of AQP function are also important for the beneficial effects found in plants over-expressing AQP genes (Maria del and Micaela,2014). Expression of AQP *PIP1*of *Vicia faba*(*VfPIP1*) in transgenic*Arabidopsis thaliana*improved drought resistance by reduction of transpiration due to stomatal closure(Cui et al. 2008). Over expression ofrelative water content gene RWC3in transgenic lowland rice (*Oryza sativa*)

showed increased resistance to polyethylene glycol (PEG) induced osmotic stress through increased root hydraulic conductivity (Lian et al. 2004). Under salinity stress, expression of *OsPIP1;1 AQP* in rice, increased in leaves but was reduced in roots (Liu et al. 2013). Therefore, the resistance of each over-expressed AQP isoform towards a specific stress depends on the rise in water uptake from soil, role of AQP in the control of water loss by transpiration and its capacity to maintain CO_2 assimilation. The effect of interaction of a particular AQP isoform on other endogenous aquaporins may show the response to the stress. The stomatal density and transpiration rate were increased in transgenic *AtPIP1b*-overexpressing tobacco plants, whereas they remained unchanged in transgenic *NtAQP1*-over-expressing *Arabidopsis*plants (Cui et al.2005) when compared to the wild-type control plants. The phenotypic changes shown by AQP over-expression play a key role in the development of tolerance during abiotic stress (Cui et al. 2008). Stress tolerance is achieved by the cooperation between the over-expressing foreign AQP and the endogenous AQPs. The expression of one AQP gene affects the expression patterns and distribution of endogenous AQP genes and this effect varies among plant species (Jang et al. 2007a). The expression of cucumber (*Cucumis sativus*) AQP genes*CsPIP1;1* or*CfPIP2;1*increased seed germination rates under high salinity.

Table 1.1: Examples of success and failures when trying to improve the plant resistance to abiotic stress by manipulation of AQP expression, for different plants.

Aquaporin gene isoform	Aquaporin over expressing plant	Promoter	Stress condition	Response	Reference
VfPIP1	*Arabidopsis thaliana*	35S	Drought (soil drying by withholding water)	Resistance	(Cui et al.2008)
AtPIP1;b	*Nicotiana tabacum*	35S	Salinity (90 mM NaCl, 40 d)	Sensitive	(Aharon et al. 2003)
VvPIP2;4N	*Vitis vinifera*	35S	Water stress (soil drying by withholding water, 14 d)	Sensitive	(Perrone et al. 2011)
StPIP1	*Nicotiana tabacum*	35S rd29A	Water stress (25% PEG 6000)	Sensitive	(Wu et al. 2009)
RWC3	*Oryza sativa*	SWPA2	Water stress (PEG treatment, 10 h)	Resistance	(Lian et al.2004)
OsPIP1;1	*Oryza sativa*	35S	Salinity (100 mM NaCl, 14 d)	Resistance	(Liu et al.2013)
SlTIP2;2	*Solanum lycopersicum*	EVO205 35S	Salinity (180–200 mM NaCl)	Resistance	(Sade et al.2012)
HvPIP2;1	*Oryza sativa*	35S	Salinity (100 mM NaCl, 2 weeks)	Sensitive	(Yu et al.2005)
TdPIP1;1	*Nicotiana tabacum*	–	Salinity (250 mM NaCl, 30 d) Water stress (300 mM mannitol, 30 d)	Resistance Resistance	(Ayadi et al. 2011)
BnPIP1	*Nicotiana tabacum*	35S	Drought (stop irrigation, 20% PEG8000)	Resistance	(Yu et al. 2005)
TaAQP7	*Nicotiana tabacum*	35S	Drought (water deprivation, 20 d) Osmotic stress (150–300 mM mannitol, 7–12 d)	Resistance Resistance	(Zhou et al.2012)
AtPIP1;4, AtPIP2;5	*Arabidopsis thaliana, Nicotiana tabacum*	35S	Water stress (100–400 mM mannitol 12–24 h, withholding water 15 d) Salinity (50 mM NaCl 14 d) Cold (10 °C, 24 h)	No effect	(Jang et al.2007a)

The expression offigleaf gourd (*Cucurbita ficifolia*) AQP gene *CfPIP2;1* raised the survival rate of *Arabidopsis*plants under extreme drought(Jang et al. 2007b). The functions of *PIP2* subfamily AQPs will be more affected than the *PIP1* subfamily, under severe abiotic stress (Jang et al. 2007b). How the foreign AQP gene modifies the function of endogenous*PIPs of Arabidopsis* is not known. Over-expression of plant AQPs did not always improve tolerance to abiotic stress (Aharon et al. 2003; Wu et al. 2009; Perrone et al. 2012). Over-expression of AQP gene *AtPIP1b* in*Arabidopsis*increased the growth rate, gas exchange and stomatal density in*Nicotiana tabacum*under favorable conditions, but showed wilting and poor growth, under drought stress conditions (Aharon et al. 2003).

1.9 Objective of the study

The present study was carried out to understand therelationships betweenphysiological responses in a hybrid aspen clone to low root temperature and osmotic stress and the role of *PIP2;5* in these processes. The link between the stress resistance and AQP function in poplar was a question. One of the main roles of AQPs in plants is to regulate water transport through roots. Most of the cell-to-cell water transport in plants occurs via AQPs, but the extent to which AQPs contribute to plant water status and affect the responses of plants under abiotic stresses such as low root temperature and osmotic stress are not clear. Over-expression of *AQPs* is one means by whichto understand the roles of these gene products in water uptake and transport and abiotic stress responses (Hussain et al. 2011).

The objectives of the study were to:

(1) Produce genetically modified hybrid aspen clones over-expressing the aquaporin *PtdPIP2;5.*

(2) Examine the effects of *PIP2;*5 over-expression on temperature responses of root water transport in the hybrid aspen clones.

(3) Investigate changes in water transport and physiological responses of the *PIP2;5* over-expressing and wild-type hybrid aspen clones under osmotic stress.

(4) Determine the functioning role of aquaporin *PIP2;5* by studying the change in the expression patterns of aquaporin transcripts between tissues of the transgenic and wild-type hybrid aspen clones that aregrowing under normal and abiotic stress conditions.

1.10 Research Hypotheses

The present study has attempted to find answers to the following questions

1. How important is the involvement of *PIP2;5* in the overall water transport of root?
2. How important is aquaporin *PIP2;5* in maintaining root water uptake of poplar plants exposed tolow root temperature and osmotic stress?
3. What are the possible relationships betweenphysiological responses in poplar plants to low root temperature and osmotic stress and the role of *PIP2;5* in these processes?
4. What happens to the expression of *PIP2;5* and the other related aquaporin isoforms, after short and long term exposure of roots to low temperature?

To address these questions, *Populus tremula* × *Populus alba*INRA clone 717 1B4 plantlets constitutively over expressing aquaporin *PIP2;5*of *Populustrichocarpa* × *Populusdeltoides* were generated. Aquaporin *PIP2;5* cloned from *Populustrichocarpa* × *deltoides* H11-11 was selected for this study as it was confirmed as a major water-transporting aquaporin in *Populus*by oocyte analysis and also showed increased expression under water stressed conditions (Almeida-

Rodriguez et al. 2010). In the present study, I investigated short and long term effects of the transpirational demand and LRT and osmotic stress on water uptake properties, physiological responses and aquaporin expression in both over-expressed and wild-type hybrid poplar clone INRA 717plantlets, as a step towards understanding the integrated mechanism of this genusto handle itself underenvironmental stresses.

In this study, the following hypotheses were tested:

(1) Over-expression of aquaporin *PIP2;5* would increase overall water transport of the root of the plant to maintain highly stable water status at low root temperatures and in consequence high physiological response rates, when their roots are exposed to low temperatures.

(2) The impact of low temperature on root water transport involves a change in the expression of *PIP2;5* and/ or related aquaporin genes.

(3) *PIP2;5* over-expressing INRA 717 clonewould enhance resistance to osmotic stress induced by PEG6000than the wild-type plants through change in aquaporin activity, water permeability and altering physiological changes.

(4) Poplar aquaporins will show differential expression patterns between tissues. Even though there is difference in the transcript profiles of different tissues, the function and expression of *PIP2;5* will be higher in transgenic lines of INRA 717 regardless of tissue under normal condition.

(5) Changes in the physiological functions and growth of the plant and transcript profile of the aquaporins in the wild-type and transgenic lines under stress conditions, will reflect the differences in the stress response of plants.

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CHAPTER 2

2.0 Generation of transgenic hybrid aspen clone INRA 717over-expressing*PtdPIP2;5*

2.1 Introduction

Poplar is an ideal model treethat could be used for*in vitro* regeneration. It is afast-growing, deciduous or semi-evergreen treegrown in diverse environmental conditions(Polle et al. 2013).A range of knowledge and techniques arecurrently available for plant transformation. Usage of viral vectors and *Agrobacteriumtumefaciens* T-DNA mediated transformation are categorized under naturally-occurring gene transfer systems. Different technologies have their own merits and demerits. Among the available techniques,*Agrobacterium-mediated* transformation is the relatively efficient one for a diversity of plant species for its low copy number of intact and the higher efficiency of incorporating transgenes into the plant genome (Gelvin, 1998). Application of the advanced knowledge of the molecular biology of *Agrobacterium tumefaciens*,particularly the role of Ti plasmid virulence (VIR)genes in transformation is efficiently being used to increase the success rate of *Agrobacterium-mediated* transformation (Pena and Seguin, 2001). The genetic transformation of poplar was successfully madefirst in 1986 (Han et al. 2013).Multiple factors influence in determining the transformation efficiency such as genotype of poplar, type of *Agrobacterium tumefaciens* strain, concentration of bacteria and the explant type used (Meilan and Ma, 2006, Ma et al. 2004,Cseke et al. 2007, Han et al. 2013). The present study was focused on the production of a transgenic plant to examine the function of an important aquaporin PIP2;5. To explore this idea, the *PIP2;5* gene, from the hybrid poplar *Populus trichocarpa*× *Populus deltoides*H11-11which promotes water transportation, was over expressed. Leaf discs of a hybrid aspen clone*Populus tremula* × *Populus alba*INRA 717 was

chosen as an ex plant for transformation to ensure an acceptable transformation efficiency. The objective of this study was to produce a genetically modified hybrid aspensover-expressing the aquaporin *PtdPIP2;5*.

2.2 Materials and methods

Construct preparation

2.2.1Addition of restriction sites to the coding sequence*PtdPIP2;5*by PCR reaction

A cDNA clone encoding PtdPIP2;5 (*PIP2:5* cloned from *Populustrichocarpa* × *P.deltoides* H11-11) was selected as it was previously confirmed (Almeida-Rodriguez et al. 2010) in a previous study to function as a water-transporting aquaporin and also showed increased expression under water limitation. To add BamHI and SacII restriction sites at the forward and reverse ends of the coding sequence respectively, a PCR reaction was performed on an Eppendoff thermocycler and the PCR conditions were 1 cycle at 94°C for 5 min, 40 cycles at 94°C for 40 s, 50°C for 40 s, and 72°C for 1.5 min, followed by 1 cycle at 72°C for 5 minutes. The PCR reaction was performed using 1μL of bacterial suspension, 2U Taq DNA polymerase (New England Biolabs® Inc. Ipswich, MA, USA), 1X Taq standard buffer, 0.2 mM dNTPs, 0.2 μM of each primers designed to add BamHI and SacII. The amplified DNA fragments were subjected to 1% agarose gel electrophoresis with 0.5X TBE buffer and visualized by SYBR safe (Sigma-Aldrich, St, Louis, MO, USA) and the DNA fragments corresponding to the expected cloning size (858 bp) for the gene was excised from the gel using the QIAquick gel extraction kit (QIAGEN Inc. Mississauga, ON, Canada) according to the manufacturer's protocol. Existence of the BamHI and SacII restriction sites at the forward and reverse ends of coding

sequence *PtdPIP2:5* were confirmed by performing a sequencing reactions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc. Foster City, CA, USA).

2.2.2DNA cloning, plasmid purification and sequencing

Ligation of purified DNA fragments and pGEM®-T easy vector (Promega Co. Madison, Wl, USA) was done and DH5α cells were transformed, according to the manufacturer's instructions. Competent cells DH5α were plated on LB agar plates (Table 2.1) to which was added 100 μg mL^{-1} ampicillin and mixed with 100μL of 100 mM IPTG and 20μL of 50mg mL^{-1} X-Gal. Plates were invertedly incubated overnight at 37°C and 10individual white colonies were randomly picked and transferred into a new manually labeled LB agar plate,added with 100 μgmL^{-1} ampicillin and previously mixed with 20μL of 50mg mL^{-1} X-Gal. The tip used for picking up each colony was then transferred into PCR tube containing PCR master mix for insert confirmation using SP6 and T7 universal primers. Amplicons were visualized in an electrophoresis of 1% agarose gel with 0.5X TBE buffer and application of SYBR safe. Two colonies with the insert were chosen for each gene and were grown in triplicate in 5ml LBliquid/ampicillin for 15h at 37°C and 220 rpm andglycerol stocks were made and stored at -80°C.

2.2.3BamHI and SacII Restriction enzyme digestions and cloning in pMJM vector

The coding sequence of *PtdPIP2:5*was inserted using BamHI and SacII enzyme digestions, into the pMJM vector(Levée et al. 2009), containing the maize (*Zea mays*) ubiquitin promoter (Christensen et al. 1992). pGEM®-T easy vector containing the coding sequence of *PtdPIP2:5* and pMJM plasmid, both were purified using GeneJET™ Plasmid Miniprep Kit.

Table 2.1: Composition of the Lysogeny Broth(LB) medium

Components	Concentration (gL^{-1})
Tryptone	10.0
Yeast extract	5.0
Sodium chloride (NaCl)	10.0
Agar	15.0

Water was added to 1 L and autoclaved.

For the LB liquid media, agar was excluded.

The light absorbance at 260nm wavelengthwas measured using a Nanodrop® ND-1000 spectrophotometer as a measure of quantifying the concentration of the plasmids.Digestion reactions for each plasmid were performed in duplicate tubes using 2µg of template, 1µL of SacII(Fermentas, Burlington, ON, Canada), 5µL of 10X buffer and RNase-free water up to a final volume of 50µL. Tubes were incubated for 3h at 37°C. The thermal inactivation of the enzymes were done by keeping the tubes at 65^{o}C water bath for 20 min. Digestion reactions for each plasmid were performed in the same duplicate tubes used for SacII digestion by the addition of 1.5µL of BamHI(Fermentas, Burlington, ON, Canada), 1X buffer 5µL and RNase-free water 3.5 µL up to a final volume of 10 µL. Tubes were incubated for 3h at 37°C in water bath. Precipitation reactions were performed to each duplicate tube by adding 0.1 volumes of 3M sodium acetate along with 2 volumes of chilled absolute ethyl alcohol and 140µL of sterile Milli Q water. After a gentle shaking, DNA was precipitated overnight by keeping the tubes at -20°C freezer. Tubes were centrifuged at 13000rpmfor 30 minutes at 4^{o}C and supernatant was removed. Pellets were washed well with 500µl of 70% ethanol and then centrifuged at 14000rpmfor 5 minutes at room temperature and the tubes were kept in an inverted position for 20 minutes, for the pellets to air dry. Then the air dried pellet was dissolved in 10µL of sterile RNase-free water and stored at -20°C. Digests and DNA were subjected to 1% agarose gel electrophoresis with 0.5X TBE buffer and visualized by SYBR safe and the DNA fragments corresponding to the expected cloning size (858 bp) were purified from the excised gel pieces using the QIAquick gel extraction kit (QIAGEN Inc. Mississauga, ON, Canada) following the manufacturer's protocol and quantified at 260nm using the Nanodrop® ND-1000 spectrophotometer.

Purified DNA fragments were ligated in pMJM vector using 2X ligation buffer and T_4 DNA ligase enzyme combination and *E. coli* DH5α cells were transformed according to the manufacturer's conditions. Competent cells DH5α were plated in LB-plates supplemented with 100μgmL^{-1} ampicillin. Plates were incubated overnight at 37°C and ten individual colonies were selected and transferred into a new manually labeled LB-plate supplemented with 100μgmL^{-1} ampicillin. The tip used for picking up each colony was then rinsed by pipetting up and down in a PCR tube containing PCR master mix (as described in the above section) for insert confirmation, using SP6 and T7 universal primers. Amplicon size of coding sequence *PtdPIP2:5* and ubiquitin promotertogether was confirmed by 1% agarose gel electrophoresis and visualization. The colonies with the proper amplicon size were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit, with the *PIP2;5* forward and reverse primers. After sequenceswere confirmed, the pMJM plasmids containing the *PtdPIP2;5*were purified as described above and quantified at 260nm using the Nanodrop® ND-1000 spectrophotometer and stored at -20^{o}C.

2.2.4SbfI restriction enzyme digestion and cloning in pCAMBIA1305.2 vector

Both coding sequence of *PtdPIP2;5* and the ubiquitin promoter were cut together and inserted into binary vector pCambia1305.2 in the sense orientation, using SbfI enzyme digestion. Both thepMJM vector containing the coding sequence of *PtdPIP2;5*and the pCAMBIA1305.2 plasmid(Levée et al. 2009)were purified using GeneJET™ Plasmid Midiprep Kit (Fermentas Canada Inc. Burlington, ON, Canada) according to the manufacturer's protocol. Concentrations of the plasmids were quantified as previously described. The pMJM vector containing the coding sequence of *PtdPIP2;5* and pCAMBIA1305.2 plasmid were both digested using SbfIrestriction enzyme (pMJM vector has two restriction sites for SbfI while pCAMBIA1305.2

vector has only one site). Digestion reactions for each plasmid were performed in duplicate using 2µg of template, 1µl of SbfI(New England Biolabs, Ipswich, MA, USA), 5µL of 10X buffer and RNase-free water up to a final volume of 50µl. Tubes were incubated for 3h at 37°C in water bath without agitation. After the thermal inactivation by keeping the tubes at 65°C water bath for 20 min, dephosphatase digestion was done. Digestion reactions were performed in the same duplicate tubes used for SbfI digestion, by the addition of 1.5µLof *Antarctic phosphatase* (New England Biolabs, Ipswich, MA, USA), 1X buffer 5µL and RNase-free water 3.5µL up to a final volume of 10µL. After the incubationfor reaction for 20 minutes at 37°C, thermal inactivation and precipitation reactions were performed as mentioned in the previous section, to get pure DNA fragments containing *PtdPIP2;5* and Ubiquitin Promoter.DNA were visualized by 1% agarose gel electrophoresis and the fragment corresponding to the expected size was purified from the excised gel pieces and quantified at 260nm. Purified DNA fragments having *PtdPIP2;5* and ubiquitin promoter were ligated in pCAMBIA1305.2 vector and *E. coli* DH5α cells, were transformed according to the manufacturer's conditions. Competent cells plated on LB-plates supplemented with 50µg mL^{-1}kanamycin were incubated overnight at 37°C and ten individual colonies were transferred into kanamycin added new manually labeled LB plate. The presence of the desired inserted DNA fragment (*PtdPIP2;5* and Ubiquitin promoter) and its sense orientation were confirmed by PCR amplification (*PtdPIP2;5* specific primers), sequencing (*PtdPIP2;5* forward and reverse primers) and restriction digestion analysis using restriction enzymes BamHI and SbfI. After the confirmation of the existence and sense orientation, the pCAMBIA1305.2 plasmids containing the *PtdPIP2;5*and ubiquitin promoter were purified, quantified at 260nm and stored at -20°C.

2.2.5*Agrobacterium* transformation

The purified plasmid DNA with the correct orientation was then transferred to competent *Agrobacterium tumefaciens*strain C58 pMP90 cells using freeze-thaw method (Holsters et al.1978**).** The plasmid also carries the *hygromycin phosphotransferase II* (HptII) gene enabling the selection of transformed tissue on hygromycin incorporated media.Single colony of *Agrobacterium tumefaciens* strain C58 pMP90 was introduced into LB medium (6mL) containing 20mgL^{-1} of gentamycin (6µL) and 100mgL^{-1} rifampicin (12µL) and allowed to agitate (200 rpm) at 28°C overnight on a gyratory shaker. Whole LB medium was transferred into a sterile 1000mL Erlenmeyer flask containing 20mgL^{-1} of gentamycin (50µL) and 100 mgL^{-1} rifampicin (100 µL) and incubated at 28°C, under agitation (200rpm) overnight.When the optical density measured at 600nm was between 0.5 and 0.9, the cultured cells were collected at the base of the flask by centrifugation for 30 min in a tabletop centrifuge at 4500 rpm at 4^{o}C. The bacterial pellet was kept on ice for 10 minutes and then dissolved in 1000µL of 20mM $CaCl_2$ solution. Then pCAMBIA vector containing the *PtdPIP2:5* and the ubiquitin promoter (200µL) was added to the tube and kept in ice for 5 minutes and then in liquid nitrogen for 5 minutes and immediately transferred to a water bath at 37°C and kept for 5 minutes. After that the tube was agitated (200rpm) at 28°C for 3 hours in the water bath and the mixture (50µL) was spread on LB agar plate containing 50mgL^{-1} of kanamycin and 100 mgL^{-1} rifampicin and 20 mgL^{-1}gentamycin on triplicate and another LB agar plate without the addition of antibiotics(Control) and incubated at 28°C for two days. The existence of the desired inserted DNA fragment (*PtdPIP2;5* and Ubiquitin promoter) was confirmed by PCR amplification (with *PtdPIP2;5* specific primers) and sequencing (with the *PtdPIP2;5* forward and reverse primers).

Table 2.2 Primers used to clone *PtdPIP2:5* coding region into pCAMBIA 1305.2vector and confirmation of its existence

Primer	Primer sequences (5'-3')
PtdPIP2:5 Forward	TCGGGTGTTCTTGAGGCTGGT
PtdPIP2:5 Reverse	ACGCCAAGCGCGCAATTAACC
BamHI	AAATAGGATCCATGGGCAAGGACATT
SacII	AAAACCGCGGTTAAATGTTGGAAGAG
pCAMBIA1305.02-Forward	ATGTGAGTTAGCTCACTCATT
pCAMBIA1305.02-Reverse	GGTGCGGGCCTCTTCGCTATT
SP6 Forward	ATTTAGGTGACACTATAG
T7 Forward	TAATACGACTCACTATAGGG
T7 Reverse	GCTAGTTATTGCTCAGCGG
HptII Forward	CAAGATGGATTGCACGCAGGTTCTC
HptII Reverse	GCGCTTCTGCGGGCGATTTG
TIF5A Forward	AACTCGCAAGGCATGTAATGG
TIF5A Reverse	ATTCCAACCTGCTGATAACACAAG

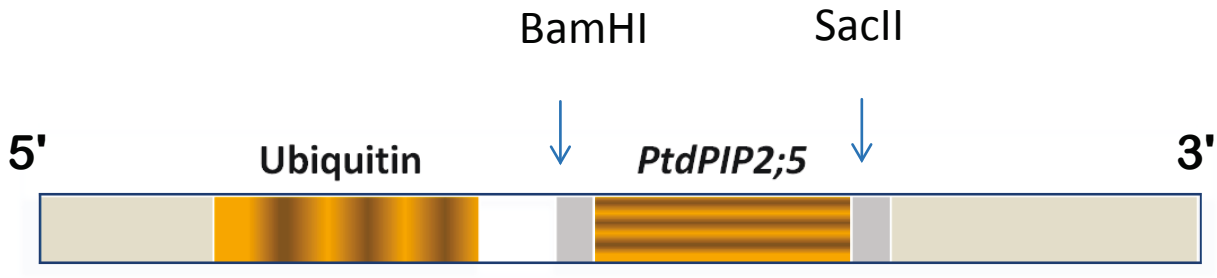

Figure 2.1:A simplified map of construct in pCAMBIA1305.2vector.The coding sequence of *PtdPIP2;5*with BamHI and SacII restriction sites at the forward and reverse ends and the ubiquitin promoter from pMJM vector were cut together and sub cloned into pCAMBIA1305.2, in the sense orientation.

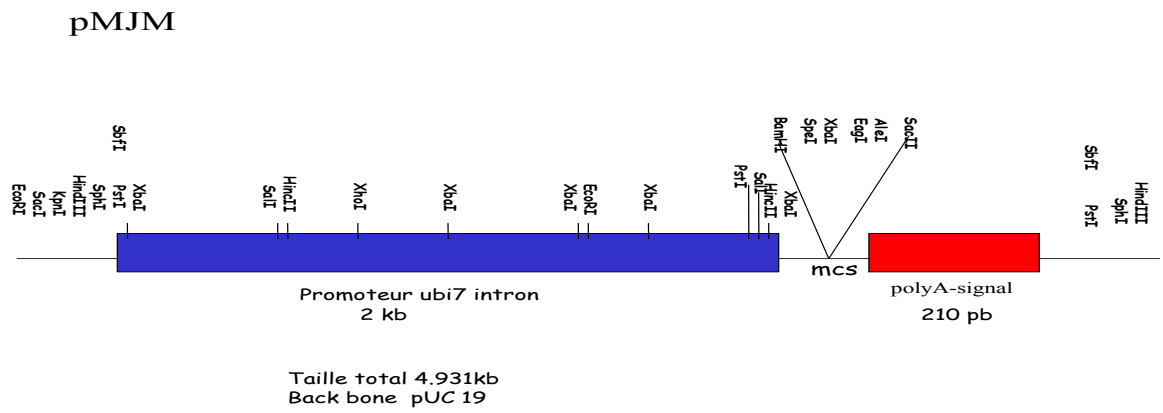

Figure 2.2A: Plasmid map of pMJM vector (Levée et al. 2009)

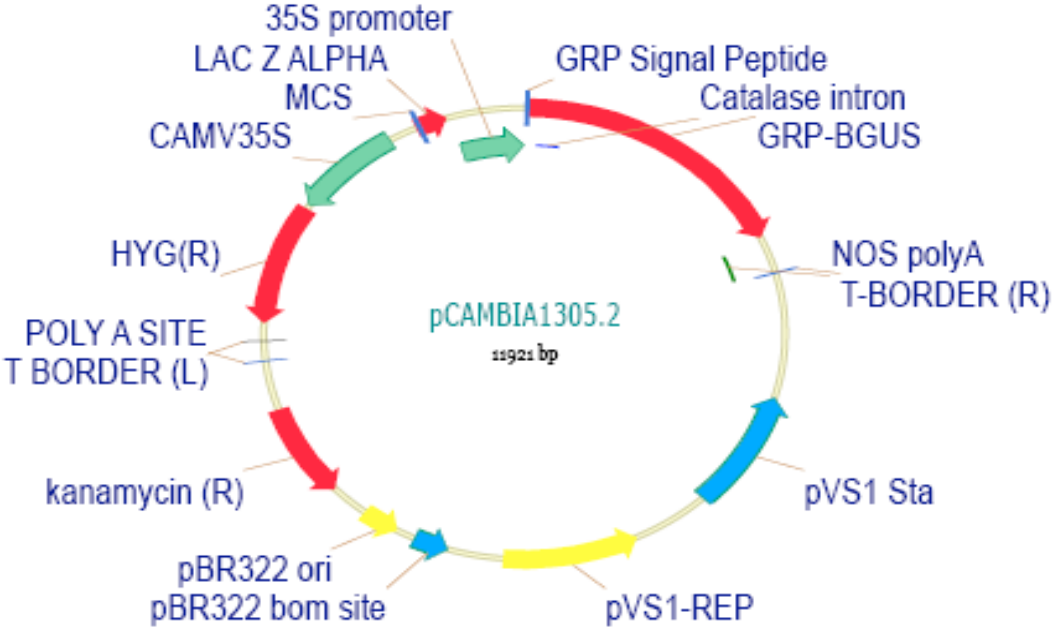

Figure 2.2B: Plasmid map of pCAMBIA1305.2 vector (Levée et al. 2009)

2.2.6Poplar transformation

2.2.6.1 Plant material and growth condition

Hybrid poplar *Populus tremula* × *P. alba* INRA Clone 717 1B4 (Leple et al.1992) were *in vitro* grown in magenta boxes (Sigma Aldrich) containing propagation medium (Table 2.3) at 25°C.

2.2.6.2 *Agrobacterium* mediated poplar transformation

Transformation by co-cultivation or contact method was performed to introduce the construct having *PtdPIP2;5* and Ubiquitin promoter into the *in vitro* grown,6-7 weeks old*Populus tremula* × *Populus alba* plantlets of INRA 717 clone. A modified poplar transformation protocol (Leple et al. 1992, Kajita et al. 1994, Cseke et al. 2007 and Levée et al. 2009) wasused to produce the transgenic lines. One loop of *Agrobacterium tumefaciens* strain C58 pMP90 containing a transformation vector was introduced into LB liquid medium (Table 2.1) containing 50mgL^{-1} of kanamycin and 100mgL^{-1} rifampicin 20mgL^{-1} of gentamycin and agitated (200 rpm) at 28°C, overnight on a shaker. Whole LB liquid medium containing grown bacterial cultures was transferred into a sterile 1-L Erlenmeyer flask, containing 50mgL^{-1} of kanamycin and 100 mgL^{-1} rifampicin and20 mgL^{-1}gentamycin and incubated at 28°C under agitation (200rpm) overnight.The cultured bacterial cells were collected at the base of the flask by centrifugation for 10 min at 4500 rpm in a centrifuge. The bacterial pellet was resuspended in liquid MSmedium (Table 2.1) with 100 mMacetosyringone and diluted until getting an O.D, between 0.3 - 0.4at 600 nm. Then *Agrobacterium*culture was poured into different sterile petridishes under sterile condition (Binnsand Thomashow, 1988). Young leaves of *in vitro* grown poplar plants were carefully taken out without contamination and cut into pieces with major leaf veins (explant), using a sterile blade and placed in petridishes containing *Agrobacterium*culture and kept for 2

hours,under a fume hood with frequent shaking of petridishes to facilitate the bacterial infection. Infected explants were then transferred onto sterile whatman paper to remove the excess *Agrobacterium* cellsand water and placed on the petridishes filled with acetosyringone added solid transformation medium (TRM, Table 2.3), covered by using aluminium foil and incubated in an inverted position at 28°C in dark for 48 h. After the co-cultivation for 48 hours, *Agrobacterium*colonies were visible on the explants and medium.Explants were washed 3-5 times with sterile liquid MS media added with 250 mgL^{-1} cefotaxime in a sterile falcon tube and placed on sterile filter paper to remove the excess moist. Then explants were transferred onto Callus Induction Medium (CIM, Table 2.3) added with cefotaxime (300 mgL^{-1}) and hygromycin (10 mgL^{-1}) and incubated for 14 days in the dark. During the incubation in dark, the medium was observed frequently for the possible *Agrobacterium* contamination and washing and transfer to new CIM continued until no bacterial growth was observed on the media. Then the leaf discs were transferred to Shoot Induction Medium (SIM, Table 2.3) containing thidiazuron and incubated in light under aseptic condition. Leaf discs that showed bacterial growth were washed and sub-cultured on the same medium continuously every week. Once the callus formed and shootsstarted to develop from the leaf discs, then the discs were transferred to Shoot Elongation Medium (SEM, table 2.3) containing benzylaminopurine to facilitate the shoot elongation. Once the shoots elongated successfully then the shoots were cut with the medium using sterile blade and transferred ontotheRoot Induction Medium (RIM, Table 2.3) and incubated under light. Some shoots rooted on RIM and some did not. The shoots that root on hygromycin-added media are most likely transformed.

Table 2.3: Products listing and the composition of the MS (Murashige and Skoog, 1962) / Transformation medium (TRM), Callus Induction Medium (CIM), Shoot Induction Medium (SIM), Shoot Elongation Medium (SEM) and Root Induction Medium (RIM).

Products	TRM/MS	CIM	SIM	SEM	RIM
MS powder		4.3g	4.3g	4.3g	
MS Macro Nu.Sol	50µL	-	-	-	50µL
MS Micro Nu.Sol	100µL	-	-	-	100µL
MS Vitamin (1000X)	1mL	1mL	1mL	1mL	1mL
Sucrose	20g	30g	30g	30g	20g
NAA stock (0.5 mg/mL)	-	3.3 mL	-	-	-
2iP stock (1mg/mL)	-	1mL	-	-	-
MES	0.25g	-	-	0.25g	0.25g
Double distilledWater	1 L	1 L	1L	1L	1 L
pH of all the media			pH 5.7		
Phytablend	8 g	8g	8g	8g	8g
		Put in bottles and autoclaved at 121^{o}C / 20 min			
Filter sterilizing stocks					
Glutamine (25 mg/mL)	8 mL/L	8 mL/L	8 mL/L	8 mL/L	8 mL/L
L-cysteine (25 mg/mL)	40 µL/L	40 µL/L	40 µL/L	40 µL/L	40 µL/L
Thidiazuron stock			100 µL/L		
Cefotaxime 250		1.2 mL/L	1.2 mL/L	1.2 mL/L	1.2 mL/L
Indole Butric Acid					100 µL/L
Benzylaminopurine (BAP)					
(1 mg/mL)				50 µL/L	
Hygromycin 25		200 µL/L	200 µL/L	200 µL/L	200 µL/L

pHwas set at 5.7 in all the media. **MS liquid media** -MS/ Transformation media (TRM) without the addition of phytoblend.

2.2.7PCR analysis of putative transformants

2.2.7.1 Genomic DNA extraction

The two lowest leaves of RIM-grown rooted healthy INRA clone 717 hybrid aspenplantletswere harvested and ground in 500 µL extraction buffer (100mM Tris pH 8.0, 50 mM EDTA, 500 mM NaCl, 0.2% (v/v) β-mercaptoethanol) using a sterile fitted plastic disposable pestle in an Eppendorf tube (1.5 mL), under sterile condition. Ten per cent SDS (6 µL) was added to the ground powder, and vortexed and incubated at 65°C in the water bath for 10-30 min. Then chilled 5 M potassium acetate(170 µL) was added and mixed well by inverting the tubes and centrifuged for 10 min at14000 rpm. The supernatant was then transferred into another sterile tube and 4 µL RNAse A (10 mg mL^{-1}) was added. The solution was again mixed well by inverting the tube and then incubated for 30 min at 37°C. To the mixture, 400 µLisopropanol was added and mixed well and then the tubes were incubated for 20 min at -80°C at water bath, to precipitate the DNA. The frozen solution was thawed for 20 minutes and centrifuged for 15 min at 14000rpm. The pellet was washed with chilled 70% ethyl alcohol, air dried for 30-40 min, redissolved in 50 µL sterile distilled water and incubated for 5 min at 65°C. The DNA was quantified by measuring its absorbance at 260 nm using Nanodrop® ND-1000 spectrophotometer and the DNA solution was stored at -20°C.

2.2.7.2 PCR analysis

The transformed lines of INRA 717 hybrid aspenplantletswere confirmed by PCR using specific primers for the hygromycin resistance gene *Hpt11*.PCR amplifications were performed on an Eppendoff thermocycler with the following amplification conditions. 1 cycle at 94°C for 5 min, 40 cycles at 94°C for 40s, 50°C for 40s, and 72°C for 1.5 min, followed by 1 cycle at 72°C for 5

min. The amplified DNA fragments were subjected to 1% agarose gel electrophoresis (60 V; 90 min) with 0.5X TBE buffer, and visualized by SYBR safe.

Rooted plantlets of the PCR-positive transformed INRA 717 hybrid aspenclones were transferred into sterile container containing sterile soil mix made up with peat and vermiculite (2:1, by volume) and sterile water was added. To maintain high humidity and to minimize desiccation, the plantlets were covered with transparent plastic bags and carefully transferred to the growth chamber and allowed to grow with a 16-h photoperiod at 24/18°C (day/night). Time of exposure of the plant to the environment was gradually increased everyday for two weeks. When new leaves started to grow, the plantlets were allowed to grow under growth chamber conditions independently. Wild-type *Populus tremula* × *Populus alba*(clone 717-1B4) plants and transgenic lines were propagated from rooted cuttings in soil mixture made up of peat and vermiculite (2:1 ratio) and grown in growth chambers at the University of Alberta. The plants were grown individually in 1.5 gallon pots and watered six days a week. The plants were fertilized weekly with 0.5 g/L of commercial fertilizer 15-30-15 (N, P, K) until 5 weeks for root growth and then1.0 g/L of 20-20-20 (N, P, K). They were treated biweekly with Thrips Eliminator (Applied BioNomics, Sidney, BC, Canada), a biological control for thrips.

2.2.8 Gene expression analysis of *PtdPIP2;5* among the transgenic lines

2.2.8.1 Growth conditions for poplars

Four week-old rooted cuttingsof *Populus tremula* × *Populus alba*INRA 717wild type and transgenic lines,were grown in pots containing a peat vermiculite mixture (2:1) in the growth room, under 16-h photoperiod with approximately 350 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR), 24/18°C (day/night) temperatures and 60% RH.

2.2.8.2 Harvesting of leaves

The 2nd and 3rd fully expanded leaves were harvested from each plant and wrapped together in aluminum foil. Tissues were immediately frozen in liquid nitrogen, and kept at -80°C until analysis.

2.2.8.3 Total RNA extraction

The frozen leaf samples stored at -80°C, were individually ground to a fine powder using sterile mortar and pestle, under sterile condition. The mortars and pestles, spatulas and the conical tubes were sterilized and chilled with liquid nitrogen. Ground sample powder was quickly transferred to the 2.0 mL Eppendorf tubes up to 0.25 mL line, closed and kept back in liquid nitrogen while the rest of the powder was kept in 15 mL sterile falcon tube (United Scientific Supplies, Inc. Waukegan, IL, U.S.A.). Then the samples were stored at -80°C again until RNA extraction. Total RNA was extracted using CTAB extraction protocol (Chang et al. 1993) with some modifications. In a labelled 15 mL Falcon tube (United Scientific Supplies, Inc. Waukegan, IL, U.S.A.),12mL of hexadecyl trimethyl ammonium bromide (CTAB) buffer (2% CTAB; 2% polyvinylpyrrolidone (PVP, MW 30000); 100 mM Tris-HCI pH 8.0; 25 mM EDTA; 2.0 M NaCI; 0.5 g L^{-1} spermidine) and 240µL of β-mercaptoethanol were added and mixed, just before the RNA extractionand kept at 65°C in a water bath. Eppendorf tubes(2.0 mL) containingground leaf sample from -80°C freezer, werebrought in liquid nitrogen and750µL of the above CTAB + β-mercaptoethanolmixture was added and votexed well to mix and the tubes were kept in the water bath at 65°C for 10 minutes with periodic vortexing. Then 600 µL of chloroform:isoamyl alcohol (24:1) mix was added to each eppendorftube and vortexed vigorously. After 10 minutes, the samples were centrifuged for 15 min at 14000 rpm in a Beckman J2-21M/E refrigerated centrifuge equipped with a JA-20 rotor (Beckman Coulter,

Brea, CA, USA). The supernatant was carefully transferred to a sterileeppendorf tubeusing a 10 mL sterile pipette. Chloroform:isoamyl alcohol addition, incubation at 65°C for 10 minutes, centrifugation and transfer of the supernatant were repeated once again to the supernatant. One-third of the sample's recovered volume of chilled 10 M LiCI was added to the aqueous phase and mixed gently by inverting the tubes for five times andthe tubes were incubated at -20°C for 60 minutes in order to precipitate the RNA. Then the tubes were centrifuged at 14000 rpmfor 15 minutesat 4°C in a Beckman JA-20 rotor to pellet the RNA and the supernatants were discarded. The pellets were washed by adding 800 µL of 80 % pre-chilled ethanol, briefly vortexed and centrifuged again at 14000 rpm at room temperature for 5 minutes. Supernatant was removed and the pellet was centrifuged again at full speed for 5 min. The pellet was air dried for 10 minutes and resuspended in sterile RNase-free baxterwater. Total RNA was dissolved in 20µL of sterile RNase-free water and quantified, at 260nm wavelength with a NanoDrop ND-1000 spectrophotometer. The ratio between A260 and A280 was also recorded to determine the purity of the RNA sample to know whether it was contaminated with protein or not. The RNA quality was determined by running 1µL of total RNA from each sample on a 1% agarose gel (0.5X TBE) for 45 minutes with 60V current and with 0.5X TBE buffer, and visualized by SYBR safe. Total RNA was aliquoted and stored at -80°C.

2.2.8.4 DNase treatment and first strand cDNA synthesis

Traces of DNA found in the prepared total RNA were removed by the DNase I treatment. Two µg of total RNA of each sample was mixed with 1 µLof RNA free DNAase I (New England Biolabs Inc. Ipswich, MA, USA) and 1 µLof 10X DNase I reaction buffer (NEB). Total volume was made up to 10 µLby the addition of sterile RNase-free water. The mixture was incubated at 37^{o}C for 15 min.DNAase enzyme was inactivated by adding 1µL of 25mM EDTA and

incubated at 65°C for 10 min. DNase inactivated sample (11µL) was added with 1 µL of 50 µM oligo-dT_{23}VN (Integrated DNA Technologies, Coralville, IA, USA) and 1 µL of 10 mM dNTP (Fermentas, Burlington, ON, Canada) and the reaction volume was made up to 15 µL by the addition of autoclaved RNase-free water. This reaction was done by incubating the mixture at 70^{o}C for 5 min in the thermocycler and quickly chilled on ice for one minute. DNA denatured sample (15µL) was added with 1 µL of SuperScript™ II Reverse Transcriptase (SSII RT; Invitrogen, Carlsbad, CA, USA),5 µL of 5X First-Strand Buffer,2 µL of 0.1 M DTT and1 µL of RnaseOUT (40U/µL)and the reaction volume was made to 25 µL by the addition of sterile RNase-free water. The contents were very well mixed by inverting the tubes for 10 times gently and incubated at 42^{o}C for 1 h and then the reaction was inactivated by heating at 70°C for 10 min.First strand cDNA was stored at -20°C. For the qRT-PCR assays, cDNA was diluted by mixing 25 µL of cDNA with 150 µL of autoclaved water.

2.2.8.5 Reference gene selection for qRT-PCR

A literature search for poplar experiments and Almeida-Rodriguez et al.(2010) helped to select the candidate reference gene. Candidate reference genes already used in *Populus* were elongation factor 1-alpha (EF1-a), transcription factor 5-A (TIF5A) and ubiquitin 10 (UBQ10). Among these reference genes, transcription factor 5-A (TIF5A) was selected and used as the reference gene in this study,because ofits constant stable expression at different experimental conditions compared to the other twogenes in the previous studies in poplar (Almeida-Rodriguez et al. 2010) and *A. thaliana* (Czechowski et al.2005). qRT-PCR primers that Almeida-Rodriguez had developedfor TIF5Awere used.

2.2.8.6 Preparation of standard curves

cDNAs encoding the *PIP2;5* and the transcription initiation factor 5-A (TIF5A) were amplified from pGEM-T easy vectors using M13F (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3') and M13R (5'-AGC GGA TAA CAA TTT CAC ACA GG-3') universal primers. Standard curves were prepared by serial dilution of the originally quantified amplicon and the concentration ranges from 4 x 10^1molecules / µL to 4 x 10^8molecules / µL. The quantity of amplicon cDNA in each well at the end ranged from 1 x 10^2 to 1 x 10^9molecules.

2.2.8.7 qRT-PCR assay

The 2X qRT-PCR Master mix is prepared and distributed by the Molecular Biology Service Unit (MBSU), Department of Biological Science, University of Alberta, Edmonton, AB, Canada. The contents of the Master mix are Tris (pH 8.3), KCl, $MgCl_2$, glycerol, Tween 20 (Applied Biosystems), DMSO, dNTPs, ROX (Invitrogen, Normalizing dye), SYBR Green (Invitrogen, Detection dye) and the Taq polymerase-Platinum Taq (Invitrogen). The qRT-PCR mix was prepared with 5 µL of 2X qRT-PCR Master mix, 2.5 µL of a mixture of the forward and reverse gene-specific primer (1.6 µM each) and 2.5 µL of cDNA (diluted ten-fold following synthesis and RNase treatment). The qRT-PCR assay was performed on the 7900 HT Fast Real-Time PCR System (Applied Biosystems) in 384-Well Clear Optical Reaction Plate with Barcode. Reagents and cDNA were pipetted in the 384-well plate by using the Biomek 3000 Robot (Beckman Coulter). Biomek 3000 Robot created three technical replicates for each sample. Expression was determined relative to translation initiation factor *TIF5A* (means ± SE, n = 3). There were three stages in the thermal profile. The first stage was one cycle of 95^{o}C for 2 min. The second stage was 40 cycles of 95^{o}C for 15 s and 60^{o}C for 1 min. In the 2nd stage, the data was collected at the end of each cycle. The third stage was a dissociation cycle of 95^{o}C for 15 s, 60^{o}C for 1 min, 95^{o}C for 15 s and 60^{o}C for 15 s. The dissociation cycle creates a dissociation curve based on the rate of change in fluorescence as an effect of temperature. This is used to detect nonspecific amplification and primer

dimer formation. Results were analyzed using SDS2.3 (Applied Biosystems). The Ct values for the serial dilution points were correlated with their known concentration (copy number of DNA molecules). According to this, a linear regression was calculated for each gene. The number of molecules for each cDNA was calculated from the corresponding Ct value, in the linear regression of the serial dilution. At first the average number of molecules from the three technical replicates was determined.This was done for each of the biological replicates for both the target and the reference genes. Then the expression levels of different transgenic lines and wild-type were determined by the ratio of target gene quantity mean over reference gene quantity mean and this was used for analysis.

2.3 Results

2.3.1 Generation of transgenic poplar

Transformation of INRA clone 717 explants with the pCAMBIA1305.2 vector containing a hygromycin selectable marker enabled selection of putative transformants by adding this antibiotic to the media. When growing on the hygromycin added shoot elongation medium, out of 1678 putatively transformed explants, 286 shoots showedelongated shoots. When the shoots were 3-5 cm long, they were transferred to hygromycinadded root induction medium containing IBA (Table 2.3) for the purpose of selecting the transformed lines during root development.It was assumed that the shoots that developed roots in the hygromycin added media were most likely transformed. When growing on the hygromycin added root induction medium, out of these 286 shoots, only 37 shoots showed root induction. Among the rooted shoots, rapid root growth was observed in some plants. The shoots that did not develop roots on the root induction medium showed comparatively slower growth than the rooted shoots. These could be ‘escapes’ that had survived by showing resistance to hygromycin. DNA was extracted from the leaves of all the rooted plantsand PCR was done using primers targeting the hygromycin resistance gene

to confirm the presence of the transgene. Twelve positive lines among the rooted plantlets were detected and confirmed as transformed lines (Figure 2.3, 2.4, Table 2.4).

2.3.2 Molecular analysis of gene expression in transgenic lines

In transgenic hybrid aspen lines, transcript levels of *PIP2;5*mRNA were analyzed by qRT-PCR. Because of the very high sequence similarity between the *PtdPIP2;5* transgene and the *PtaPIP2;5* endogenous gene, the primers used for qRT-PCR amplified cDNAs representing both the transgene and the endogenous gene. RNA was extracted from the leaves of all of the transgenic poplar lines and wild-type plants. Among the transformed lines, two lines exhibiting the highest expression of *PIP2;5*(the sum of the endogenous *PtaPIP2;5* and transgene *PtdPIP2;5,* 121, 31) were selected (Figure 2.5). These lines were multiplied by rooted cuttings, to generate *ex vitro* plants for experimentation. The level of expression of the *PIP2;5* varies among the transgenic lines. A range of *PIP2;5*gene expression levels was detected in transgenic lines, including plants expressing high (Transgenic lines over-expressing *PtdPIP2;5*121,31 and 452), medium (Lines 115,1151,147,321,341,372 and 441) or low levels (Lines 932 and 941). Wild-type plants yielded the expression of only the endogenous *PtaPIP2;5*. The expression of the reference gene *TIF5A* was not significantly different ($p - 4.09\times10^{-3}$)across the treatments.

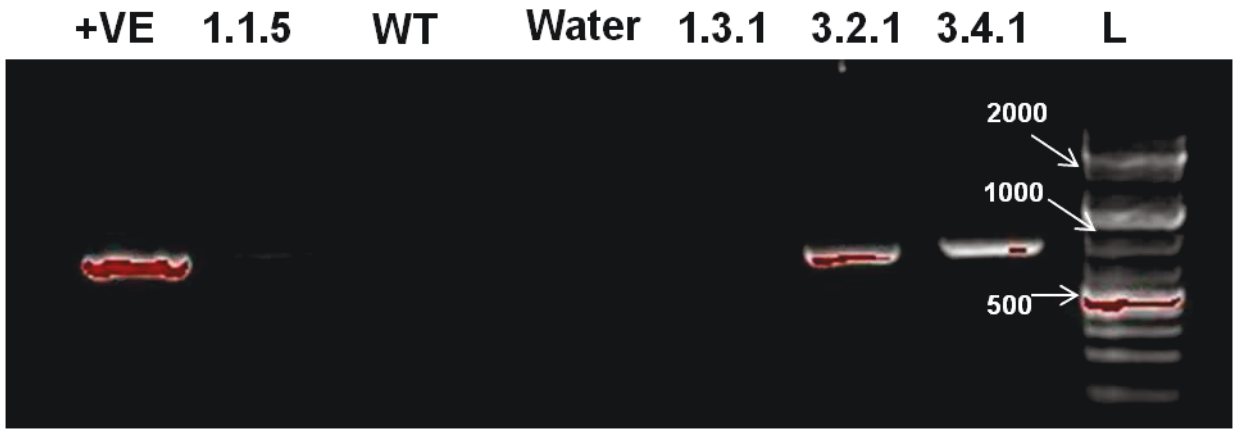

Figure 2.3: Gel showing the amplified PCR fragments of the genomic DNA of the rooted plantlets of INRA 717 clone transferred to growth chamber, using hygromycin resistant gene primers (Expected size: 865 bp). Purified pCambia 1305.2plasmid was used as a positive control. Lines 121 and 31 are transformed and lines 115 and 131 are escapes. + ve – Positive control (Plasmid), WT - Wild-type, L- DNA Ladder.

Table 2.4: Transformation summary of *Populus tremula×P. alba* INRA Clone 717 1B4 transformed by the co-cultivation method of *Agrobacterium*-mediated transformation. Fully expanded second and third young leaves from in vitro plantlets were used as explants

Total number of explants	Number of explants forming callus	Number of explants forming shoots	Percentage of regeneration (%)	Number of explants surviving on hygromycin	Number of lines containing transgene shown by PCR	Transformation frequency (%)
1678	713	286	17.04	37	12	0.71

$$\text{Percentage of Regeneration (\%)} = \frac{\text{No of explants formed shoots} \times 100}{\text{Total number of explants}}$$

$$\text{Transformation Frequency (\%)} = \frac{\text{No of transformed lines} \times 100}{\text{Total number of explants}}$$

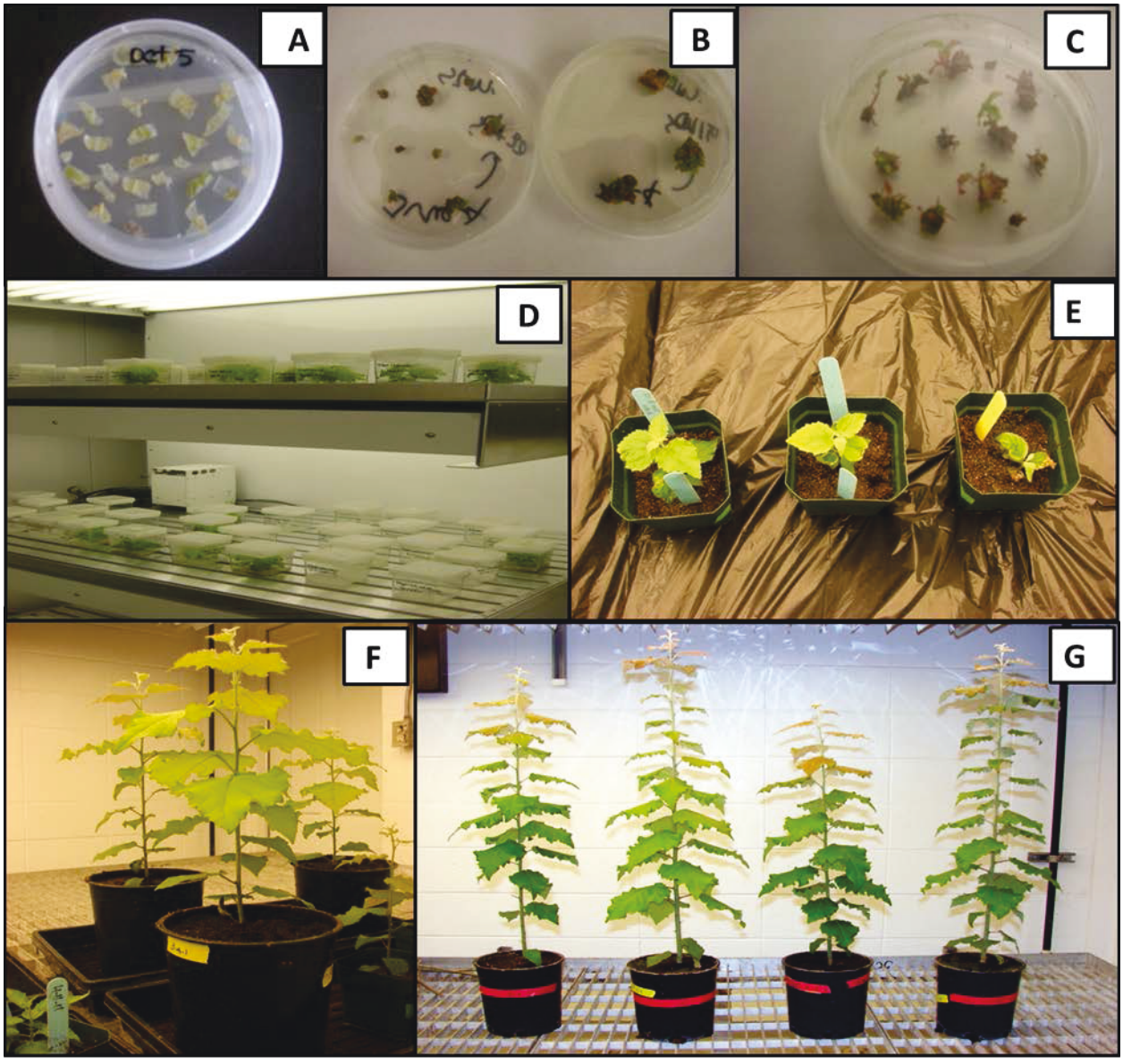

Figure 2.4: Different stages of the poplar hybrid 717 transformation process.

A)*Agrobacterium*-co- cultivation of leaf discs of hybrid poplar INRA 717 clone. B) Induction of shoot buds from leaf discs on the SIM medium. C) Development of shoot bud clumps on the SEM medium. D) Elongated shoots rooted on RIM medium. E)The young seedlings transferred to soil mixture before transferring to the growth chamber in polythene bags.F)Transgenic plants in soil mixture after 25 weeks. G) Growth of plants after 29 weeks.

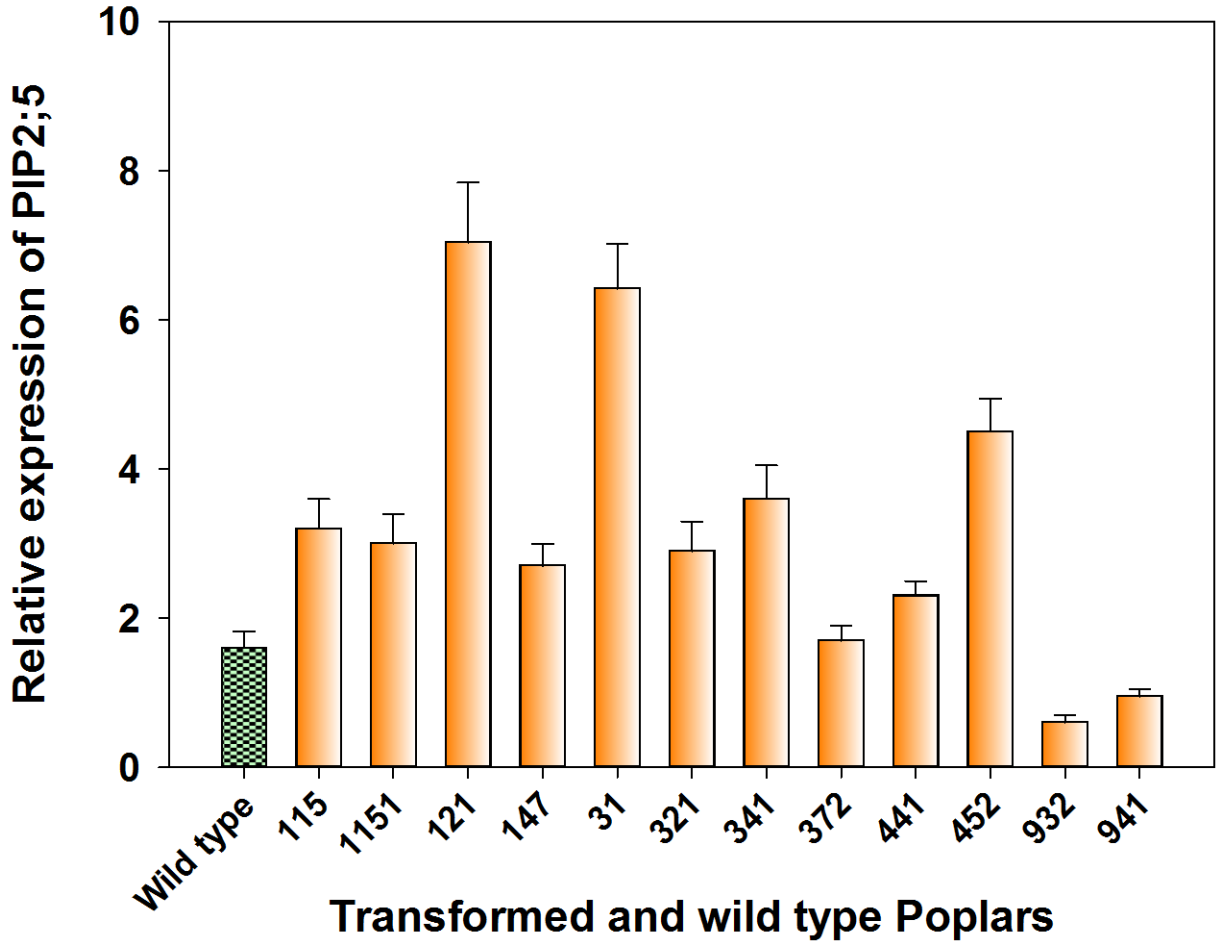

Figure 2.5: Relative transcript abundance measured by qRT-PCR of *PIP2;5* in 2nd and 3rd fully expanded leaves of 15 weeks old 12transformed lines and wild-type hybrid poplar INRA 717 growing in soil mix in the growth chamber. Expression was determined relative to translation initiation factor *TIF5A* (Means ± SE, n = 3).

2.4 Discussion

Leaf discs from the lines of *Populus tremula* × *P. alba*, INRA clone 717 (Levée et al. 2009) were co-cultivated with *Agrobacterium tumefaciens* carrying the binary vector pCAMBIA. The vector contained a hygromycin resistance gene (hptII) and the selectable marker. The *P. tremula* × *P. alba* INRA clone 717 was chosen because it is highly amenable to *Agrobacterium*-mediatedtransformation (Leple et al.1992).The Murashige and Skoog medium (Murashige and Skoog, 1962)was modified with supplementary 6-benzylaminopurine and naphthalene acetic acid to achieve a high regeneration efficiency ofthis clone.

Themaize ubiquitin (Ubi-l) promotor has been shown to be highly active in monocots. Therefore the constructs with ubiquitin are useful for higher gene expression to facilitate efficient transformation.Further, theUbi-l promotor has been used in expression studies to drive expression of reference reporter genes and in transgenic plants,it provides expression of some protein products which are biotechnologically important(Christensen and Quail, 1996).35S promoter ofthe cauliflower mosaic virus (CaMV) is one of the widely used promoters in monocots but its strength is comparatively less in dicots and it is sometimes inactive in some plant parts, e.g. pollen (Christensen et al. 1992). The Ubi-1 promoter is a very useful alternative to the CaMV 35S as it isvery active in young metabolically active tissues of woody plantsand significantly more efficientand strongerthan the CaMV 35S promoter when compared directly in angiosperm (McElroy et al. 1990; Christensen et al. 1992; Cornejo et al. 1993; McElroy and Brettell, 1994).

A variety of efficient and improved transformation systems for poplars have been followed in several research reports (Leple et al.1992; Tsai et al.1994; Tzifra et al. 1997; Han et al.2000).

Efficiency of transformation in poplars depends on multiple factors such asthe explant chosen, *Agrobacterium* strain used, physiological conditions for regeneration, hormones, the protocol followed and the combination of all (Han et al.2000, Cseke et al. 2007). The transformation efficiency varies greatly among the poplars and hybridsand range from 0.7 to 60 percent. The transformation efficiency of*P. nigra* and *P. trichocarpa x P. deltoides* hybrids falls between 3.5 and 20% (Confalonieri et al.1995; Han et al.2000) and*Populus x euramericana* and *P. alba x P. grandidentata* showed a very lowtransformation efficiency (0.7 %)(Liang et al.2001; McCown et al.1991).

Even though an optimised protocol that has been modified to improve the transformation efficiency was used in this study, the transformation efficiency was low.The reason for getting a lower transformation efficiency might be due to the continuous infection of *Agrobacterium* and the increased number of washings of ex plants to get rid of the bacterial growth.Acetosyringine was added to the *Agrobacterium* cultureas indicated in the protocol, to further increase the transformation efficiency, before the co-cultivation with poplar leaf discs. Phenolics exudatesof the plant wounded site regulate the *Agrobacterium VIR* genes that induce the transfer of the TDNA(Stachel et al.1986; Rogowsky et al.1987). Acetosyringone, aphenolic signal molecule, is an important component of the wound exudates improves the transformation efficiency of leaf explants in *Populus nigra* (Confalonieri et al.1995). The poplar regenerants were selected on media containing hygromycin and passed through a secondary hygromycin screen to trap any escapes.

The PCR with the hygromycin resistance gene (*Hpt11*)was usedto detect the putative transformants among the rooted regenerants. To exclude contamination in PCR-generated data,

sterile waterwas used as a negative control in the analysis. PCR with hygromycin resistance gene primers helped to identify the transgenic poplars. PCR was used to confirm the integration of the transgene with the plant genomic DNA, butthe regular PCR cannot be used to identify how the transgene is expressed. Therefore, the quantitative RT-PCR technique was used to study the expression of the *PIP2;5* and related AQP genes in both the transgenic and wild-type lines. Even though the expression of the selected AQP genes was quantified using qRT-PCR, there were some limitations in this approach.An important limitation in qRT-PCR technique was the inability to differentiate between transcripts corresponding to the transgene *PtdPIP2;5* and the endogenous gene *PtaPIP2;5* because of the sequence similarity. The qRT-PCR technique was used to quantify the gene expression as the traditional methods such as Northern blot or PCR products on a gel are unreliable and not very precise therefore they were not used.

Transcript abundance corresponding tothe transgene and other *AQP* genes varied among the different transgenic lines. The variation in the expression of transgene is often related to position effect and itis due to thevariation in the structure and function of the chromatin into which the transgene isadded (Gelvin, 1998). Sometimes expression of the transgene might be missed and this is due to gene silencing. If a functional transgene is found in the genome but the expression of the transgene is turned off then this phenomenon is calledgene silencing. Gene silencing is the ability of a cell to prevent the expression of a certain gene and it can occur during either transcription or translationwhere transgene RNA will fail to accumulate after transcription (Hood, 2004). Levels of transgene expression in the transgenic plants vary among independent transformants. There were differences in the gene expression profile between the *PtdPIP2;5ox* lines used and this may be due to variation that occursalone or in combination withthe plant genome after transformation. Loss of expression of the transgene, rearrangements in the copy

number of the transgene, loss of a transgene, random integration site of thetransgene in the genome, position effect and rearrangements caused by a foreign gene in the host genome are some of the reasons for the differences in the expression level of the transgene and other related genes among the different transgenic lines. The site of integration of the gene into the plant genome is random and nonspecific. Therefore the integration of a new gene might alter other genes and their expression might get affected(Ahuja, 1988). The differences in the expression level between the twelve *PtdPIP2;5ox* plants may be due to functional differences that arose due to the differences in the transgene ligation in the genome (Almeida-Rodriguez et al. 2010) or the differences in tissue distribution (Alexandersson et al. 2005).

Inoculated explants were transferred into a series of growth media containing cefotaxime and hygromycin. Cefataxime is a broad spectrum antibiotic and it affect the peptidoglycan synthesis in bacterial cell walls, thus inhibiting cell wall biosynthesis and killing the bacteria(Mittal et al. 2009). Once the transformation step was over, cefotaxime was used to remove the *Agrobacterium* completely. Until reaching a point of no *Agrobacterium* on the growth media, explants were washed with cefotaxime added liquid MS solution every week. The *Agrobacterium* infection was very severe until shoots were transferred onto the RIM and washing of the explants using cefotaxime added MS solution was needed until bacterial growth was completely eliminated. The excessive and frequent usage of concentrated cefotaxime on the explants, was one of the main reason of getting less number of transformants resulting in low transformation efficiency. Hygromycin is an antibiotic that prevents translocation thus inhibits polypeptide synthesis of bacteria (Kauffman, 2009). Hygromycin was added to each growth media to confirm the selection of transgenic lines. As the construct has hygromycin resistant

gene, the transgenic bacteria can resist and grow well on the media that is incorporated with hygromycin.

2.5 Conclusion

Gene *PIP2:5* cloned from *Populustrichocarpa* × *Populus deltoides* H11-11 was selected as it was previously confirmed as a water-transporting aquaporin and also showed increased expression under water limitation. Rooted plantlets of the twelve transformed lines of *Populus tremula* × *P.alba*INRA Clone 717 1B4over-expressing water-transporting AQP*PtdPIP2;5* were confirmed by performing conventional PCR, from the leaves of all the rooted plants, using primers targeting the hygromycin resistance gene in the pCambia1305.2 vector. Among the transformed lines, quantitative RT-PCR was used to identify two lines (Line 121 and line 31) exhibiting the highest expression of *PtdPIP2;5*. These lines were selected to generate *ex vitro* plants for physiological experimentation. A gene that expressed at relatively high levels in wild-type was picked for transformation, so even if comparatively good expression of the transgene is observed, it does not lead to a large fold change increase.

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CHAPTER 3

3. Physiological responses of hybrid aspenclone INRA 717 over-expressing *PIP2;5*aquaporin to short term low root zone temperatures

3.1 Introduction

Low soil temperature is one of the major environmental factors that affect growth and yield of the plants growing in the temperate and boreal region (Wan et al. 2001). Cold sensitive plants show low root water uptake ratesat low temperatures relative to cold tolerant plants. The reductions in water uptake, transport and gas exchange are the principal factors responsible for lower growth of plants at low soil temperatures (Wan et al. 1999;Lee et al. 2005a). Reduced water flux at low temperatures may be due to higher viscosity of the water (Muhsin and Zwiazek, 2002), inhibition of hydraulic conductivity of root cells (Wan et al. 2001; Lee et al. 2005a, b; 2012) and/orreduced cellular metabolic rate (Kamaluddin and Zwiazek, 2001). Plant treatments that facilitate root water uptake and/or reduce plant water loss improve water balance at low root temperature (LRT) (Aroca et al. 2005; Lee et al. 2008). LRT showed no or very low effect on cell hydraulic conductivity in roots of low-temperature-tolerant *Cucurbita ficifolia*(Lee et al. 2008) and this shows that the ability of plants to maintain the transmembrane water flow is linked to chilling tolerance. When the root temperature drops below a critical level (15°C), then the root hydraulic conductivity(L_p) will decline very fast (Murai-Hatano et al. 2008).Hydraulic conductivity and leaf water potential of root cortical cells decreased with decreasing root temperature (Kuwagata et al. 2012;Lee et al. 2012). These results show that the ability of water uptake by theroots is a key factor for sound physiological activities and productivity of the plant under low temperature conditions.

Aquaporins (AQP), the water channel proteins, help to maintain plant water homeostasis in response to various environmental stresses (Maurel et al. 2002). AQP-mediated water transportis affected by the abundance of AQPs, subcellular trafficking and gating behavior (Aroca et al. 2005). Phosphorylation and dephosphorylation are the two main steps involved in the AQP opening and closing (i.e. gating) (Johansson et al.1996).Tetramerization (Chaumont et al. 2000), cytoplasmic pH (Tournaire-Roux, 2003, Sutka et al. 2005), divalent cations (Gerbeau et al.2002), phytohormones and reactive oxygen species (Luu and Maurel, 2005) are some of the other factors that control AQP gating and function. Extensive studies have been carried out on the effects of root water status andon the AQP function and regulation (Tournaire-Roux et al. 2003, Jang et al. 2004, Bramley et al. 2007, Murai-Hatano et al. 2008, Aroca et al. 2012, Lee et al.2012). Many of these studies concluded that environmental stresses significantly affectthe AQP abundance in roots and shoots. However, the functions and responses of plant AQP to transpirational water demand and LRT have not been well studied.

Plasma membrane intrinsic proteins (PIP) are a subfamily of AQPs that typically have the capacity to mediate water movement.*Arabidopsis* plants over-expressing *AtPIP1;4* and *AtPIP2;5* were subjected to LRT stress (10°C) and the role of AQPs on the growth rate and L_p of root cortical cells were studied (Lee et al. 2012). The study concluded that the over-expression of *AtPIP1;4* and *AtPIP2;5* in *Arabidopsis* alleviates the short term LRT effects on L_p in root cells. Further, it was concluded that the calcium dependent protein phosphorylation /dephosphorylation are involvedin the effect of low temperature on root water transport (Lee et al. 2012). Both *AtPIP1;4* and *AtPIP2;5* have been confirmed as water transporting AQPs (Alexandersson et al. 2005). More recently, Almeida-Rodriguez et al. (2010) demonstrated that the putative *Populus* orthologue of *AtPIP2;5* is also a functional water channel. The present

study has attempted to find answers to the questions concerning: 1)The role of poplar *PIP2;5*AQP in root temperature responses 2)Responses of poplar to different root temperatures 3)The expression of *PIP2;5* and 4) the other related AQP isoforms in poplar plants exposed to LRT.

To address these questions, we have generated *Populus tremula* × *Populus alba*INRA 717 1B4 linesconstitutively over-expressing *PIP2;5* aquaporin from*Populustrichocarpa* × *P. deltoides* H11-11, designated *PtdPIP2;5*. *PtdPIP2;5*was selected for this study as it was confirmed to be a major water-transporting aquaporin in *Populus*and because it showed increased expression under drought stress conditions (Almeida-Rodriguez et al. 2010). In the present study, we investigated short term effects of the transpirational demand and LRT on water uptake properties, physiological responses and AQP expression in theplantlets of the INRA 717 clone, as a step toward understanding the integrated mechanisms of stress resistance in poplars. The objective of the study was to compare the physiological responses and the AQP expression of the *PtdPIP2;5*-over-expressing lines(*PIP2;5ox*)with the wild-type clonesubjected to different low root temperatures. The following hypothesis wastested: Over-expression of *PtdPIP2;5* would increase water transport of the plant to maintain stable water status at low root temperatures and as aconsequence, high transpiration and photosynthetic rates would also be maintained.

3.2 Materials and methods

3.2.1 Plant material

Plantlets oftwo transgenic INRA 717 lines *PIP2;5ox1* and *PIP2;5ox2*(Line 121 and Line 31, Figure 2.5) and the wild-type were used in this study. The generation of these transgenic plants is described in Chapter 2. Plantlets for experimentation were vegetatively propagated from the primary transformants or wild-type by means of rooted cuttings of the stem segments. Sufficient amount of young stem pieces of 10-12 cm length with nodes and young leaveswere placed in small pots filled with a moist peat: vermiculite (2:1) mixture, after dipping in IBA powder (Sigma Aldrich). Pots were placed in trays with adequate water and covered with plastic to create a humid atmosphere for rooting. After 15 days of growth in the growth chamber, the plastic was gradually opened up to expose the plants to the growth chamber condition. After several weeks, the cuttings were transplanted into four-liter pots with the same mixand were grown under controlled conditions. All plantletswere grown for threeweeks reaching a height between 30 and 40 cm and 3-6mm in stem diameter at the root collar before the temperature treatment was applied. The plantlets were watered six times per week and fertilized weekly with 1g L^{-1} NPK (15:30:15) commercial fertilizer, before the temperature treatment experiment. Environmental conditions in the growth chamber room were maintained at 24/18°C (day/night) temperature, $65 \pm 10\%$ relative humidity and 16h photoperiod with 350 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the top of the plantlets provided by the full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ONTARIO, Canada).

3.2.2 Low root temperature treatments

3.2.2.1 Solution culture and temperature treatment

Six plantlets of each two transgenic lines (*PIP2;5ox1* and *PIP2;5ox2*) and wild-type produced by rooted cuttings, were gently washed with deionized water and placed in the Hoagland's

mineral solution, kept in the 30 L circulating water bath (Thermomix BU coupled to Frigomix U, B. Braun Biotech International, Melsungen, Germany). A complete randomized design was used to test the effect of two treatments (temperature and plant type) on the responses of the plantlets. Twenty equal sized holes were made at regular intervals on flat styrofoam plates and these styrofoams were used to keep the plantlets chosen by random number table, in the solution culture. Foam plugs were fitted around the stems and inserted into the holes to hold the stems in place upright while the roots were immersed in solution, with the stems protruding through the holes. The plants (6 plantlets of each from transgenic lines *PIP2;5ox1* and *PIP2;5ox2* and wild-type) were grown at 20^{o}C for 3 weeks in the circulating water baths with aerated full strength modified Hoagland solution (Zhang et al. 2013) in three replicates. After 3weeks of growth at 20°C, the solution temperature was gradually decreased and maintained for 30 minutes at the desired temperature (20, 15, 10, 5 and again 20°C) while the shoots of the plantlets were exposed to high transpirational demand conditions (24°C day temperatures) and roots were made to expose to that desired solution temperatures. Styrofoamdirections were twisted to 180 degrees twice a week to minimize edge effects and the plants were inspected for the presence of mites and thrips, with any infected leaf being manually cleaned using cotton swabs. Hoagland's solution was changed every week and aerated continuously.

3.2.3 Measurements

3.2.3.1 Root hydraulic conductivity

Root hydraulic conductivity (L_p) of all the rootswasdetermined after 30 minutes of exposure of each tested temperature, by excising the root with approximately 2 cm of the stem remaining above the root and attached to the high pressure flow meter (HPFM, Dynamax Inc, Houston, TX, USA) using compression couplings and rings. Water was then forced inside stems at

gradually increasing pressure. The computer connected with HPFM recorded water flow rate (Q), the applied pressure (P) and computed root hydraulic conductance (K_r). To determine L_p, root volumes were determined for each root system using the volume displacement method and L_p was expressed on the root volume basis (Voicu and Zwiazek 2004). Activation energy (Ea) for L_p was calculated for all plants from the slopes of Arrhenius plots where the natural log of root L_p was plotted against the inverse of absolute temperature (T = 281–298 K), because: ln L_p =-Ea/R [1=T] (Wan and Zwiazek, 1999).

3.2.3.2 Gas exchange measurements

Stomatal conductance (g_s), net photosynthesis (P_n) and transpiration rates(E)were measured after 30 minutes, on the second fully expanded leaf of each plantlet using a portable open-flow infrared gas analyzer (LI-6400, Li-Cor Inc., Lincoln, NE) equipped with an incorporated auxiliary LI-6400B red/blue LED light source (chamber area = 6 cm^2). Light intensity was set at 350 μmol photon m^{-2} s^{-2} and reference CO_2 was set at constant 386 μmol mol^{-1}using the 6400-01 CO_2 mixer; air temperature was 21 ± 0.15°C and vapour pressure deficit (VPD) 1.3 ± 0.3 kPa. Sample and reference infrared gas analyzers were matched prior to measure every plant and data were logged every 30s during a 10 min period (Voicu et al. 2008).

3.2.3.3 qRT-PCR study for gene expression

Young roots of each plantlet were harvested after 30 minutes exposure to 20 and 5°C, flash frozen in liquid nitrogen,andmanually ground in a mortar and pestle in liquid nitrogen. Total RNA was extracted from the ground root samples of transgenic and wild-type plants using the Plant RNeasy extraction kit (Qiagen, Valencia, CA, USA). DNase I treatment was done to remove the possible DNA traces present in the samples with the total RNA. SuperScript™II

Reverse Transcriptase (SSII RT; Invitrogen, Carlsbad, CA, USA) was used to synthesize cDNA using the manufacturer's instructions. cDNA was diluted fivefold prior to use in real time PCR. qRT-PCR primers for *PIP1:1, PIP1:2, PIP2:1, PIP2:2, PIP2:3, PIP2:4* and *PIP2:5* previously designed and validated by Almeida-Rodriguez et al. (2010) were used.To compare the transcript abundance of the *PIP* genes,qRT-PCR was performed on a 7900 HT Fast Real-Time PCR system (Applied Biosystems) in 384-well clear optical reaction plate with barcode. Reagents and cDNA were pipetted in the 384 well plate by using the Biomek 3000 Robot (Beckman Coulter). PCR was carried out in a volume of 10 µl including a final concentration of 50 ng cDNA, 1× master mix containing 0.2 mM dNTPs, 0.3 U Platinum Taq Polymerase (Invitrogen), 0.25× SYBR Green and 0.1× ROX. There were three stepsin the thermal profile. The first step was one cycle of 95°C for 2 minutes. The second step was 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data was collected at the second step in each cycle. The third step was a dissociation cycle of 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds and 60°C for 15 seconds. The dissociation cycle resulted in a dissociation curve which helped to detect nonspecific amplification and primer dimers. Samples were subjected to auto Ct (cyclic threshold) for analysis, and dissociation curves were verified for each of the genes. Results were analyzed using SDS2.3 (384-well plates; Applied Biosystems). Expression was determined relative to translation initiation factor *TIF5A* (Means ± SE (n = 3)). After normalization of the transcript levels of the target genes using *TIF5A*as a reference gene, the expression level of each AQP gene in plants under low-temperature conditions (5°C) was calculated by comparing the expression level with plants exposed to 20°C root temperature. *TIF5A* expression did not change significantly across samples (p= 0.09) that were compared in this study.

$$X_{test}/X_{control} = 2^{\Delta\Delta CT} = 2^{(C_T X - C_T R)\,control - (C_T X - C_T R)\,test}$$

Here C_T,X is the threshold cycle of the *PIP2;5* and C_T,R is the threshold cycle of the *TIF5A*. Test refers to the test cDNA sample and Control refers to the control cDNA sample.

3.2.4 Statistical analysis

Statistical analyses were performed using R2.15.3 statistical software (R Development Core Team, 2011)($\alpha = 0.05$). The data sets were checked for the parametric assumptions of normality (Shapiro-Wilk test) and homogeneity of variances (Bartlett's test). When necessary to meet the assumptions of normality and homogeneity of variance, the data were transformed either by log transformation or square root transformation. The data were analyzed using ANOVA. Tukey's multiple comparison test was used to determine significant differences at $p \leq 0.05$.

3.3 Results

3.3.1 Temperature effects on root hydraulic conductivity (L_p)

At 20°C, there were no differences in the L_p values of the two transgenic lines and wild-type plants. Decreasing temperature from 20°C to 5°C, significantlydecreased the $L_p$in wild-type (p=0.05), but there was no significant effect of decreasing temperature on $L_p$in the transgenic lines. When the temperature was reduced to 10ºC, wild-type showed a significant reduction in L_p that was greater than that of the two transgenic lines. A similar observation was made when the temperature was reduced to 5°C. When the temperature was raised back to 20°C, *PtdPIP2;5ox*linesshowed significantly higher L_p values than the wild-type (Figure 3.1). Recovery of the L_p in the transgenic line*PtdPIP2;5ox1* at 20°C was faster than the wild-type.

3.3.2 Activation energy for root water transport

Activation energy (Ea) for L_p was 36.4 kJ mol^{-1} in the wild-type (Table 3.1). In both *PIP* over-expressionglines, Ea values for L_p were below that of the wild-type (13.6 and 22.2 kJ mol^{-1} Table 3.1).

3.3.3 Temperature effects on gas exchange properties

Stomatal conductance is the measure of the rate of passage of carbon dioxide entering for respiration or water vapor exiting for photosynthesis through the stomata.At 20°Croot temperature, net photosynthetic rate (P_n) was similar in the wild-type and transgenic *PIP2;5ox*lines (Figure 3.2). Decreasing temperature significantly decreased the $P_n$in wild-type compared tothe transgenic lines. When the temperature was reduced to 10^{o}C, there was a significant reduction in P_n in wild-type than the transgenic lines (p=0.05). When plants were exposed to 10°C for 30 minutes, *PIP2;5ox1*line showed significantly higher P_n than the*PIP2;5ox2* and wild-type. Both the transgenic lines showed significantly higher $P_n$than the wild-type, when the temperature was reduced to 10 and 5^{o}C. When the temperature was raised back to 20°C,P_n of the transgenic lines were higher than the wild-type and P_n of the transgenic line*PIP2;5ox1* was significantly higher than the wild-type. Recovery of the P_n in the transgenic line *PIP2;5ox1* at 20°C was faster than the *PIP2;5ox2* and wild-type. At 20°C root temperature, transpiration rate (E) was similar in the wild-type and transgenic *PIP2;5ox*lines(Figure 3.3). Decreasing temperature significantly decreased the Ein wild-type plants than the transgenic lines. When the temperature was reduced from 20 to 5°C, there was no significant difference in E between the transgenic lines and wild-typein any of the temperatures tested(Figure 3.3). However, E values at 5°C root temperature were comparatively higher in the transgenic lines then the wild-type. When the roots were transferred from 5°C to 20°C for 30 min, *PtdPIP2;5ox* transgenic lines showed improvedE values than the wild-type plants.

3.3.4 Water use efficiency (WUE)

At 20°C root temperature, WUE was similar in the wild-type and transgenic *PIP2;5ox*lines(Figure 3.4). WUE showed an increase at 10°C in both transgenic and wild-typelinesand then started to decline until 5°C. When the plantletss were treated at 5°C for 30 minutes, the WUE values for thewild-typewere lower than the transgenic lines. However WUE of all the plantlets were higherwhen the temperature was raised back to 20°C than at 5°C. At 5 and 10°C temperatures, the WUE values of the wild-type were lower than the *PIP2;5ox* lines.

3.3.5 PIP expression analysis

Primers previously validated as specific to *PIP1;1, PIP1;2, PIP2;1*, *PIP2;2*, *PIP2;3,PIP2;4* and *PIP2;5*were used to determine transcript abundance corresponding to these genes. Primers designed to amplify *PIP2;5* amplified both the endogenous *Populus tremula* x *P. albaPIP2;5* (*PtaPIP2;5*) as well as the *Populus trichocarpa* x *P. deltoidesPIP2;5* transgene (*PtdPIP2;5*). As such, transcript abundance corresponding to *PIP2;5* represents the sum of transcript abundance for both the endogenous *PtaPIP2;5* and transgene *PtdPIP2;5*. Transcript abundance corresponding to *PIP1;1, PIP1;2, PIP2;1*, *PIP2;2*, *PIP2;3*and*PIP2;4*did not significantly differ between wild-type and *PIP2;5ox* lines, nor did transcript abundance for these genes significantly differ between the 20°C and 5°C treatments. However, both over-expressing lines had previously been demonstrated to show considerably greater levels of *PIP2;5* transcript abundance than wild-type plants (see Chapter 2).Transgenic lines showed significantly higher expression of *PIP2;5* AQP at favorable growth temperatures and did not change when the roots of poplar plants were exposed to 5ºC for a short period of time (Figure 3.5). In this expression study, when the roots were exposed to 5°C for 30 minutes, the expression of *PIP2;5* remained significantly higher in transgenic lines (2.5 fold in *PIP2;5ox1* and 1.75 fold in *PIP2;5ox2*) than

the wild-type. The expression of the reference gene *TIF5A* was not significantly different (p - 5.7×10^{-16})across the treatments.

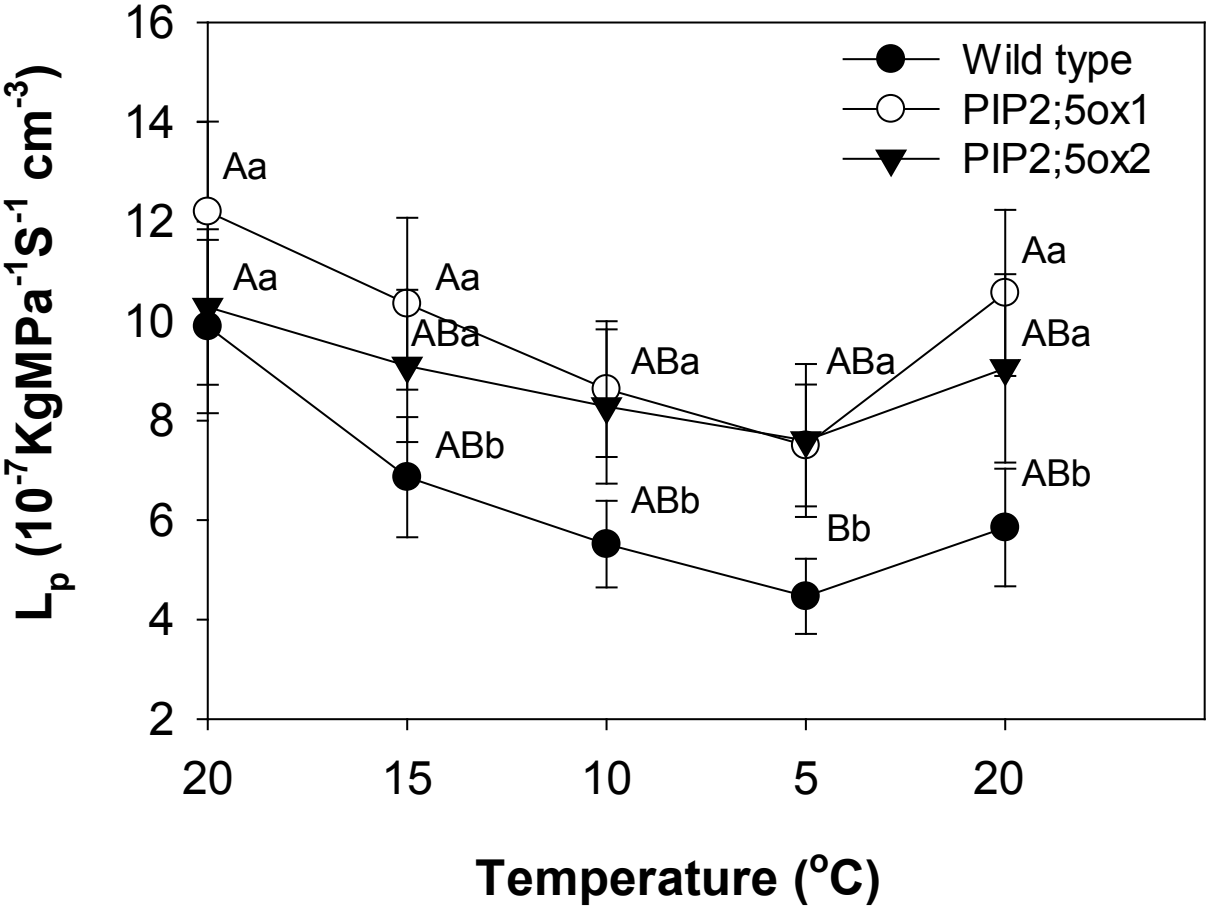

Figure 3.1: Root hydraulic conductivity (L_p) of *PIP2;5* over-expressing and wild-typeINRA 717 clonesexposed to root temperatures of 20, 15, 10 and 5°C for 30 minutes. Means ± SE (n = 6) are shown. Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, whilelower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

Table 3.1: Activation energy (Ea) for root water flow was measured at the temperature range of 281 to 298 K. Different letters for the wild-type and transgenic poplar linesindicate significant differences at P = 0.05. Values are means ± SE (n = 6).

Plant	Activation energy (Ea) (KJ Mol $^{-1}$)
Wild-type	36.4 ± 6.45 a
PIP2;5ox1	13.6 ± 2.02 b
PIP2;5ox2	22.2 ± 2.68 b

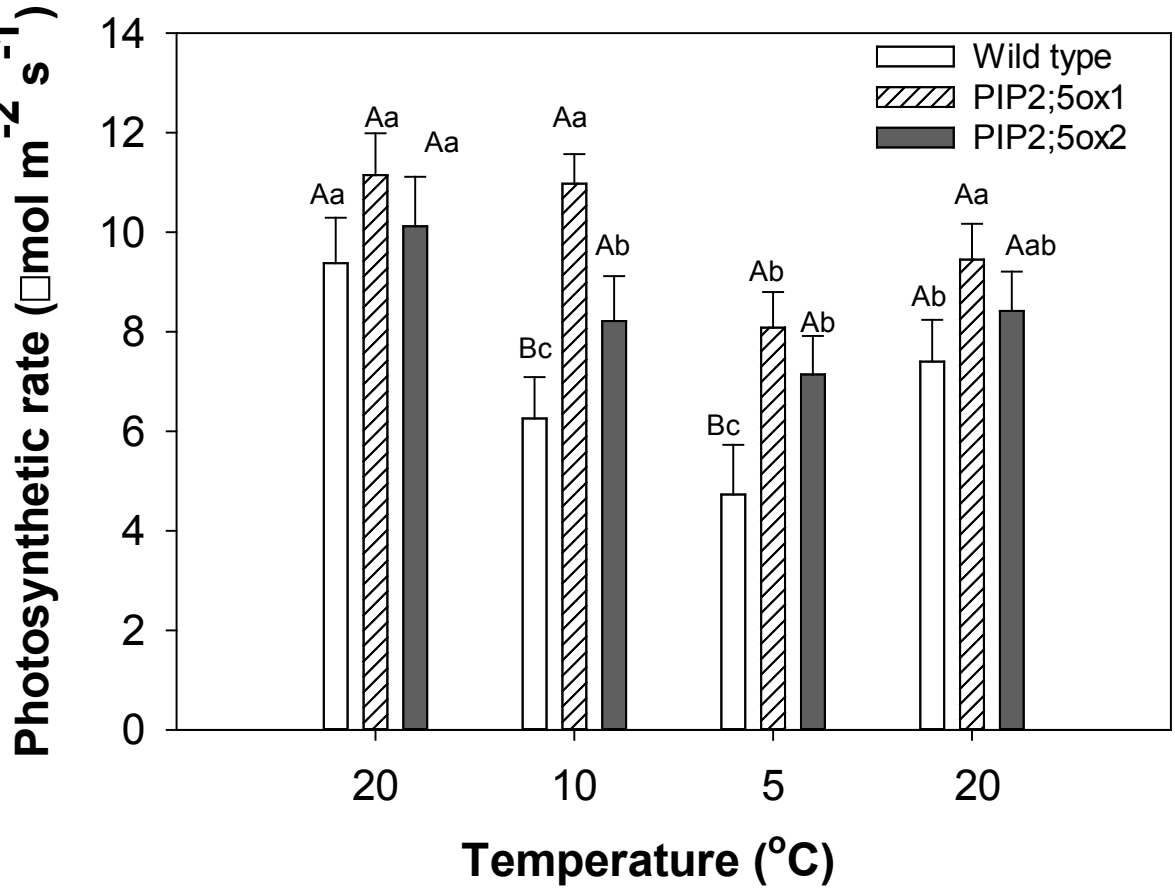

Figure 3.2: Net photosynthetic ratesof *PIP2;5* over-expressing lines *PIP2;5ox1*and *PIP2;5ox2* and wild-typeINRA 717 clonesexposed to root temperatures of 20, 10, 5°C for 30 minutes. Means ± SE (n = 6) are shown. Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

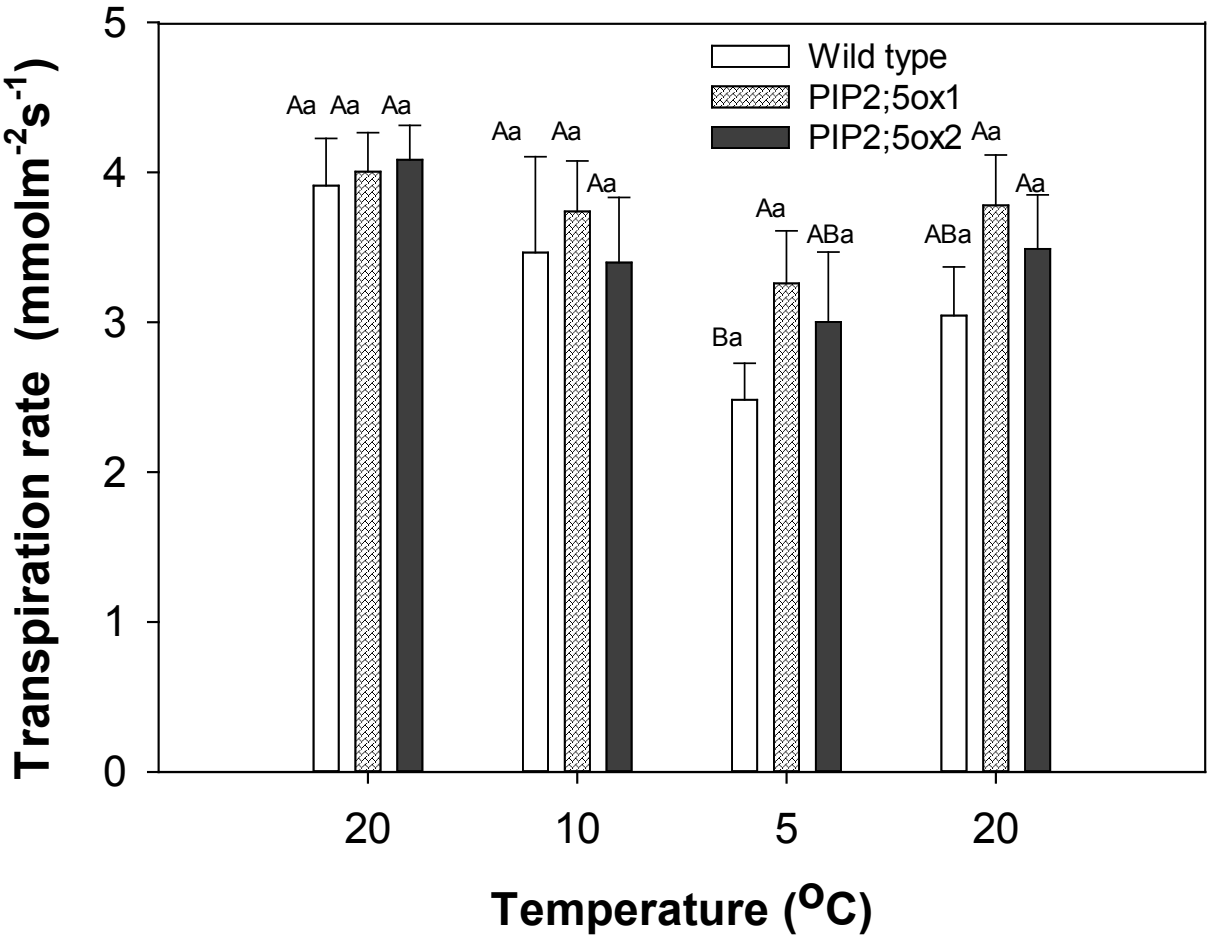

Figure 3.3: Transpiration rate of *PIP2;5* over-expressing lines *PIP2;5ox1*and *PIP2;5ox2* and wild-typeINRA 717 clonesexposed to root temperatures of 20, 10, 5°C for 30 minutes.Means ± SE (n = 6) are shown. Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

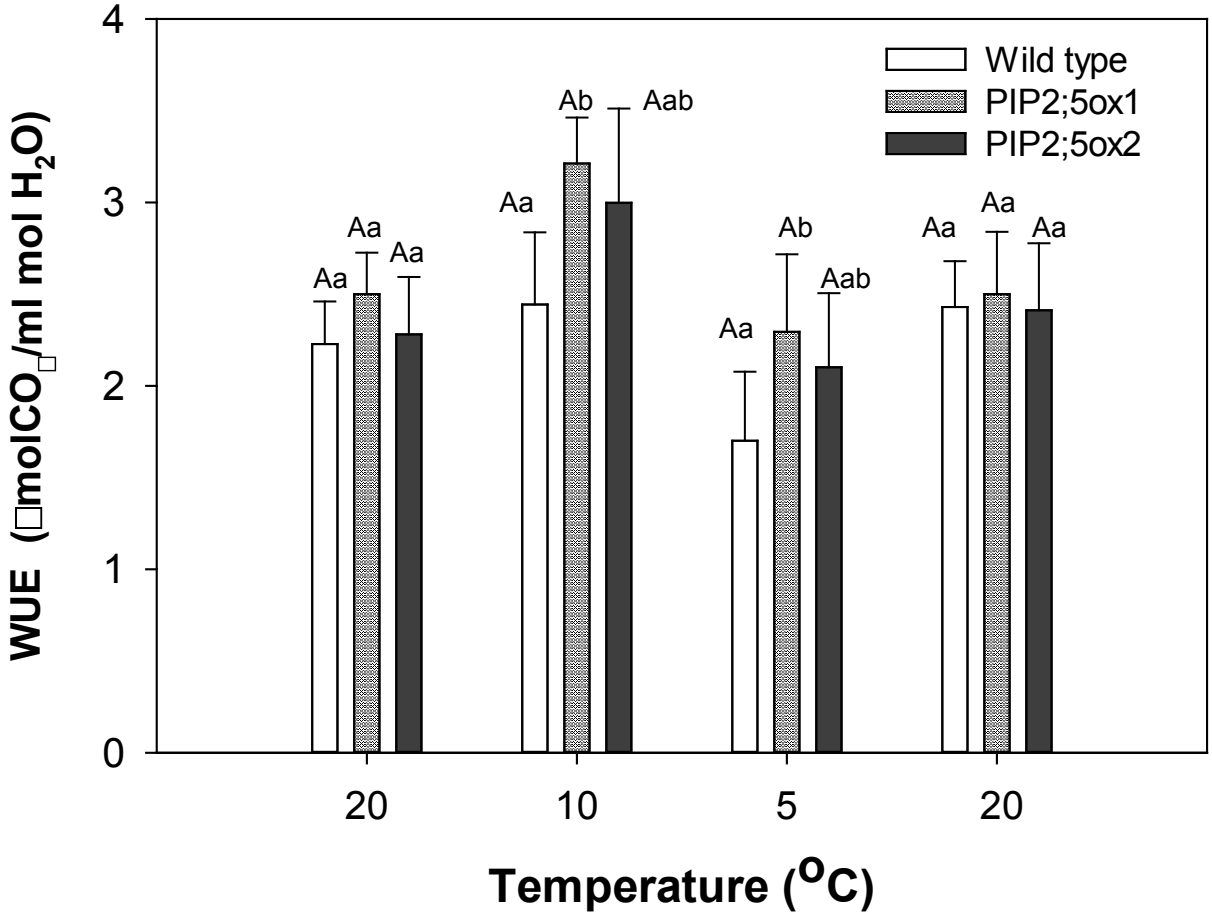

Figure 3.4: Water use efficiency (WUE) of *PIP2;5* over-expressing lines *PIP2;5ox1*and *PIP2;5ox2* and wild-typeINRA 717 clones exposed to root temperatures of 20, 10, 5°C for 30 minutes.Means ± SE (n = 6) are shown. Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

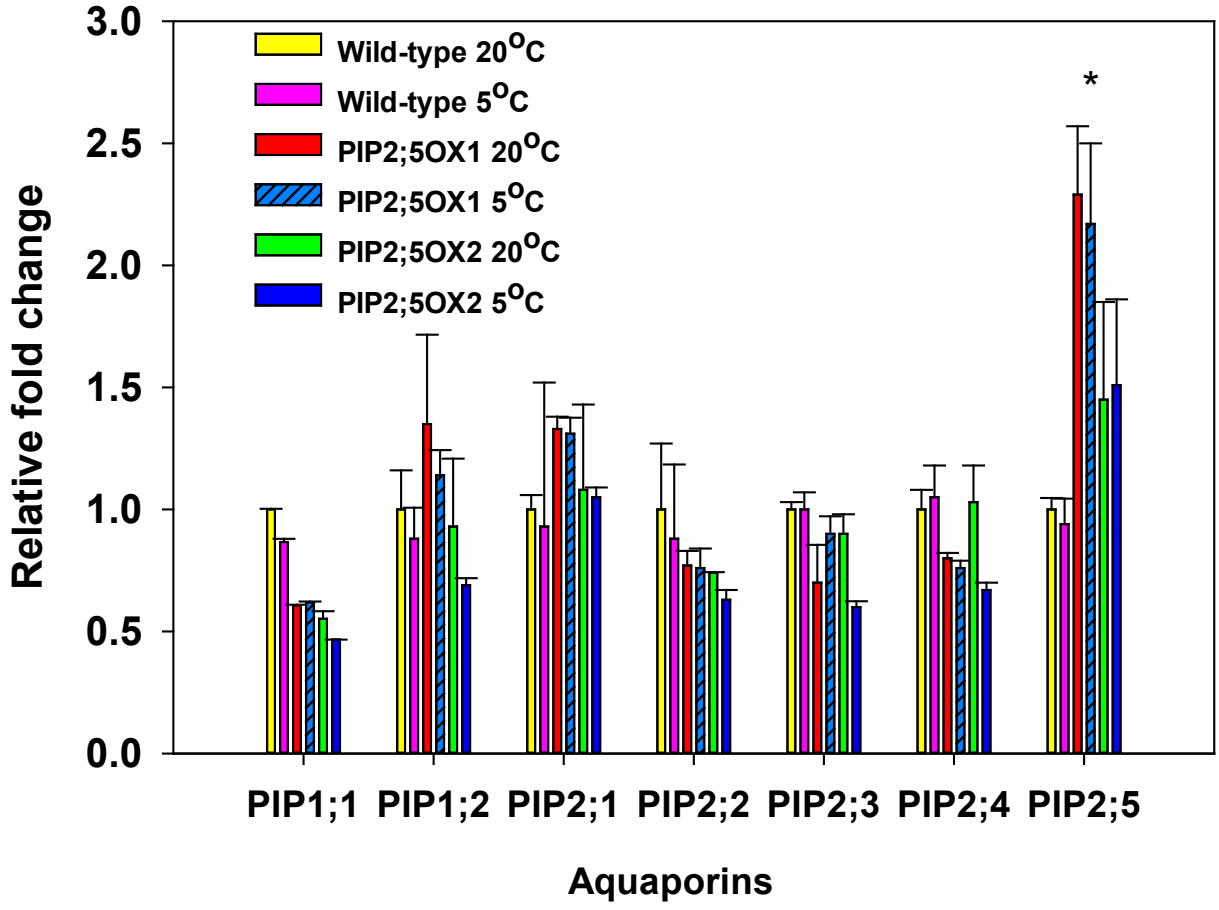

Figure 3.5: Relative expression profile of the seven*PIP* genes in roots of wild-typeand*PIP2;5* over-expressing INRA 717 hybrid aspen linesat 20°C and after 30min exposure of roots to 5°C. Means ± SE are shown(*n* = 3). Astric indicates significant difference at P = 0.05.

3.4 Discussion

The present study characterized the effects of LRT on the root water transport properties, gas exchange and AQP expression in poplar. When the root zone temperature was reduced from 20° to 15°, 10° and then 5°C, root $L_p$showed adecreasing trend in the wild-type.However, L_p values of *PIP2;5ox* lines are significantly greater than those of wild-type at 5°Cshowsthat over-expressing *PIP2;5* is still affecting root hydraulic conductivity. Similarly to *AtPIP2;5* over-expressing and *AtPIP1;4* over-expressing *Arabidopsis* plants (Lee et al. 2012), L_p was not affected by LRTinthe plantlets of INRA 717 hybrid aspenclone over-expressing *PIP2;5*AQP. The decline in L_p at LRT may be a consequence of increased water viscosity and decreasein apoplastic and (or) cell-to-cell water transport (Wan and Zwiazek 1999). At low soil temperatures, reduction in root water uptake causes reduction in the transpiration rates and plant water status (Wan et al. 1999, Wan and Zwiazek, 1999). The change in the root water flow due toLRT is relatively rapid (Fennell and Markhart, 1998). Water stored in the stem can temporarily supply water to overcome the effect (Goldstein et al. 1998). In *Cucurbita ficifolia*, LRTreducedthe root cell hydraulic conductivities. Therefore, it is surmised that the cell-to-cell pathway is the principal target of LRTwhich will lead to inhibition of root water flow (Lee et al. 2008). As the apoplastic pathway of water movement is blocked by the casparian strips of the endodermis, the endodermis plays a key role in cell-to-cell water movement (Steudle, 2000). Root water transport in *Populus tremuloides*was inhibited by mercurybecause mercuryinhibitsthe temperature sensitive metabolic reactionsand this suggests that the functioning of AQP channels is temperature dependent (Wan and Zwiazek, 1999). LRT can induce conformational changes of the AQP and affect the gating behaviour (Tyerman et al. 1999). This may be connected to respiration (Kamaluddin and Zwiazek, 2001) and

phosphorylation (Maurel, 1997).In thisstudy, the reduction of L_p at LRT in the wild-type was not fully reversible by increasing the temperature back to 20°C in 30 minutes. When the temperature was raised from 4°Cto 25°C and then returned back to 4°C, L_p was higher in the descending temperature order compared with the ascending temperature order in *Populus tremuloides*plantlets (Wan et al. 2001). Root respiration is inhibited byLRT and this may be indirectly responsible for the decline in root water flow. When the measurements started at 20°C and the temperature was reduced to 5°C and then returned to 20°C, water flow rates at the lower temperatures were higher in the descending sequence at each temperature tested, compared with the ascending sequence. This could indicate that root energy reserves were quickly filled up again at higher temperatures and that some were still available for the AQP function when the temperature was lowered. Maintenance of a stable hydraulic conductivity in the *PIP2;5ox*lines showed that these lines were less sensitive to short term LRT treatments than the wild-type.

In this study, high Ea (36.4 kJ mol^{-1}) was measured in the wild-type plants, demonstrating a high sensitivity of root water transport to temperature in *Populus tremula* x *P. alba*INRA 717 clone roots. Therefore, the 3- to 1.5-fold lower Ea values measured in *PIP2;5ox1*and *PIP2;5ox2* lines provide evidence for the importance of AQP in root water transport at LRT. Ea depends on the nature of the barrier for water movement and on the interactions of the water-pore with water (Verkman et al. 1996). Water transporting membranes have low Ea for water transportbecause movement of water across a channel does not need to overcome a higher energy barrier. When water moves through the lipid bilayer, it needs to overcomea high energy barrier because of the hydrophobic lipid phase (Tyerman et al. 1999). In higher plants and algae, the Ea values at the cell and membrane level varies in a range between 18 to 48 kJ mol^{-1}(Gerbeau et al. 2002; Lee et al. 2005). As an exception, the Ea valuesof leaf epidermal tissue of *Tradescantia virginiana* and

root cortical cellsof cucumberwere 186 kJ mol^{-1} (Tomos et al. 1981) and 100 kJ mol^{-1} (Lee et al. 2005a) respectively. The differences in Ea values may be due to the differences in measurement methods, diversity in the morphology and physiology of the planttissues. However, in thisshort term LRT experiment, the higher Ea values of the wild-type and lower Ea values of the *PIP2;5*ox lines obtained showed high temperature sensitivity pointing to the significant involvement of AQPs in root water transport.

LRT reduced E, g_s and P_n in wild-type more than in the over-expressing lines *PIP2;5ox1* and *PIP2;5ox2*. The maintenance of the soil-plant-air continuum requires a water flux control system. AQPs regulate the plant water balance together with the stomatal conductance (Wan et al. 1999; Javot and Maurel, 2002).At low soil temperatures, root water uptake limits the transpiration rates and CO_2 uptake (Wan et al. 1999, Wan and Zwiazek, 1999). Gas exchange measurements demonstrated that AQP-mediated water transport inhibits gas exchanging capacity of the plants growing in LRT condition. These results emphasize the significant role of AQPs in plant stress responses. Many environmental stresses directly or indirectly upset plant water balance (Sutka et al. 2011). When the root temperatures were gradually reduced, wild-type showed a highly decreasing trend in the gas exchanging properties than the *PtdPIP2;5ox*linestested. The change observed in the gas exchange of the plantlets when the roots were exposed to 5°C and then transferred to 20°C indicates that the inhibition of root water flow was a slowly reversible process. When the root temperature was reduced from 20°C and held at 15, 10 and 5°C for 30 min and then raised back to 20°C, P_n and E increased to the values close to the initial level after 30 min whileg_s showed a slower recovery. This relatively slow stomatal response may be due to the osmotic adjustment of the guard cells and resulting turgor maintenance during the reduced water supply. Therefore, the results indicate that theinhibition

of plant physiological functions by LRT is associated with water stress due to decreased root water uptake. *PIP2;5ox* plants showed reductions in gas exchangeunder LRT stress providing an evidence for a link between plant gas exchange responses and AQP function at LRT. Similar observation was made in wild-type*Populus trichocarpa×P. deltoides* (Laur andHacke, 2013). Recovery in water potential is driven by the increase inroot water flow. When aspen (*Populus tremuloides*) seedlings were grown at 10°C and 5°C in solution culture, net assimilation, stomatal conductance and shoot water potential decreased and when the seedlings were kept at 20°C, these parameters increased(Wan et al. 1999). The difference between Wan et al (1999)results and thismight derive from differences between the two plant species and in the experimental conditions. As the plantlets were exposed to low temperature for a short term, growth was not monitored. In a plant, synchronization of leaf and root water transport is essential for CO_2uptake (Kaldenhoff et al. 2008). Considering that LRT severely reduced water uptake and transpiration during short term LRT, LRT probably causes stronger water stress on the *PtdPIP2;5ox* plants than the wild-type because of the altered biochemical changes in the transgenic lines. LRT may be accompanied by relatively high air temperature with high transpirational demand and this may lead to challenges in maintaining the water balance. Therefore LRT tolerance is often correlated with drought resistance (Janowiak et al. 2002; Aroca et al. 2005). Occurance of reduced water flux at LRT may be due to higher water viscosity (Muhsin and Zwiazek, 2002), the inhibition of transmembrane water transport (Wan et al. 2001; Lee et al. 2005), reduced enzyme activities (Johansson et al. 1998) and reduced root respiration/metabolism (Kamaluddin and Zwiazek, 2001).

The expression profile of the *PIP*s demonstrated that the expression levels of all the AQP isoforms tested, remained the more or less same and did not differ significantly, after the short

term exposure to low root temperature 5°C. In a study in *Arabidopsis*, expression levels of *AtPIP1;4* and *AtPIP2;5* in the wild-type plants increasedafter 1- and 24-h exposures of roots to 10°C (Jang et al. 2004). In an expression study in 3-week-old wild-type *Arabidopsis* seedlings, when the roots were exposed to 10°C for 1 and 24 h, the expression of *PIP1;2, PIP1;4, PIP2;5* and *PIP2;6* increased and *PIP2;6* showed the greatest increase (8-fold) in plants treated for 24 h (Lee et al. 2012). In a similar experiment in *Arabidopsis*, some *PIP* genes were down-regulated, others up-regulated and others unaltered (Aroca et al. 2007; Ruiz-Lozano et al. 2009). For this study, water transporting AQPs previously shown to have relatively greater expression in roots (Almeida-Rodriguez et al. 2010) were tested for the effect of temperature and *PIP2;5* over-expression on their expression levels. Exposure of roots to LRT for 30 minutes did not significantly change the expression of any of the tested aquaporin genes. Based on the expression studies, it is difficult to assign a role for *PIP* genes in regulating L_p during stress condition. As previously indicated, L_p behaviour is regulated partially by AQP function, specifically by *PIP*s (Postaire et al. 2010). However, based on the other studies, it is very challenging to find a common response of root *PIP*AQP expression and PIP protein abundance under stress conditions. Each *PIP* gene could have shown a specific function under specific stress circumstances. For example, the over-expression of a certain *PIP*AQP gene induced tolerance to some environmental stresses but sensitivity to others (Jang et al. 2007). Similarly, the over-expression of an*ArabidopsisPIP*AQP gene in transgenic tobacco improved plant vigour under conducive conditions but not under any form of stress (Aharon et al. 2003). There were no significant changes in the expression levels of several PIP and TIP proteins in root tissues of rice during the first 5 h of low temperature treatment, whereas hydraulic conductivity declined rapidly (Murai-Hatanoet al.2008). We are making the assumption that transcript levels are at least somewhat correlated with protein abundance, but we recognize that this is often not the

case. Therefore, it could be suggested that a decrease in hydraulic conductivity could be due to closure of AQP pores rather than by a decrease in protein amounts. Closure of AQP might be due to acidification of the cytosol during low temperature periods (Kawamura, 2008). Therefore, the response of PIP AQPs to LRT stress was not be the only explanation for the difference in hydraulic conductivity behaviour between the transgenic and wild-type lines. The difference between other experimental results and minemay be due to the differences between the plant species, experimental conditions and measurement methods. Studies on the protein abundances corresponding to all the PIPs and tonoplast intrinsic proteins (TIPs) might answer the question of the change in the hydraulic conductivities in transgenic and wild-type poplars when they are exposed to low root temperature for a short period of time.

The differences in low-root temperature responses between the two *PtdPIP2;5ox*lines may be due to functional differences that arose due to the ligation in the genome and the differences in tissue distribution (Alexandersson et al. 2010). Levels of transgene expression vary among independent transformants. The over-expression of *PIP2;5* was effective in maintaining high water permeability of poplar roots when measured after exposing the plants for 30 min to 15°C, 10°C and 5°C root temperature. The differences in LRT responses between the *PtdPIP2;5ox* lines and the wild-type plants may be due to the difference in abundance and activity of AQPs in different plant tissue (Jang et al. 2004, 2007; Almeida-Rodriguez et al. 2010) or the differences in tissue distribution (Alexandersson et al. 2010) or differences in the protein – protein interaction or possibilities in the differences in regulation mechanisms between endogenous and exogenous *PIP2;5*s or in their water-transporting capacity (Lee et al. 2012) or difference in the function of *PIP2;5*.

3.5 Conclusion

Over-expression of *PIP2;5* in the INRA clone 717 *Populus tremula*× *Populus alba*, was effective in alleviating the short term effects of low root temperature on the root hydraulic conductivity. The inhibition of L_p by low root temperature could be partially prevented by the function of over-expressing *PIP2;5*, suggesting that the effect of low root temperature on root water transport may involve stress responses developed by one or moreAQPs and other altered physiological processes. The temperature sensitivity of root water transport was reflected by a reduction in the water transport in the wild-type compared with *PtdPIP2;5ox*lines exposed to low root temperatures for short term. Based on the L_p and gas exchange measurements of the transgenic lines over-expressing *PIP2;5* and the wild-type hybrid aspen(*Populustremula* × *Populus alba*)717-1B4, transgenic lines show more resistance to LRT than the wild-type. These results provide evidence for a positive relationship between responses in gas exchange to low root temperature and aquaporin-mediated root water transport in *Populus*and suggest that the low temperature sensitivity of root water transport may be connected to AQP activity.

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CHAPTER 4

4. Effects of prolonged low root temperature on growth, gas exchange and root water transport of the over-expressing *PIP2;5* aquaporin in two lines of INRA 717 hybrid aspen clones

4.1 Introduction

Among the major environmental factors that affect the growth and productivity of the plants growing in the temperate and boreal regions, the effect of cold climate is very important (Wan et al. 2001). Cold sensitive plants show low root water uptake rate at low temperatures. The reductions in water uptake, transport and gas exchange are the principal factors responsible for growth reduction of the plants growing at low soil temperatures (Wan et al. 1999; Lee et al. 2005a). When the plant roots are cooled below a critical temperature, there will be a decline in root hydraulic conductivity (L_p) (Murai-Hatano et al. 2008) and leaf water potential (Kuwagata et al. 2004; Kuwagata et al. 2012; Lee at al. 2012). High transpirational demand will be created by the relatively high air temperature. Therefore, the ability of the plant to transport water is a critical factor for growth and productivity in low root temperature condition.

Aquaporins (AQPs) are water channel proteins that facilitate the movement of water through membranes and play a key role in plant water homeostasis in response to various environmental stresses (Maurel et al. 2002). Generally plants have a large number of AQP genes (Gomes et al. 2009). AQPs are classified into five subfamilies including plasma membrane intrinsic proteins (PIP), tonoplast intrinsic proteins (TIP), nodulin-26 like intrinsic proteins (NIP), small basic intrinsic proteins (SIP) and X intrinsic proteins (XIP). The water transporting ability of poplar plants through the roots can be critical for the growth and production of plants, especially under

water stress conditions (Almeida-Rodriquez et al. 2010). Fifty five AQP genes have been identified in poplar (Gupta and Sankararamakrishnan, 2009; Almeida-Rodriquez et al. 2010). AQP-mediated water transport can be affected by various environmental factors which alter the abundance of AQPs in the cell membranes, their subcellular trafficking and gating behaviour (Aroca et al. 2005). The role of the AQPs *PIP1;4* and *PIP2;5* in the growth and L_p responses to low temperature of root cortical cells was studied in *Arabidopsis* plants over-expressing *AtPIP1;4* and *AtPIP2;5* (Lee et al. 2012). The study concluded that the over-expression of *AtPIP1;4* and *AtPIP2;5* in *Arabidopsis* was effective in alleviating the short term effects of LRT on L_p in root cells and phosphorylation /dephosphorylation processes involve in the effect of low temperature on root water transport (Lee et al. 2012). AQP *PIP2;5* functions as one of the important water transporting *PIPs* in *Arabidopsis* and poplar (Almeida-Rodriguez et al. 2010, Lee et al. 2012). Extensive studies have been carried out to understand the functions and regulations of AQPs (Tournaire-Roux et al. 2003, Jang et al. 2004, Alexandersson et al. 2005, Bramley et al. 2007, Murai-Hatano et al. 2008, Lee et al. 2012, Aroca et al. 2012). Most of the studies indicate that the abundance of the AQPs in roots and shoots is affected by the diverse environmental stress conditionssuch as low root temperature (LRT), salinity, water stress and flooding. However, the responses of the plant AQPs to transpirational water demand and long term LRT are still poorly understood.

To examine the role of poplar AQP *PIP2;5* in the response of plants to long term LRT stress, *Populus tremula* × *Populus alba*INRA Clone 717 plants constitutively over-expressing *PIP2;5* AQP of *Populustrichocarpa* × *Populus deltoides* H11-11 were generated (See chapter 2). AQP *PIP2;5* cloned from *Populustrichocarpa* × *Populus deltoides* H11-11 was selected for this study as it was confirmed to be a major water-transporting AQP in poplars and because it showed

increased expression under drought stress conditions (Almeida-Rodriguez et al. 2010). In the present study, effects of the transpirational demand and prolonged LRT on water uptake properties, growth, physiological responses and AQP expression in *PIP2;5ox* lines versus wild-type poplar plants were investigated, as a step towards understanding the development of integrated stress.It was hypothesized that the over-expression of AQP *PIP2;5* would increase root hydraulic conductivity enabling the plants to maintain sufficiently high rates of water transport to meet transpirational demands at continuouslow root temperatures which, in turn will help maintain high growth and gas exchange rates compared with the wild-type poplars.

4.2 Materials and methods

4.2.1 Plant material and growth conditions

The plantlets of two transgenic poplar lines *PIP2;5ox1* and *PIP2;5ox2* and wild-type INRA 717 clones were used in this study. The generation of these transgenic plants is described in Chapter 2. Plantlets for experimentation were vegetatively propagated from the primary transformants or wild-type by means of rooted cuttings of the stem segments. Young stem pieces of 10-12 cm length plantlets of all three hybrid lines with nodes and young leaf, were placed in the trays filled with peat: vermiculite (2:1) mix, after dipping in IBA powder (Sigma Aldrich) and covered by polythene. After 15 days of growth in the growth chamber, polythene cover was gradually opened to expose the plantlets to the growth chamber condition. After the steady development of theroots, the cuttings were replanted in four-liter pots with the same soil composition and were grown under semi-controlled conditions. They were grown for 3 weeks reaching a height between 30 and 40 cm and 3-6 mm in stem diameter at the root collar before temperature treatment was applied.The plants were watered six times per week and fertilized

weekly with NPK (1g/L) 15:30:15 commercial fertilizer, before the temperature treatment experiment. Environmental conditions in the growth room were maintained at 24/18°C (day/night) temperature, 65 ± 10 % relative humidity and 16 h photoperiod with 350 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the top of the seedlings provided by the full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

4.2.2 Long term low root temperature treatments

4.2.2.1 Solution culture

The cuttings of transgenic lines *PIP2;5ox1* and *PIP2;5ox2* and wild-type poplars were gently washed with distilled water and transplanted in the 30-L opaque plastic tubs (6 plants of each line in each of the three replicated tubs) filled with aerated full strength modified Hoagland solution (Zhang et al. 2013) and grown at 20°C for 3 weeks.A split plot design with randomizationwas used. Twenty 8-cm diameter holes were made at regular intervals in flat styrofoam boards, and these styrofoam were used to keep the plants in the plastic tubs filled with solution culture. Snugfitting foam plugs were fitted around the stems, and inserted into the holes to hold the stems in place while the roots were immersed in solution, with the stems protruding through the holes. All tubs had spouts installed into their sides to facilitate drainage, aeration and circulation of the nutrient solution via plastic tubing. Low root temperature treatment was applied after 3 weeks of growth of plants at 20°C. Each treatment consisted of three 30-L plastic tubs containing nutrient solution at a particular temperature (5, 10 and 20°C for 3 weeks), were connected to a circulating water bath (Thermomix BU coupled to Frigomix U, B. Braun Biotech International, Melsungen, Germany) using flexible tubings. This maintained stable temperature in the three tubs and continuous circulation of the solution among the water bath and tubs. An

aeration pump (Model 9.5 950GPH, Danner MFG Inc., New York, USA) was immersed inside of all the containers to maintain continuous aeration and uniform circulation of the solution inside the container. Dissolved O_2 during the experiment was maintained above 5 mg L^{-1}. The solution culture tubs, tubing, and pumps were cleaned once per week to prevent algal and bacterial buildup. The shoots of the plants were exposed to high transpirational demand conditions (24°C day temperatures), while the roots were made to expose to that desired solution temperatures for three weeks. Styrofoamtray orientations were rotated 180 degrees twice a week to minimize edge effects. The plants were inspected for the presence of mites and thrips and the infected leaves were manually cleaned using cotton swabs. Hoagland's solution was changed every week and aerated continuously.

4.2.3 Measurements

4.2.3.1 Growth

The height of all the plantlets was measured after 3 weeks of growth, from the base of the stem to the shoot tip for each plant. The dry weight determination of roots and shoots of individual plants was obtained after drying the plant part in an oven at 85°C for 72 h. The relative shoot height growth (RSHG) was calculated according to the following formula: RSHG = [(Final height –Initial height)/Initial height] x 100(Hoffmann and Poorter, 2002).

4.2.3.2 Gas exchange measurements

After the end of 1st, 2nd and 3rd weeksof LRT treatments, gas exchange measurements including net photosynthesis (P_n), transpiration rates (E)and stomatal conductance (g_s) were performed on the 2nd fully expanded leaf of each plant using a portable open-flow photosynthesis system (LI-6400, Li-Cor Inc., Lincoln, NE) equipped with an incorporated auxiliary LI-6400B red/blue

LED light source (6 cm^2 chamber area). PPFD was set at 350 μmol m^{-2} s^{-2} and reference $CO_2$was set at constant 386 μmol mol^{-1}using the 6400-01 CO_2 mixer; air temperature was 24± 0.15°C and vapour pressure deficit (VPD) 1.3 ± 0.3 kPa. Sample and reference infrared gas analyzers were matched prior to measure every plant, and data were logged every 30 s during a 10-min period (Voicu et al. 2008).

4.2.3.3 Root water transport

The root hydraulic conductivities were measured every week by attaching excised root system with approximately 2 cm of the stem remaining, to the high pressure flow meter (HPFM, Dynamax Inc, Houston, TX, USA) using compression couplings and rings. Water was then forced inside stems at increasing pressure. The computer recorded water flow rate and the applied pressure wasused to compute root absolute hydraulic conductance. Root volumes were measured for each root system using volume of displaced water after immersing the root in a graduated cylinder (Voicu and Zwiazek, 2004).

4.2.3.4 qRT-PCR study for gene expression

Young roots of the plantlets(6 per treatment) were harvestedafter they were exposed to 20°C and 5°C for 3 weeks andmanually ground to a fine powder using mortar and pestle. Total RNA was extracted from the ground root samples of transgenic and wild-type plants using the Plant RNeasy extraction kit (Qiagen, Valencia, CA, USA). Possible DNA traces present in the samples were removed from the total RNA by the DNase I treatment. SuperScript™ II Reverse Transcriptase (SSII RT; Invitrogen, Carlsbad, CA, USA) was used to synthesize cDNA using the manufacturer's instructions. qRT-PCR primers for *PIP1:1, PIP1:2, PIP2:1, PIP2:2, PIP2:3,*

PIP2:4 and *PIP2:5* previously designed and validated by Almeida-Rodriguez et al. (2010) were used.To compare the transcript abundance of the *PIP* genes,quantitative RT-PCR was performed on a 7900 HT Fast Real-Time PCR system (Applied Biosystems) in 384-well Clear Optical Reaction Plate with Barcode. Reagents and cDNA were pipetted by using the Biomek 3000 Robot (Beckman Coulter). Three biological replicates, each with three technical replicates, were assayed for each sample. PCR was carried out in a volume of 10 µl including a final concentration of 50 ng cDNA, 1× master mix containing 0.2 mM dNTPs, 0.3 U Platinum Taq Polymerase (Invitrogen), 0.25× SYBR Green and 0.1× ROX. The thermal profile consisted of three stages. The first stage was one cycle of 95°C for 2 minutes. The second stage was 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data was collected at the end of each cycle during stage 2. The third stage was a dissociation cycle of 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds and 60°C for 15 seconds. Samples were subjected to auto Ct (cycle threshold) for analysis, and dissociation curves were verified for each of the genes. The dissociation curve was generated using the first derivative of the rate of change in fluorescence as a function of temperature. Dissociation curve drawn from the dissociation cycle helped to detect nonspecific amplification and formation of primer dimers. Results were analyzed using SDS2.3 (384-well plates; Applied Biosystems). Expression was determined relative to translation initiation factor *TIF5A* (means ± SE, n = 3). After normalization of the DNA content using the *TIF5A* gene expression pattern in each sample, the expression level of each gene in plants under low-temperature conditions was calculated by comparing the expression level with plants exposed to 20°C root temperature.

4.2.4 Statistical analysis

Statistical analyses were performed using R version R2.15.3 statistical software (R Development Core Team, 2011), at $\alpha = 0.05$ confidence level. The data sets were checked for the parametric assumptions of normality (Shapiro-Wilk test) and homogeneity of variances (Bartlett's test). When necessary to meet the assumptions of normality and homogeneity of variance, the data were transformed by log transformation. The data were analyzed using ANOVA. Tukey's multiple comparison test was used for pairwise comparisonat $P \leq 0.05$.

4.3 Results

4.3.1 Growth

Relative shoot height growth (RSHG) decreased significantly (p=0.05) with decreasing root temperatures from 20° to 10° and 5°C and the effect was similar in the transgenic and wild-type lines (Figure 4.1). Root dry weights of all the transgenic and wild-type lines grown in 5°C were significantly lower compared to those grown in root temperatures 10° and 20°C (Figure 4.4). There were no significant differences between the transgenic and the wild-type linesin RSHG (Figure 4.1), shoot dry weight (Figure 4.3),root dry weight (Figure 4.4) and total dry weight (Figure 4.2)in any of the temperatures tested.

4.3.2 Gas exchange

The net photosynthetic rate (Pn) of the transgenic and wild-type poplars exposed to 10 and 5°C root temperature were significantly lower (p=0.05) compared with 20°C, after 1 week, 2 weeks and 3 weeks of root temperature treatments (Figure 4.5 A,B,C). The*PIP2;5ox1*line showed significantly higher P_n than the wild-type at 10 and 5°C, after 1^{st}, 2^{nd} and 3^{rd} weeks (Figure 4.5 A, B and C)whereas *PIP2;5ox2* line showed significantly higher P_n than the wild-type at 10 and 5°C after 2^{nd} week and at 5°C after 3^{rd} week (Figure 4.5 B,C). The transpiration rate (E) of the

transgenic and wild-type poplars exposed to 10 and 5°C root temperature were significantly lower compared with 20°C, after 1 week, 2 weeks and 3 weeks of root temperature treatments(Figure 4.6A, B, C). *PIP2;5ox*linesshowed significantly higher E than the wild-type at 10 and 5°C after 2 weeks (Figure 4.6 B).Transgenic*PIP2;5ox2*lines showed significantly higher E than the wild-type at 10°C and both the *PIP2;5ox*linesshowed significantly higher E than the wild-type at 5°C, after 3 weeks of LRT treatment (Figure 4.6C). Water use efficiency (WUE) values of all the lines growing at 5°C were lower compared with the plantlets at 20°C after 1, 2 and 3 weeks of root temperature treatments. WUE values werehigher in the *PIP2;5ox1*line than the wild-typeat 10°C, after one week (Figure 4.7A) and 10 and 5°C,after 2 weeks (Figure 4.7 B).At all the temperatures tested, the WUE values of the wild-typewere relatively lower than the *PIP2;5ox* lines, after 3 weeks (Figure 4.7C). After 3 weeks, when the decrease in WUE at 10°C compared with 20°C was again significant for the wild-type (Figure 4 C).

4.3.3 Root hydraulic conductivity (L_p)

The root hydraulic conductivity (L_p) of the transgenic and wild-type poplars exposed to 10 and 5°C root temperatures were significantly lower compared with the plants exposed to 20°C for 1, 2 and 3 weeks (Figure 4.8 A,B,C). There were no differences in $L_p$between plants of the wild-type and *PIP2;5ox* lines in any of the tested root zone temperatures after 1 week (Figure 4.8 A). However, after 2 and 3 weeks, transgenic plants exposed to lower root temperatures had higher L_p compared with the wild-type plants (Figure 4.8 B, C).

4.3.4 PIP expression

Transcript abundance profiling indicated that there was an enhanced level of *PIP2;5* transcript in the over-expressing lines compared with the wild-type at 20°C root temperature (Figure 4.9 A).

There were no other significant differences in *PIP* root expression between plants of the different lines at 20°C (Figure 4.9 A). When the roots of plants were exposed to 5°C for 3 weeks, PIP2;1 transcript level in *PIP2;5ox1*linewas significantly higher compared with the wild-type and *PIP2;5ox2*line and there was no difference in *PIP2;5* levels between the different lines (Figure 4.9 B). Transcript abundance corresponding to *PIP1;1, PIP1;2, PIP2;1*, *PIP2;2*, *PIP2;3*and*PIP2;4*aquaporins did not significantly differ between wild-type and *PIP2;5ox* lines, nor did transcript abundance for these genes significantly differ between the 3 weeks treatment of 20°C and 5°C. The expression of the reference gene *TIF5A* was not significantly different (p - 5.4×10^{-16})across the treatments.

4.4 Discussion

The transgenic and wild-type plants grown in 5°C showed significant reduction in the root dry weight compared to those grown in root temperatures 10 and 20°C. Relative shoot height growth (RSHG), root and shoot dry weight and total dry weight of the wild-type poplars showed significant reductions from 20 to 5°C (Figure 4.1, 4.2, 4.3, 4.4). Similarly to the present study, LRT inhibited shoot and root growth in poplar (Landhäusser and Lieffers, 1998, Wan et al. 1999) and *Arabidopsis* (Lee et al. 2012). Transgenic lines did not respond differently from the wild-type poplars in RSHG, shoot and root dry weight and total dry weight at any temperatures tested. Growth of plant roots generally declines with decreasing root temperature in the 5–30°C range and reduced whole-plant growth and photosynthetic rate may be a result of reduced water and nutrient uptake (Bowen, 1991). Decline in L_p due to LRT has been linked to reductions in shoot and root growth (Wan et al. 1999). Inhibition of growth may partly be due to decreased root metabolic activity (Kramer and Boyer, 1995).

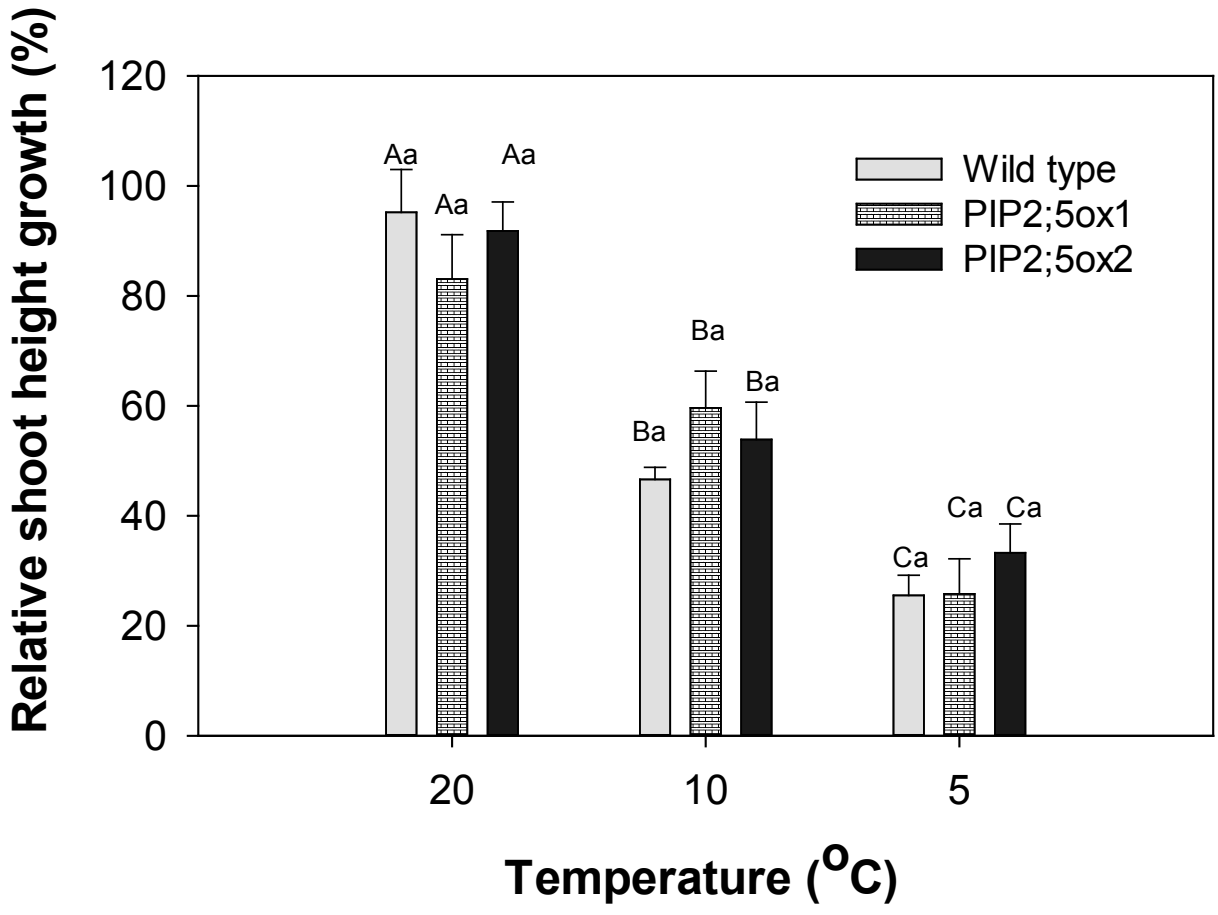

Figure 4.1: Relative shoot height growth of the two *PIP2;5ox* lines and wild-type poplar plants exposed to 20, 10 and 5°C root temperatures for threeweeks in mineral solution culture (Means ± SE, n = 6). Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

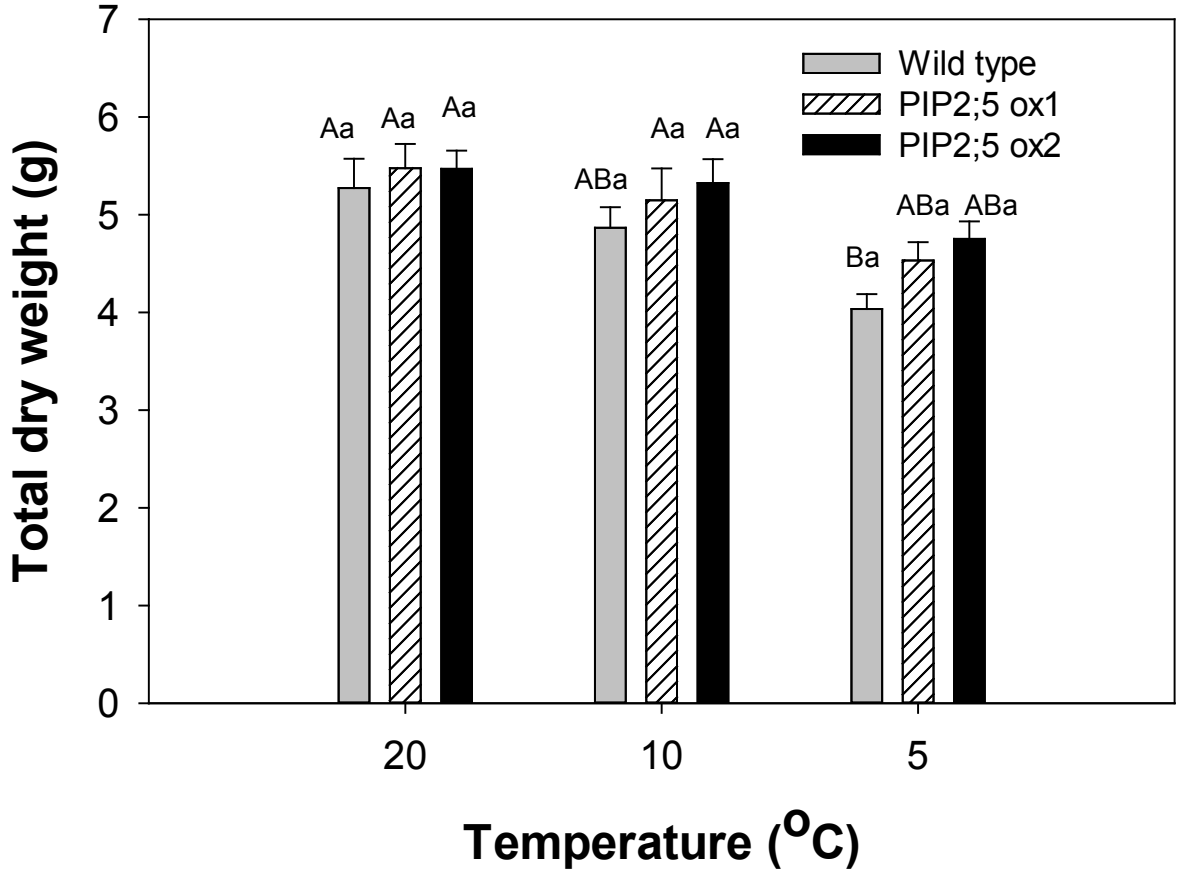

Figure 4.2: Total dry weight of the two *PIP2;5ox* lines and wild-type poplar plants exposed to 20, 10 and 5°C root temperatures for threeweeks in mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

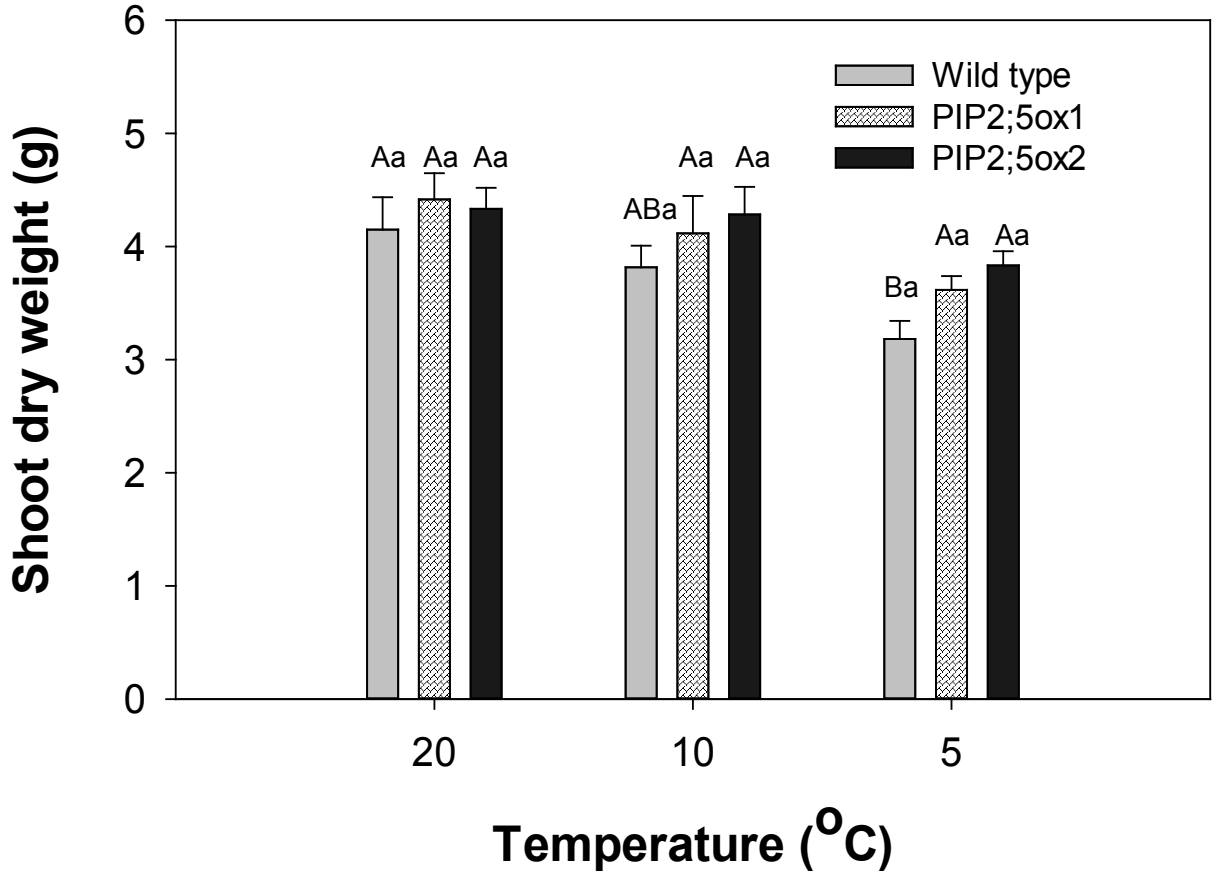

Figure 4.3: Shoot dry weight of the two *PIP2;5ox* lines and wild-type poplar plants exposed to 20, 10 and 5°C root temperatures for threeweeks in mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

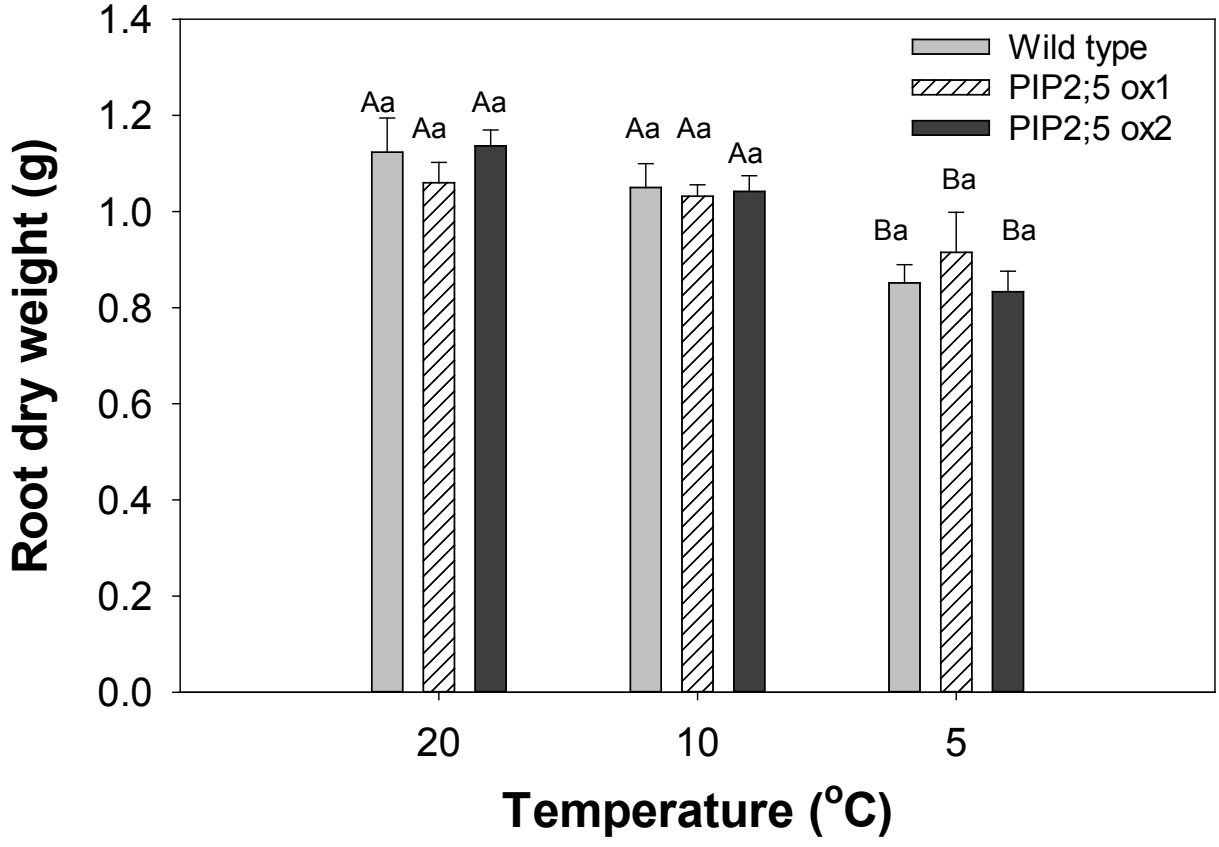

Figure 4.4: Root dry weight of the two *PIP2;5ox* lines and wild-type poplar plants exposed to 20, 10 and 5°C root temperatures for threeweeks in mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

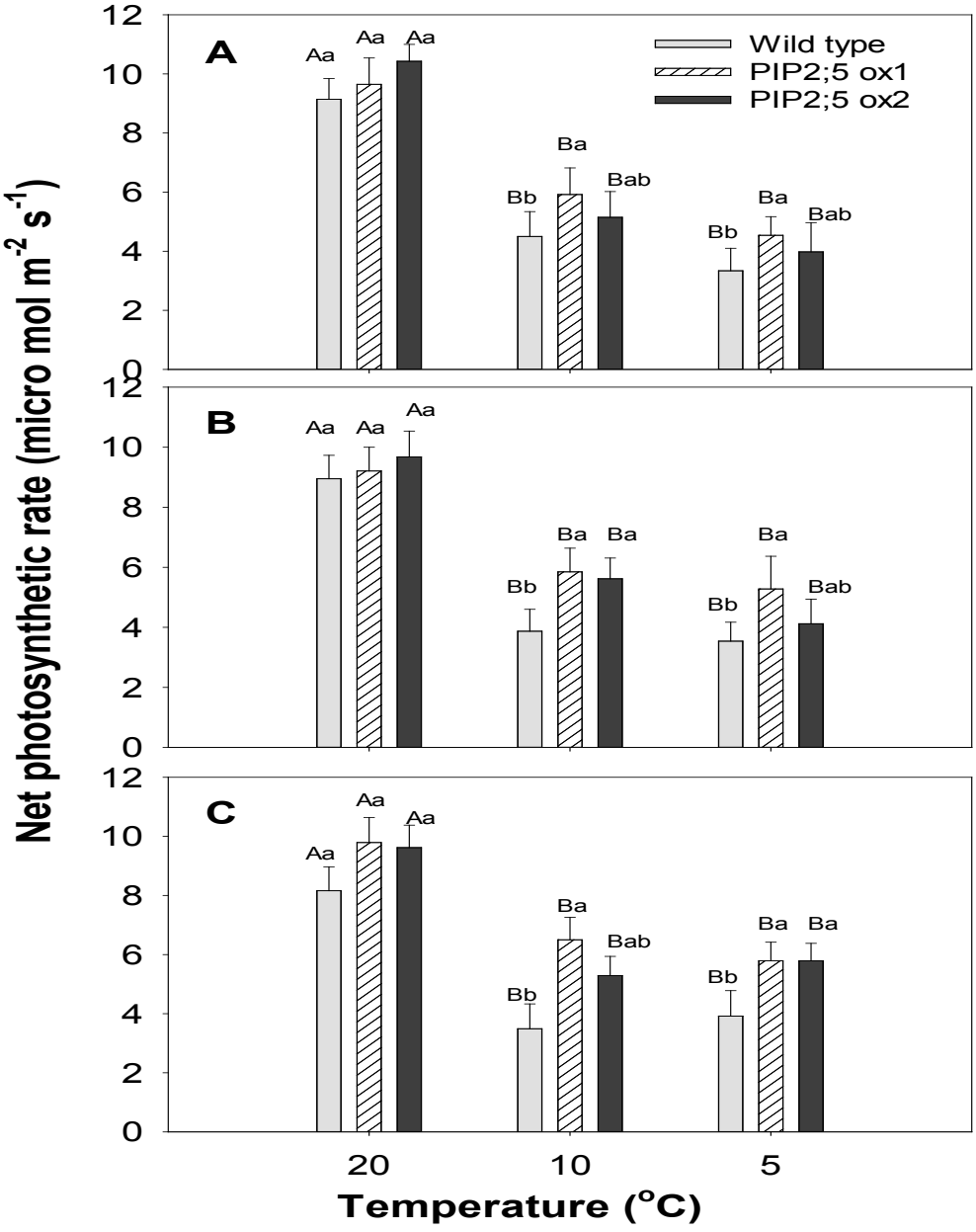

Figure 4.5: Net photosynthetic rateof the two *PIP2;5ox* lines and wild-type poplar plants exposed to 20, 10 and 5°C root temperatures for 1 week (A), 2 weeks (B) and 3 weeks (C) (Means ± SE, n = 6). Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

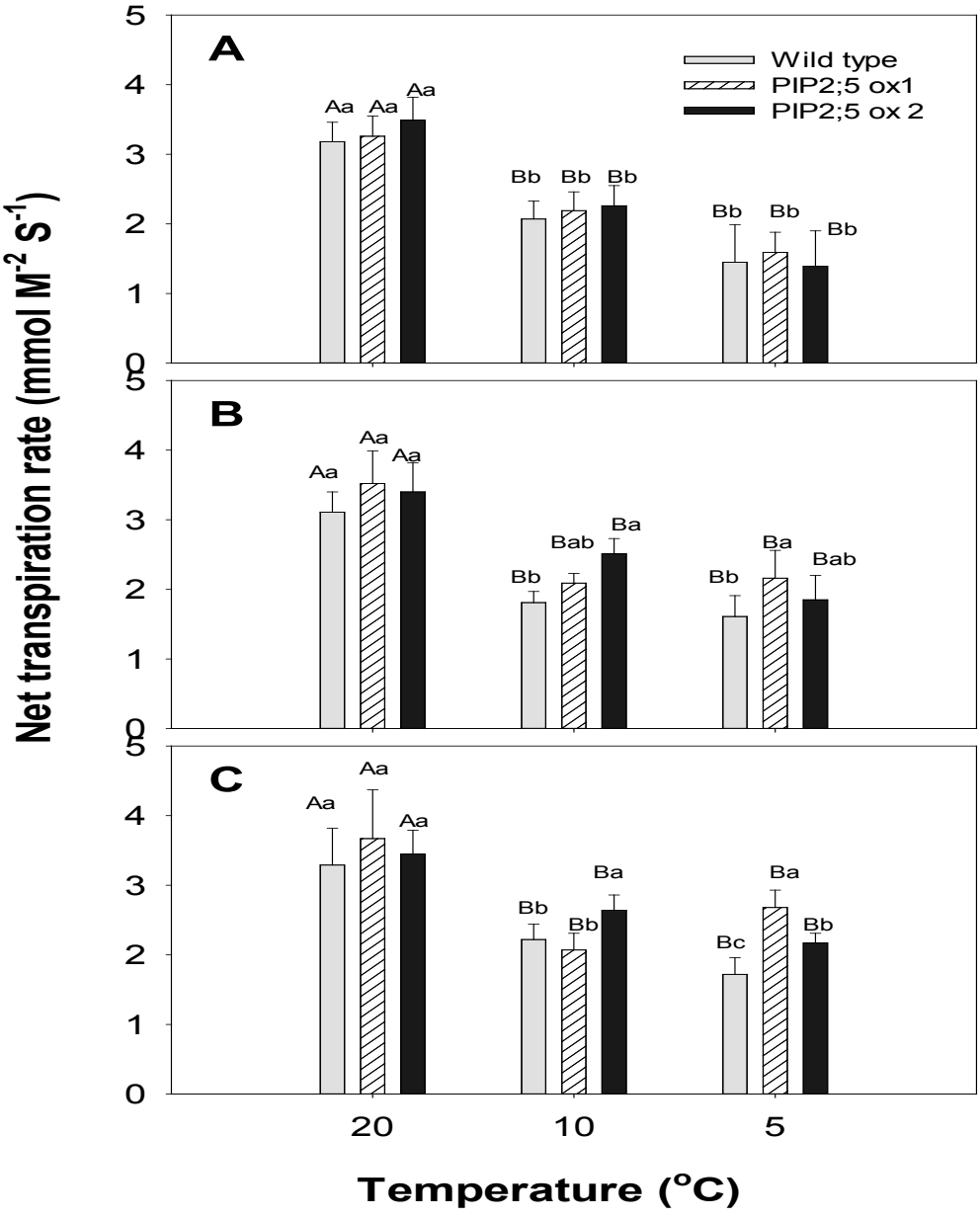

Figure 4.6: Net ranspiration rate of the two *PIP2;5ox* lines and wild-type poplar plants exposed to 20, 10 and 5°C root temperatures for week (A), 2 weeks (B) and 3 weeks (C) mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

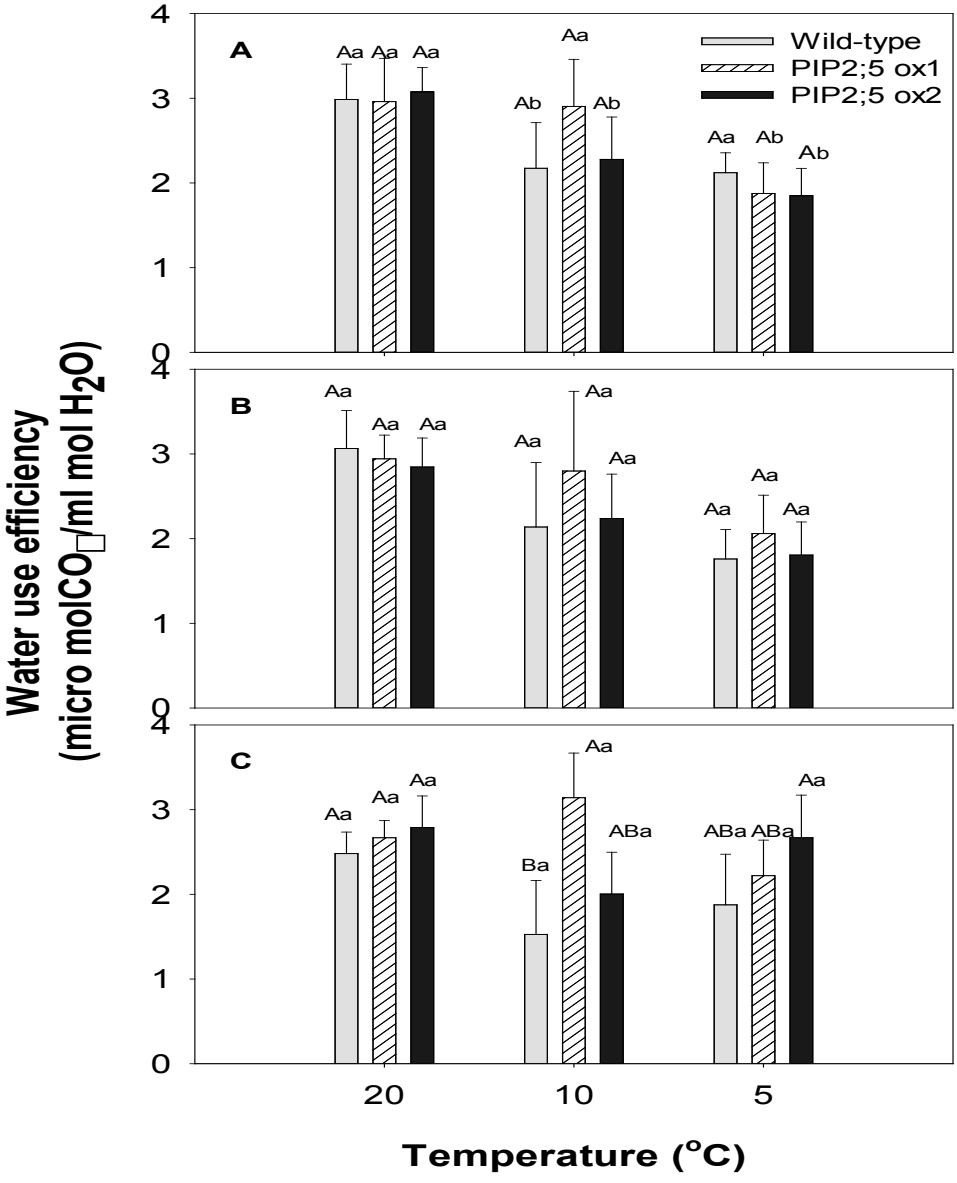

Figure 4.7: Water use efficiency (WUE) of the two *PIP2;5ox* lines and wild-type poplar plants exposed to 20, 10 and 5°C root temperatures for 1 week (A), 2 weeks (B) and 3 weeks (C) mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences ($P \leq 0.05$) between temperature treatments within a genotype, while lower case letters denote significant differences ($P \leq 0.05$) between genotypes at a given temperature treatment.

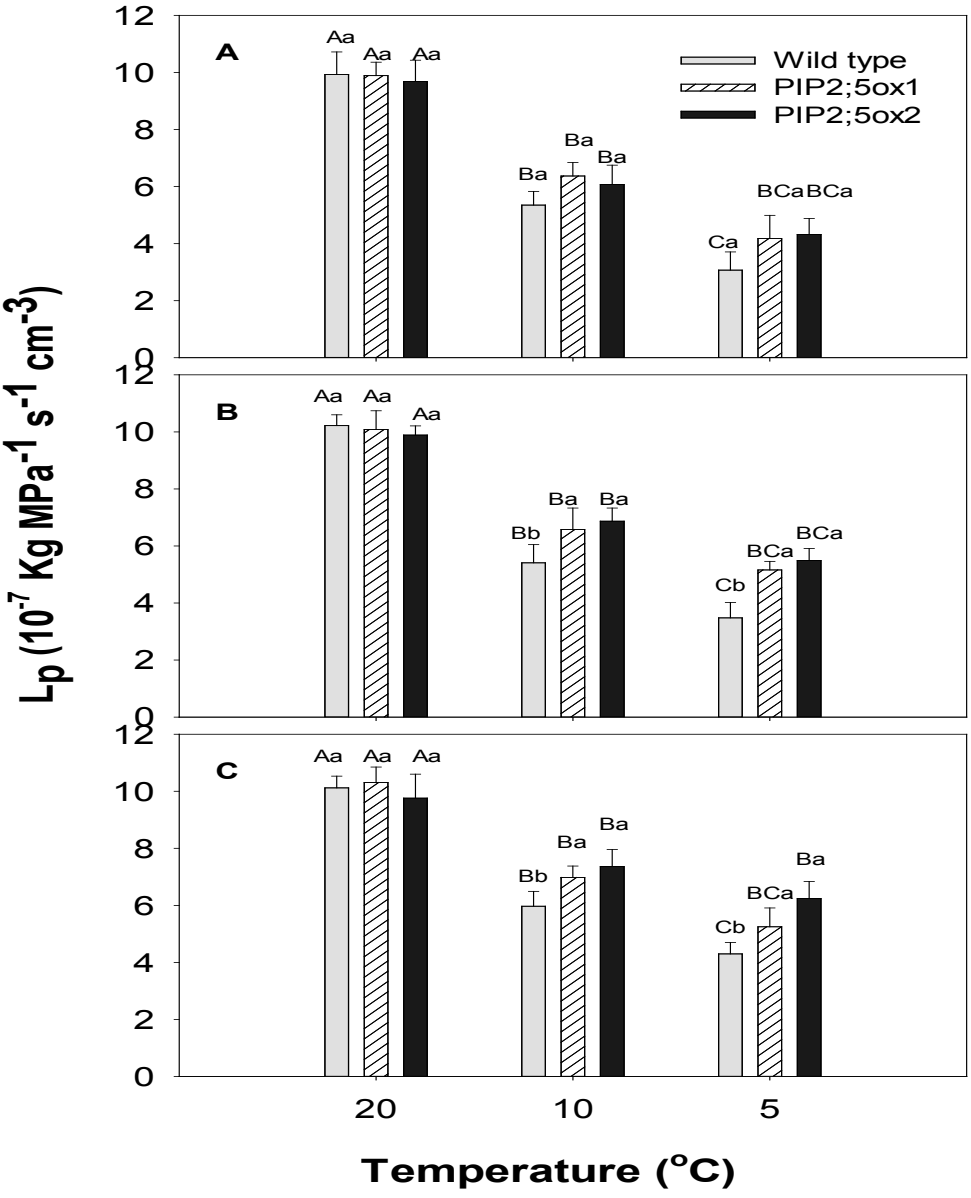

Figure 4.8: Root hydraulic conductivity (L_p) of the two *PIP2;5ox* lines and wild-type poplar plants exposed to 20, 10 and 5°C root temperatures for 1 week (A), 2 weeks (B) and 3 weeks (C) in mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences (P≤0.05) between temperature treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given temperature treatment.

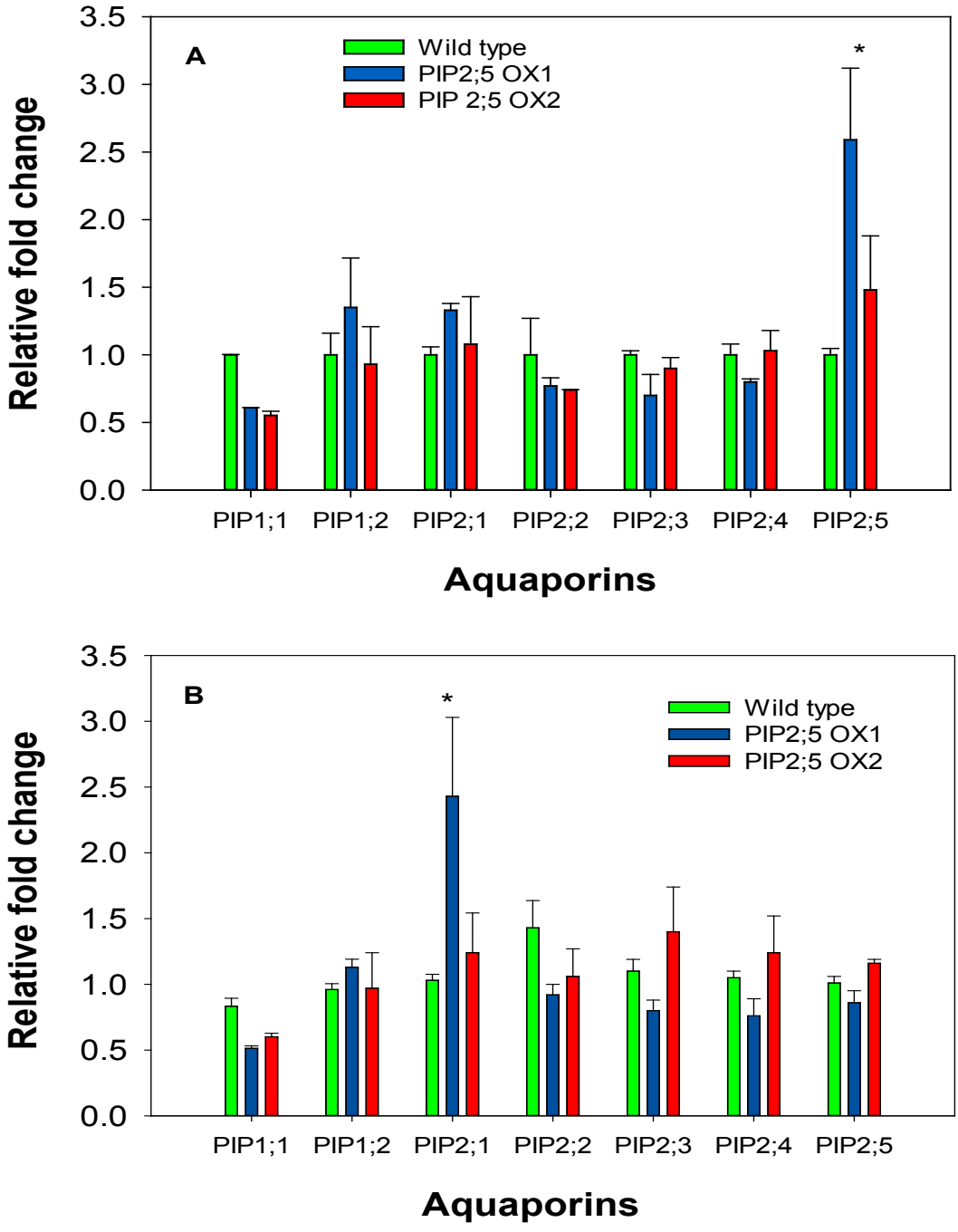

Figure 4.9: Analysis of expression. Relative expression profiles of the 7 *PIP* genes in roots of wild-type and*PIP2;5* over-expressing poplars A. Exposed to root temperature 20°C B. Subjected to LRT 5°C treatment for three weeks. (Means ± SE, *n*= 3). Asterics indicate significant differences ($P \leq 0.05$, Tukey's multiple comparison after ANOVA).

This may help explain lower root transpiration rates observed in poplar plants exposed to 10°C and 5°C compared with those grown at 20°C (Figure 4.6). However, three weeks of temperature treatments may not be sufficiently long to produce growth differences in relatively slow growing poplars. This is different than the earlier reports in aspen and other poplars (Wan and Zwiazek, 1999; Wan et al. 2001). The hybrid poplar *Populus tremula* × *Populus alba* INRA clone 717 used in this study might have shown higher resistance to LRT stress conditions. In this study, LRT had a greater effect on transpiration rates and net photosynthetic rates in wild-type compared with *PIP2;5ox1* and *PIP2;5ox2* over-expressing lines and the reduction was greater at lower temperature (Figure 4.5 and 4.6). LRT suppressed dry matter production and decreased leaf area and the response of leaf area to LRT was accompanied by changes in the relative water content in *Arabidopsis* (Lee et al. 2012) and in rice (Nagasuga et al. 2011). The maintenance of the soil-plant-air continuum (SPAC) requires a water flux control system. AQPs function as a regulatory system of the water flux for plants together with the stomatal conductance (Wan et al. 1999; Javot and Maurel, 2002).At low soil temperatures, root water is one of the main factors that limit transpiration rates and CO_2 uptake of the plant (Wan et al. 1999; Wan and Zwiazek 1999). Comparison of gas exchange measurements in high and low RT demonstrated that AQP-mediated water transport is one of those factors that is responsible for the inhibition of gas exchange capacity in plants growing in LRT. Poplars showed reductions in gas exchange values when exposed to low root temperature and this provides an evidence for a link between gas exchange responses and AQP function at LRT. Higher gas exchange rates were observed at 10 and 5°C root temperature in plants over-expressing *PIP2;5* than the wild-type. These results also emphasize the key role of AQPs in stress responses of plants, because many environmental stress conditions directly or indirectly affect plant water balance (Sutka et al. 2011). The WUE values were higher in the *PIP2;5ox* lines than the wild-type at 10°C, after one and two weeks.

The WUE values of the wild-type were lower than the *PIP2;5ox* lines, after 3 weeks, at all the temperatures. Stomatal conductance was also higher in transgenic lines than wild-type poplars, at 5°C, after 2 and 3 weeks of root temperature treatments (data not shown). The stomatal conductance was low during LRT treatment and it could be explained by osmotic adjustment of the guard cells and resulting turgor maintenance during the stages of reduced water supply. Higher transpiration rates in transgenic plants were possible due to faster water flow through the roots. These observations suggest that the suppression of plant physiological functions by LRT is associated with decreased water uptake ability of the root. This shows that there is a direct link between gas exchange responses and AQP function at LRT. Similar observation was made in wild-type *Populus trichocarpa* × *Populus deltoides* (Laur and Hacke, 2013). When aspen (*Populus tremuloides*) seedlings were grown at 10°C and 5°C in solution culture, net assimilation, stomatal conductance and shoot water potential were decreased and these parameters increased when the seedling roots were exposed to 20°C (Wan et al. 1999). In *Arabidopsis*, the shoot and root growth of the plants over-expressing *PIP2;5* AQP was less sensitive to LRT compared with the wild-type plants (Lee et al. 2012). The discrepancies between the results of similar studies might be due to the differences in the plant species, growth conditions, physiology of plant, duration of experimental period and the type and degree of the stress employed. Low soil temperature is a common factor that inhibits plant growth (Wan et al. 2001) and upsets plant water balance by reducing root water flux to the transpiring leaves (Wan and Zwiazek, 1999; Wan et al. 2001). In a plant, synchronization of leaf and root water transport is needed to maintain stomatal conductance that is essential for CO_2 uptake (Siefritz et al. 2002; Kaldenhoff et al. 2008). The temperature sensitivity of root water transport was reflected by a greater reduction in the growth of poplars at 5°C root temperature for 3 weeks. All of these

observations support the hypothesis that the reduction of plant growth by LRT is associated with water uptake, which is reduced in plants grown at LRT.

Low temperatures can induce conformational changes of the AQPs and affect their gating behaviour (Tyerman et al. 1999). This may be linked to respiration (Zhang and Tyerman 1999, Kamaluddin and Zwiazek 2001) and phosphorylation (Lee et al. 2005). Root respiration is inhibited at LRT and this may be indirectly responsible for the decline in root water flow. When the root temperature was reduced from 20°C to temperatures below that, the root respiration was likely reduced leading to the reduction in water flow rates and consequently, gas exchange rate and growth of the plant. This suggests that root energy reserves were quickly restocked at higher temperatures and that available energy for the AQP function was low when the temperature was lowered. After the drop of gas exchange rates and root water flow rates in all of the plant types when they were exposed to 5°C, these parameters started to increase slowly with time as acclimation progressed. After two weeks of exposure to 5°C, transgenic lines showed higher gas exchange rates and root hydraulic conductivity than the wild-type plants and it indicates the direct or indirect role of *PIP2;5* in increasing the root water flux under LRT.

Root water uptake capacity is controlled mainly by the hydraulic conductivity of the roots. In this study, the root hydraulic conductivity (L_p) of the poplars growing at 10 and 5°C was significantly lower than the plants at 20°C, after 1 week (Figure 4.8 A). Both transgenic linesshowed significantly higherL_p values than the wild-type at 10 and 5°C after two weeks (Figure 4.8 B).When the *Arabidopsis* roots were exposed to 10°C for 5 days, L_p was reduced in wild-type plants, whereas there was no effect on L_p in *PIP2;5* over-expressing plants (Lee et al. 2012). Up-regulation of *PIP2;5* and the other related AQPs induced during the LRT exposure

might have contributed to the increased root water flux and it subsequently affects the water transport in the transgenic lines. Therefore, over-expression of *PIP2;5* is likely to be effective in improving gas exchange and water transport properties of poplars in soils of cold climate.

Of the *PIP* genes in the poplar genome, seven highly-expressed *PIP* genes were selected for the present study (Almeida-Rodriguez et al. 2010). Of the seven examined *PIP* genes, *PIP2;5* was the most responsive to water deficit conditions and showed relatively low expression in response to LRT. *PIP2;5* expression differs among plants based on the species, growing conditions and treatment type. Studies on the post transcriptional level and the analysis of all of the *PIP* and *TIP* aquaporin genes might answer the question of their relevance to changes in root hydraulic properties of the transgenic and wild-type poplars exposed to LRT. The effect of the reduced gas exchange properties was correlated with altered expression of some AQP genes stimulated by over-expressing *PIP2;5* in the transgenic lines, and may have contributed to partial recovery/restoration of the reduced gas exchange properties. In contrast, in wild-type plants, these altered physiological responses were lacking. Since transpiration is a major driving force for the root water uptake from the soil, up-regulation of AQP *PIP2;1* and other AQPs showing up-regulation are expected to play an important role in facilitating water uptake through the roots to the shoots by increasing the water permeability of the plasma membranes and facilitating water transport via the cell-to-cell pathway.

4.5 Conclusions

These results demonstrate the importance of *PtdPIP2;5* AQP in facilitating the maintenance of the root water transport and stable water status by altering the water permeability of the plasma membrane. Transgenic lines did not respond differently from the wild-type poplars in RSHG,

shoot androot dry weight and total dry weight at any temperatures tested, but they showed improved gas exchangeproperties and root hydraulic conductivities than the wild-type at LRT stress condition after three weeks.This suggests that the effects on growth may take a longer time than just threeweeks. Compared tothe wild-type plants,the transgenic lines over expressing *PtdPIP2;5* poplars may be more resistant to continuous low root temperature treatment. There is a relationship between gas exchanging properties at low root temperature and AQP-mediated water transport in INRA 717aspen clone and there is a possibility that AQP are part of an integrated LRT stress response of this cloneto continuouslow root temperature treatments.

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CHAPTER 5

5. Characterization of transgenic hybrid aspen clone INRA 717 over-expressing aquaporin *PIP2;5* under PEG induced osmotic stress

5.1 Introduction

Water uptake and transport across the membrane is important not only for the plant growth under favourable conditions but also for the ability of the plant to tolerate and survive during adverse environmental conditions (Steudle and Peterson, 1998). Water transport across the membranes is efficiently facilitated and regulated in almost all higher organisms in nature by water channel proteins called aquaporins (AQPs), which belong to a family of major intrinsic proteins (MIPs) (Maurel et al. 2008). AQPs are classified into five subfamilies including plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), small basic intrinsic proteins (SIPs), nodulin 26-like intrinsic proteins (NIPs) and X intrinsic proteins (XIPs) (Baiges et al. 2002; Maurel et al. 2008; Gupta and Sankararamakrishnan, 2009). Cell-to-cell water movement through AQPs plays an important role in coping with environmental stresses that lead to reduced transpiration. This feature makes osmotic forces predominant for water flow and root water uptake of the plant (Vandeleuret al. 2005;Kaldenhoffet al. 2007).

Water stress (drought) is an important abiotic stress that influences the distribution of woody plants and limits their productivity across the globe (Han et al, 2013). Water-stressed plants may also be more sensitive to other abiotic and biotic stresses such as chilling, air pollution and pathogen attack (Caruso et al. 2008). Plants may use different morphological, physiological and biochemical defense mechanisms to cope with inadequate water supply such as changes in

growth rate, stomatal conductance, osmotic potential and antioxidant defenses (Kozlowski and Pallardy 2002, Yordanov et al. 2003, Caruso et al. 2008,Xu et al. 2008).Trees are long-lived organisms that are often exposed to various environmental stresses during their life-time. *Populus*are an ideal model tree system to apply genetic engineering technology and stress treatments since they are easy to vegetatively propagate by rooted cuttings and to produce a large biomass in a short time length. Drought resistance varies considerably between genotypes of*Populus*, both inter and intra-specifically (Gebre and Kuhns, 1993;Cochard et al. 1996; Tschaplinski et al.1998; Marronet al.2002) suggesting that the genus is a good choice to investigate the adaptive responses to drought stress. Experimental drought is mainly obtained by withholding water. A similar water stress effect can also be produced by an osmotic constraint induced by addition of a non-permeant osmolyte such as polyethylene glycol (PEG) (Jacominiet al. 1988, Chen et al. 2002). As the water stress affects the expression of plant AQPs (Aharon et al. 2003), it could be suggested that AQPs that involve in transpiration-driven water transport are linked to stomatal movements.

Poplar AQPs were characterized and their functions in differential drought responses of two clones of *Populus balsamifera* and *Populus simonii* × *Populus balsamifera* leaves were studied by comparing their responses to mild and severe drought stress conditions and drought recovery treatments (Almeida-Rodriguez et al. 2010). Five AQPs were functionally characterized for water channel activity and *PIP1;3* and *PIP2;5* were among the most highly expressed leaf AQPs. Expression of *PIP1;3* and five other AQPs increased in response to drought in the leaves of a *Populus simonii* × *Populusbalsamifera*clone but not in a *Populusbalsamifera*clone, suggesting that this AQP favours the transpiration-driven water flow (Almeida-Rodriguez et al. 2010). In *Arabidopsis thaliana* leaves, expression of several *PIP* genes was down-regulated in

response to drought stress induced by withholding water, but AQPs *AtPIP1;4* and *AtPIP2;5* were exceptionally up-regulated (Alexandersson et al. 2005). Expression of *AQP*s in roots and aerial parts of *A. thaliana* plants responded differently to gradual water stress (Alexandersson et al. 2005). Transgenic banana plants constitutively over-expressing *MusaPIP1;2* showed improved water stress survival characteristics (Sreedharan et al. 2013).There are variations in induction of drought stress under laboratory conditions and the resistance strategies differ diversly among plants under different drought levels (Siemens and Zwiazek, 2004). PlantAQP expression and physiological response in plants to water deficit stress varies depending on the species, developmental stage, growth conditions and severity of drought (Alexandersson et al. 2005). These differential responses of *AQP*s to drought stress are likely because the functions of AQPs differ in various tissues under stress. Most of the stress related experiments suggest that AQPs play roles in maintaining water balance in plants (Luu and Maurel, 2005). However, the role of AQPs in plant water transport under water stress conditions remains unclear (Heinen et al. 2009).

In this study, the role of a major water-transporting AQP in hybrid aspen clone INRA 717 and its response to water stress by comparing how physiological responses in plants over-expressing AQP*PIP2;5* differ from those of the wild-type were evaluated. For this study, osmotic stress was applied by adding polyethylene glycol (PEG 6000) in the liquid culture of rooted cuttings of the AQP*PIP2;5* over-expressing transgenic lines and wild-type INRA 717 hybrid clone *Populus tremula*× *Populus alba*, under controlled environmental conditions. PEG has been successfully applied before to induce osmotic stress showingthat the molecular weight of PEG above 3000 will not penetrate the cells (Lawlor, 1970; Caruso et al. 2008). PEG of low molecular weight

(e.g. PEG 600) can be absorbed by the plants and cause toxic effects (Ehlert et al. 2011). PEG of high molecular weight (e.g. PEG 16 000) can block the water movement into the plant and thus cause a disproportionate decrease in the water potential of the plant. PEG 6000 was chosen in this study, because it is neither readily absorbed by the plant cells nor does it efficiently block water passage into the plants and has been successfully used in many studies as an osmotic stress inducer(Lawlor, 1970; Jacominiet al. 1988, Chen et al. 2002; Mujtaba et al. 2005; Ehlert et al. 2011).

The objective of the study was to investigate the effects ongrowth, water transport and the physiological responses of the *PIP2;5* over-expressing and wild-type poplars subjected to osmotic stress. When a plant is subjected to osmotic stress, the water potential gradient will be upset. Therefore the plant cells with more AQPs should lose water more quickly. Therefore the transgenic lines over-expressing *PIP2;5*would lose the resistance.The tested hypothesis was that *PIP2;5ox* lineswould show enhanced resistance to osmotic stress induced by PEG6000 through changes in growth, water permeability and altering physiological changes as compared to the wild-type. Several plant AQPs will act as a membrane-selective pathway for small uncharged solutes such as glycerol, urea, ammonia, carbon dioxide, hydrogen peroxide etc(Chaumont and Tyerman, 2014). AQP's also transport ions in addition to waterand these ions might play a role in the development of resistance in the transgenic lines. Water transport and physiological properties of the AQP over-expressed transgenic plants may bealtered by the changes in xylem sap ionic concentration and alteration in the membrane selective pathway for the movement of the uncharged solutes, under osmotic stress and this might lead to the changes in the physiology to develop resistance to osmotic stress. For example, when *Populus tremuloides*trees were

exposed to water stress, root hydraulic conductivity was up-regulated (Siemens and Zwiazek, 2004).

5.2 Materials and methods

5.2.1 Plant material

Poplar AQP*PIP2:5* cloned from *Populustrichocarpa* × *P. deltoides* H11-11 was selected to produce transgenic plants, as *PIP2:5* was previously confirmed to function as a water-transporting AQP and showed increased expression under water limitation (Almeida-Rodriguez et al. 2010). The coding sequence of *PtdPIP2:5* was cloned into the expression vector pCambia1305.2, under the control of the Ubiquitin promoter. *Agrobacterium*-mediated transformation was carried out (Levée et al. 2009) to introduce the construct into the *in vitro* grown hybrid poplar *Populus tremula* × *Populusalba* INRA Clone 717 1B4 obtained from INRA-Versailles, France. Rooted plantlets of transformed poplar produced from leaf sections grown through callus, were transferred to the growth chamber.

Transformed lines were confirmed by PCR using primers against the hygromycin resistance gene. Among the transformed lines, quantitative RT-PCR performed on the pooled 2nd and 3rd young fully expanded leaves, was used to identify two lines exhibiting the highest expression of *PtdPIP2;5*. These lines were selected and multiplied by rooted cuttings from stems and used for experimentation. The plants of two transgenic lines *PIP2;5ox1* and *PIP2;5ox2* and wild-type were used in this study. These were produced by rooted cuttings of the stem segments. 10-12 cm length young stem pieces with nodes and young leaf were placed in the trays filled with moist peat:vermiculite (2:1) after dipping in IBA powder and covered by polythene. After 15 days of

growth in growth chamber, polythene cover was gradually opened to expose the plants to the growth chamber condition. After steady rooting, the cuttings were replanted in four-liter pots with the same soil mixand were grown under semi-controlled conditions. They were grown for 3 weeks reaching a height between 30 and 40 cm and 3-6 mm in stem diameter at the root collar before PEG treatments were applied. The plants were watered six times per week and fertilized weekly with NPK ((1g/L) nitrogen-phosphorus-potassium) 15:30:15 commercial fertilizer, before the PEG treatment experiment. Plants were inspected for the presence of mites and thrips, with any infected leaf being manually cleaned using cotton swabs. Environmental conditions in the growth room were maintained at 24/18 °C (day/night) temperature, 65 ± 10 % relative humidity and 16h photoperiod with 350 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the top of the seedlings provided by the full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

5.2.2 Solution culture and PEG treatment

Each treatment consisted of three 30-L independent containers (Total of 9 tubs with 3 treatments). The plantlets (6 of each transgenic line (*PIP2;5 ox1* and *PIP2;5 ox2*)and wild-type in each plastic tub for the total of 18 plantlets) were grown at 20°C in aerated full strength modified Hoagland's solution culture (Zhang et al. 2013).A split plot design with randomizationwas used. An aeration pump (Model 9.5 950GPH, Danner MFG Inc., New York, USA) was immersed inside the containers to maintain continuous aeration and uniform circulation of the solution. This resulted in dissolved O_2 values > 5 mg L^{-1} while root systems were immersed in treatment solutions. In each container, a circulating pump (Model 9.5 950GPH, Danner MFG Inc., New York, USA) was immersed to continuously circulate the solution. The solution culture tubs, tubing and pumps were cleaned once per week to prevent

algal and bacterial buildup. Plantlets were gently washed with distilled water and transplanted into the solution culture. Twenty 8-cm diameter holes were made at regular intervals on flat styrofoam plates and these styrofoams were used to keep the plants in the plastic tubs filled with solution culture. Foam plugs were fitted around the stems and inserted into the holes to hold the stems in place while the roots were immersed in solution. All tubs had spouts installed into their sides to facilitate drainage, aeration and circulation of the nutrient solution via plastic tubing. After 3-weeks, plants were subjected to treatments with three different polyethylene glycol (PEG6000- Sigma Aldrich) concentrations (0, 20 and 50 g L^{-1}. Corresponding osmotic potential values were -0.10 to -0.14, -0.16 to -0.18 and -0.20 to -0.25MPa) by gradually adding PEG6000 to the nutrient solution over 36 h. Appropriate PEG concentration of solutionswere replaced every week. Styrofoamdirections were twisted by 180 degrees twice a week to minimize the edge effects.

5.2.3 Measurements

5.2.3.1 Height and weight

Plantlet heights were measured from the root collar to the shoot tip for each plant,every week. The heights were measured. The dry weight determinations of roots and shoots of individual plants were obtained after three weeks by drying the plants in an oven at 85°C for 72 h. The relative shoot height growth (RSHR) was calculated according to the following formula: RSHR = [(Final height –Initial height)/Initial height] x 100 (Hoffmann and Poorter, 2002).

5.2.3.2 Leaf size

Leaf expansion was estimated non-destructively by measuring the lengths and widths of the uppermost (youngest) leaves in control and PEG-treated plants. Initial leaf area of selected

leaves was 7.2±0.05 cm^2. Approximately ≤ 15% of the final leaf area of mature leaves developed in the control plants. The same leaf was observed and the length and width were measured in every week until the 3rd week. Width and length values were used to calculate actual leaf area values from a previously determined linear regression between leaf length (L) × width (W) and projected leaf area (A): A = 0.6712 L ×W (r^2= 0.99, n = 20). For the regression, twenty leaves from top, bottom and middle parts of one wild-type poplar, were randomly picked and length and width of each leaf was measured. Each leaf was scanned and the leaf area was measured using image analysis software.

5.2.3.3 Gas exchange measurements

After threeweeks of PEG6000 treatments, gas exchange measurements including stomatal conductance (gs), net photosynthesis (P_n) and transpiration rates (E) were performed on the 2nd fully expanded leaf of each plant using a portable open-flow photosynthesis system (LI-6400, Li-Cor Inc., Lincoln, NE) equipped with an incorporated auxiliary LI-6400B red/blue LED light source (chamber area = 6 cm^2). Light intensity was set at 350 μmol photon m^{-2} s^{-2} and reference CO_2 was set at constant 386 μmol mol^{-1}using the 6400-01 CO_2 mixer; air temperature was 21 ± 0.15°C and vapour pressure deficit (VPD) 1.3 ± 0.3 kPa. Sample and reference infrared gas analyzers were matched prior to every plant and data were logged every 30 s during a 5-min period (Voicu et al. 2008).

5.2.3.4 Root water transport

The root hydraulic conductivity was measured after three weeks, by attaching excised root system to the high pressure flow meter (HPFM, Dynamax Inc, Houston, TX, USA) using compression couplings. Water was then forced inside stems at increasing pressure. The

computer recorded the water flow rate and the applied pressure asused to compute root absolute hydraulic conductance. Root volumes were measured for each root system using volume displacement of water in a graduated cylinder (Voicu and Zwiazek 2004).

5.2.4 Statistical analysis

Statistical analyses were performed using R2.15.3 statistical software (R Development Core Team, 2011), at $\alpha = 0.05$ confidence level. The data sets were checked whether they keep the parametric assumptions of normality (Shapiro-Wilk and Kolmogorov-Smirnov tests) and homogeneity of variances (Bartlett's test). When necessary to meet the assumptions of normality and homogeneity of variance, the data were transformed, either by log transformation or square root transformation. The data were analyzed using ANOVA. Tukey's multiple comparison test was used to determine significant differences at $p \leq 0.05$.

5.3 Results

5.3.1 Growth and dry weight

Relative shoot height growth (RSHG) of the transgenic and wild-type poplars showed significant differencesin response to increasing PEG concentrations (Figure 5.1). Increasing concentrations of PEG from 0 to 50 g L^{-1}significantly reduced RSHG and both the transgenic lines showed significantly higher RSHG than the wild-type, after three weeks of treatment with 20 and 50 g L^{-1} PEG (Figure 5.1). There was no significant difference in RSHG between the two *PIP2:5* over-expressing lines subjected to 0 and 20 g L^{-1} PEG treatments, after threeweeks of growth (Figure 5.1). Total plant dry weight showed a decreasing trend with the increasing concentrations of PEG in the nutrient solution (Figure 5.2C). When subjected to 20 and 50 g L^{-1} PEG for three weeks, both transgenic lines showed significantly higher total plant dry weights

compared to the wild-type plants (Figure 5.2C). Similar decreases, which were greater in wild-type compared with transgenic plants, were observed for shoot (Figure 5.2A) and root (Figure 5.2B) dry weights.

5.3.2 Leaf area

When *PIP2;5ox* and wild-type lineswere exposed to 0 g L^{-1} PEG (control) for threeweeks, there were no difference in the leaf area values atthe end of the 3rdweeks (Figure 5.3A). When the plants were exposed to 20 g L^{-1} PEG for 3 weeks in nutrient solution, wild-type showed a smaller leaf area values relative to 0 g L^{-1} PEG compared to the *PIP2;5ox* plants (Figure 5.3B). The leaf area values in the two *PIP2;5ox* lines were similar when the plants were exposed to 20 g L^{-1} PEG for 3 weeks. Similar trend was observed for the leaf area values, when the plants were grown in 50 g L^{-1} PEG for 3 weeks in the solution culture (Figure 5.3C).

5.3.3 Gas exchange

Net photosynthetic ratesof the transgenic and wild-type poplars exposed to50 g L^{-1} PEG were significantly lower compared with that of 0 g L^{-1} PEG (Figure 5.4A). There were no significant differences in net photosynthesis between wild-type and *PIP2;5ox* lines when they were exposed to 0 g L^{-1} PEG (Figure 5.4A). When the plants were subjected to 20 g L^{-1} PEG treatment, wild-type plants showed a significant reduction in net photosynthesis compared with the *PIP2;5ox* plants (Figure 5.4A). Similar net photosynthesis responses were measured in plants subjected to 50 g L^{-1} PEG treatment (Figure 5.4A). Transpiration rate (Figure 5.4B) and stomatal conductance (Figure 5.4C) responded to PEG treatments similarly to net photosynthesis. Both the transgenic lines showed significant increases in the transpiration rates compared with the wild-type poplars when exposed to 50 g L^{-1}PEG (Figure 5.4B and 5.4C).

However transgenic line *PIP2;5ox1*only showed significant increase in the transpiration ratecompared with the wild-type poplars when exposed to 20 g L^{-1}PEG (Figure 5.4B).Both the transgenic lines showed significant increase in the stomatal conductance compared with the wild-type poplars when exposed to 20 and 50 g L^{-1}PEG (Figure 5.4C). There was no significant differencein WUE of the transgenic and wild-type poplars with the increasing concentration of PEG (Figure 5.4D). There were no significant differences in the WUE values between the wild-type and transgenic lines at any of the tested PEG concentrations (Figure 5.4D). The PEG treatment lowered the gas exchange rates of the transgenic lines *PIP2;5ox1* and *PIP:2;5ox2* and wild-type poplars. In addition, both transpiration rates and stomatal conductance were higher in the transgenic lines than the non-transgenic poplarsafter 3 weeks of 20 and 50 g L^{-1} PEG treatments (Figure 5.4B and 5.4C).

5.3.4 Root hydraulic conductivity

There was a significant reduction in the root hydraulic conductivities of the *PIP2;5ox* and wild-type plants with increasing PEG treatment concentrations (Figure 5.5). There were no significant differences in the root hydraulic conductivities (L_p) between the plants of wild-type and *PIP2;5ox* lines when exposed to 0 g L^{-1} PEG in the nutrient solution for 3 weeks (Figure 5.5). However, when the plants were treated with 20 and 50 g L^{-1} PEG for 3 weeks, transgenic line *PIP2;5ox1* showed significantly higher L_p values, than the wild-type plants. There were no significant differences in the root hydraulic conductivities between the *PIP2;5ox2*lines and wild-type plants in any of the PEG treatments (Figure 5.5).

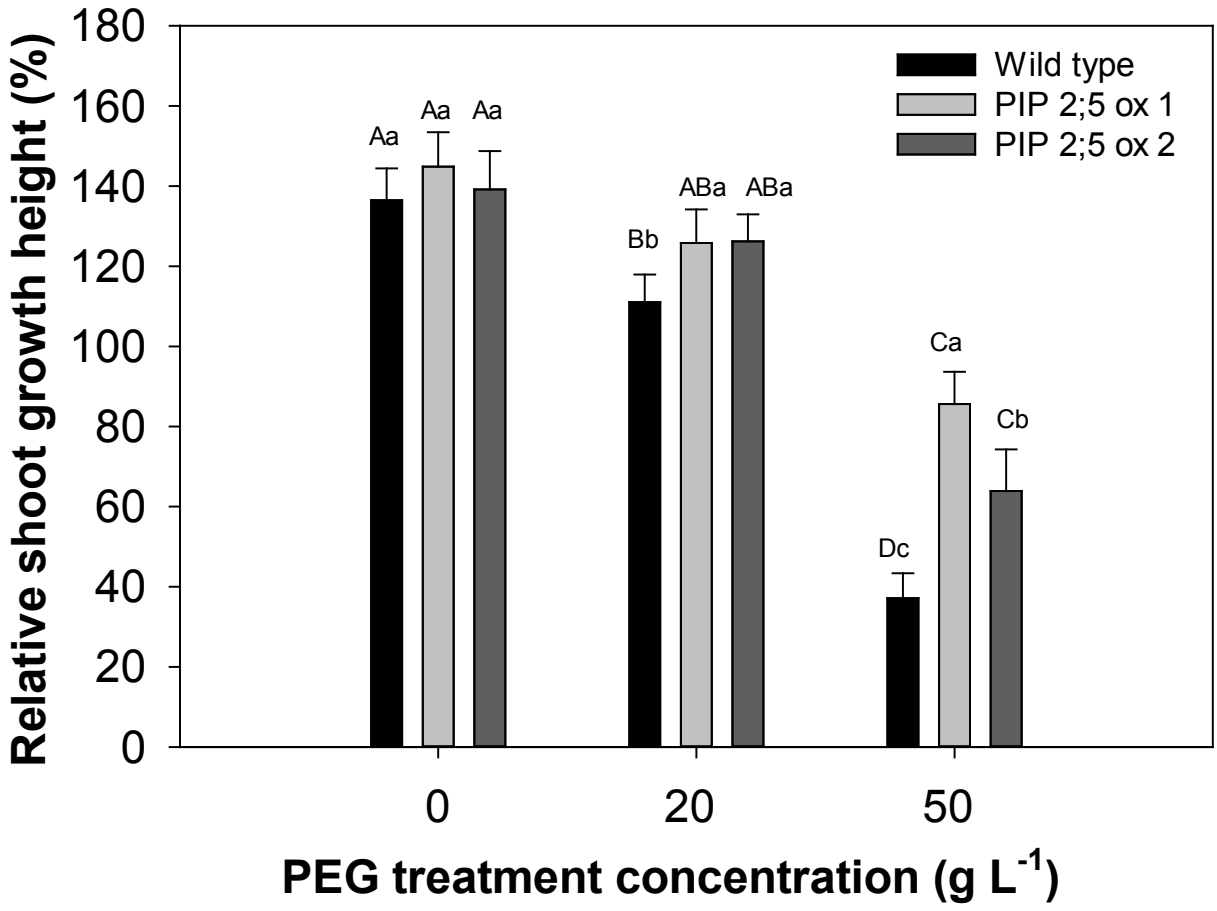

Figure 5.1: Relative shoot height growth (RSHG) of the two *PIP2;5ox* lines and wild-type poplar plants grown in poly ethylene glycol treatment concentrations 0, 20 and 50 g L^{-1} for 3 weeks in mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences (P≤0.05) between PEG treatments within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given PEG treatment.

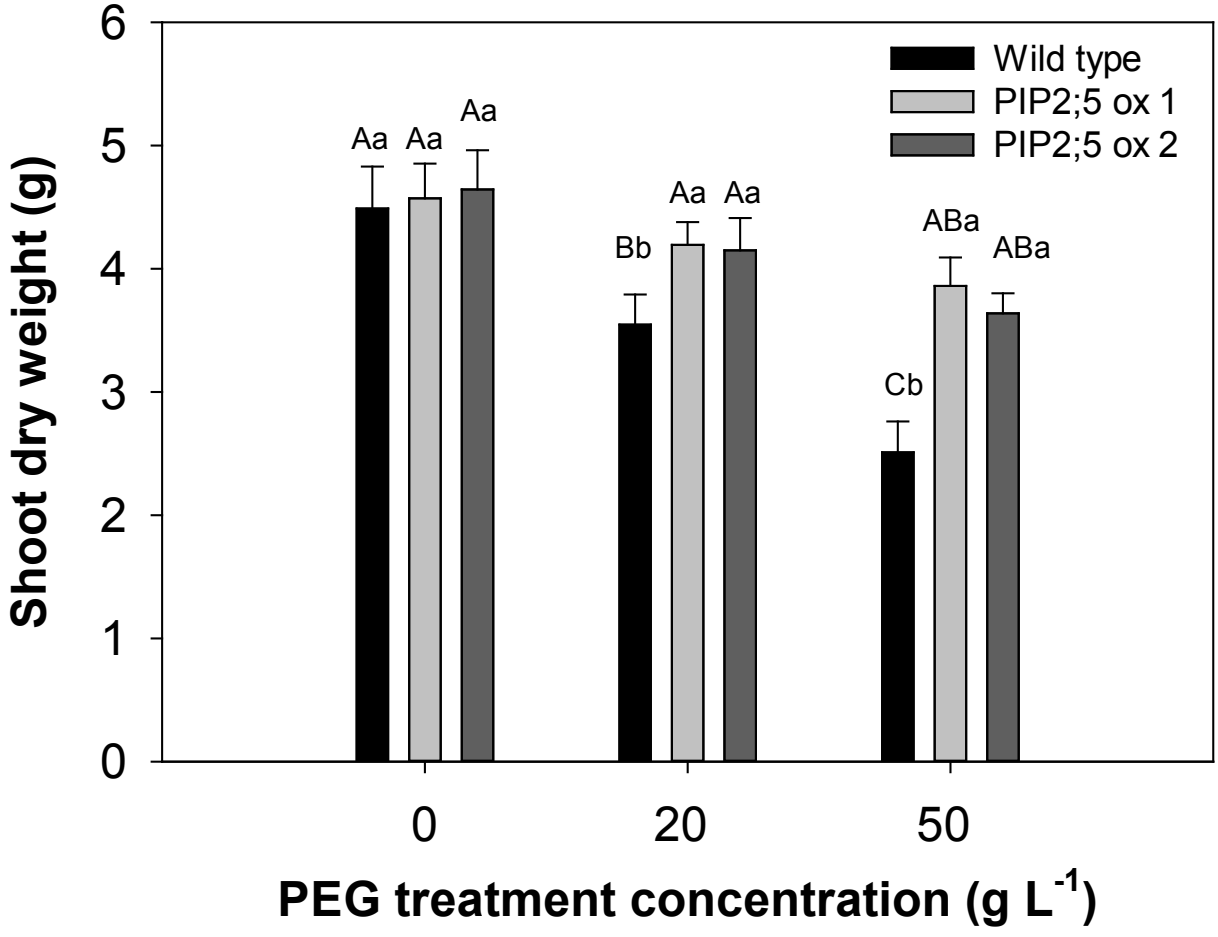

Figure 5.2A: Shoot dry weight of the two *PIP2;5ox* lines and wild-type poplar plants grown in poly ethylene glycol treatment concentrations 0, 20 and 50 gL^{-1} for 3 weeks in mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences ($P \leq 0.05$) between PEG treatments within a genotype, while lower case letters denote significant differences ($P \leq 0.05$) between genotypes at a given PEG treatment.

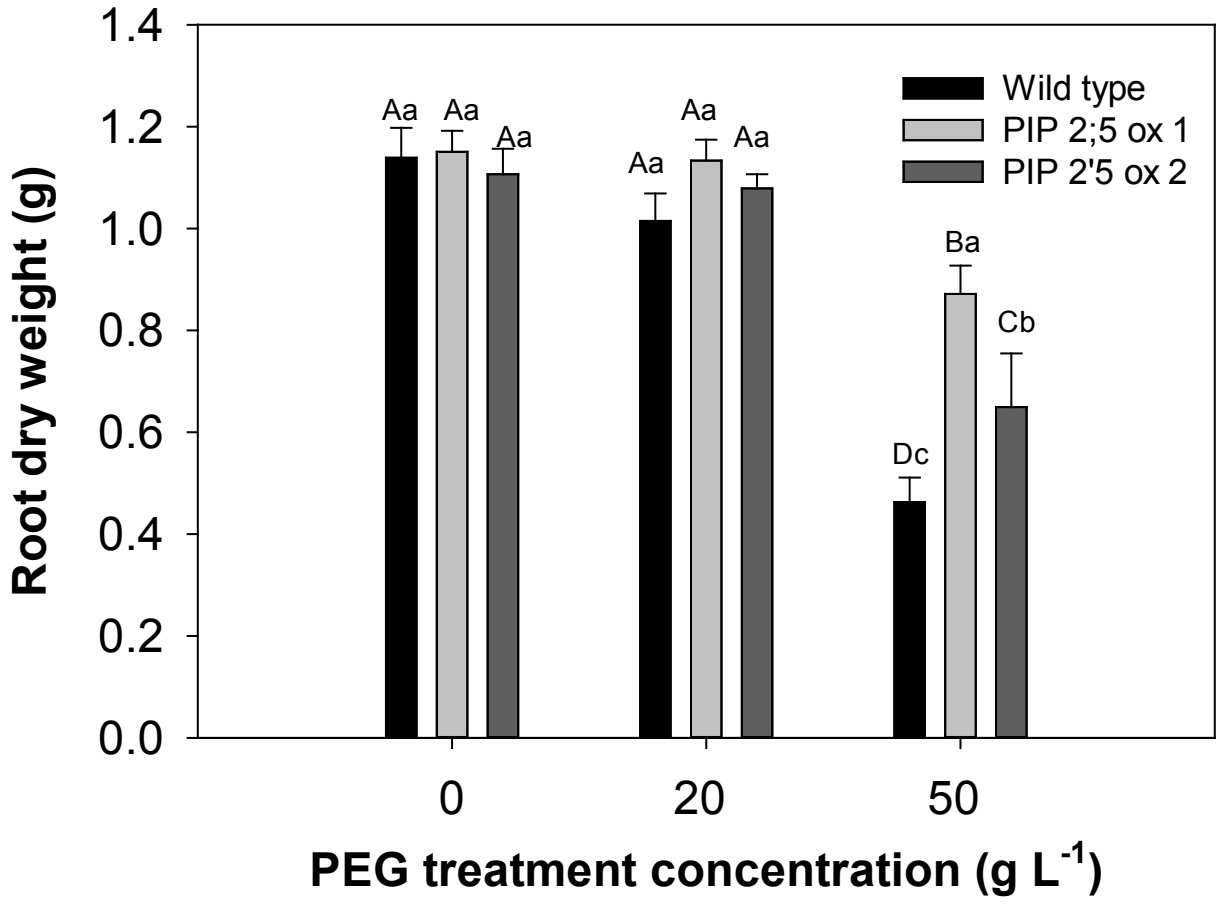

Figure 5.2B: Root dry weight of the two *PIP2;5ox* lines and wild-type poplar plants grown in poly ethylene glycol treatment concentrations 0, 20 and 50 gL^{-1} for 3 weeks in mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences ($P \leq 0.05$) between PEG treatments within a genotype, while lower case letters denote significant differences ($P \leq 0.05$) between genotypes at a given PEG treatment.

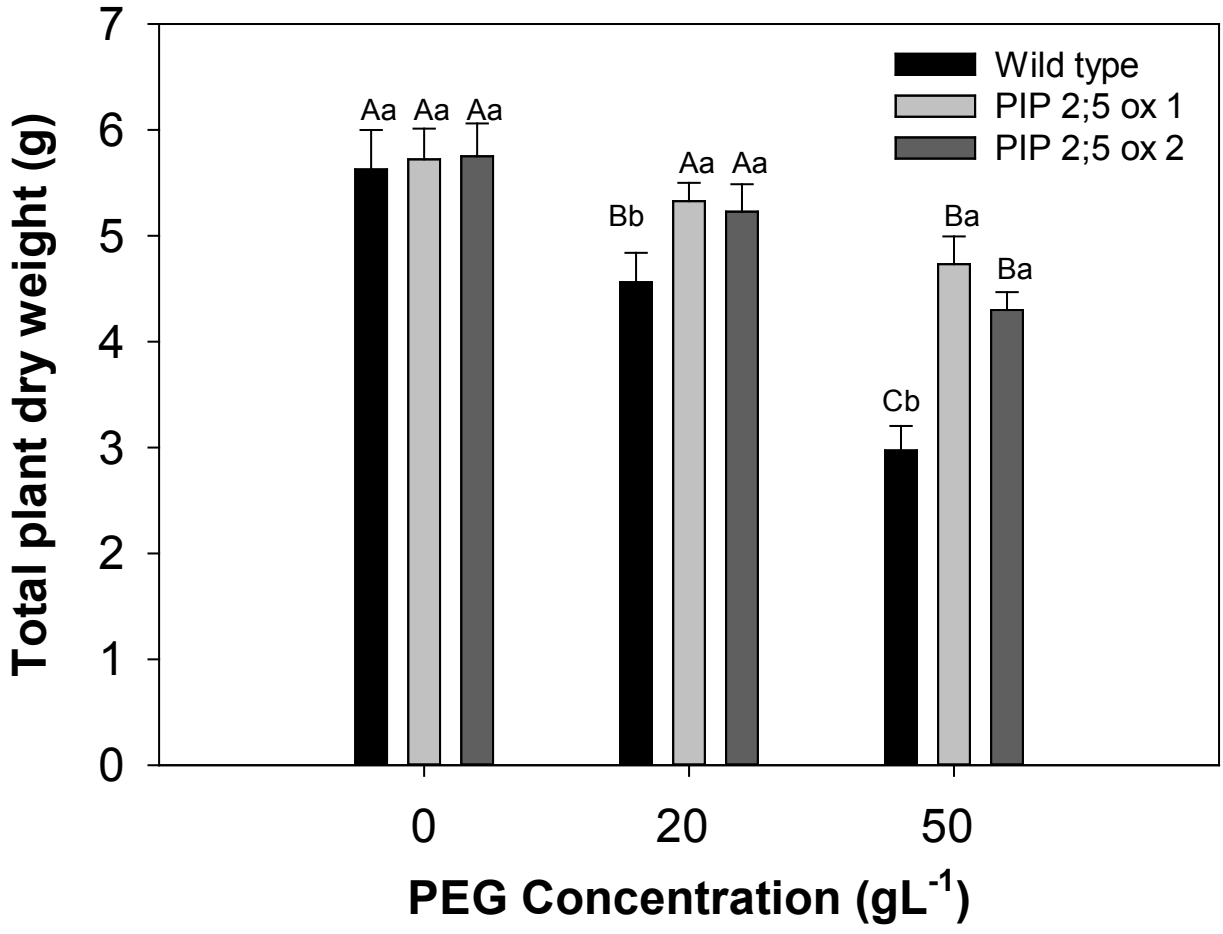

Figure 5.2C: Total plant dry weight of the two *PIP2;5ox* lines and wild-type poplar plants grown in poly ethylene glycol treatment concentrations 0, 20 and 50 gL^{-1} for 3 weeks in mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences ($P \leq 0.05$) between PEG treatment concentrations within a genotype, while lower case letters denote significant differences ($P \leq 0.05$) between genotypes at a given PEG treatment concentration.

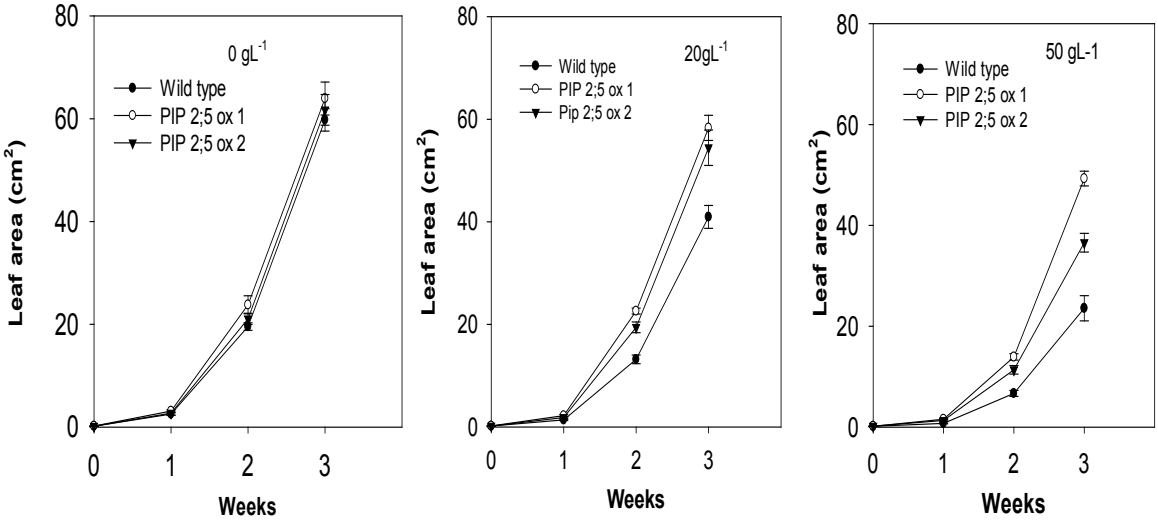

Figure 5.3: Areas of the growing leaves over time in the two *PIP2;5ox* lines and wild-type poplar plants subjected to 0, 20and 50 g L^{-1} of poly ethylene glycol treatment concentrations in mineral nutrient solution culture for up to three weeks. (Means ± SE, n = 6) are shown.

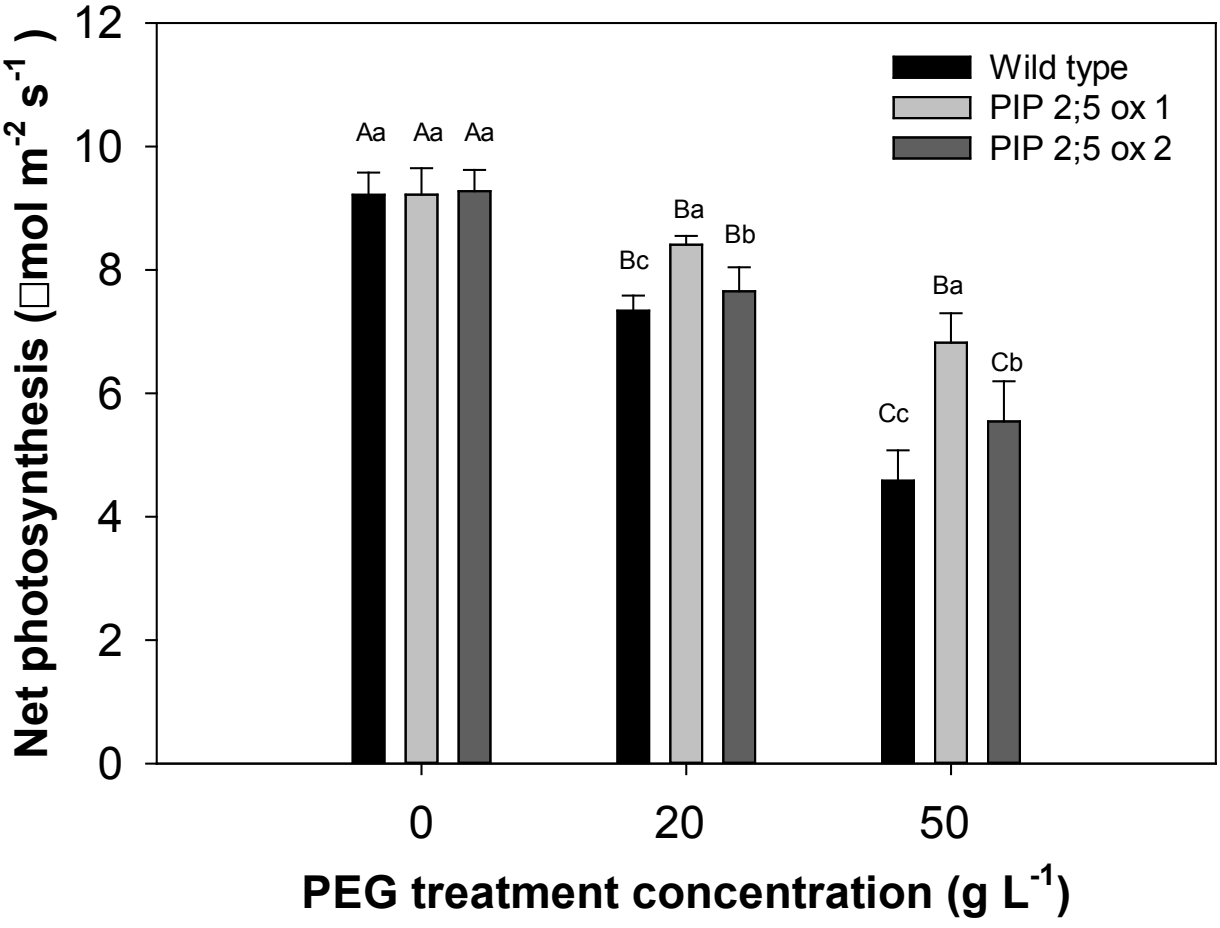

Figure 5.4A: Net photosynthesis of the two *PIP2;5ox* lines and wild-type poplar plants grown in poly ethylene glycol of concentrations 0, 20 and 50 gL^{-1} for 3 weeks in mineral nutrient solution (Means ± SE, n = 6). Upper case letters denote significant differences (P≤0.05) between PEG treatment concentrations within a genotype, while lower case letters denote significant differences (P≤0.05) between genotypes at a given PEG treatment concentration.

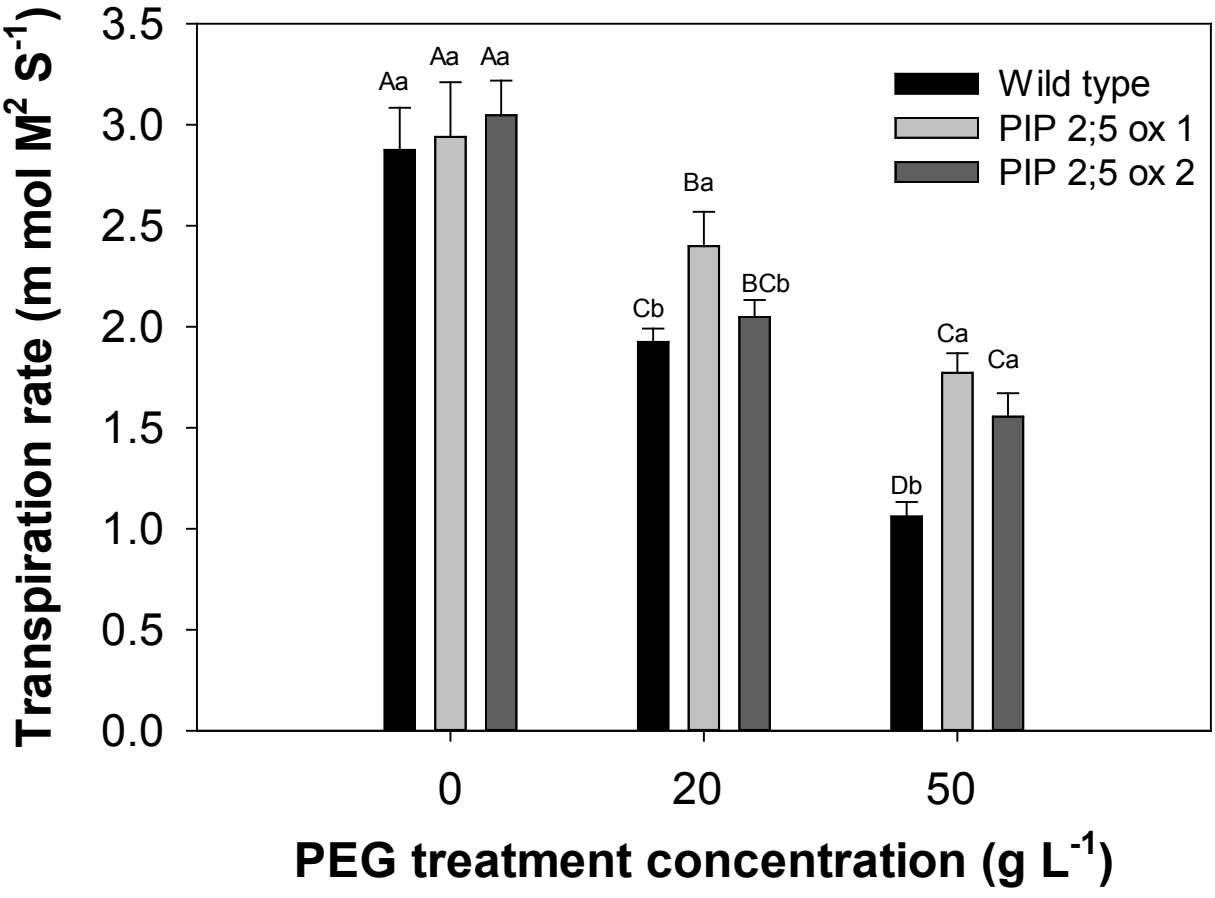

Figure 5.4B: Transpiration of the two *PIP2;5ox* lines and wild-type poplar plants grown in poly ethylene glycol of concentrations 0, 20 and 50 gL^{-1} for 3 weeks in mineral solution (Means ± SE, n = 6). Upper case letters denote significant differences ($P \leq 0.05$) between PEG treatment concentrations within a genotype, while lower case letters denote significant differences ($P \leq 0.05$) between genotypes at a given PEG treatment concentration.

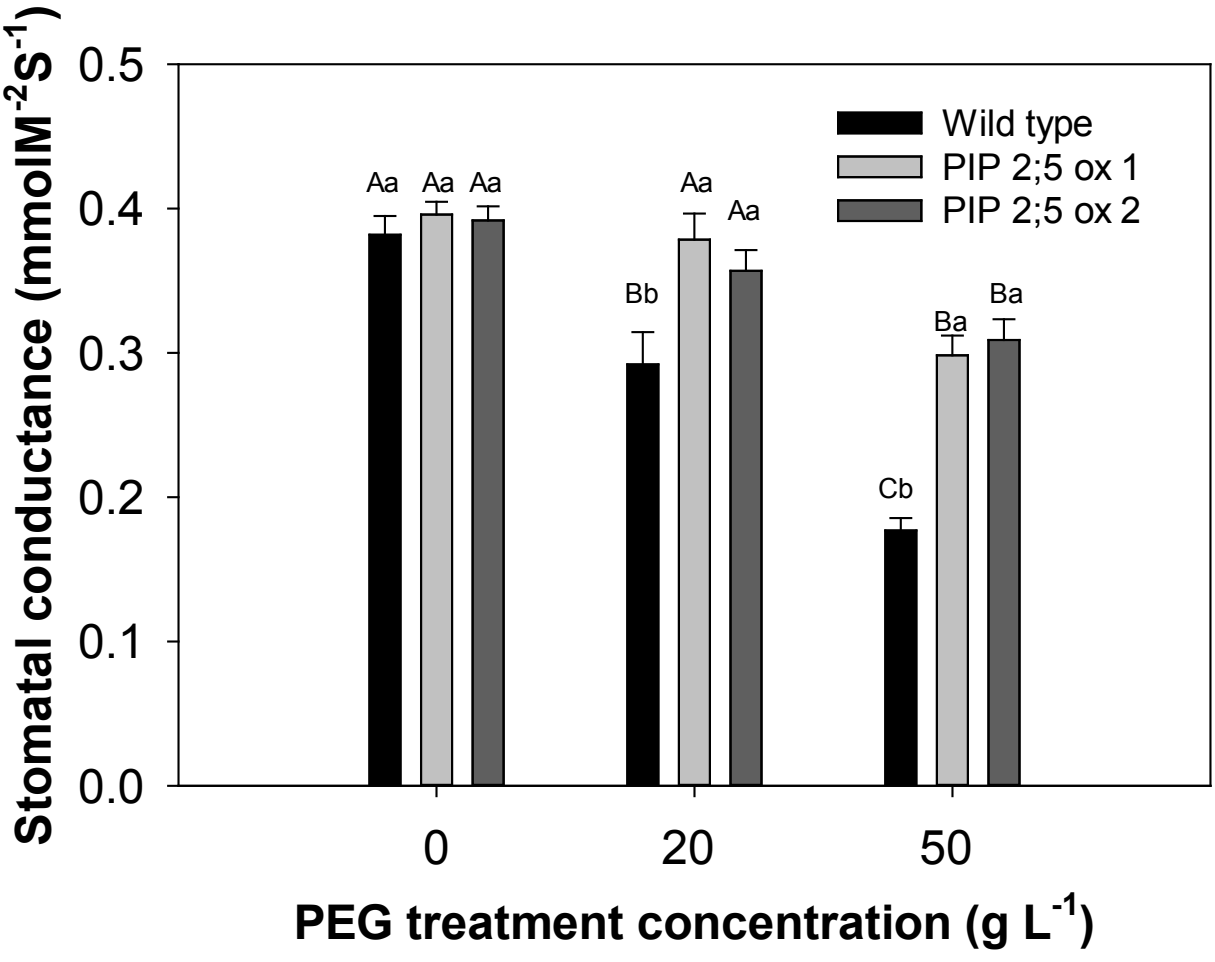

Figure 5.4C: Stomatal conductance of the two *PIP2;5ox* lines and wild-type poplar plants subjected to 0, 20 and 50 g L^{-1} PEG treatment concentrations in mineral nutrient solution for 3 weeks (Means ± SE, n = 6). Upper case letters denote significant differences ($P \leq 0.05$) between PEG treatment concentrations within a genotype, while lower case letters denote significant differences ($P \leq 0.05$) between genotypes at a given PEG treatment concentration.

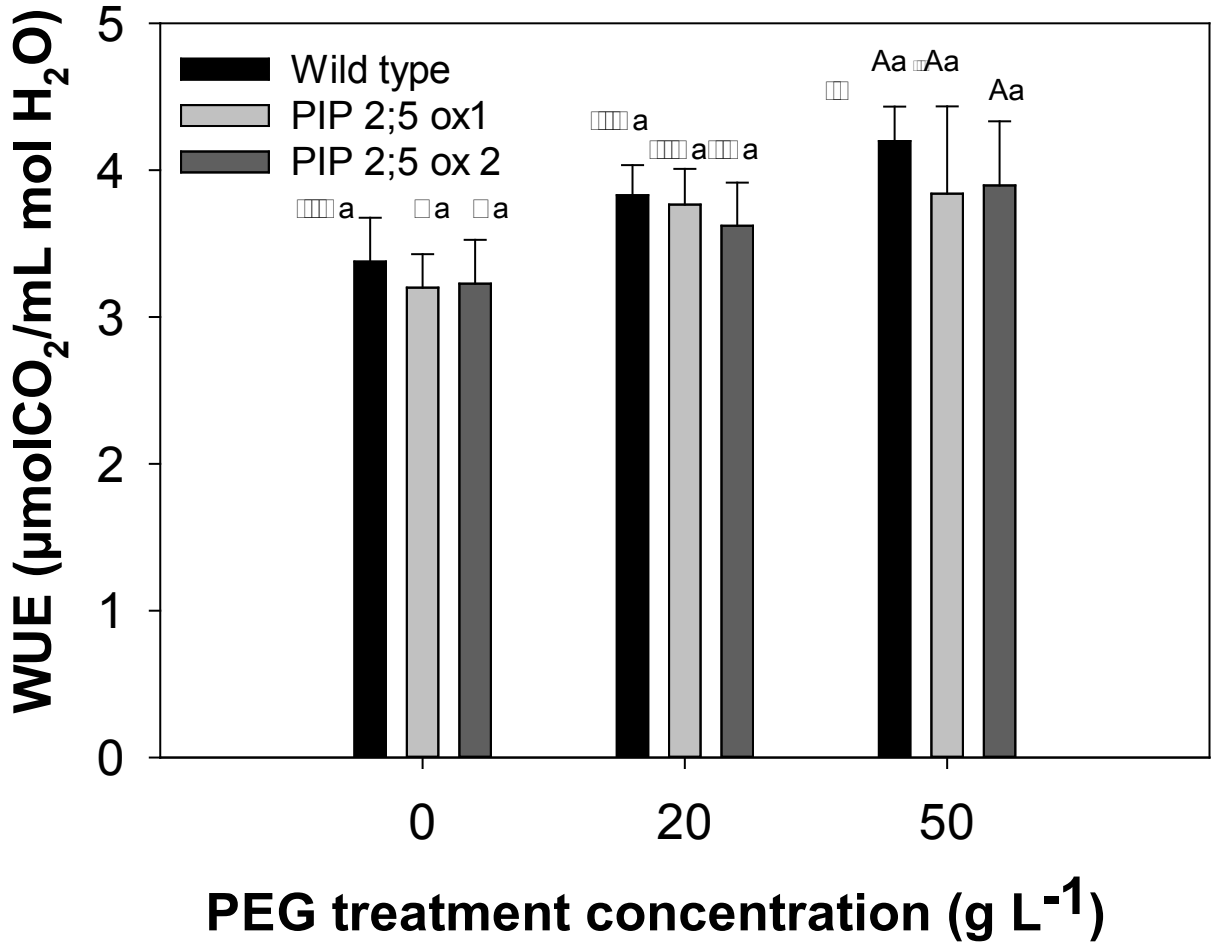

Figure 5.4D: Water use efficiency of the two *PIP2;5ox* lines and wild-type poplar plants subjected to 0, 20 and 50 g L^{-1} PEG treatment concentrations in mineral nutrient solution for 3 weeks (Means ± SE, n = 6). Upper case letters denote significant differences ($P \leq 0.05$) between PEG treatment concentrations within a genotype, while lower case letters denote significant differences ($P \leq 0.05$) between genotypes at a given PEG treatment concentration.

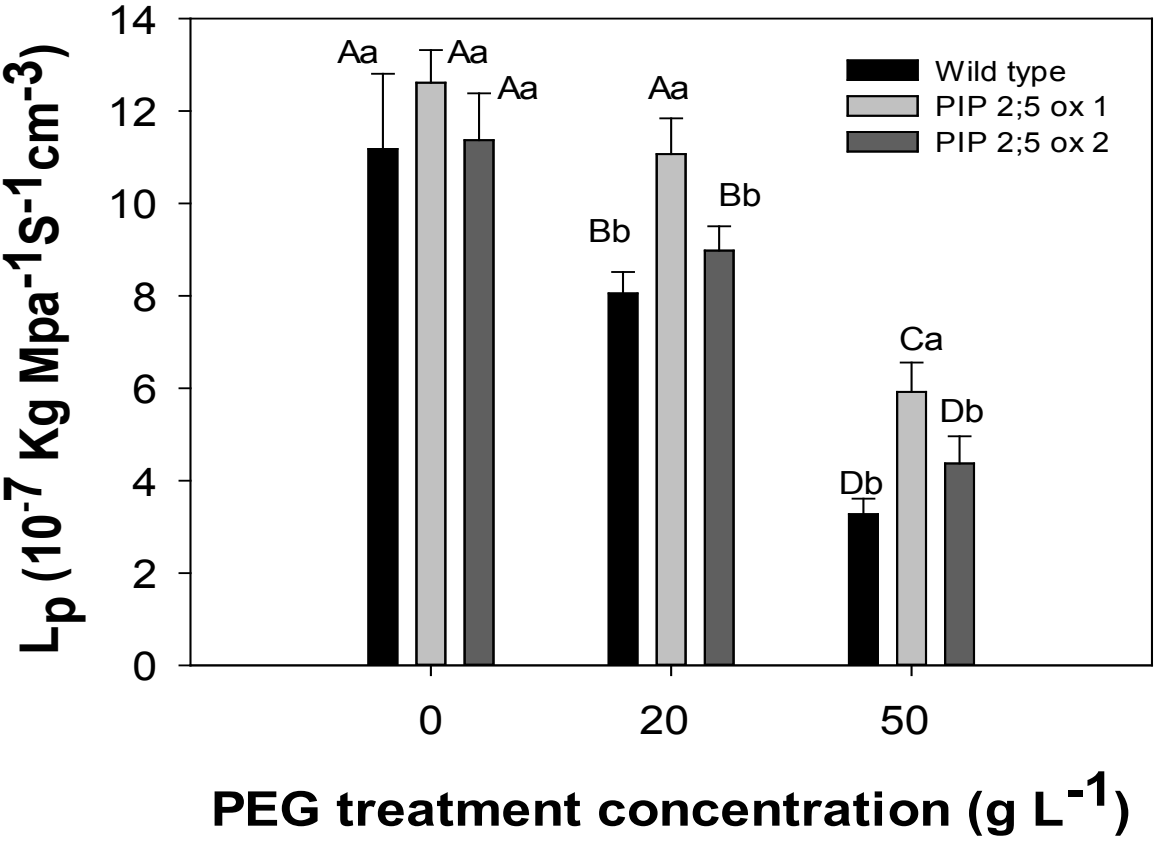

Figure 5.5: Root hydraulic conductivities of the two *PIP2;5ox* lines and wild-type poplar plants grown in PEG treatment concentrations 0, 20 and 50 gL^{-1} for 3 weeks in mineral nutrient solutions (Means ± SE, n = 6). Upper case letters denote significant differences ($P \leq 0.05$) between PEG treatment concentrations within a genotype, while lower case letters denote significant differences ($P \leq 0.05$) between genotypes at a given PEG treatment concentration.

5.4 Discussion

Plants were osmotically stressed by the addition of different concentrations of PEG to the aerated nutrient solution culture. PEG is commonly used as an external osmoticum to reduce the water potential of the medium (Verslues et al. 2006, Ehlert et al. 2011).PEG treatment induces a plant response similar to that induced by natural drought and causes a depression in plant growth (Dhanda et al. 2004; Mujtaba et al. 2005).

In this study, *PIP2;5ox* lines were more resistant to PEG-induced osmotic stress than wild-type with regard to plant height growth, plant dry weight and leaf size. Treatment with 50 g L^{-1} PEG caused significant reductions in root and shoot dry weight of wild-type poplars than *PIP2;5ox* lines (Figures 5.1, 5.2, 5.3). PEG induced osmotic stress is one of the important growth limiting factor that decreases the growth of poplars at the selected stage. Growth parameters markedly decreased in both transgenic and wild-type poplars due to PEG induced osmotic stress. Growth is inhibited by the effects on cell elongation and inhibition of stomatal conductance as a result of PEG induced osmotic stress.Growth is under the control of numerous genes whose expression may be differentially modulated by osmotic stress, thereby affecting the rate of growth of different parts. Root/shoot fresh weight ratio was reduced in transgenic *AtPIP1b* – tobacco plants relative to the non-transformed control plants, whereas plants over-expressing *VfPIP1* had longer lateral and primary roots than controls which affected root hydraulic conductivity (Cuiet al. 2005). Therefore root growth is directly related to the whole plant water transport efficiency and the phenotypic changes produced by over-expression of AQPs are key factors that influence the stress tolerance. At the same time, plants will show response to the stress by the activation of complex signal transduction networks (Bartels and Souer, 2003). Increase in the endogenous jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) in response to water stress

explains the involvement of these hormones in the development of stress resistance (Hamayun et al. 2010). Transgenic banana over-expressing Musa*PIP1;2* constitutively showed faster growth after cessation of water stress stimuli (Sreedharan et al. 2013).The over-expression of durum wheat *TdPIP1;1* in tobacco plants enhanced stress tolerance through increased root growth and leaf area of the plants (Ayadi et al. 2011).

The net photosynthesis and transpiration rates were significantly higher in transgenic poplars than in wild-type plants at 50 gL^{-1} PEG concentrations after 3 weeks (Figure 5.4A, 5.4B). Gas exchange capacity of plants was reduced because of the slow water flow rate of the plant and the reduction in the amount of water reaching the leaf and this resulting in adjustment of stomatal opening. When the amount of water reaching the guard cell reduces, the turgour pressure will decrease and that will lead to closure of stomata. Decrease in the gas exchange capacity will considerably reduce the photosynthesis and transpiration rates. Because of the comparatively less abundance and activity of AQPs in wild-type poplars, the rate of water loss would be higher in wild-type than the transgenic lines, thus the photosynthetic and respiratory rates will be considerably reduced in wild-type poplars compared to the transgenic lines. Stomatal conductance was higher in transgenic lines than wild-type poplars, at $50gL^{-1}$, after 3 weeks of PEG treatment. The stomatal conductance was reduced in all types of poplars during the $50gL^{-1}$ of PEG treatment and this is due to the osmotic adjustment of the guard cells and maintenance of turgor pressure at reduced water supply conditions. Inhibition of plant physiological functions by higher PEG concentration is associated with decreased water uptake and transport abilities of the root. This shows that there is a direct link between gas exchange responses and AQP function at PEG induced stress.Reductions in plant growth due to water stress are often associated with decreases in photosynthetic activities (Greenway and Munns, 1980). This is

because, a decreased amount of water and increased content of Na^+ in the cytosol can irreversibly inactivate PSI and PSII (Allakhverdiev et al. 2000). Stomatal closure results in a decrease in the leaf CO_2 concentrations. This might cause a decrease in the concentration of NADP+ available to accept electrons from photo system I/II and thus initiate O_2 reduction with the concomitant generation of activated oxygen species (AOS) (Hernandez et al. 2000). The over-expression of wheat *TaAQP7* gene, in tobacco increased detoxification enzymes such as SOD and CAT that reduces the H_2O_2 levels under drought/osmotic stress (Zhou et al. 2012). Under prolonged soil water deficit, the over-expression of ice plant (*Mesembryanthemum crystellinum*) AQP *McMIPB* resulted in a decreased rate of photosynthesis. The induction of *McMIPB* expression ameliorated the decreases of stomatal conductance and net photosynthesis rate under water deficit compared with those observed in non-transformed plants (Kawase et al. 2013). Even though the over-expression of the barley AQP gene, *HvPIP2;1* enhanced internal CO_2 conductance and CO_2 assimilation in rice (Hanba et al. 2004). However, in both *McMIPB* and *HvPIP2;1* over-expressing plants, a reduced shoot/root biomass ratio was observed under stress. Therefore plant responses to stress differ among plants depending on multiple factors. Increasing water use efficiency, carbon gain and the promotion of plant water retention by absorption are the critical goals in the improvement of plant tolerance to abiotic stress (Tsuchihira et al. 2010).Plants detect water deficit conditions, which triggers molecular signaling that result in change in growth, gas exchange reduction, osmoregulation and adjustments of water use efficiency (Barbieri et al. 2012, Bogeat-Triboulot et al.2007). There was an increase in WUE of all type of poplars with the increasing concentration of PEG (Figure 4D). This shows the tendency of poplars developing resistance to osmotic stress generally increases with the increasing PEG concentration.

The reduction in the gas exchange rate when poplars were exposed to PEG induced osmotic stress shows an evidence for a link between gas exchange responses and AQP function at osmotic stress condition.Higher gas exchange rates were observed at 20 and 50 gL^{-1} PEG concentration in the transgenic lines than the wild-type emphasize the key role of AQPs in osmotic stress responses of plants. Many environmental stress conditions directly or indirectly affect plant water balance (Sutka et al. 2011). The stomatal density was increased in transgenic AtPIP1b-tobacco plants, enhancing transpiration, whereas it remained unmodified in transgenic NtAQP1-*Arabidopsis* plants (Cui et al. 2005), compared to the wild-type. There may be root-to-shoot signals that influence the effect of AQP over-expression in the stomatal behavior. This might have caused the response of the transgenic plant to osmotic stress. Phenotypes of AQP over-expressing plant, water transporting capacity, rate of transpiration and stomatal size and plant anatomyare some factors that could be involved in the plant resistance to osmotic stress when AQPs are over-expressed.

When the plants were treated with 20 and 50 g L^{-1} PEG for three weeks, transgenic line *PIP2;5 ox1* showed significantly higher L_p values, than the wild-type (Figure 5.5). When the transpiration is restricted due to osmotic stress, the cell-to-cell path plays a major role in water movement. At the same time, a reduction of overall root hydraulic conductivity took place (Vandeleur et al. 2009) because of the suberin and lignin depositions restricting the apoplastic water flow which was not compensated by the cell-to-cell path. However, the decrease of whole root L_p is correlated with an increase in the water movement by the apoplastic path way, under water stress conditions (Siemens and Zwiazek, 2004). To have water flux, there should be a driving force resulting from water potential gradient. If there is no gradient, there will be no water flux. The water may leak from the roots if the gradient goes the other way.Even though

transpiration proceeds in a reduced amount under osmotic stress, water absorption will also take place simultaneously to compensate the water loss from the leaf. As a compensation effect, treangenic plants might have produced a smaller root system to cope up the osmotic stress. An inability of root water uptake to satisfy the water demand of the aerial parts was expressed in different ways in different plants, under different conditions.In transgenic low land rice (*Oryza sativa* L.) plants over-expressing *RWC3* gene,resistance to osmotic stress imposed by polyethylene glycol was produced through higher root L_p and lower leaf water potential (Lian et al. 2004). When the anisohydric (plants that keep their leaf water potential variable during the day) grapevine cultivar Chardonnay showed increased expression of *PIP1* gene in roots under drought, resulted in an increase in the cortical cell hydraulic conductivity (Vandeleur et al. 2009).

There were differences in the osmotic stress responses between the two transgenic lines used and this may be due to variations that occur in the plant genome after transformation, such as loss of expression of the transgene, rearrangements of the transgene in the host genome. As the site of integration of the gene into the plant genome is random and nonspecific, the integration might alter other gene functions differently in different plants (Ahuja, 1988). The expression in one plant species of an AQP gene from a different species may affect differentially the expression patterns depending on the target PIP isoform, nature of the foreign protein, stress condition (Janget al. 2007a). The expression of *CfPIP2;1*, a figleaf gourd (*Cucurbita ficifolia*) AQP gene, increased the survival rate of *Arabidopsis* plants under drought (Jang et al. 2007b). Degree of tolerance shown by poplars to stressful environments varies considerably among species, populations and clones because of their genetic diversity (Zhang et al. 2007, Yang et al. 2010). Even in the same experiment, some PIP genes were down-regulated,

othersup-regulated and others unaltered (Aroca et al. 2007; Ruiz-Lozano et al. 2009). Results revealed that the adaptive responses to osmotic stress vary between the two poplar transgenic lines.

Over-expression of AQP isoforms that are induced by stress may lead to plant stress resistance (Sade et al. 2010).This diversity of responses shown by different plants indicates a complex AQP regulation mechanism, where the altered expression of a *PIP* gene is required to cope with stress. The over-expression of a foreign AQP may improve osmotic stress tolerance through different ways such as cooperation with endogenous AQPs, interaction with other membrane AQPs and co-expression and formation of heterotetramers between PIP1 and PIP2 (Zelazny et al. 2007).

5.5 Conclusion

The height growth, leaf size and gas exchange as well as root hydraulic conductivity were less affected by PEG-induced osmotic stress in *PIP2;5*-over-expressing lines of poplar compared with the wild-type plants. These results suggest that the activity of *PIP2;5ox* poplars may be more resistant to prolonged treatment of 50 gL^{-1}PEG, compared to the wild-type.A combination of characteristics like higher root hydraulic conductivity leading to improved water flux, a higher stomatal conductance and altered biochemical reactions contribute to PEG induced stress resistance of *PIP2;5ox* lines than wild-type poplars. The ability of each over-expressed AQP isoform to confer resistance to osmotic stress may depend on its contribution to the plant control of water loss by transpiration or its ability to maintain CO_2 assimilation or increase water uptake

and water transport properties. The mode of function of a particular isoform on other endogenous AQPs may produce an overall response to the osmotic stress.

5.6 References

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CHAPTER 6

6. Conclusions

6.1 Summary of outcomes from this study

6.1.1 Completion of study objectives

The goal of this research was to accomplish the objectives presented in the Chapter 1 of this dissertation. The results presented in Chapter 2 addressed the first objective, which was to produce genetically modified poplars over-expressing the aquaporin (AQP)*PtdPIP2;5*. The results in Chapter 3 addressed the second objective, which was to examine the short term physiological responses and the AQP expression of the *PIP2;5ox* and the wild-type poplars when poplar roots were kept at low temperature. The results presented in Chapter 4 addressed the secondobjective further, which was to examine the physiological responses, low root temperature stress response, AQP expression and the growth of *PIP2;5ox* with the wild-type poplars when the roots were kept at continuous low temperature for three weeks. The results presented in Chapter 5 addressed the thirdobjective, which was to investigate the change in growth, water transport and the physiological responses of the transgenic and wild-type poplars, under PEG6000-induced osmotic stress.Expression profiles of AQP*PIP2;5*and other *PIP1* and *PIP2* genes demonstrated in Chapters 2, 3 and 4 were a step towards the fourthobjective, which was to determine the function of AQP*PIP2;5* by studying the expression patterns of AQPgenesin tissues of the transgenic and wild-type hybrid aspen clones that were subjected to short term and continuous low root temperature (LRT) and osmotic stress.

6.1.2 Summary of findings

Over-expression of *PIP2;5* in *Populus tremula×P. alba* was effective in alleviating the effects of short term exposure of LRT on hydraulic conductivity (L_p), even though LRT did not result in short term down-regulation of genes encoding *PIP1* or *PIP2* AQPs in wild type plants. The inhibition of L_p by LRT could be partially prevented by over-expressing *PIP2;5*, suggesting that the effect of LRT on root water transport and cell-to-cell water transport processes involving this aquaporin. The temperature sensitivity of root water transport was reflected by a reduction in the water transport in the wild-type compared with *PtdPIP2;5ox* plants exposed to LRT. Based on the L_p and gas exchange measurements, transgenic lines showed more resistance to LRT than the wild-type plants. These results provide a evidence for a positive relationship between responses in gas exchange to LRT and AQP-mediated root water transport in poplar and suggest that the LRT sensitivity of root water transport may be connected to the AQPactivity rather than abundance, since transcript abundance corresponding to *PIP1* and *PIP2* AQPs was unchanged. The transgenic lines showed higher growth as well as improved gas exchange and water transporting qualities compared with the wild-type poplars when exposed to higher concentrations of PEG for long term. These results suggest that the activity of *PIP2;5ox* poplars may be more resistant to long term treatment of $50gL^{-1}$PEG, compared to the wild-type. A combination of characteristics like higher $L_p$leading to improved water flux, higher stomatal conductance and altered biochemical reactions contributed to higher PEG induced osmotic stress resistance of *PIP2;5ox* lines compared with wild-type poplars.

6.1.3 Verification of the tested hypotheses

6.1.3.1 LRT stress resistance of the transgenic lines through increase inroot water transport

Over-expression of AQP*PIP2;5* can increase overall water transport of the plant to maintain highly stable water status at LRT's and in consequence, high physiological response rates when their roots are exposed to low temperatures.

The results shown in Chapters 3 and 4 support the first hypothesis of this research by demonstrating the differences in the physiological response rates and root water transport, between *PtdPIP2;5ox*lines and wild-type poplars. Decreasing temperatures during periods of less than 2 hours significantly decreased L_p in wild-type plants, but had no significant effect on L_p in the transgenic lines. Recovery of $L_p$to pre-stress levels in the transgenic lineswas faster than the wild-type poplars. When the roots were exposed to 5°C for 30 min in solution culture, wild-type plants showed significantly lower net photosynthetic rates and L_p compared with the *PtdPIP2;5ox* plants and the transpiration rate of the wild-type plants were also relatively lower.Relative shoot height growth, shoot androot dry weight and total dry weight did not respond differently between transgenic lines and the wild-type poplars at any temperatures. However the transgenic lines showed improved gas exchanging properties and root hydraulic conductivities than the wild-type at continuous LRT stress treatment for three weeks.AQPs play a role in many abiotic stress conditions directly or indirectly and affect water balance in plants. AQP-mediated water transport involves maintaining the water balance in the plant body and in transgenic lines, this water balance was likely to be reached faster than in the wild-type plants in an efficient way using the function of over-expressing AQP*PIP2;5*. Because of the AQP *PIP2;5*over-expression,rate of root water flow might have increased in transgenic lines but not in wild-type poplars.The results suggest that the transgenic poplars may be more resistant to LRT compared with the wild-type and provide a evidence for a positive relationship between gas exchange responses to LRT and AQP-mediated root water transport in poplar. The quick recovery of the $L_p$in transgenic lines compared with the wild-type, when the temperature was

raised back to 20°C from 5°C, suggests AQP mediated trans-membrane water transport. However, it was not possible to identify an exact relationship between the physiological responses related to the temperature-dependent $L_p$to obtain a possible role of AQPs in this process. This likely was due to the density of the plant tissues and how root water transport pathways adjust under LRT stress.

6.1.3.2 LRT changes root water movement and aquaporin expression

The impact of low temperature on root water transport involves changes in the expression of *PIP2;5* and/or related AQP genes.Results shown in Chapters 3 and 4 tested the second hypothesis of this research. The functional significance of the AQP isoform *PIP2;5*, highly expressed at favorable growth temperatures, did not change at LRT exposure in the short term. When the roots were kept at 5°C for 30 minutes, the expression of *PIP2;5* remained significantly increased as in 20°C, in transgenic lines compared with the wild-type. The enhanced level of *PIP2;5* transcript abundance observed at favorable growth temperatures was diminished in both over-expressing lines when the roots were exposed to LRT for 3 weeks. Therefore, the physiological responses exhibited by plants might be due to the integrated effect of different AQP genes which express differently at LRT conditions. In contrast, *PIP2;5ox* linesmight have differential expression of *AQPs* in different tissues like leaves and roots, depending on its strategy in response to handle LRT stress. The differentially expressed *AQPs* in both the transgenic lines (*PIP2;5ox1*, *PIP2;5ox2*) and wild-type might play a significant role in the responses of the respective plants to handle the LRT stress. Non-stressed plants showed similar transcript abundance levels and plants exposed to stress showed different transcript abundance, which supports the above statement.

6.1.3.3 Osmotic stress resistance of the transgenic lines

PIP2;5 over-expressing poplars showed enhance resistance to osmotic stress induced by PEG6000 relative to wild-type plants by maintaininghigher rate of water transport and altering gas exchange properties.The results shown in Chapter 5 support the third hypothesis of this research by demonstrating differences in physiological responses between the *PIP2;5ox*lines and wild-typehybrid poplars to osmotic stress. In these findings, transgenic lineshave shown more efficient management of water resources compared to the wild-type poplar and maintained higher net photosynthesis, stomatal conductance and root water transport during mild and severe osmotic stress. The higher stomatal conductance and net photosynthesis exhibited by the transgenic lines under osmotic stress is likely advantageous to these plants by enabling them to maintain growth rates and attain greater leaf size relative to the wild-type plants. Transgenic lines showed improved WUE and water transport capacities than the wild-type when exposed to osmotic stress. Stomatal responses might also be related to the development of resistance in the *PIP2;5ox* lines to osmotic stress and xylem cavitation.The transgenic poplar might be better able to increase WUE and water retention capacities compared with the wild-type plants when exposed to more severe osmotic stress. Reductions in transpiration in plants might be related to xylem hydraulic protection when the amount of water in the xylem decreases below a critical point of water potential during water deficit due to osmotic stress and due to the involvement of ions that are accumulated and transported through the xylem.

6.1.3.4 Higher PIP2;5 expression in transgenic lines under non-stress condition

Even though there are differences in the relative abundance of *PIP2;5*transcript corresponding to the endogenous gene plus transgene in leaves and roots examined in this study, the expression of *PIP2;5* was higher in transgenic lines relative to wild type plants in all tissues, under non-stress

conditions.There is a difference in *PIP2;5*expressionin shoot and root and this may be due to differences in the morphology and function of the tissues and diverse function of genes in response to different abiotic stress experienced by roots and shoots. *PIP2;5*expression was profiled in leaf tissue when the poplars were grown in the growth chamber for one month after the transfer from culture media to soil mix. Using leaves instead of young roots for qRT-PCR to confirm over-expression of the transgene avoided damage of roots at the early stage of establishment,avoiding the issue of plant mortality. Expression profiles of *PIP2;5*and six other *PIP1* and *PIP2* genes were obtained from the root tissue before and after the LRT stress experiment. In spite of differing expression levels of the combined endogenous gene plus transgene *PIP2;5*between the leaves and root tissues of *PIP2;5ox* lines, expression of *PIP2;5* was higher in both of the selected transgenic lines regardless of the tissue and developmental stage, under non-stress conditions. This confirms the existence and efficient expression of *PtdPIP2;5* in the transgenic plants. Among the seven *PIPs* examined from the root tissue,*PIP2;5* showed significantly increased levels of transcript abundance in both the transgenic lines at 20°C and *PIP2;1*showed significantly increased levels of transcript abundance in one transgenic line, after 3 weeks of RT treatment at 5°C. Highly expressed genes showed contrasting expression levels in both transgenic lines (*PIP2;5ox1*, *PIP2;5ox2*)and in the wild-type poplars.Some low abundance AQPs(*PIP2;1*) were up regulated significantly in response to long term LRT. High and low expression levels of AQPgenes during LRT stress might be related to functional differentiation of AQPs. Highly expressed AQPsmight be related to water redistribution within cells, while lowerexpressed AQPsmight be involved in the avoidance of water loss from tissues. Lower expressed AQPsmay also exhibit restricted spatial expression patterns within the root, leading to a seemingly low abundance. The expression levels of AQPs among poplar tissues and in response to LRT stress conditions might be due to different

pressures of selection, driving the functional diversification of this protein's family members in poplars. Variation in the AQP gene expression might depend on the cell-type, substrate specificities, tissue type, severity of the stress and environmental conditions. The change in the expression level of differentAQPsunder LRT stressmay be a part of the mechanism of role in plant survival under LRT stress conditions.

6.1.3.5 Different plants handle stressresistance differently under stress

Changes in the physiological processes and growth of the plant and AQP transcript profiles in the wild-type and transgenic lines under stress conditions may reflect the differences in plant response to stress. Based on the results shown in Chapters 4 and 5, relative shoot growth height, L_p and gas exchange measurements, during the stress response process of the *PIP2;5ox* lines and the wild-type hybrid poplar to low root temperature and osmotic stresses, suggests that the transgenic lines are more resistant to LRT and PEG 6000 induced osmotic stress than the wild-type plants. According to the transcript profiling, there were significant differences in the transcript abundance of *PIP2;5* and *PIP2;1* between wild-type and *PIP2;5ox1*transgenic lineat the end of the 3 weeks of LRT stress relative to the control plants. These results provide evidence for a positive relationship between the responses to long term stress and AQP-mediated root water transport in transgenic poplar compared to wild-type plants. Root growth and leaf size are directly related to the whole plant water transport efficiency, highlighting that the changes produced by AQP over-expression might have influenced the resistance development to the abiotic stress in transgenic lines compared to the wild-type plants during the stress. Increasing WUE and the promotion of plant water retention are critical goals in the improvement of plant tolerance to LRT and osmotic stresses. In the transgenic lines, the over-expression of a foreign AQP gene*PIP2;5*may improve stress resistance development through cooperation with

endogenous AQPs, stimulation of other AQP genes, which is lacking in wild-type plants. Therefore the changes in the morphological, anatomical and physiological organization of the different poplar lines will lead them to respond differently to different stress conditions. This was reflected in the difference in the stress response process between the two transgenic lines and the wild-type plants.

6.2 Room for improvement

In all research projects, time and other constraints limit the amount and type of work that can be carried out. The lessons that are learnt as the research is carried out also provide good feedback for the researcher to improve future research. Looking back at the project, I have found several things that could have improved the quality of the study including:

1. Measuring the cell to cell water transport under stress conditions
2. Studying the AQP expression in different tissues
3. Measuring changes in the AQP protein contents under stress

6.3 Suggestions for future research

1. Investigation of the changes in growth, AQP gating, expression and the physiological responses of the *PIP2;5* over-expressing and wild-type poplars, under salt and drought stress separately to study the role of AQP in salinity and drought stress.
2. The generation of transgenic poplar down regulating AQP*PIP2;5* and examining the functional role of this AQP by studying cell to cell water transport, expression and the physiological responses, under various stress conditions.

3. The generation of poplar over-expressing and down regulating other water transporting AQPsand examining how their water transport, physiology, growth and development, stress resistance changes in response to different abiotic stresses.
4. The identification of expression patterns of the poplar AQPsin different tissues of the transgenic and wild-type poplar exposed to different stress conditions might provide information required to validate the putative role of these proteins in responses to stress.
5. Tightly link research on improvement of wood properties, biomass production for industrial applications with research on poplar tolerance to environmental stresses.

APPENDIX A

ANOVA : Effect of short term low root temperature on root hydraulic conductivity

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	197	98.51	8.566	4.46E-04	***
Temperature	4	159.7	39.93	3.473	1.17E-02	*
Genotype:Temp	8	14.4	1.8	0.156	9.96E-01	
Residuals	75	862.5	11.5			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of short term low root temperature on net photosynthetic rate

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	105.76	52.88	17.673	9.24E-07	***
Temperature	3	117.6	39.22	13.107	1.09E-06	***
Genotype:Temp	6	8.07	1.34	0.449	8.43E-01	
Residuals	60	179.53	2.99			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of short term low root temperature on net transpiration rate

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	3.59	1.793	2.839	6.64E-02	.
Temperature	3	9.56	3.188	5.047	3.49E-03	**
Genotype:Temp	6	2.12	0.354	0.56	7.60E-01	
Residuals	60	37.89	0.632			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of short term low root temperature on water use efficiency

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	10.78	5.392	7.052	1.78E-03	**
Temperature	3	0.31	0.103	0.134	9.39E-01	
Genotype:Temp	6	3.98	0.664	0.868	5.24E-01	
Residuals	60	45.88	0.765			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of short term low root temperature on the transcript abundance of the selected aquaporins

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Temperature	1	0.153	0.1525	2.310	0.131	
AQP	6	7.34	1.2233	18.525	7.3e-15	***
AQP:Temp	6	0.067	0.0111	0.169	0.0985 .	
Residuals	112	7.396	0.0660			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

APPENDIX B

ANOVA: Effect of long term low root temperature on the relative shoot height growth

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	165	82	0.374	6.90E-01	
Temperature	2	34803	17401	78.89	1.91E-15	***
Genotype:Temp	4	1049	262	1.19	3.28E-01	
Residuals	45	9915	220			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA: Effect of long term low root temperature on the total dry weight

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	1.801	0.9	3.001	5.98E-02	.
Temperature	2	11.586	5.793	19.309	8.82E-07	***
Genotype:Temp	4	0.449	0.112	0.374	8.26E-01	
Residuals	45	13.501	0.3			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA: Effect of long term low root temperature on the shoot dry weight

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	2.046	1.023	3.453	4.03E-02	
Temperature	2	6.1	3.0502	10.296	2.08E-04	***
Genotype:Temp	4	0.507	0.1269	0.428	7.87E-01	
Residuals	45	13.332	0.2963			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA: Effect of long term low root temperature on the root dry weight

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	0.0466	0.0233	1.912	1.60E-01	
Temperature	2	0.8849	0.4425	36.277	4.14E-10	***
Genotype:Temp	4	0.0175	0.0044	0.36	8.36E-01	
Residuals	45	0.5488	0.0122			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA: Effect of long term low root temperature on the net photosynthetic rate (1st week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	134.3	67.15	9.947	2.65E-04	***
Temperature	2	72.9	36.45	5.399	7.91E-03	**
Genotype:Temp	4	20.09	5.02	0.744	5.67E-01	
Residuals	45	303.79	6.75			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the net photosynthetic rate (2nd week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	265.42	132.71	33.143	1.42E-09	***
Temperature	2	48.93	24.47	6.11	4.49E-03	**
Genotype:Temp	4	2.84	0.71	0.177	9.49E-01	
Residuals	45	180.19	4			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the net photosynthetic rate (3rd week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	222	111	28.272	1.12E-08	***
Temperature	2	34.42	17.21	4.384	1.82E-02	*
Genotype:Temp	4	29.25	7.31	1.862	1.34E-01	
Residuals	45	176.68	3.93			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the net transpiration rate (1st week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	32.42	16.211	232.49	< 2e-16	***
Temperature	2	0.96	0.481	6.903	2.43E-03	**
Genotype:Temp	4	0.28	0.071	1.018	4.08E-01	
Residuals	45	3.14	0.07			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the net transpiration rate (2nd week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	21.989	10.995	100.64	< 2e-16	***
Temperature	2	2.067	1.034	9.46	3.72E-04	***
Genotype:Temp	4	0.587	0.147	1.342	2.69E-01	
Residuals	45	4.916	0.109			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the net transpiration rate (3rd week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	13.423	6.712	66.326	3.82E-14	***
Temperature	2	0.836	0.418	4.128	2.26E-02	*
Genotype:Temp	4	2.621	0.655	6.476	3.33E-04	***
Residuals	45	4.554	0.101			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the root hydraulic conductivity (1st week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	330.4	165.18	38.134	2.06E-10	***
Temperature	2	5.1	2.57	0.593	5.57E-01	
Genotype:Temp	4	17.1	4.28	0.989	4.23E-01	
Residuals	45	194.9	4.33			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the root hydraulic conductivity (2nd week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	269.19	134.59	33.969	1.02E-09	***
Temperature	2	73.88	36.94	9.323	4.10E-04	***
Genotype:Temp	4	8.11	2.03	0.512	7.27E-01	
Residuals	45	178.3	3.96			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the root hydraulic conductivity (3rd week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	75.11	37.55	6.712	1.02E-09	**
Temperature	2	65.82	32.91	5.882	5.38E-03	**
Genotype:Temp	4	155.74	38.93	6.959	1.89E-04	***
Residuals	45	251.77	5.59			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the water usage efficiency (WUE) (1st week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	16.38	8.188	5.763	5.91E-03	**
Temperature	2	14.22	7.112	5.006	1.09E-02	*
Genotype:Temp	4	2.74	0.686	0.483	7.48E-01	
Residuals	45	63.94	1.421			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the water usage efficiency (WUE) (2nd week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)
Genotype	2	3.15	1.5765	1.641	2.05E-01
Temperature	2	2.43	1.2135	1.263	2.93E-01
Genotype:Temp	4	1.13	0.2816	0.293	8.81E-01
Residuals	45	43.23	0.9606		

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the water usage efficiency (WUE) (3rd week)

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Genotype	2	5.55	2.7728	3.617	3.49E-02	*
Temperature	2	4.02	2.0119	2.624	8.36E-02	.
Genotype:Temp	4	2.94	0.734	0.957	4.40E-01	
Residuals	45	34.5	0.7666			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of long term low root temperature on the change of transcript abundance of the selected aquaporins

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
Temperature	1	0.015	0.0153	0.189	0.66431	
AQP	6	6.122	1.0203	12.602	8.03e-11	***
AQP:Temp	6	1.538	0.2564	3.167	0.00659	**
Residuals	112	9.068	0.0810			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

APPENDIXC

ANOVA : Effect of PEG induced osmotic stress on relative shoot growth height

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
PEG Concentration	2	178281	89140	167.101	< 2e-16	***
Plant type	2	15705	7852	14.72	1.42E-06	***
PEG Con:Plt type	4	8884	2221	4.163	3.14E-03	**
Residuals	153	81618	533			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of PEG induced osmotic stress on shoot dry weight

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
PEG Concentration	2	40.95	20.476	54.711	< 2e-16	***
Plant type	2	15.8	7.902	21.114	7.99E-09	***
PEG Con:Plt type	4	7.94	1.985	5.304	3.14E-03	***
Residuals	153	57.26	0.374			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of PEG induced osmotic stress on root dry weight

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
PEG Concentration	2	6.533	3.267	140.391	< 2e-16	***
Plant type	2	0.53	0.265	11.386	2.47E-05	***
PEG Con:Plt type	4	0.629	0.157	6.755	4.96E-05	***
Residuals	153	3.537	0.023			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of PEG induced osmotic stress on total plant dry weight

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
PEG Concentration	2	79.17	39.59	97.354	< 2e-16	***
Plant type	2	23.18	11.59	28.506	3.00E-11	***
PEG Con:Plt type	4	13.44	3.36	8.264	4.62E-06	***
Residuals	153	62.21	0.41			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of PEG induced osmotic stress on net photosynthetic rate

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
PEG Concentration	2	34.46	17.23	24.06	8.24E-10	***
Plant type	2	74.26	37.13	51.84	< 2e-16	***
PEG Con:Plt type	4	32.55	8.14	11.36	4.23E-08	
Residuals	153	109.58	0.72			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of PEG induced osmotic stress on net transpiration rate

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
PEG Concentration	2	60.33	30.17	186.26	< 2e-16	***
Plant type	2	4.8	2.39	14.81	1.32E-06	***
PEG Con:Plt type	4	2.44	0.611	3.72	5.91E-03	**
Residuals	153	24.78	0.162			

Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of PEG induced osmotic stress on stomatal conductance

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
PEG Concentration	2	0.456	0.2279	164.8	< 2e-16	***
Plant type	2	0.184	0.0921	66.6	< 2e-16	***
PEG Con:Plt type	4	0.084	0.021	15.2	1.74E-10	***
Residuals	153	0.211	0.0014			

Significant codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

ANOVA : Effect of PEG induced osmotic stress on root hydraulic conductivity

	D.O.Fdm	Sum of square	Mean square	F value	Pr(>F)	
PEG Concentration	2	1433.4	716.7	177.21	< 2e-16	***
Plant type	2	169.3	84.6	20.93	9.24E-09	***
PEG Con:Plt type	4	20.9	5.2	1.29	2.75E-01	
Residuals	153	109.58	0.72			

Significant codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1