Aquaporin regulation in poplar and spruce trees under environmental change

by

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

Doctor of Philosophy

Forest Biology and Management

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ABSTRACT

This dissertation describes a series of experiments that examined: 1) hydraulic responses of *Populus trichocarpa* x *deltoides*, *Populus trichocarpa* and *Picea glauca* plants to change in their surrounding environment; 2) Changes of aquaporin expression in response to such changes.

In the first study, we demonstrated that changes of the transpirational demand is related to fine adjustment of root water uptake that is associated with up-regulation of plasma membrane intrinsic proteins isoforms (PIPs) in hybrid poplar saplings. PIP1 proteins are mostly localized in the endodermis where they may facilitate water movement to the stele. In the second study, we investigated the dynamics of leaf hydraulics in *P. trichocarpa* saplings exposed to a dehydration-rewatering episode. Fast leaf recovery was associated with an increase in expression of several tonoplast intrinsic proteins isoforms (TIPs) localized in xylem parenchyma. In the third study, we considered the physiological importance of foliar water uptake in *P. glauca* plants exposed to drought. In order to study the role of aquaporin in needle water uptake, we characterized the aquaporin family in white spruce. Our findings are consistent with the hypothesis that aquaporins facilitate radial water movement from the atmosphere towards the needle vascular tissue, therefore providing an alternate water source for embolism repair in conifers.

These results suggest the several roles of aquaporin regulation in the dynamic and fine adjustment of tree-water relations.

PREFACE

This thesis is an original work by Joan Laur.

Chapter 2 of this thesis has been published as J. Laur and U. G. Hacke, "Transpirational demand affects aquaporin expression in poplar roots." Journal of Experimental Botany, vol. 64, 2283-2293. I equally shared with my supervisor, Uwe G. Hacke, the responsibility for conceiving, designing and writing the paper. I performed the experiments.

Chapter 3 of this thesis has been submitted to PLOS ONE as J. Laur and U. G. Hacke, "The role of water channel proteins in facilitating recovery of leaf hydraulic conductance from water stress in *Populus trichocarpa*". I equally shared with my supervisor, Uwe G. Hacke, the responsibility for conceiving, designing and writing the paper. I performed the experiment.

Chapter 4 of this thesis has been published as J. Laur and U. G. Hacke, "Exploring *Picea glauca* aquaporins in the context of needle water uptake and xylem refilling." New Phytologist, vol. 203, 388-400. I equally shared with my supervisor, Uwe G. Hacke, the responsibility for conceiving, designing and writing the paper. I performed the experiment.

"As the water reappeared, so there reappeared willows, rushes, meadows, gardens, flowers, and a certain purpose in being alive"

The man who planted trees, Jean Giono

"En même temps que l'eau réapparut réapparaissaient les saules, les osiers, les prés, les jardins, les fleurs et une certaine raison de vivre."

L'homme qui plantait des arbres, Jean Giono

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Dr. Uwe Hacke, for mentoring my PhD research. Uwe, I am very grateful for your constant guidance and support over the last four years. This work would not have been possible without your help.

I want to thank Pr. Janusz Zwiazek and Dr. Enrico Scarpella, the members of my supervisory committee, for their assistance and feedback along those years. I also extend my thanks to Pr. Phil Comeau and Dr. Christophe Maurel for acting as thesis examiners.

The completion of my research would not have been possible without the help of several collaborators (in alphabetical order): Julie Gravel-Grenier, Dr. Krystyna Klimaszewska, Pr. Stefan Mayr, Arlene Oatway and of course my colleagues in the lab: Cayla Brocious, Sabrina Chamberland, Rachel Hillabrand, Lenka Plavcova, Stefan Schreiber. I would also like to express my gratitude to Gail Rankin, Gabor Botar and Kelley Dunfield who made my teaching assistantship such a rewarding experience.

I thank Clémentine. Despite the geographical distance, she was the best labmate ever!

Finally I thank my growing family: Louise and Nicolas.

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LIST OF ABBREVIATIONS AND SYMBOLS

aa	amino acid
ABA	abscissic acid
AQP	aquaporin
A_L	leaf area
ANOVA	analysis of variance
ar	aromatic
AXS	Artificial xylem sap
BS	Blocking solution
BSA	bovin serum albumin
cDNA	complementary DNA
CTAB	Cetyltrimethylammonium bromide
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DW_r	root dry weight
ER	endoplasmic reticulum
EST	expressed sequence tag
FAA	formalin acetic acid alcohol
GMO	genetically modified organism

g_m	mesophyll conductance
g_s	stomatal conductance
ISH	in situ hybridization
K_{flush}	stem hydraulic conductivity after flushing
K_h	stem hydraulic conductivity
K_L	leaf-specific hydraulic conductivity
K_{max}	maximal hydraulic conductivity
K_{native}	native hydraulic conductivity
K_R	root hydraulic conductivity
K_S	xylem-specific hydraulic conductivity
MIP	major intrinsic protein
mRNA	messenger RNA
NIP	NOD-26 like intrinsic protein
NJ	Neighbor Joining
N:P:K	Nitrogen (N):phosphorus (P):potassium (K)
ns	non-significant
P	probability
P50	the pressure at 50% loss of hydraulic conductivity
PBS	phosphate-buffered saline
pCa	potential of Calcium
PCR	polymerase chain reaction

pH	potential of Hydrogen
PIP	plasma membrane intrinsic protein
PLC	percent loss of conductivity
PM	plasma membrane
Q_R	root water flow
qRT-PCR	quantitative real-time PCR
QTL	quantitative trait loci
RH	relative humidity
RNA	ribonucleic acid
ROS	reactive oxygen species
RWC	relative water content
SD	standard deviation
SE	standard error
SIP	small intrinsic protein
TIP	tonoplast intrinsic protein
XIP	uncategorized X intrinsic protein
Ψ_L	leaf water potential
Ψ_S	stem water potential

I. General Introduction and literature review

Water is the universal solvent. It plays a fundamental role for survival, growth and the proper function of living creatures. Within the confined space of our body, water constitutes a matrix involved in every single biochemical reaction.

70% of the human body weight is composed of water; a 70 kg sedentary adult consumes 2.9 L of water on a daily basis (Kleiner, 1999): 0.04 L per kilogram. Water is essential to the maintenance of our metabolism: it regulates our temperature; flushes waste products; transports oxygen, minerals, vitamins and organic nutrients within our enclosed vascular system.

In plants, requirements are even greater (compare the two parts of Fig1-1): a plant transports up to 1000 times its dry body weight of water over its lifetime (Hsiao and Xu, 2000). For autotrophic organisms, water is also essential for leaf photosynthesis to reduce atmospheric carbon dioxide (CO_2) into organic compound. For every kilogram of organic matter a plant produces, 500 litres of water has to be transpired into the atmosphere (Black, 1973). Indeed, the vascular system of plants is not isolated from its surrounding environment; plants continuously absorb and lose a considerable amount of water. Transpiration at the stomata level is intimately linked to CO_2 uptake from the air, but it also drives the passive movement of water and minerals in a continuous column throughout the whole plant body.

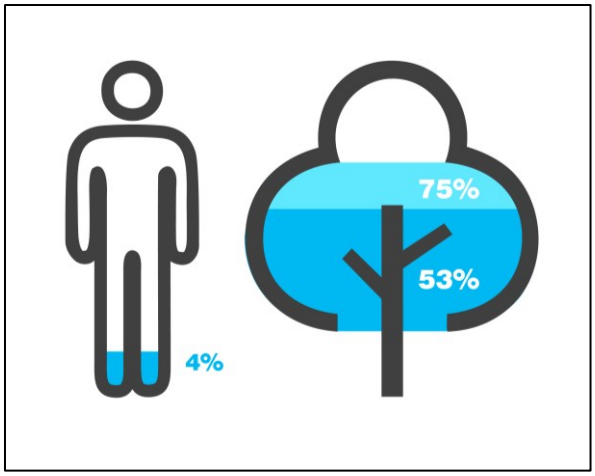

Figure 1-1: Daily consumption of water in human and plant.

Water consumption is reported as relative (%) to the organism fresh body weight according to Kleiner (1999) and Hinckley et al. (1993).

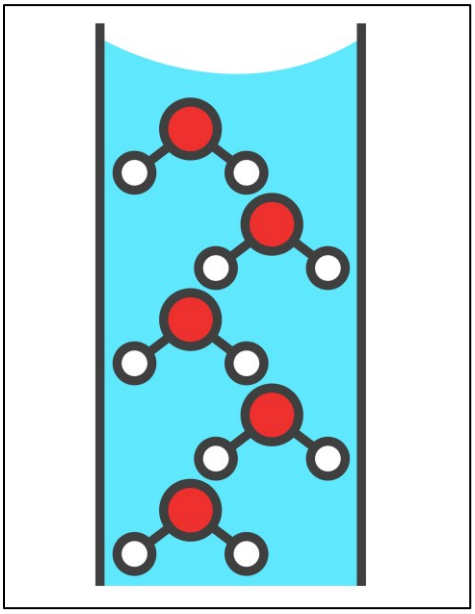

Figure 1-2: Schematic representation of a water molecules column.

Atoms of oxygen are represented in red, hydrogens in white. The water column adheres to the negatively charged biological membrane (adhesion). Hydrogen bonding attracts water molecules together (cohesion) while surface tension occurs at the air-liquid interface (see the meniscus at the top of the water column).

While several parameters affects transpiration rate (species, growth stage, environment), Hinckley *et al*. (1993) estimated that a four-year old poplar tree might lose 20 to 51 L of water per day: 0.53 to 0.75 L per kilogram (total fresh weight index according to Johansson and Hjelm, 2012), which is about 16 to 20 times more than the human body on a per mass basis. In his Vegetable Staticks (1727), Hales speaks about seventeen times more. Plants and—particularly—trees must move such a tremendous amount of water from the soil to the foliage several tens of meters higher, and this without the help of a heart-like pumping mechanism. How is this even possible?

The polar structure of a water molecule (Figure 1-2) gives it such unique characteristics that it is the universal solvent; the exact same physical properties allow the maintenance of the water column integrity.

The water molecule is relatively small (~ 3 ångströms): it consists of an oxygen atom covalently bonded to two hydrogen atoms. Because oxygen tends to be more electronegative, water is a dipolar molecule (partially charged) and the two O—H bonds form a distorted angle of 104.5°. Hydrogen bonding—the electrostatic Van der Waals attraction between water molecules—makes it a highly structured liquid with a high tensile strength: water molecules are attracted to each other (cohesion) and this is exacerbated at the air-liquid interface resulting in the phenomenon of surface tension. There is also the adhesion of water to the negatively charged biological membrane—or in the case of the tree's vascular system—cellulose molecules lining the wood conduit walls. However the capillary

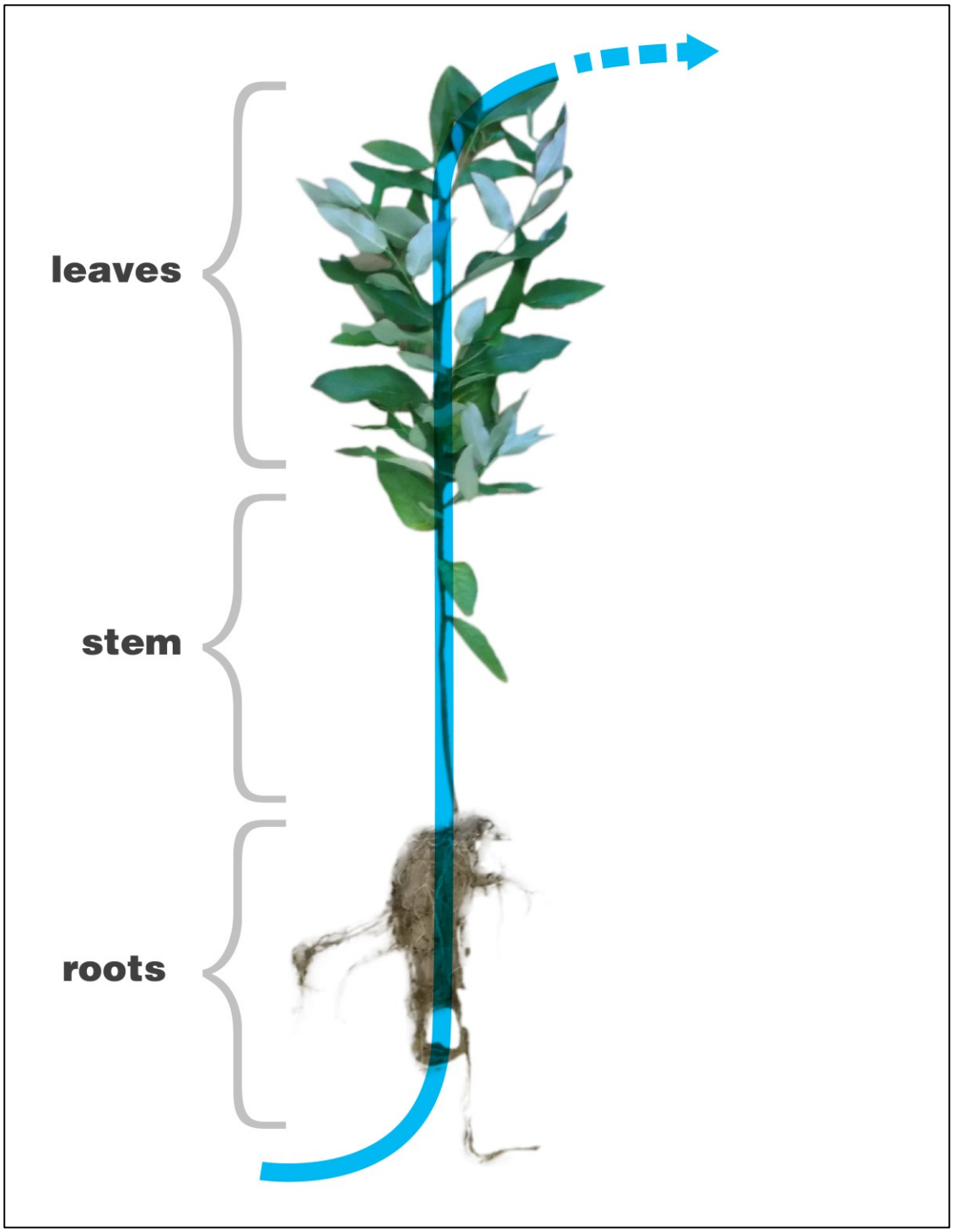

Figure 1-3: Water movement within the plant body.

Water is transported in the vascular system as a continuous column driven by the negative pressure created by transpiration at the leaf level.

action resulting from adhesion and surface tension is not strong enough to explain how water moves up the entire plant body. The weight of the water column will limit the capillary rise to 1 meter in a typical xylem vessel that constitutes the wood inner vasculature (Koch *et al.*, 2004).

1. Water movement through the plant

The water column within the vascular system of a plant creates a connection between the soil, the plant and the atmosphere: the soil-plant atmosphere continuum in which the ascent of sap is passively pulled up under tension (or negative pressure) by a gradient of decreasing water potential generated via transpiration at the stomata level according to the well-supported cohesion-tension theory (Dixon, 1914; Zimmerman, 1983; Tyree, 1997). The cohesion-tension theory relies on the transpiration driving force, the lignified structure of the xylem conduits and the properties of water to explain its ability to remain in a metastable liquid phase within the xylem conduit system until its evaporation in the stomatal region (Baker, 1989).

The long-distance water movement occurs passively within the plant vascular system, however to get a more detailed picture of its much more complex pathway it should be broken down into the different plant organs (Figure 1-3): water uptake by roots, exit in leaves, and long distance flow through the xylem. In each of these, water movement must be finely adjusted to match the requirements

of the surroundings.

a. Water uptake in roots

The permeability of roots to water is variable. Its close contact with the soil is necessary for maintaining the soil-plant-atmosphere continuum but water uptake should also adjust to environmental factors and avoid the introduction of unwanted compounds. Water absorption occurs in the root hair zone. In its radial path to the stele and the xylem within the stele, water has to traverse a series of living cell layers: the epidermis, cortex and endodermis, each resisting more or less to the water flow (Figure 1-4).

- The epidermis: the root hairs arise from a single layer of epidermal cells that may surround an inner hypodermis exhibiting Casparian strips on the radial cell walls (hydrophobic suberin and cutin deposits); a hypodermis with Casparian bands is named an exodermis (Perumalla and Peterson, 1986). These materials restrict the movement of water outside the cells which is referred to as the apoplastic pathway (in intercellular spaces, in the cell walls and the lumen of dead cells). Depending on the species, the developmental stage or the environmental conditions, the exodermis could constitute a barrier of variable resistance to water flow (Ferguson and Clarkson, 1976; Peterson, 1988).
- the cortex, on the contrary, consists of a number of cell layers without suberin; water can flow across the thin cell walls with ease.

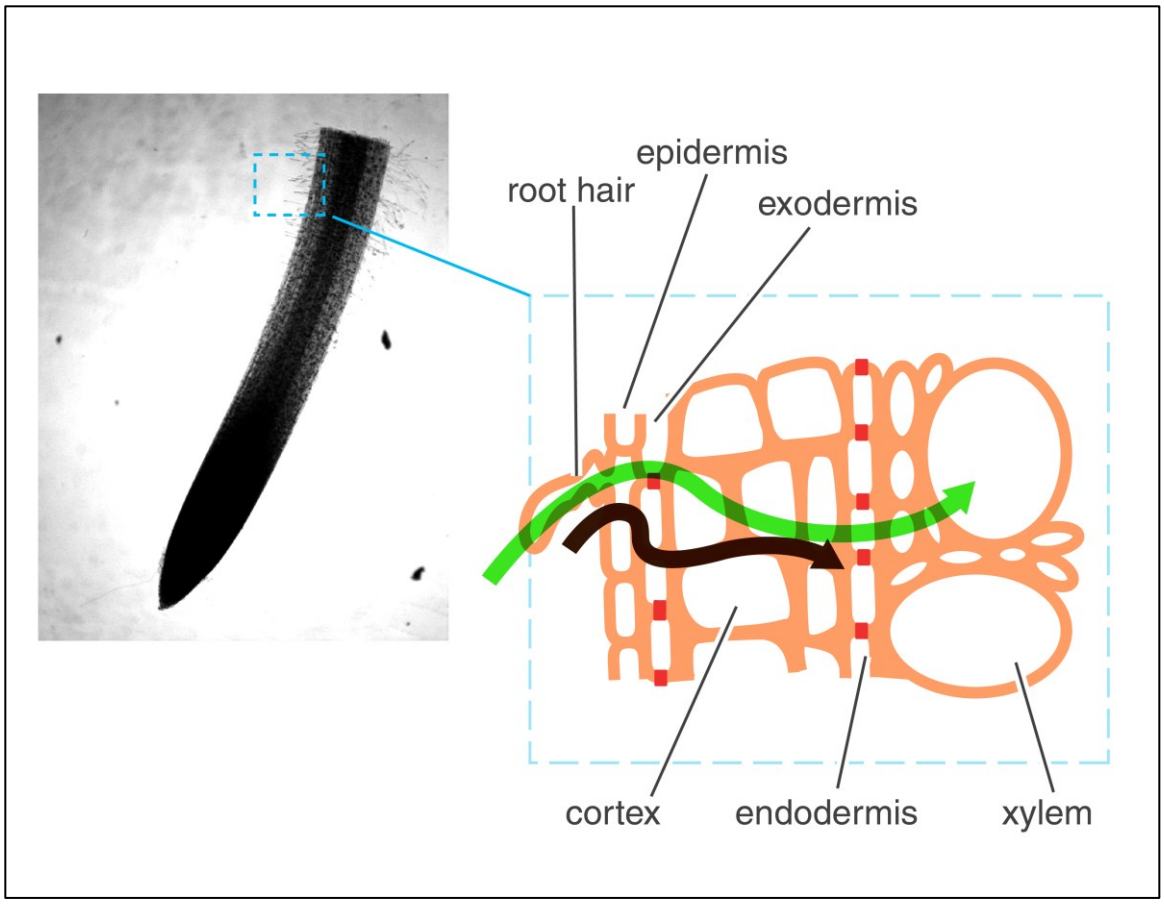

Figure 1-4: Water movement in root.

The left photograph shows water uptake through the root hair zone. The schematic transverse section on the right shows the two pathways before reaching the vascular system (i.e. xylem conduits): in black the apoplastic path across the cell walls; in green the cell-to-cell path where water moves throughout the intracellular continuum (symplastic pathway) or cross the cell membranes (transcellular pathway). The later route has to be taken in order to pass the hydrophobic barrier of the Casparian strip (red) in the endodermis and possibly the exodermis.

- the endodermis forms another apoplastic barrier, a single cell layer that surrounds the stele (Moreshet and Huck, 1991); its structure is similar to the exodermis.

To enter the inner tissues, water has to cross the cell membrane (transmembrane pathway) of those cells. The transmembrane pathway cannot be easily separated experimentally from the symplastic pathway (from cell to cell through plasmodesmata). The transmembrane and the symplastic paths are referred as the cell-to-cell pathway (Maurel, 1997). Water uses a combination of the apoplastic and the cell-to-cell pathway as it moves to the root xylem (the contribution of each is variable and stills a subject of debate (Steudle and Frensch, 1996; Murphy, 2000). The cell-to-cell pathway provides an opportunity to selectively control water uptake to match the whole plant's requirements (Almeida-Rodriguez *et al*., 2011; Sakurai-Ishikawa *et al*., 2011; Laur and Hacke, 2013).

b. Water flow through the leaves

Most of the water absorbed in roots is bound to the foliage where it has to leave the vasculature and, similar to the root pathway, flow through apoplastic and cell-to-cell routes until it reaches the site of transpiration in the sub-stomatal cavity located in the mesophyll (Figure 1-5). In most angiosperm species the inner vascular system is not directly surrounded by the mesophyll but by a tight bundle sheath and parenchyma cells. In conifers, this anatomical pattern is even more similar to roots: the phloem, xylem and transfusion tissues are enclosed by an endodermis-like

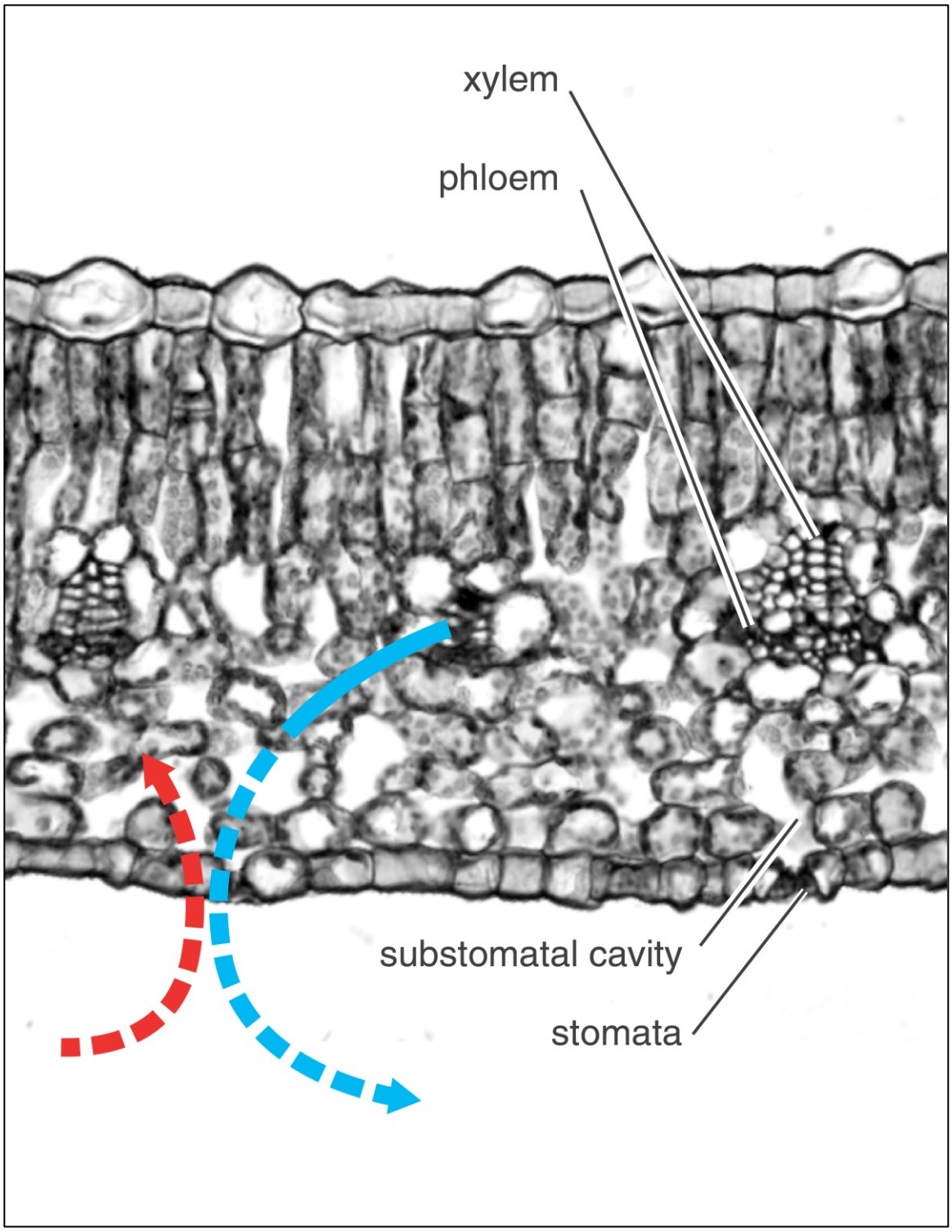

Figure 1-5: Water flow in leaf.

The blue line shows typical water flow in the leaf, from the xylem to the atmosphere via the stomata, the counter current CO_2 uptake is in red.

bundle sheath. A more or less thick waxy cuticle envelops the leaf in order to limit evaporation under stressful conditions.

The whole plant's water flux is controlled at the stomata level: by decreasing the aperture, plants tend to contain dehydration, but photosynthesis is also reduced. Plants may or may not close their stomata in order to maintain their leaf water status. But, to maximize the daily photosynthetic rate, an intermediate water-use strategy is often in use under minimal water stress. There is some evidence that both isohydric (strict control of stomata aperture) and anisohydric (little or no control of stomata aperture) behaviours occurring within plant group or species such as grapevine and poplar (Schultz, 2003; Almeida-Rodriguez *et al*., 2010) depending on the availability of water in their natural environment (Sade *et al*., 2012). Recovery from dehydration has been observed within hours in leaves of poplar (Laur and Hacke, unpublished), rice (Stiller *et al*,. 2005) and sunflower (Trifilo *et al*., 2003) but is inhibited by mercuric compounds—implicating the involvement of proteins (Macey 1984; Wayne and Tazawa 1990) in this adjustment to environmental changes that occur daily in the field.

c. Water pathway through the stem xylem

Both water entry and exit points are important checkpoints of the water path. In its long distance move to the foliage, water flows apoplastically within the xylem. Xylem consists of a network of heavily lignified dead cell walls connected end to end to form the apoplastic water conducting pipelines. In trees, the wood (secondary

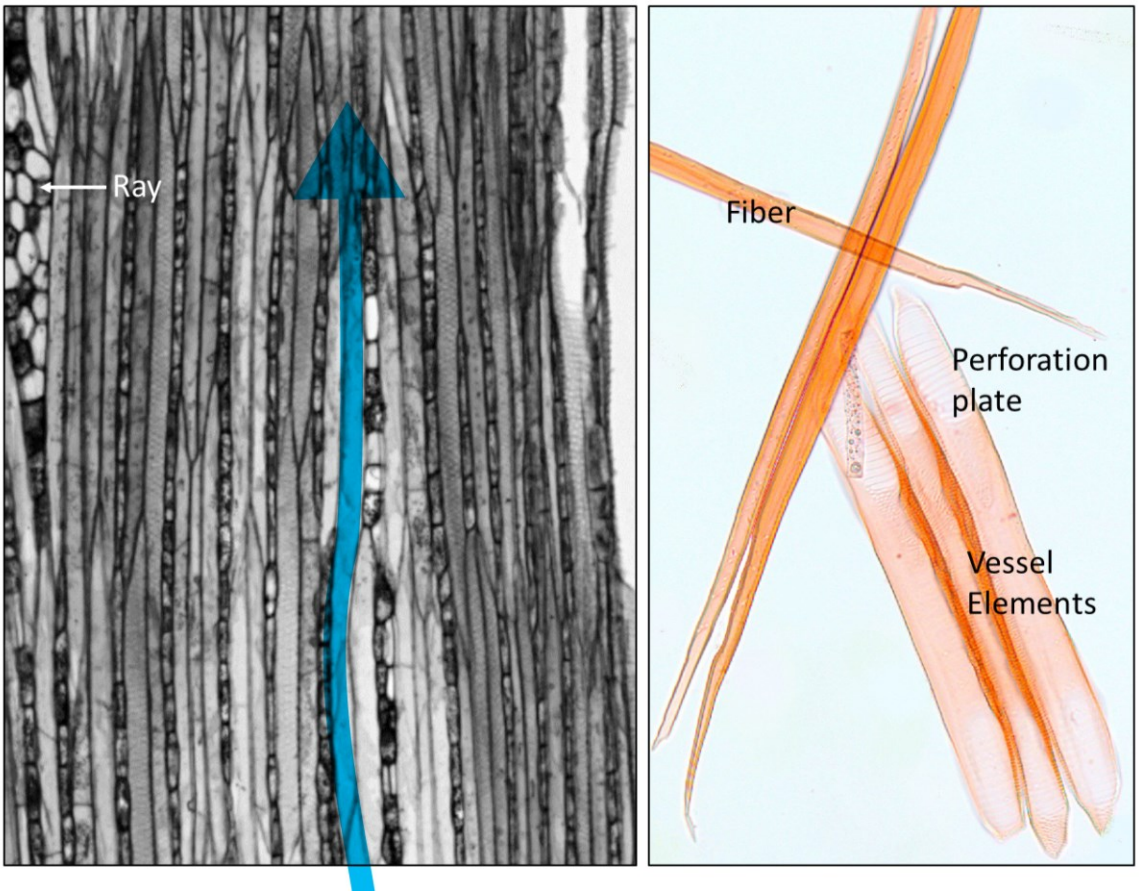

Figure 1-6: Tangential section and cells of angiosperm wood.

The picture on the left is a tangential section of angiosperm wood (*Tilia sp.*); ray cells are indicated as well as ascendant water flow (in blue) along a continuous xylem vessel. On the right, fibers and vessel elements (wood maceration) are stained with safranin.

xylem) is anatomically designed to be efficient in terms of water transport and to support the whole plant body. To do so, the wood is composed of specialized cells (Figure 1-6):

- Tracheids are narrow elongated cells (up to 3 millimetres long in *Picea glauca* (Beaulieu, 2003)) connected through porous pits in their overlapping end walls. In gymnosperms, wood is made up of as much as 95 % tracheids that also act to provide structural support to the plant body. The last 5 % are constituted of resin canals and living parenchyma cells arranged in rays that allow for storage and radial translocation of water and other compounds (Kozlowski and Pallardy, 1997). Angiosperm wood is more complex with more highly specialized cells, the vessel elements.

- Vessel elements are the chief water-conducting cells in angiosperms. They are wide and short cells forming tubular conduits through their disintegrated end walls: the vessels. Vessels walls are relatively thin; the dense fibers act as supportive elements and constitute more than 50% of poplar wood (Balatinecz and Kretschmann, 2001).

In both angiosperms and gymnosperms, living parenchyma cells play an important role in radial translocation of water and nutrients; they may be also important to maintain the integrity of the water column. Xylem is constituted of a dead hollow cell wall in which the water is in a fragile metastable state. A xylem conduit can cavitate (water phase change from liquid to vapour as the cohesive force between molecules is disrupted) because of adverse environmental conditions such as drought or freeze-thaw events (Tyree and Sperry, 1989; Schreiber *et al.*,

2013). Recent work has focused on the refilling mechanisms of embolized xylem in which adjacent parenchyma cells and foliar water uptake may be involved (Secchi and Zwieniecki, 2010; Mayr *et al*. 2014; Laur and Hacke, 2014).

d. Regulation of water movement

Well-designed anatomical features are responsible for the passive movement of water within the plant body but only active mechanisms can explain the dynamic adjustments to an ever-changing environment. While the plant has little control on water flow in the apoplast, the transmembrane path provides the opportunity for regulatory control since membrane permeability can be actively modulated.

Root water uptake adjustment, rapid leaf recovery and the use of foliar water uptake to facilitate xylem refilling, are the three phenomena that I examine in this thesis. All of them occur in the vicinity of different "living" parts of the plant. *A priori*, all of them can be controlled by the regulation of cell membrane permeability to water. Cell membranes consist primarily of a lipid bilayer with embedded proteins.

Peter Agre's group identified in the early 1990s the first aquaporin, a water channel protein, CHIP28 expressed constitutively in the red blood cell membrane (Smith and Agre, 1991; Preston *et al*., 1992). These results changed the view of how water moves across the lipid bilayer of a biological membrane and led to the 2003 Nobel Prize in chemistry awarded for "the discovery of water channels" (see www.nobel.se/chemistry/laureates/2003).

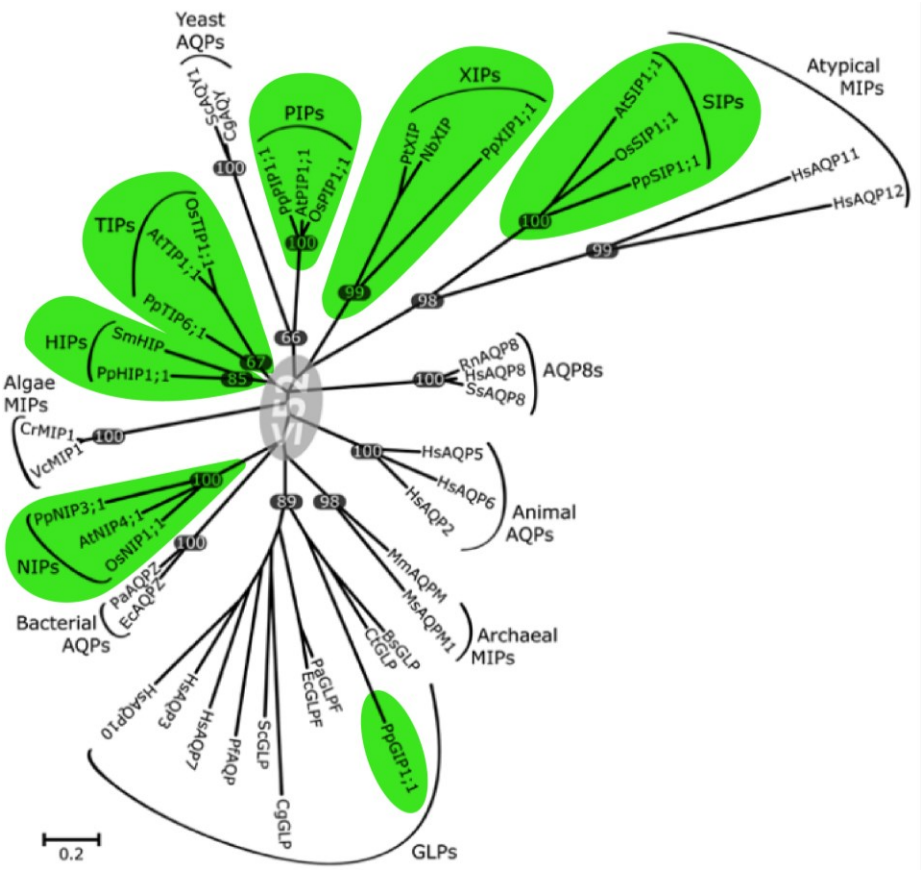

Figure 1-7: Phylogenetic analysis of MIPs.

13 different subfamilies are supported by high bootstrap values in a Neighbor-Joining analysis of 44 representative MIPs from *Arabidopsis thaliana* (At), *Bacillus subtilis* (Bs), *Candida glabrata* (Cg), *Chlamydomonas reinhartii* (Cr), *Clostridium tetani* (Ct), *Escherichia coli* (Ec), *Homo sapiens* (Hs), *Methanosphaera stadtmanae* (Ms), *Nicotiana benthamiana* (Nb), *Oryza sativa* (Os), *Physcomitrella patens* (Pp), *Populus trichocarpa* (Pt), *Pseudomonas aeruginosa* (Pa), *Rattus norvegicus* (Rn), *Saccharomyces cerevisiae* (Sc), *Selaginella moellendorffii* (Sm), *Sus scrofa* (Ss), *Volvox carteri* (Vc), *Zea mays* (Zm). The shading in the middle of the tree marks the uncertainty of the positioning of the central nodes as inferred from bootstrap values ≤ 52%. Plants MIPs subfamilies are indicated in green. This figure is an adaptation from Danielson & Johanson, 2010.

2. Aquaporins (AQPs)

Aquaporins are water channel proteins that belong to the ubiquitous Major Intrinsic Proteins (MIPs) family. They can constitute up to 15 % of total membrane proteins (Johansson *et al*., 1996; Maurel *et al*., 2008) and their active regulation influences the passive movement of water across cell membranes, tissues and organs (cell-to cell pathway).

a. Significance of the MIP family in Plant-Water relations

A remarkably large number of MIPs are expressed in plant cells. Their patterns of expression are complex, varying between species, organs and tissues. They are often expressed in tissues associated with high water permeability: some isoforms are encountered in primary roots (Hachez *et al*., 2006); fine roots and/or main roots (Marjanovic *et al*., 2005); epidermis, cortical cells, xylem or the root endodermis (Javot *et al*., 2003; Suga *et al*., 2003; Almeida *et al*., 2010); but also leaves (Fraysse *et al*., 2005; Flexas *et al*., 2006; Postaire *et al*., 2010) and wood (Secchi and Zwieniecki, 2010).

So far only 13 MIPs have been discovered in the human genome (Gonen and Walz, 2006), but 35 MIPs in *Arabidopsis thaliana* (Johanson *et al*., 2001) and *Physcomitrella patens* (Danielson and Johanson, 2008), 33 in *Oryza sativa* (Sakurai *et al*., 2005), more than 50 in *Populus trichocarpa* (Gupta and Sankararamakrishnan, 2009), 71 in *Gossypium hirsutum* (Park *et al*., 2010).

Gene	Effect	Reference
AtPIP1;2	KO: ↑ root:shoot ratio	Kaldenhoff et al., 1998
	↓CO_2 diffusion	Uehlein et al., 2012
	OE: ↑ growth, transpiration, stomatal density	Aharon et al., 2003
	↓ drought tolerance	
AtPIP1;4	OE: ↓ drought tolerance	Jang et al., 2007
	↑ water flow, germination under cold	
	↑ cold tolerance of root cells	Lee et al., 2012
AtPIP1;2+AtPIP2;3	KO: ↑root:shoot ratio; drought tolerance	Martre et al., 2002
	↓root and leaf protoplast *K*h	
AtPIP2;2	KO: ↓root *K*h	Javot et al., 2003
AtPIP2;5	OE: ↓ drought tolerance	Jang et al., 2007
	↑ water flow, germination under cold	
	↑ cold tolerance of root cells	Lee et al., 2012
AtPIP2;1	KO: ↓leaf water transport, rosette hydraulic conductivity	Prado et al., 2013
	OE: ↑ rosette hydraulic conductivity, vein protoplast conductivity (not mesophyll)	
BjPIP1	OE: ↑ drought tolerance	Zhang et al., 2008
	↓water loss, transpiration, g_s	
BnPIP1	OE: ↑ drought tolerance	Yu et al., 2005
	KO: ↓growth, germination, drought tolerance	
CsPIP1;1	OE: ↑salt tolerance	Jang et al., 2007
	↓ drought tolerance	
CfPIP2;1	OE: ↑ drought tolerance	Jang et al., 2007
JcPIP1	KO: ↓drought tolerance	Jang et al., 2013
JcPIP2	KO: ↓ drought tolerance	
GhPIP2s	KO: ↓ fibre elongation	Li et al., 2013
GhPIP2;7	OE: ↑ drought tolerance	Zhang et al., 2013
HvPIP2;1	OE: ↑ root *K*h;	Katsuhara et al., 2003
	↓ root:shoot ratio	
	↓ salt tolerance	
	↑ g_s, CO_2 diffusion, CO_2 assimilation	Hanba et al., 2004
LlPIP1	OE: ↑ *K*h, stomatal density, stomatal aperture	Ding et al., 2004
MaPIP1;1	OE: ↑ root growth, salt and drought tolerance	Xu et al., 2014
McMIPB (PIP1)	OE: ↑ CO_2 assimilation, CO_2 diffusion	Kawase et al., 2013
MusaPIP1;2	OE: ↑ abiotic stress tolerance	Sreedharan et al., 2013
NtAQP1 (PIP1)	OE: ↑ photosynthesis, g_m	Flexas et al., 2006
	KO: ↓ photosynthesis, g_m	
	OE: ↑ leaf growth, water & CO_2 membrane permeability	Uehlein at al., 2003
	OE: ↑ photosynthesis, g_m, g_s; under drought: L_{pr}	Sade et al., 2014
	OE: ↑ salt stress tolerance	Sade et al., 2010
	KO: ↓*K*h; drought tolerance	Siefritz et al., 2002
NtAQP1 + AtHXK1	OE: ↑ stress tolerance, productivity	Kelly et al., 2014
OsPIP1;1	OE: ↑ drought and salt stress tolerance	Guo et al., 2006
	OE: ↑ salt and osmotic stress tolerance	Liu et al., 2013
OsPIP1;3	OE: ↑ root *K*h, leaf Ψ	Lian et al., 2004
	OE: ↑ cold tolerance	Matsumoto et al., 2009
OsPIP2;2	OE: ↑ drought and salt stress tolerance	Guo et al., 2006
OsPIP2;7	OE: ↑ transpiration rate, cold tolerance	Li et al., 2008
RcPIP2; 1	OE: ↑ dehydration rate, leaf size, mesophyll cell size	Peng et al., 2008

	↓cold tolerance	
RcPIP2;2	OE: ↑ dehydration rate, leaf size, mesophyll cell size	Peng et al., 2008
	↓cold tolerance	
RhPIP2;1	KO: ↓ petal cell expansion	Ma et al., 2008
RsPIP1s + RsPIP2s	KO: ↓growth, photosynthesis	Tsuchihira et al., 2010
RsPIP2;1	OE: ↑ growth, photosynthesis	Tsuchihira et al., 2010
StPIP1	KO: ↓cellular water transport, drought tolerance	Wu et al., 2009
	↑ root biomass	
PtPIP1s	KO: ↑ leaf hydraulic resistance	Secchi & Zwieniecki, 2013
	↓CO_2 mesophyll conductance	
	KO: ↑ xylem vulnerability	Secchi & Zwieniecki, 2014
	↓embolism recovery, drought tolerance	
TaAQP8 (PIP1)	OE: ↑ salt tolerance	Hu et al., 2012
TaAQP7 (PIP2)	OE: ↑ drought tolerance	Zhou et al., 2012
TdPIP1;1	OE: ↑ drought and salt tolerance	Ayadi et al., 2011
TdPIP2;1	OE: ↑ drought and salt tolerance	Ayadi et al., 2011
VfPIP1	OE: ↑ drought tolerance	Cui et al., 2008
VvPIP2;4	OE: ↑ growth, root hydraulic conductance	Perrone et al., 2012
AtTIP1;1	KO: plant death	Ma et al., 2004
	KO: no clear effect	Beebo et al., 2009
	KO: no clear effect	Schussler et al., 2008
AtTIP1;1 + AtTIP1;2	KO: no clear effects	Schussler et al., 2008
BoTIP	OE: ↑vacuole & cell size	Reisen et al., 2003
GsTIP2;1	OE: ↓salt, drought tolerance	Wang et al., 2011
NtTIP1;1	OE: ↑ cell growth	Okubo-Kurihara et al., 2009
SlTIP2;2	OE: ↑growth, yield, transpiration, stress tolerance	Sade et al., 2009
PgTIP	OE: ↑ growth, seed size	Lin et al., 2007
	OE: ↑ growth, salt stress tolerance, drought tolerance	Peng et al., 2007
	↓ cold tolerance	
TaTIP2;2	OE: ↓ drought, salt stress tolerance	Xu et al., 2013
TsTIP1;2	OE: ↑ drought, salt, oxidative stress tolerance	Wang et al., 2014

Table 1-1: Impact of *in planta* deregulation of PIP and TIP isoforms.

Up to date overview of *in planta* genetic modification that indicates their importance in plant-water relations. For reasons of clarity, heterologous expression and overexpression are included in the OE designation; knockout, RNAi lines and knockdown mutants are included in the KO designation.

Among the many phylogenetic subgroups that form the MIP superfamily (Figure 1-7), seven are plant specific (Gustavsson *et al*., 2005): the GIPs (GlpF-like intrinsic proteins), HIPs (hybrid intrinsic proteins), NIPs (NOD26-like intrinsic proteins), PIPs (plasma membrane intrinsic proteins), SIPs (small basic intrinsic proteins), TIPs (tonoplast membrane intrinsic proteins) and the XIPs (uncategorized X intrinsic proteins).

MIPs can be permeable to a wide range of solutes and gases such as CO_2, glycerol, H_2O_2, metalloids, nitrate, urea and water to name a few (reviewed in Carbrey and Agre, 2009; Wudick *et al*., 2009). PIP (mostly found in plasma membrane), divided into PIP1 and PIP2 subgroups, and the TIP (mostly expressed in the vacuole membrane) isoforms are true water channel proteins (Kaldenhoff and Fisher, 2006) and are the most studied (Figure 1-7). Their transcription is significantly affected by several abiotic stresses through miRNA (Zhang *et al*. 2014), hormones and transcription factors. Drought (Liu *et al*., 2014), flooding (Calvo-Polanco *et al*., 2014), low temperature (Chen and Arora, 2014) and salinity (Xu *et al*., 2014) have all been shown to impact aquaporin transcription. Their role in plant-water relations is elegantly demonstrated through transgenic manipulations: *in planta* gene manipulation experiments are summarized in Table 1-1. The pioneer observations of Kaldenhoff *et al*. (1998), as well as numerous other studies, illustrate AQPs involvement in abiotic stress response. To date, more than 50 studies have investigated the effect of AQP genetic deregulation on plant-water relations. OE (over-expression) often increased hydraulic conductivity (Lian *et al*.,

2004), plant growth and tolerance to water stress whilst loss-of-function manipulation reduced overall plant fitness (Martre *et al*., 2002; Ma *et al*, 2004). Similarly, antisense NtAQP1 plants showed reduced root hydraulic conductivity and lower water stress tolerance (Siefritz *et al*., 2002), although some results do not follow this rule: for example AtPIP1;2 gain-of-function in tobacco caused plants to wilt faster under drought (Aharon *et al*., 2003). Before AQPs can be used efficiently as a selection marker or in the development of stress-tolerant GMOs (genetically modified organism), it will be necessary to compile a more complete amount of knowledge. The diversity of plant AQP isoforms implies different functional roles. A detailed analysis of AQP properties may help to elucidate their significance in the physiology of water transport.

b. AQP structure

The continuity of the water column is maintained in the cell-to-cell component of the plant water path because of AQPs that form proteic pores in biological membranes. The pores of AQPs are believed to be narrow so that hydrogen bonds between water molecules are disrupted and the molecules can move through in single file (Murata *et al*., 2000). The AQP polypeptide consists of six transmembrane α-helices connected by five loops and the amino (NH_2) and carboxy (COOH) termini located on the cytoplasmic side of the membrane. A number of residues are conserved in the majority of cases. Oocyte swelling assays (exogenous AQPs are expressed on the oocyte membrane, which has an intrinsically low permeability to

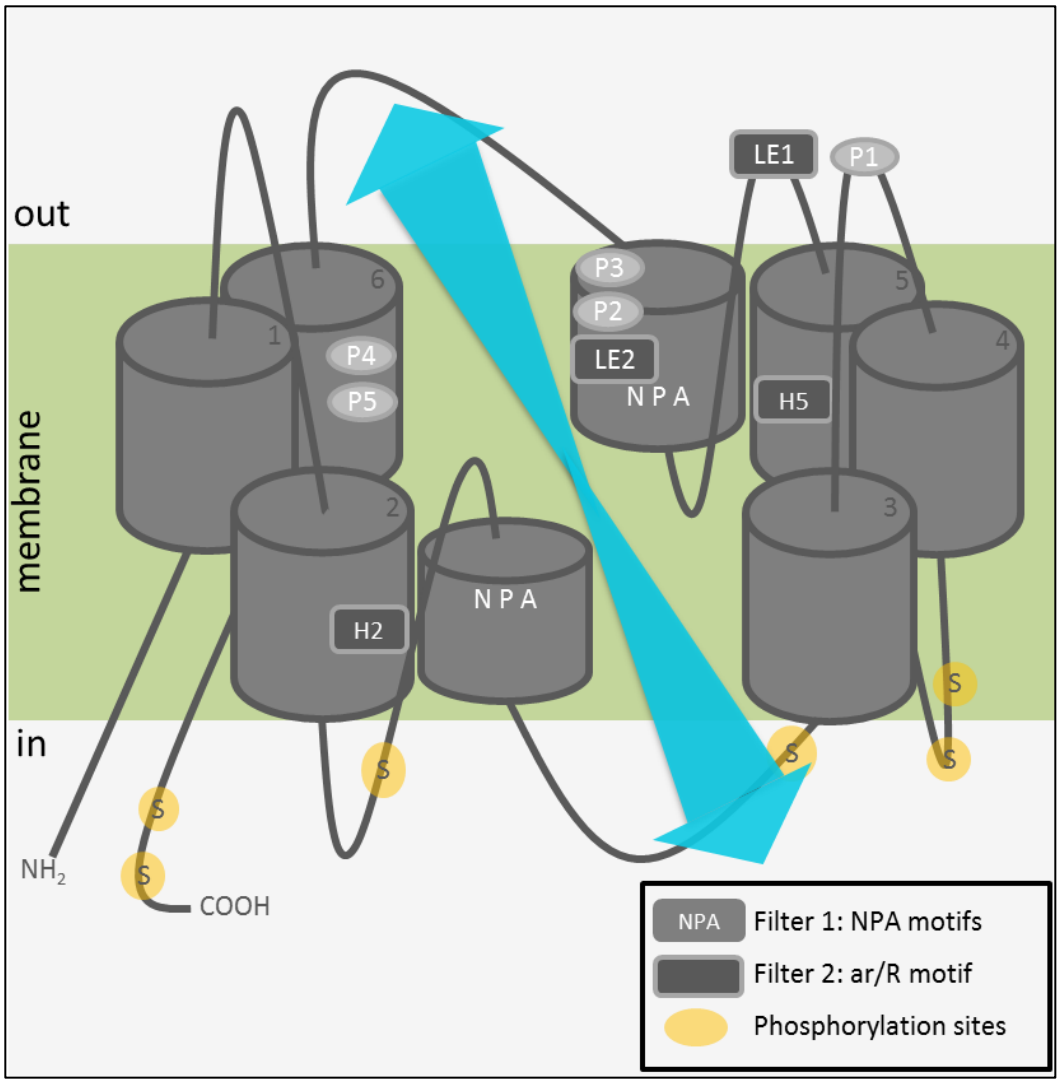

Figure 1-8: Schematic transmembrane structure of an aquaporin protein.

In this schema, the numerated grey cylinders represent the transmembrane α-helices domains and the grey lines the extramembrane loops. The blue double arrowed hourglass represents the water flow through the pore. The positions of the two NPA motifs responsible are indicated in white, the five ar/R residues are in the dark grey rectangles. Finally, the yellow circles indicate putative sites (Serine) of posttranslational regulation (Tornroth-Horsefield et al., 2006; Van Wilder et al., 2008).

water) and sequence analyses have led to the characterisation of several amino-acid residues for their importance in solute specificity (Bansal and Sankararamakrishnan, 2007; Hove *et al*.; 2011): the P1-P5 residues (Froger *et al*., 1998), the aromatic/arginine (ar/R) selectivity filter, and the two asparagine-proline-alanine (NPA) boxes contained in loops B and E (LB, LE). Loops B and E contain short α-helical domains and fold into the membrane forming a seventh "broken" helix that creates the symmetrical hourglass-shaped pore.

The NPA motifs are conserved in numerous MIPs from animals, fungi, yeast and plant PIPs and TIPs. Site-directed mutagenesis (Kong and Ma, 2001) established their importance in the maintenance of an adequate pore aperture through proton exclusion. Among the four residues of the ar/R filter that form a size restriction region of the pore, the highly conserved Arginine in Loop E is thought to provide hydrogen bonds important to the bidirectional trafficking of water or glycerol, while the five P1-P5 residues differ drastically between water and glycerol MIP channels.

Generally the 25-30 kDa AQPs form tetramers through interaction between different isoforms with each monomer acting as an independent water-channel (Smith and Agre, 1991; Murata *et al*., 2000; Sui *et al*., 2001; Törnroth-Horsefield *et al*., 2006; Secchi *et al*., 2009).

c. Translational and post translational regulation of plant AQP proteins

Changes in the membrane protein density and activity are regulated through the

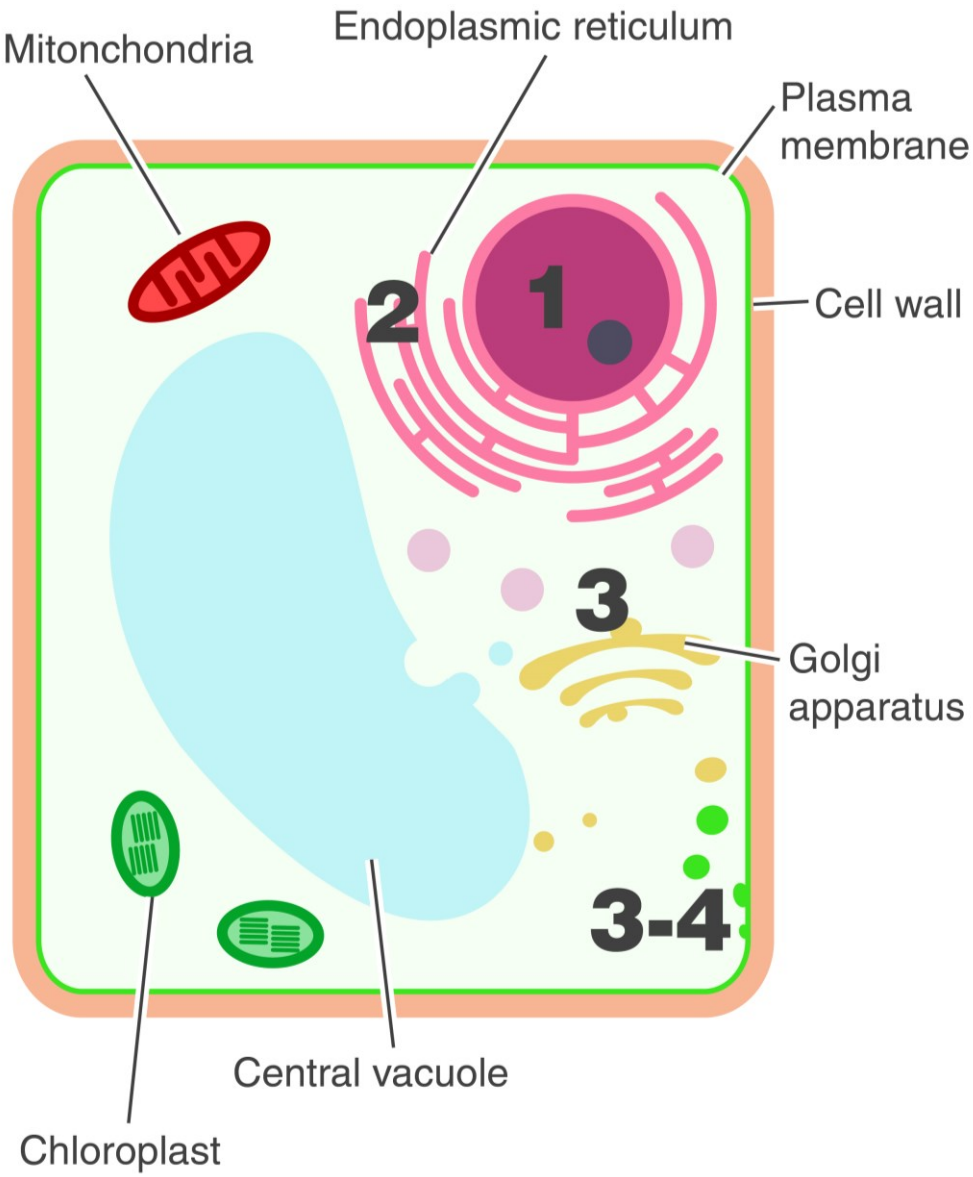

Figure 1-9: Summary of the regulatory mechanisms of plant AQPs.

The cellular localisations of plant aquaporins regulatory mechanisms are numerated. (1) Gene transcription may be regulated by several environmental factors. (2) Transcriptional regulation by microRNAs has been extensively described for mammals *AQPs*; recent work suggests this could also occur in plants. Transcriptional and posttranscriptional regulation of aquaporin expression can both affect the cell membrane permeability through the resulting AQPs density. AQPs density at the membrane is also affected by the regulation of protein trafficking (and/or degradation) through posttranslational modifications and heteromerization (3-4) that can also change their gating behaviour.

modulation of gene transcription (as described above; in Figure 1-9, '1' indicates transcriptional regulatory mechanisms and '2' the posttranscriptional processes) and at the protein level. Interestingly, the tetramerization of aquaporin monomers is an active regulatory mechanism of cell water permeability (indicated as '4' in Figure 1-9) (most recent reference: Jones *et al*., 2014; reviewed in Chaumont *et al*., 2005; Maurel 2007; Chaumont and Tyerman, 2014).

- Hetero-tetramerization positively affects the water-channel function of PIP and TIP isoforms (Harvengt *et al*., 2000; Fetter *et al*., 2004) and their cellular trafficking. Tetramerization is an example of post-translational regulation that affects both the protein subcellular localization and its structure. The physical interaction between isoforms can change the *conformation* of the monomers and therefore their transport properties. Notably, PIP1 relocation to the plasma membrane and its activity is enhanced when co-expressed with PIP2s (Zelazny *et al*., 2007; Secchi and Zwieniecki, 2010); this suggested the importance of PIP1 isoforms in finely regulated mechanisms such as xylem refilling (Secchi and Zwieniecki, 2014).

AQP proteins are regulated through several post-translational modifications (indicated as '3' in Figure 1-9) that affect the protein location:

- McTIP2;1 glycosylation redistributes the protein to non-tonoplast endosomic membrane fractions (Vera-Estrella *et al*., 2004)
- salt-dependant dephosphorylation of Ser283 induces the internalisation of AtPIP2;1 (Prak *et al*., 2008)

and their gating (opening and closing of the pore):

The kinase-dependant phosphorylation of Serine residues, notably Ser115, Ser 274 in the PIP subfamily, is widely reported to enhance AQP activity through changes in the cytosolic pCa (determined by Ca^{2+} concentration) or pH (Johnson and Chrispeels, 1992; Maurel *et al*., 1995; Johansson *et al*., 1998; Törnroth-Horsefield *et al*., 2006; reviewed in Chaumont *et al*., 2005; Li *et al*., 2013).

- Similarly the pH dependant protonation of a loop D Histidine residue (His193) of the spinach SoPIP2;1 cause the AQP to close (Tournaire-Roux *et al*., 2003).

Possible co-translational *acetylation* or *methylation* (Santoni *et al*., 2006) has also been reported for plant AQPs but their specific impact is not yet characterized.

Ultimately, MIP *degradation* via the proteasome can be regulated by E3 ubiquitin ligase targeting as Lee *et al*., (2009) has shown.

3. Summary

Unlike animals, the sessile nature of plants forces them to adjust to their environment. One of the major challenges is to maintain an adequate water supply to the foliage where most of the water loss occurs through the stomatal apertures. According to the cohesion-tension theory, the water column is pulled up by transpiration within the whole plant body. To avoid hydraulic failure, water use must be constantlly adjusted.

Water channel proteins, the aquaporins, discovered 25 years ago, are key-components of this fine-tuning. They form a remarkably large and conserved family in plants. PIPs and TIPs are the most studied subfamilies that actively control several developmental and plant hydraulic parameters through their own regulation, which occurs both at transcriptional and translational levels. Indeed, the activity of PIP proteins, mostly located in the plasma membrane, can modulate water flow through tissue and organs with high water permeability while TIPs, mostly located in the vacuole membrane, are important for cell osmotic adjustment. Their roles, in response to water stress, can range from the control of water uptake at the root endodermis level, to the facilitation of xylem refilling in stem and the overall maintenance of hydraulic functions in leaf.

4. Research objectives

The literature review shown above indicates that AQPs play important roles in plant-water relations. Water channel proteins are relatively well studied in model plants like Arabidopsis, but their functions in tree-water relations are much less understood. In this context, I studied how AQPs impact water transport in poplar and spruce (two dominant trees in Canada). Specifically, I assessed the possible roles of AQPs in (Figure 1-10):

- Physiological adjustment of root to changes in the above-ground environment (**Chapter 2**),

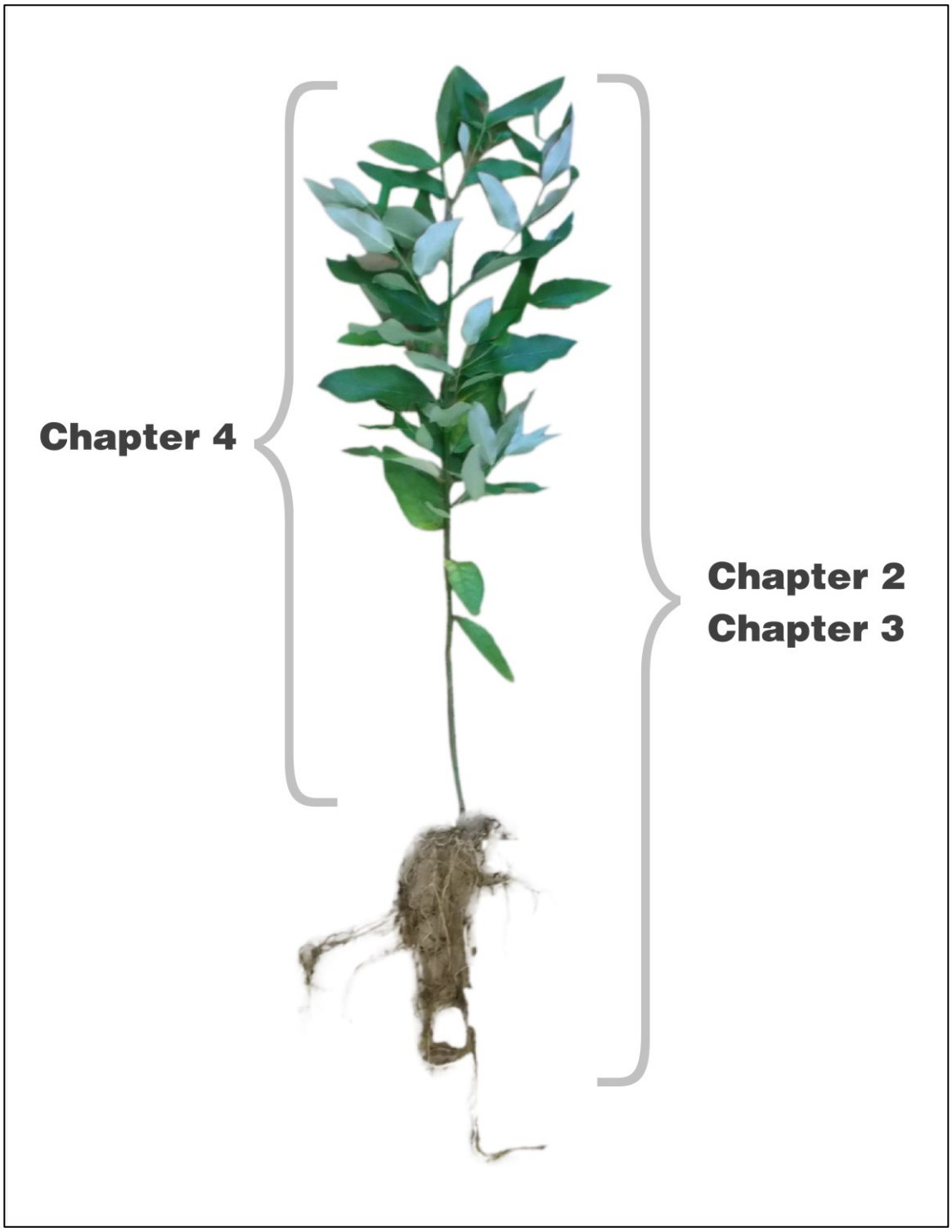

Figure 1-10: Thesis outline.

- the fast recovery of leaf from moderate drought stress (**Chapter3**),
- foliar water uptake (**Chapter4**).

In this thesis I also tried to answer the following questions:

- What is the absolute range of AQP transcript in poplar roots? Poplar leaves? Spruce needle?
- Is the protein regulation correlated with transcription in the tested conditions?
- How many AQPs are present in the spruce genome?

The present study attempts to increase our knowledge concerning the biology of trees, long-living organisms, which have to face several unfavourable environmental conditions. In the context of global warming, there is a real urge for more comprehensive management of the forest industry. The larger scope is to acquire information that will have a future impact on two economically important tree species, including a more comprehensive view of the tree-water relations and the selection of poplar and spruce genotypes with superior water stress response abilities.

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II. Transpirational demand affects aquaporin expression in poplar roots.

1. Introduction

Plants face ever-changing environmental conditions. Throughout their lifetime, trees may not only experience gradual changes in soil moisture, temperature, and other variables, but also have to respond to sudden changes in light and transpirational demand. Dynamic physiological adjustments are required to respond to sudden environmental changes, for example the opening of a gap in the canopy.

In isohydric plants, active stomatal control of water loss maintains leaf water potential relatively constant during periods of water stress (Jones and Tardieu, 1998). By dynamically controlling stomatal conductance, plants can effectively regulate long-distance water flow and water potential over the short term (Jones and Sutherland, 1991; Sperry and Pockman, 1993; Hacke and Sauter, 1995). However, plants can also modulate water uptake in a dynamic fashion. Water taken up by roots flows through living cells, and root water flow (Q_R) is influenced by the modulation of aquaporin abundance and regulation of aquaporin activity (Henzler *et al*., 1999; Kamaluddin and Zwiazek, 2004; Aroca *et al*., 2012).

Aquaporins are water channel proteins and are present in a wide range of animal, microbial, and plant membranes (Henzler *et al*., 1999; Baiges *et al*., 2002). Fifty-six full-length aquaporin sequences have been identified in the *Populus trichocarpa* genome (Gupta and Sankararamakrishnan, 2009; Almeida-Rodriguez *et al*., 2010; Lopez *et al*., 2012). The plasma membrane intrinsic protein subfamily

(PIPs), with their phylogenetic subgroups PIP1 and PIP2, is composed of 15 members in poplar (Supplementary Fig. 2S1). Both PIP1-type (Siefritz *et al*., 2002; Postaire *et al*., 2010) and PIP2-type aquaporins (Vandeleur *et al*., 2009) show significant water transport activity *in planta*. Moreover, PIP1 and PIP2 aquaporins may interact to increase water permeability (Zelazny *et al*., 2007; Secchi and Zwieniecki, 2010). PIPs are generally localized in organs and tissues characterized by high fluxes of water, including root tissues (Javot and Maurel, 2002; Gomes *et al*., 2009; Secchi *et al*., 2009). Thus, plants have the ability to adjust their water uptake capacity to changing environmental conditions by regulating aquaporins in the plasma membrane of root cells. How dynamic above-ground changes are perceived by roots and how root aquaporins are subsequently regulated is not well understood.

In rice, root-specific aquaporins, such as *OsPIP2;3*, *OsPIP2;4*, and *OsPIP2;5* were strongly induced by transpirational demand (Sakurai-Ishikawa *et al*., 2011); these aquaporins could play important roles in the adjustment of radial water transport in rice roots. That transpirational demand can strongly affect K_R has also been shown in poplar (Almeida-Rodriguez *et al*., 2011) and other woody plants (McElrone *et al*., 2007). Almeida-Rodriguez *et al*. (2011) identified gene candidates in poplar that could play similar roles to those of the rice genes mentioned above. However, in their study, plant responses were measured 40–46h after plants were exposed to higher light levels, providing little temporal resolution of molecular and physiological changes that occurred prior to this time.

The first objective of this present study was to measure absolute transcript abundance of key *PIP1* and *PIP2* genes 4 and 28 h after hybrid poplar plants were exposed to an increase in transpirational demand, and to assess how transcriptional responses correspond with changes in Q_R and other parameters of water relations. The second objective was to determine whether changes in gene expression and Q_R would require an increase in light level *per se*, or whether such changes could also be triggered by lowering relative humidity (RH) at a constant light level.

To test this, plants were grown under contrasting irradiance and RH conditions, and were subsequently exposed to a sudden increase in transpirational demand with or without changing the light level. It was hypothesized that a step change in environmental conditions would lead to a transient perturbation of the water potential homeostasis, but that transcript accumulation of key *PIPs* and associated dynamic changes in Q_R would correspond with at least a partial recovery of water potentials.

2. Materials and Methods

a. Plant material and growing conditions

Saplings of hybrid poplar (*Populus trichocarpa* × *deltoides*, clone H11-11) were produced in 2 liters pots from rooted cuttings and maintained in a growth chamber under the following growing conditions: 18/6h day/night cycle; 24/18 °C day/night temperature; ~75% RH. Plants were watered daily and fertilized on a weekly

basis with a 2 g L^{-1} solution of 15:30:15 N:P:K. Plants were grown in turface calcined clay in order to facilitated the separation of roots from soil particles (Almeida-Rodriguez et al., 2011).

After a 2 month period of sapling establishment, plants were randomly assigned to one of three groups and were kept under specific growing conditions for 6 weeks. A control group (subsequently referred to as 'light control') was kept at an irradiance level of 350 μmol m^{-2} s^{-1} (measured at plant level) under the same growing conditions as outlined above. A second group of plants (subsequently referred to as 'shaded plants') was placed in shading structures, which resulted in 80% reduction in irradiance from 350 μmol m^{-2} s^{-1} to 70 μmol m^{-2} s^{-1} at plant level. A third group of plants (subsequently referred to as 'high humidity plants') was placed in a humidified box. The humidified box allowed the RH to be increased to 95% while light level, temperature, and day/night cycles remained the same as in control conditions.

b. Experimental treatments

Experiments were designed to examine changes in hydraulic parameters and aquaporin gene expression in response to an increase in light (shaded plants) and a decrease in relative air humidity (high humidity plants), respectively. A subset of plants was removed from the shade and high humidity boxes at 07:00 h. This was always done at the same time to minimize any effect of time of day on the physiological and molecular measurements. Measurements (or tissue sampling in

the case of gene expression and immunolocalization assays) were carried out 4 h (same day) and 28 h (next day) after shaded and high humidity plants had been removed from their respective environment. All measurements were conducted between 10:30 h and 11:30 h. Control plants were also measured at this time.

c. Plant morphology

Morphological measurements included plant height above pots, root dry weight, and total leaf area. Root dry weight was measured after washing and drying entire root systems at 70°C for 48 h. Leaf areas were determined with a LI-3100C leaf area meter (Li-Cor Inc.; Lincoln, NE, USA). The root dry weight to leaf area ratio is considered as a measure of biomass partitioning (Blake and Filho, 1988; Barigah *et al.*, 2006).

d. Stomatal parameters

The youngest fully expanded leaf of five plants per treatment was used for measurements of stomatal length, density, and pore aperture. Images were recorded in eight randomly selected fields of view of each leaf. Fields of view were located near the point of maximum leaf width on the abaxial (lower) leaf surface. Images were recorded with a digital camera (DFC420C, Leica, Wetzlar, Germany) attached to a light microscope (DM3000, Leica) at ×400 magnification. Analysis was performed with Fiji software (Schindelin *et al.*, 2012). To test if there was an effect of growing conditions on stomatal responses to abscissic acid (ABA), ABA was

applied to detached leaves as described by Nejad and van Meeteren (2007) and Arend *et al*. (2009). Leaf samples were pre-incubated for 2 h under light (~100 μmol m^{-2} s^{-1} photosyntheic photon flux density) in a stomata-opening medium (10mM MES-KOH, pH 6.15, 50 mM KCl) to achieve stomatal opening. Stomatal closure was induced by supplementing the solution with 100 μM ABA (Sigma-Aldrich, St Louis, MO, USA) for 1 h.

e. Water potential and stomatal conductance

Water potential of leaves (Ψ_L) and stems (Ψ_S) were measured using a Scholander-type pressure chamber (Model 1000; PMS Instruments, Albany, OR, USA). One leaf per plant was measured, from five plants per group. Stem water potential was measured after leaves had been sealed in aluminium foil and plastic bags the night before harvesting to promote equilibration of water potentials. Stomatal conductance and transpiration were measured with a steady state porometer (LI-1600, Li-Cor) on five plants per group. High humidity plants were removed from the humidity box (and kept inside the growth chamber) immediately prior to measurements. Stomatal conductance and transpiration could not be measured in the humidity box because the high RH was outside the recommended operating range of the LI-1600. To minimize potential artefacts which might be caused by water desorption from the leaf surface immediately following a transition from high to low RH, leaf surfaces were wiped with Kimwipes (laboratory tissues) prior to measurements.

f. Root water flow

The Q_R of five plants per group was measured according to the hydrostatic pressure method (Kamaluddin and Zwiazek, 2004). Entire root systems were immersed in a beaker filled with measuring solution (20 mM KCl, 1 mM $CaCl_2$) and placed in a pressure chamber. A constant pressure of 0.3 MPa was applied. This pressure allowed stable flow rates to be recorded within ~15 min. The protruding stem was fitted to a graduated pipette and the volume of exudate was measured. Q_R was normalized by the total leaf area of each plant. Normalizing by leaf area provides a measure of the 'sufficiency' of the roots to supply water to leaves (Lo Gullo *et al.*, 1998; Tyree *et al.*, 1998).

g. Gene transcript measurements by quantitative real time PCR

For molecular analysis, representative root samples were collected, immediately frozen in liquid nitrogen and stored at -80°C until analysed. Total RNA was extracted from root tissue of 3-4 plants per treatment using the RNeasy Plant Extraction Mini Kit (Qiagen, Valencia, CA, USA) with hexadecyltrimethylammonium bromide extraction buffer. RNA quality was assessed on an agarose gel and quantified with a spectrophotometer (Nanodrop ND-1000; Thermo Scientific; Wilmington, DE, USA). A 1 µg aliquot of total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and used as template for first-strand cDNA synthesis

with SuperScript II (Invitrogen) following the manufacturer's instructions. cDNA quality was checked by PCR with intron spanning actin (POPTR_0001s45780) primers (TCCCTCAGCACTTTCCAACAG/ACAAGCCATATTACTCGGCCTCAC).

Candidate genes were selected according to their expression patterns in previous experiments (Secchi et al., 2009; Wilkins *et al.*, 2009; Almeida-Rodriguez *et al.*, 2011) and due to their close similarity to rice genes induced by transpirational demand (Sakurai-Ishikawa *et al.*, 2011) (Supplementary Table 2-S1; Supplementary Figure 2-S1). Specific primers (Supplementary Table 2-S2) were designed according to Rutledge and Stewart (2010) using the QuantPrime online tool (Arvidsson *et al.*, 2008). PCR efficiency (E) was determined from a five-point cDNA serial dilution, according to: E = 10[-1/slope]. All selected primer pairs showed correlation coefficients of $R^2 > 0.98$ and primer efficiency values ranging between 1.95 and 2.01.

Real-time qPCR was performed on a 7900 HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using cDNA equivalent to 2.5 ng of RNA following instructions provided by Rutledge and Stewart (2008) and using lambda genomic DNA as a quantitative standard. Each reaction was carried out in triplicate using master mix containing 0.2 mM dNTPs, 0.3 U Platinum Taq polymerase, and 0.25x SYBR Green. The PCR conditions were as follows: 15 min activation at 95°C, 40 cycles of 95°C for 10 s, 65°C for 2-min and a dissociation stage including two cycles of 95°C for 15 s, 60°C for 1 min. Each run was completed with a melting curve analysis to confirm the specificity of amplification and absence of primer dimers. Data analysis was performed according to the sigmoidal method with LRE (linear

regression of efficiency) analyser software (Rutledge, 2011) to assess the absolute quantity of transcripts expressed as number of molecules per ng of total RNA.

h. Immunolocalization

Root segments were fixed in formaldehyde–acetic acid medium (FAA; 10% formaldehyde, 5% acetic acid, 50% ethanol) under vacuum for 1 h and stored in FAA for 16 h at 4 °C. Next, samples were embedded, sectioned, dewaxed, and rehydrated as described before (Almeida-Rodriguez *et al*., 2011). Before the first immunoreaction, sections were incubated for 45 min with blocking solution [BS; 1.5% glycine, 5% (w/v) bovine serum albumin, 0.1% Tween-20 in phosphate-buffered saline (PBS)] following the protocol of Gong *et al*. (2006). Primary antibody directed against the first 42 N-terminal amino acids of AtPIP1;3 (Kammerloher *et al*., 1994; Henzler *et al*., 1999) was applied overnight at 4 °C. Slides were washed as described previously (Gong *et* al., 2006). DyLight 549-conjugated rabbit anti-chicken secondary antibody was pre-absorbed with plant tissue extract (1:500 in BS) before it was applied for 2 h at 37 °C. Slides were rinsed several times and were coverslipped with Permount. Controls with no primary and/or secondary antibody were also prepared. Images were taken with a Leica DMRXA fluorescence microscope (filter cube N2.1, excitation range 515–560 nm, suppression filter LP 590nm) equipped with a Nikon DXM1200 camera (Melville, NY, USA) at a standardized exposure time.

Experimental treatment	Height (m)	DW_R (g)	A_L (m^2)	A_L:DW_R (m^2 g^{-1})
Light control	0.98 (0.03) [a]	1.14 (0.12) [a]	0.32 (0.04)[a]	0.28 (0.01) [a]
Shade	0.73 (0.03) [b]	0.61 (0.06) [b]	0.16 (0.02) [b]	0.27 (0.02) [a]
High RH	1.21 (0.05) [c]	1.61 (0.19) [a]	0.27 (0.04) [a,b]	0.16 (0.01) [b]

Table 2-1: Morphological traits of hybrid poplar saplings grown under control ('Light control'), shade, and high humidity ('High RH') conditions.

The standard error of the mean is given in parentheses, n = 5. Different letters indicate significant differences between treatments (P < 0.05). Variables shown are plant height above pots, total root dry weight (DW_R), total leaf area (A_L), and leaf area to root dry weight ratio (A_L:DW_R).

Experimental treatment	Stomatal length (µm)	Stomatal density (no. per mm^2)	Pore aperture (µm) before/after application of ABA
Light control	35.18 (0.58) [a]	132.6 (4.5) [a]	6.83 (0.36) [a] / 4.20 (0.36) [a]
Shade	32.84 (0.53) [a]	118.1 (8.7) [a]	6.81 (0.30) [a] / 4.18 (0.20) [a]
High RH	39.36 (0.77) [b]	161.7 (6.1) [b]	8.55 (0.10) [b] / 7.07 (0.22) [b]

Table 2-2: Stomatal characteristics of hybrid poplar saplings grown under control ('Light control'), shade, and high humidity ('High RH') conditions. All parameters were measured on abaxial leaf surfaces.

The standard error of the mean is given in parentheses. Values are grand means of five plants. Different letters indicate significant differences between treatments (P < 0.05). In the case of pore apertures, two separate statistical analyses were conducted; one on apertures measured before application of ABA and one after ABA application. i.e., apertures were not compared before and after ABA application.

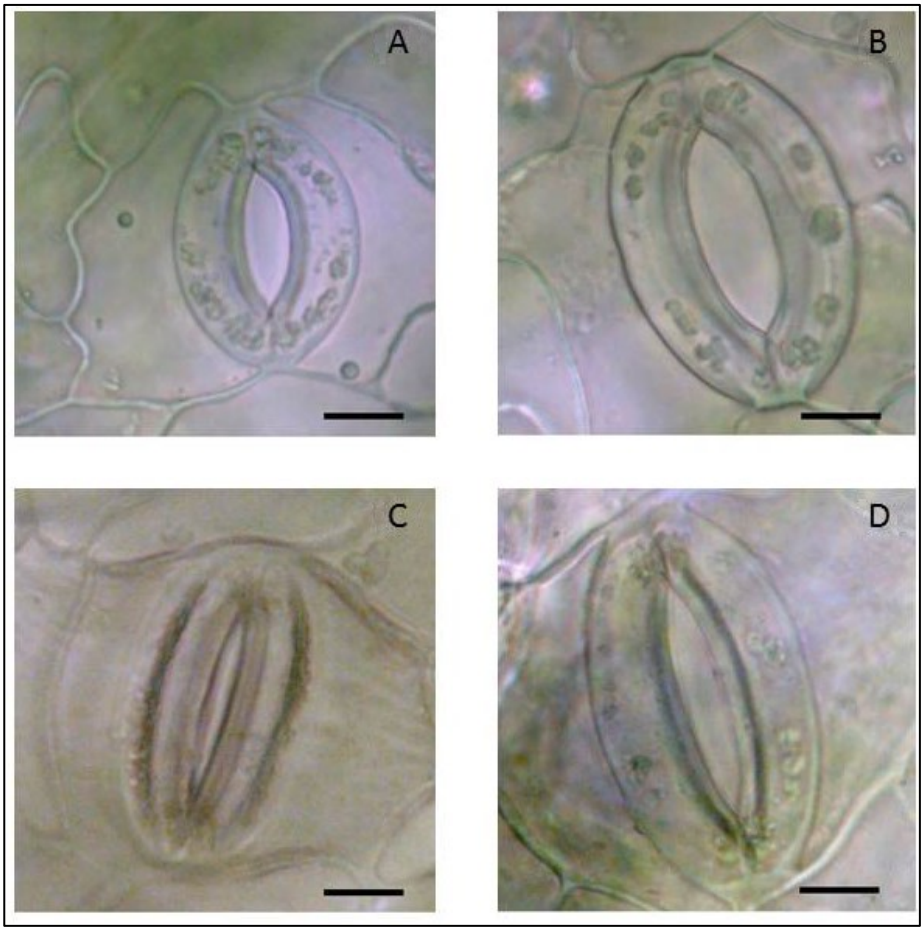

Figure 2-1: **Light microscope images of stomata from poplar leaves growing in moderate (~75% RH) (A) and high (95% RH) relative humidity (B).** The images were taken from the abaxial side of the leaves. Leaves that developed under high RH had larger stomatal length and aperture. While application of 100 µM ABA triggered stomatal closure in plants growing at moderate RH (C), the large stomata of high humidity grown plants failed to close fully (D). Bars = 10 µm.

i. Statistical analysis

Differences due to the effect of treatments and growing conditions were analysed using a one-way analysis of variance (ANOVA) followed by a Tukey's test. Data are presented as means ± SE. Differences were considered significantly different at $P \leq 0.05$. All statistical analyses were carried out using SigmaPlot 12.3 (Systat, Point Richmond, CA, USA).

3. Results

a. Morphology and stomatal characteristics

Morphological traits of the different plant groups are shown in Table 2-1. Shaded plants had 54% lower root dry mass (DW_R) and 50% lower leaf area (A_L) than control plants. As a result of this proportional decrease, the A_L:DW_R ratio did not differ between shaded and control plants. Plants growing at high humidity had the lowest A_L:DW_R ratio of any plant group.

Stomatal characteristics did not differ between shaded and control plants (Table 2-2), although stomatal density of shaded plants tended to be more heterogeneous than in controls. High humidity plants had larger stomata and pore apertures as well as higher stomatal densities than other plant groups. Moreover, after application of 100 µM ABA to leaves, the pore apertures of high humidity plants remained larger than those of other plant groups; that is, stomata of high

humidity plants exhibited incomplete closure (Fig. 2-1, Table 2-2).

b. Water potential and stomatal conductance

Control plants had a Ψ_S of -0.57 ± 0.01 MPa (Fig. 2-2A, 'Light control'). At 4 h after shaded plants were exposed to an increase in light level, their Ψ_S dropped from -0.51 ± 0.02 MPa to -0.71 ± 0.03 MPa (Fig. 2-2B). Leaf water potential showed a similar drop (data not shown). At 28 h after the increase in light level, Ψ_S recovered to -0.46 ± 0.03 MPa. Plants experiencing a sudden drop in RH showed a very similar Ψ_S pattern (Fig. 2-2C).

Shaded plants exhibited a temporary increase in stomatal conductance 4 h after the increase in light level (Fig. 2-3B). In contrast, plants that were exposed to decreasing RH maintained high stomatal conductances and transpiration rates throughout the experiment (Fig. 2-3C; Supplementary Figure 2-S2).

c. Root water flow and aquaporin expression patterns in light-exposed plants

Q_R increased in response to increased evaporative demand. In shaded plants, this increase was significant 28 h after the increase in light level, but not after 4 h (Fig. 2-4B). The delayed increase in Q_R corresponded with aquaporin expression patterns (Fig. 2-5). The total amount of PIP transcripts and the relative proportions of transcripts remained unchanged after 4 h (Fig. 2-5A, compare 'Shade' and 'Light

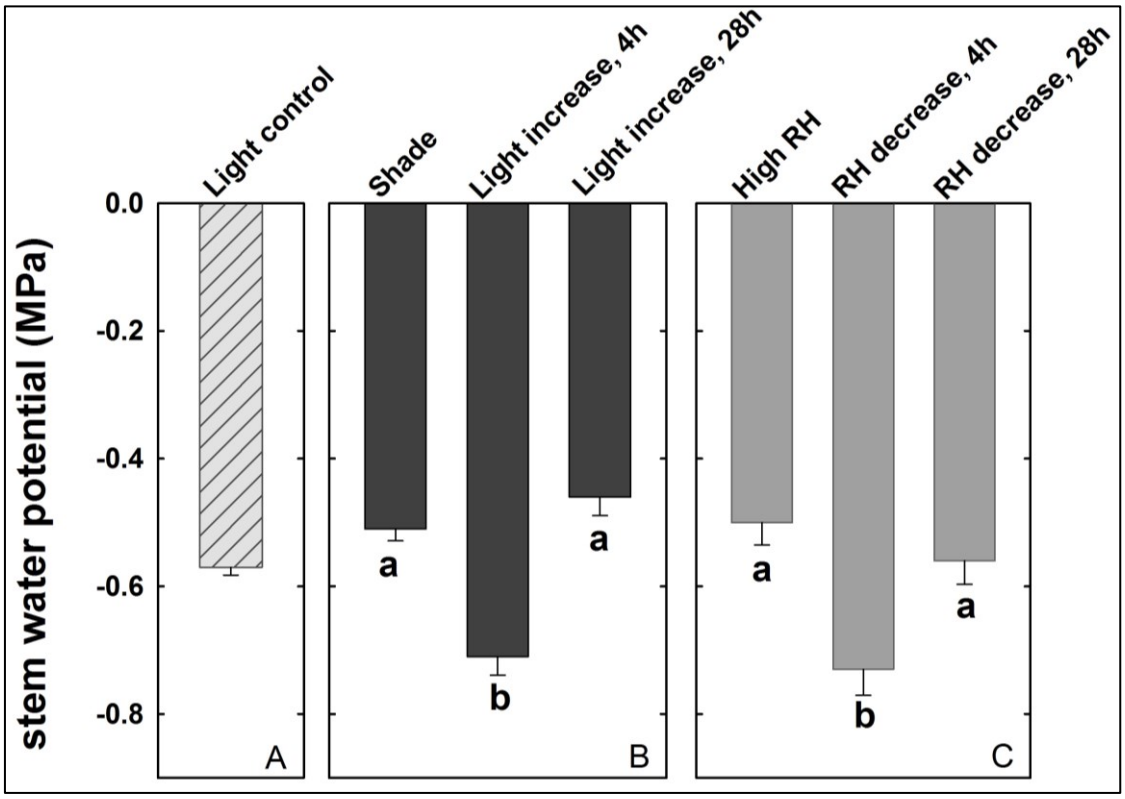

Figure 2-2: Effect of a sudden change in transpirational demand on stem water potential.

(A) Stem water potential of control plants grown under full light conditions in the growth chamber ('Light control'). (B) Stem water potentials of shaded plants ('Shade'), of plants removed from shade after 4 h ('Light increase, 4h'), and of plants removed from shade after 28 h ('Light increase, 28h'). (C) Stem water potentials of plants growing at high relative humidity ('High RH'), of plants removed from high RH after 4 h ('RH decrease, 4h'), and of plants removed from high RH after 28 h ('RH decrease, 28h'). Data shows means + SE; n = 5 plants. Significant differences are indicated by unique letters ($P < 0.05$).

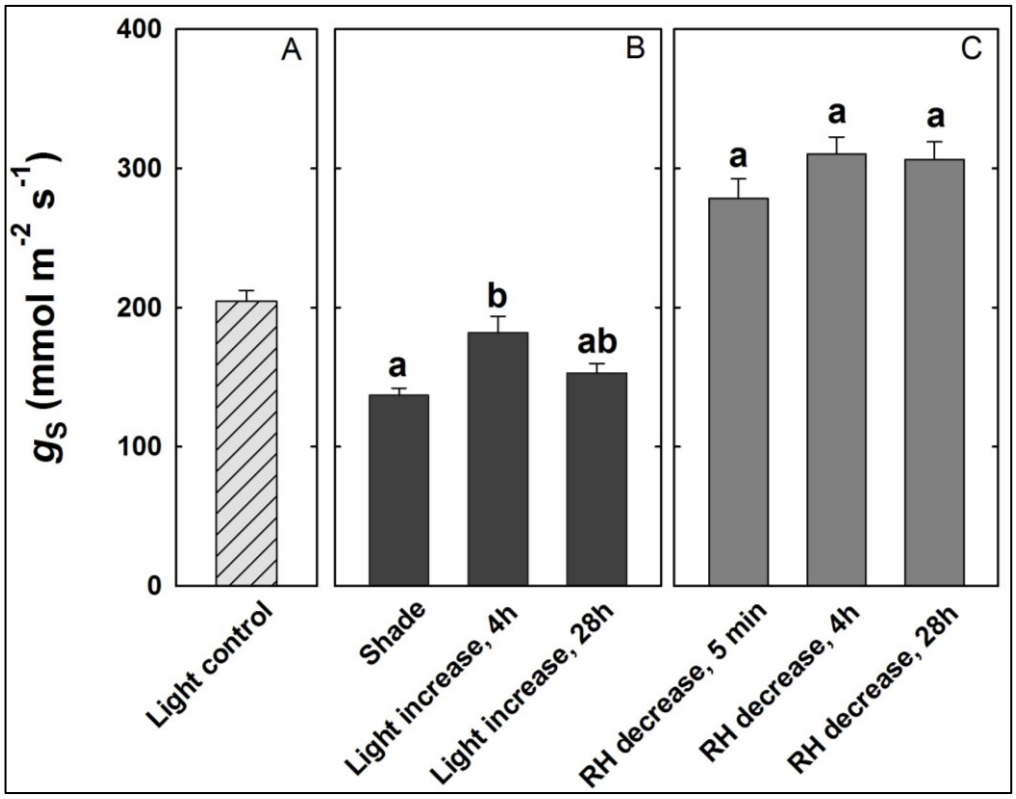

Figure 2-3: Effect of a sudden change in transpirational demand on stomatal conductance.

(A) Stomatal conductance of control plants ('Light control'). (B) Stomatal conductance of shaded plants ('Shade'), of plants removed from shade after 4 h ('Light increase, 4h'), and of plants removed from shade after 28 h ('Light increase, 28h'). (C) Stomatal conductance of plants growing at high relative humidity ('High RH'), of plants removed from high RH after 4 h ('RH decrease, 4h'), and of plants removed from high RH after 28 h ('RH decrease, 28h'). Data shows means + SE; n = 5 plants. Significant differences are indicated by unique letters ($P < 0.05$).

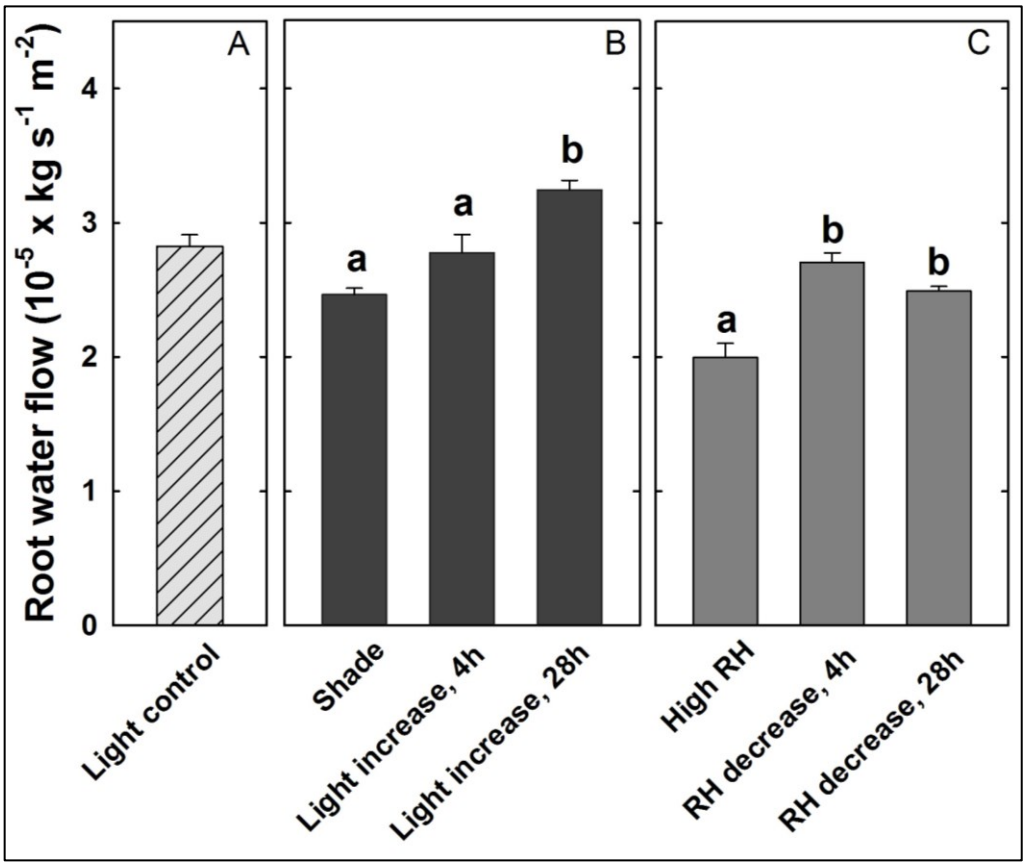

Figure 2-4: Effect of a sudden change in transpirational demand on root water flow (scaled by leaf area).

(A) Root water flow of control plants ('Light control'). (B) Root hydraulic conductance of shaded plants ('Shade'), of plants removed from shade after 4 h ('Light increase, 4h'), and of plants removed from shade after 28 h ('Light increase, 28h'). (C) Root hydraulic conductance of plants growing at high relative humidity ('High RH'), of plants removed from high RH after 4 h ('RH decrease, 4h'), and of plants removed from high RH after 28 h ('RH decrease, 28h'). Data shows means + SE; n = 5 plants. Significant differences are indicated by unique letters ($P < 0.05$).

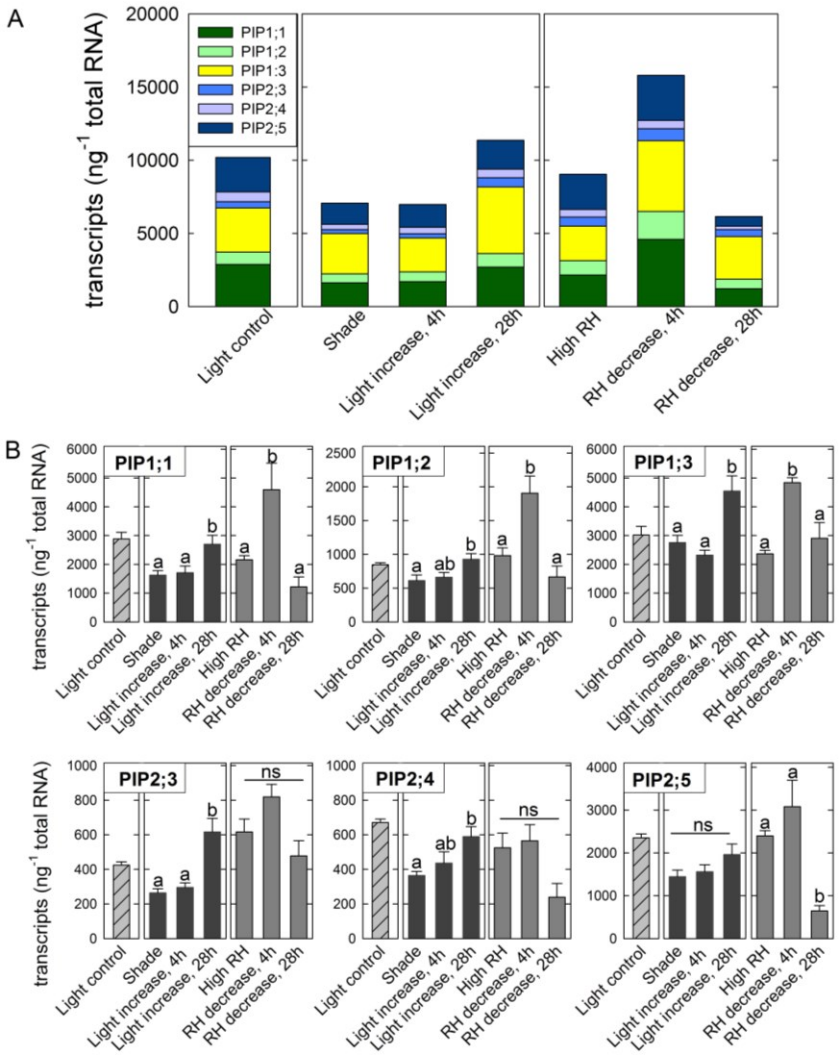

Figure 2-5: Effect of a sudden change in transpirational demand on aquaporin transcript amounts in poplar roots.

(A) Cumulative aquaporin transcript amounts in roots. Individual genes are labeled with different colors. One subset of plants was grown at adequate light level in the growth chamber ('Light'). Other subsets of plants were grown in shade ('Shade') or in a humidified box at ~95% relative humidity ('High RH'). Shaded plants were exposed to a ~four-fold increase in light level. Gene expression was measured 4 h ('Light increase, 4h') and 28 h ('Light increase, 28h') after the increase in light level. Plants growing at high humidity were removed from their humidified box and were exposed to a ~four-fold increase in vapor pressure deficit while light levels remained adequate. Gene expression was measured 4 h and 28 h after the decrease in relative humidity. (B) Transcript abundance of PtPIP1;1, PtPIP1;2, PtPIP1;3, PtPIP2;3, PtPIP2;4, and PtPIP2;5. Values are means + SE from three biological samples which were tested in triplicate. Significant differences are indicated by unique letters ($P < 0.05$).

increase, 4h'), but increased by 60% after 28 h (Fig. 2-5A, 'Light increase, 28h'). Of the aquaporin genes studied here, *PtPIP1;3* ranked first in terms of its proportion to the total number of mRNA molecules (Fig. 2-5A, yellow portion of the bars). Moreover, this gene contributed substantially to the dynamic response shown in Fig. 2-5A. *PtPIP2;5* was also highly expressed in roots (Fig. 2-5A, dark blue portion of the bars), but did not show significant changes in expression in response to an increase in light level.

Fig. 2-5B shows the expression patterns of individual genes. All of the three *PIP1* genes exhibited a significant 52-66% increase in expression after 28 h relative to plants that remained in shade; expression of *PtPIP2;3* even increased >2-fold after 28 h (Fig. 2-5B, black bars).

d. Root water flow and aquaporin expression patterns in plants experiencing a sudden drop in humidity

In plants that were removed from the high humidity environment, Q_R increased by 35% after 4 h and remained unchanged after 28 h (Fig. 2-4C). The rapid increase in Q_R corresponded to a 75% increase in the cumulative transcript copy numbers of all six *PIPs* (Fig. 2-5A). This increase in transcripts after 4 h was mainly due to a 2-fold increase in the transcript copy numbers of the three *PIP1* genes (Fig. 2-5B, grey bars). No significant changes in the expression of *PIP2s* occurred after 4 h.

After 28 h, expression levels of *PIP1* genes had returned to values found prior to the change in RH while Q_R remained relatively high. While transcript copy

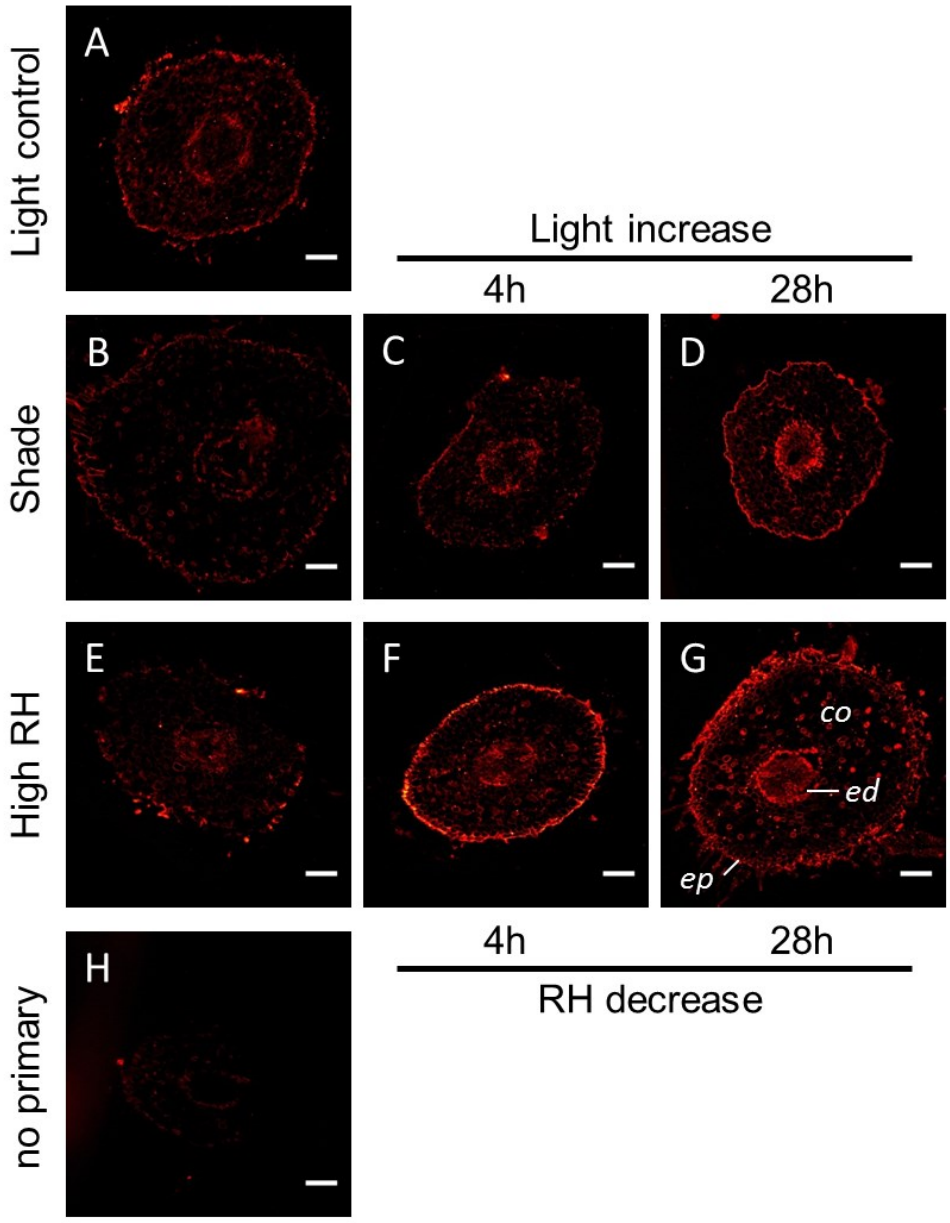

Figure 2-6. Immunolocalization of PIP1 protein in root cross-sections.

Transverse sections were taken at 25–30 mm from the root tip. PIP1 antibody is specific to all PIP1s. (A) Roots of control plants growing at full light in the growth chamber. (B–D) Roots of shaded plants before (B) and after a step change in light level (C, D). (E–G) Roots of plants growing at high relative humidity before (E) and after a step change in humidity (F, G). (H) Control with no pimary antibody indicates minimal background autofluorescence. co, cortex; ed, endodermis; ep, epidermis. Bars=100 μm

numbers of *PtPIP2;3* and *PtPIP2;4* did not change significantly in response to the change in humidity, transcript numbers of *PtPIP2;5* had decreased sharply after 28 h (Fig. 2-5B).

e. Immunolabelling

Immunofluorescence labelling was performed on cross-sections taken at 25–30mm from the root tip (Fig. 2-6). The intensity of the red colour is equivalent to the abundance of PIP1 protein. In roots of control plants, PIP1 was present in epidermis and cortex cells as well as in the endodermis and in vascular tissue (Fig. 2-6A). Weak labelling was observed in roots of shaded plants (Fig. 2-6B). In contrast, root sections taken after the increase in light level exhibited strong immunolabeling of the epidermis, endodermis, and of cells adjacent to the endodermis. Labelling was particularly abundant after 28 h when a continuous fluorescence signal occurred in the epidermis (Fig. 2-6D). A similar trend was observed in plants that were exposed to decreasing humidity (Fig. 2- 6E-G), although strong signals were already detected after 4 h (Fig. 2-6F). Controls without primary antibody exhibited minimal fluorescence (Fig.2- 6H).

4. Discussion

Although much has been learned about the possible physiological roles of aquaporins in plants, many questions remain unanswered (Baiges *et al.*, 2002; Aroca

et al., 2012). The present study was conducted to gain a better understanding of how aquaporins in roots are regulated and how their function relates to whole-plant–water relations in woody plants (Hacke *et al*., 2012).

a. Aquaporin gene expression and root hydraulics are affected by changes in transpirational demand

The first objective of this present study was to measure absolute transcript abundance of key PIP1 and PIP2 genes 4 h and 28 h after hybrid poplar plants were exposed to an increase in transpirational demand, and to assess how transcriptional responses correspond to changes in Q_R and other parameters of water relations. To minimize the effect of a circadian rhythm (Henzler *et al*., 1999; Clarkson *et al*., 2000; Lopez *et al*., 2003) on the data collected in this present study, all measurements were conducted between 10:30 and 11:30 h.

Among the 11 *PIP* gnes that were studied by Almeida-Rodriguez et al. (2011), the authors reported the differential expression of nine *PIP* genes in roots of poplars exposed to different light regimes. Based on this and on available literature data (Secchi *et al*., 2009; Supplementary Table 2-S1),e six *PIPs* that were highly expressed in roots were chosen for gene expression analysis.

The three *PIP1* genes exhibited remarkably similar expression patterns (Fig. 2-5B). Interestingly, these genes are orthologs of the rice *OsPIP1s* whose transcription in roots increased with transpirational demand (Sakurai-Ishikawa *et al*., 2011). Furthermore, the closely related *PtPIP1;1* and *PtPIP1;2* (95% amino-acids

identity; Supplementary Fig. 2-S1) were found to be induced in response to xylem embolism (Secchi and Zwieniecki, 2010) and by osmotic stress (Bae *et al*., 2010). Expression changes of the studied PIP2 genes were smaller and more variable than those of the three PIP1 genes, a pattern which has also been described in drought-stressed stems of *P. trichocarpa* (Secchi and Zwieniecki, 2010).

In terms of transcript copy numbers, *PtPIP1;1* and *PtPIP1;3* ranked first among the *PIP* genes measured in this study (Fig. 2-5B). The transcripts of all three *PIP1* genes represented nearly three-quarters of the total transcript amount while Q_R increased. It is therefore suggested that these genes play crucial roles in modifying root water uptake in poplar in response to changes in transpirational demand. Aquaporin activity is regulated at both the transcriptional and the post-translational levels. While the present study focused on transcriptional regulation, it is noted that responses to a change in environmental conditions can also be realized by other mechanisms, including aquaporin gating, translocation of aquaporins into the membrane, and interactions of membrane proteins (e.g., Hedfalk *et al*., 2006; Zelazny *et al*., 2007; Maurel *et al*., 2008;). Nonetheless, the fact that expression patterns, particularly those of *PIP1* genes, closely corresponded with changes in Q_R, suggests that transcriptional control was an important mechanism involved in the regulation of root physiology.

Striking differences between trends in transcript abundance and Q_R only occurred 28 h after plants were transferred to lower humidity (compare Fig. 2-4C and Fig. 2-5A 'RH decrease, 28h'). At that time, transcript copy numbers of several

genes reached low levels (Fig. 2-5B, grey 'RH decrease, 28h' bars) while Q_R was still nearly as high as it was 4 h after the change in humidity (Fig. 2-4C, grey bars). It is suggested that the peak in transcription seen 4 h after the change in humidity resulted in an accumulation of water channel proteins, and that proteins were still present 24 h later. This conclusion is supported by immunolabeling experiments, which revealed that PIP1 protein remained highly abundant in root cross-sections 28 h after the transfer to lower humidity (Fig. 2-6)

b. Differences between plants grown in shade and in high humidity

The adaptive significance of aquaporin-mediated changes in whole-plant hydraulic conductance is that it would provide plants with a mechanism to maintain their water potential homeostasis despite changing environmental conditions through modifying water transport in roots. While the present study focused on roots, it is noted that whole-plant hydraulic conductance will probably also be affected by aquaporins in leaves (Heinen *et al*., 2009). A fine-tuned balance between water loss and water uptake is especially important in plants that are vulnerable to xylem cavitation and lack efficient mechanisms to repair xylem dysfunction. The poplar clone studied here (H11-11) is very vulnerable to cavitation. In a previous study (Plavcova and Hacke, 2012) on H11-11 plants growing under similar conditions, 50% loss of hydraulic conductivity occurred at -1.14 MPa and -0.62 MPa in basal and distal stem segments, respectively. This is close to or within the range of stem

water potentials measured in the present study. It is therefore concluded that the recovery of stem water potentials 28 h after the increase in transpirational demand was necessary to prevent excessive and irreversible levels of embolism.

Shaded plants would likely have benefited from a faster increase in Q_R to take advantage of increased light levels (Almeida-Rodriguez *et al*., 2011). The relatively slow increase of Q_R in shaded plants may be due to the stressful growing conditions that these plants experienced. Poplars are light-demanding plants, and shade-grown plants were probably energy-starved. To the degree that new expression and activation of aquaporins are energy dependent, water uptake dynamics may have been constrained by limited resources in the roots of shaded plants.

Interestingly, changes in the transcript levels of *PIP1* genes and in Q_R occurred sooner in high humidity plants than in shaded plants. This may in part be due to the fact that stomatal conductance in high humidity plants remained high throughout the experiment (Fig. 2-3; Supplementary Fig. 2-S2). Stomata of these plants were larger and more frequent than in other plant groups, and were unable to close (Fig. 2-1; see also Arve *et al*., 2013). Hence, fast aquaporin-mediated responses of Q_R to changes in the above-ground environment may have compensated for a lack of stomatal control.

c. An increase in light level is not required to trigger changes in gene expression and root hydraulics

The second objective of this study was to determine whether changes in gene expression and Q_R would require an increase in light level *per se*, or whether such changes could also be triggered by lowering RH at a constant light level. Altering RH without changing irradiance had a profound effect on both *PIP* transcript levels and Q_R (see above). It was therefore concluded that an increase in light level is not required to trigger changes in *PIP* expression and Q_R in poplar. This conclusion agrees with recent work on rice (Sakurai-Ishikawa *et al*., 2011). Levin *et al*. (2009) found that some aquaporin genes were differentially expressed in *Arabidopsis thaliana* plants subjected to low RH. How exactly changes in the above-ground environment are transmitted to and sensed by roots remains unknown. The most parsimonious hypothesis is that root cells sense xylem pressure pulses (McElrone *et al*., 2007) or changes in water potential (Levin *et al*., 2009), and/or cell turgor (Hill *et al*., 2004), which all would correspond with changes in transpirational demand.

In conclusion, hybrid poplar plants were subjected to a sudden increase in transpirational demand, either by increasing light level or by reducing RH. Both treatments led to a transient perturbation of water potentials. At 28 h after plants were removed from shade or from their high humidity environment, respectively, stem water potentials recovered to their original values (measured prior to treatments). The recovery of water potentials was associated with an increase in Q_R and an increase in the transcript abundance of aquaporin genes in roots. In both experiments, transcript levels of three *PIP1* genes closely matched trends in Q_R. While stomata of plants grown in high humidity were unable to close properly, the

Q_R of these plants quickly responded to increased transpirational demand. In contrast, the Q_R of shaded plants increased 28 h after the increase in light, but not 4 h after the removal from the shade environment. The fact that aquaporin gene expression and Q_R responded to a drop in RH while light levels were unchanged indicates that an unknown signal was involved in this case of shoot-root communication. Future work will probably be directed at unravelling the nature of this signalling process and will study how the signal is perceived by root aquaporins.

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III. Dynamics of leaf hydraulic conductance and aquaporin expression in *Populus trichocarpa* leaves with dehydration and rehydration.

1. Introduction

High gas exchange rates can only be sustained when leaves are kept well hydrated. This, in turn, depends on the properties of the xylem pipeline and on the way in which water moves through living cells in roots and leaves (Tyree and Sperry, 1988; Sperry *et al.*, 2002). Leaf hydraulic conductance is emerging as an important component of whole-plant hydraulic conductance (Brodribb and Holbrook, 2004; Heinen *et al*., 2009; Scoffoni *et a*l., 2012; Prado and Maurel, 2013; Nardini and Luglio, 2014). Like in roots and stems, hydraulic conductance of leaves declines as the water potential becomes more negative. This loss of hydraulic conductance is due to embolism formation in leaf veins (Stiller et al., 2003; Johnson et al., 2011), collapse of xylem conduits (Brodribb and Holbrook, 2005), and/or to decline in the permeability of extra-xylary tissues (Shatil-Cohen *et al*., 2011). Compared with stems, leaves (Brodribb *et al*., 2003) and roots (Hacke *et al*., 2000) are often more vulnerable to hydraulic dysfunction. In some cases, however, the hydraulic conductance of these plant organs may also be able to quickly recover from the effects of drought (Stiller *et al*., 2005; Scoffoni *et al*., 2012).

This recovery of hydraulic function may be facilitated by the activity of aquaporin (AQP) water channels (Martre *et al*., 2002; North *et al*., 2004; Galmés *et al*., 2007; Jang *et al*., 2013; Laur and Hacke, 2014). AQPs belong to the major intrinsic protein (MIP) superfamily, a family of protein pores present in the membranes of almost all biological cells to facilitate the diffusion of a wide range of

small uncharged solutes. Plant MIPs form a particularly large family of proteins, with 28 members in *Vitis vinifera* (Fouquet *et al*., 2008), ≥30 members in *Arabidopsis thaliana*, *Picea glauca* and *Oryza sativa* (Quigley *et al*., 2002; Sakurai *et al*., 2005; Laur and Hacke, 2014), and >50 members in *Populus trichocarpa* (Gupta and Sankararamakrishnan, 2009). The plant-specific plasma membrane intrinsic proteins (PIPs), with their highly conserved phylogenetic subgroups PIP1 and PIP2, and tonoplast intrinsic proteins (TIPs) show significant water transport activity *in vitro* and *in planta* (Daniels *et al*., 1994; Vandeleur *et al*., 2009; Postaire *et al*., 2010). Regulation of AQPs via transcription, translation, post-translational modifications or trafficking allows plant cells and organs to respond to hydraulic changes in their surrounding environment (Chaumont and Tyerman, 2014).

In this present study, *Populus trichocarpa* plants were exposed to moderate drought and then rewatered. The objective was to study the recovery of K_{leaf} from water stress at both physiological and molecular levels. We hypothesized that leaves would quickly (i.e., within hours) recover from water stress, and that this would be associated with modulation of AQP activity. To test this hypothesis, we monitored K_{leaf} and Ψ_{leaf} during a dehydration-rehydration episode. We also explored the regulation of 12 leaf-expressed *AQP* isoforms as well as the tissue-specific location of PIP1, PIP2 and TIP2 proteins. Recovery of K_{leaf} was assessed in two ways: (i) intact plants were taken through a drying-rewatering cycle, and (ii) detached leaves were bench-dried and subsequently xylem-perfused with AQP inhibitors.

2. Materials and Methods

a. Plant material and growing conditions

All experiments were carried out with *P. trichocarpa* clone 664042 (IUFRO collection). Rooted cuttings were produced and established in the greenhouse for 2 months in 3.8 L containers with sunshine mix 4 (Sun Gro Horticulture Canada Ltd.) under semi-controlled conditions (22/20 °C day : night cycle, 18/6 h light : dark, watered daily, and fertilized (2g L^{-1} NPK 15-30-15) once a week).

b. Leaf hydraulic conductance measurements

Leaf hydraulic conductance was measured using the evaporative flux method (Sack and Scoffoni, 2012) on six plants per treatment. A filtered (0.2 μm) 20 mM KCl + 1 mM $CaCl_2$ solution (subsequently referred to as 'artificial xylem sap', AXS) was used for these measurements. Flow rate through leaves was measured with a balance (model CP 224S, Sartorius, Göttingen, Germany), which logged data every 30 s to a computer. The air was well stirred by a fan as explained by Sack and Scoffoni (2012). Leaves were illuminated with ~1000 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at the leaf surface by an LED worklight (Husky, distributed by Home Depot, Atlanta, GA, USA). Leaf temperature was monitored by a thermocouple. Leaf water potential was measured using a pressure chamber (PMS Instruments, Albany, OR, USA). Leaf area was determined with a scanner. A leaf vulnerability curve was generated with plants experiencing different

levels of water stress following methods of Sack and Scoffoni (2012). Fully expanded leaves corresponding to leaf plastochron index (LPI) 8 (Larson and Isebrands, 1971) were used to measure Ψ_{leaf}; K_{leaf} was then measured on leaves corresponding to LPI 9. The curve was fitted with a Weibull function.

c. Recovery of leaf hydraulic conductance after dehydration

To study the recovery of K_{leaf} in intact plants, plants were randomly assigned to different watering regimes in the greenhouse. One group of plants was kept well watered (control). Another group of plant was subjected to a drought treatment. Water was withheld for several days until plants reached a Ψ_{leaf} of -0.77 ±0.05 MPa (mean ±SE, n=6). This Ψ_{leaf} was associated with a substantial reduction in K_{leaf}. A subset of drought-stressed plants was then rewatered, and Ψ_{leaf} and K_{leaf} were re-measured 2 h and 26 h after rewatering.

To assess the effect of AQP inhibitors and abscisic acid (ABA) on the recovery of K_{leaf}, excised leaves were bench-dried for 1h and then perfused for 2 h with AXS, AXS + 0.2 mM $HgCl_2$, AXS + 50 mM H_2O_2 or AXS + 50 µM ABA. Solutions were introduced into the transpiring leaf by immersing the petiole inside 50 mL containers. Leaves were placed near a fan; light was provided at a light level of ~1,000 µmol m^{-2} s^{-1} PAR. Mercury chloride and H_2O_2 have been widely used as AQP inhibitors; ABA may also reduce AQP activity in leaves (Shatil-Cohen *et al.*, 2011; reviewed in Chaumont and Tyerman, 2014). Control leaves were always kept hydrated and were perfused with pure AXS for 2 h. Immediately after perfusion with

these solutions, K_{leaf} was determined using the evaporative flux method as described above. All measurements were conducted at the same time of day (10:00 – 11:30 h).

After perfusion with the different solutions, stomatal pore aperture of leaves was measured as described in Laur and Hacke (2013). Images were recorded in six randomly selected fields of view of each leaf. Fields of view were located near the point of maximum leaf width on the abaxial leaf surface.

d. Dye uptake experiments

The extent of dye uptake in excised leaves was used as an additional method to assess xylem refilling during the rehydration phase. We also used the dye uptake experiments in an attempt to study how embolism reversal in leaf veins is impacted by mercury and ABA, respectively. Excised leaves were bench-dried for 1 h and rehydrated for 2 h by immersion of the petioles in filtered safranin solutions. Transpiration during dye uptake was promoted by placing leaves near a fan at a light level of ~1,000 µmol m^{-2} s^{-1} PAR (i.e., conditions similar to the protocol used to measure K_{leaf}). Dye (0.1 % (w/v) safranin) was dissolved in pure AXS, AXS + 0.2 mM $HgCl_2$. Control leaves were excised from well-watered plants and then perfused for 2 h with 0.1 % safranin-containing AXS without prior dehydration treatment.

e. Gene transcript measurements by quantitative real-time PCR

Fully expanded leaves corresponding to LPI 7-10 were collected, immediately

frozen in liquid nitrogen and stored at -80°C until analyzed. Samples were always collected between 10:00 h and 11:30 h to minimize any diurnal effect on AQP expression. Total RNA was extracted from 3 plants per treatment following the CTAB method of Pavy *et al*. (2008). RNA quality was assessed on an agarose gel and quantified with a spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Wilmington, DE, USA). RNA was treated as previously described (Laur and Hacke, 2014). cDNA quality was checked by PCR with intron-spanning actin primers. Putative leaf-expressed AQP genes were selected (Wilkins *et al*., 2009; Almeida-Rodriguez *et al*., 2010; Cohen *et al*., 2013), specific primers (Table 3-S1) were designed according to Rutledge and Stewart (2010) using the QuantPrime online tool (Arvidsson *et al*., 2008). PCR efficiency was 100±7% for all primer pairs and specificity was checked using melting curves. Real-time qPCR was performed on a 7900 HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) as described previously (Laur and Hacke, 2013). Relative gene expression was measured according to Livak and Schmittgen (2001) using the 2ΔΔC(t) method. The expression values were normalized to the geometric mean of four housekeeping genes (actin (POPTR_0001s31700), cyclophilin (POPTR_0005s26170), TIP4-like (POPTR_0009s09620.1) and ubiquitin (POPTR_0005s09940)). Relative gene expression was determined as the fold change of an AQP isoform at a given condition relative to its expression under control conditions. Real-time PCR was carried out using three biological replicates each with three technical replicates.

f. Immunolocalization

Samples were fixed in formaldehyde-acetic acid and embedded in paraffin as described previously (Almeida-Rodriguez *et al*., 2011). Transverse sections, 10 µm thick, were prepared with a microtome. Immunoreactions were performed following the protocol of Gong *et al*. (2006). Primary antibodies directed against the 42 N-terminal amino acids of AtPIP1;3 (Kammerloher *et al*., 1994) and the conserved 10 amino acids of the C-terminal of PIP2s (Laur and Hacke, 2014) were used. In addition, we applied a commercially available anti-TIP2 antibody (Sakurai *et al*., 2008); Agrisera AB, Sweden; alignment shown in Fig. S1). AlexaFluo 488-conjugated goat anti-chicken, anti-mouse and anti- rabbit secondary antibodies (Life Technologies Inc., Burlington, ON, Canada) were respectively applied for 2 h at 37°C. Slides were mounted with Permount. Images were taken with a Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

g. Statistical analysis

All statistical analyses were carried out using SigmaPlot 11.0 (Systat, Point Richmond, CA, USA). Differences due to the effect of treatments and growing conditions were analyzed using a one-way ANOVA followed by a Tukey's test for physiological data, and a one-way ANOVA followed by Bonferroni's post test for the gene expression analysis. For all tests, differences were considered significant at $P \leq 0.05$.

3. Results and Discussion

a. Leaf hydraulic conductance is highly sensitive to drought

To assess how K_{leaf} declines as a function of Ψ_{leaf}, we first constructed a vulnerability curve. Water was withheld from plants in the greenhouse until plants reached different levels of water stress. Leaves were highly vulnerable with 50% and 80% loss of hydraulic conductance occurring at Ψ_{leaf} = -0.45 MPa and -0.70 MPa,respectively (Figure 3-1, insert). The drought-induced loss in K_{leaf} shown in Figure 3-1 may have been due to xylem cavitation, reduced water permeability of cell membranes and/or other factors (Heinen *et al*., 2009; Prado *et al*., 2013). The water potentials at 50% and 80% loss of hydraulic conductance (P_{50} and P_{80}, respectively) are well within the range of water potentials that trees experience under natural conditions (Pezeshki and Hinckley, 1982; Sparks and Black, 1999). It therefore appears that K_{leaf} is subject to substantial diurnal changes under natural conditions, similar to what has been observed in rice and other species (Trifilò *et al*., 2003; Stiller *et al*., 2005; Scoffoni *et al*., 2012). Our data also indicates that leaf hydraulic conductance is more sensitive to decreasing water potentials than the hydraulic conductance of stems (Sparks and Black, 1999). However, since we only worked with young greenhouse-grown plants, it remains to be seen whether leaves of field-grown trees are similar in their response to water stress.

b. Leaves of intact plants quickly recover from drought

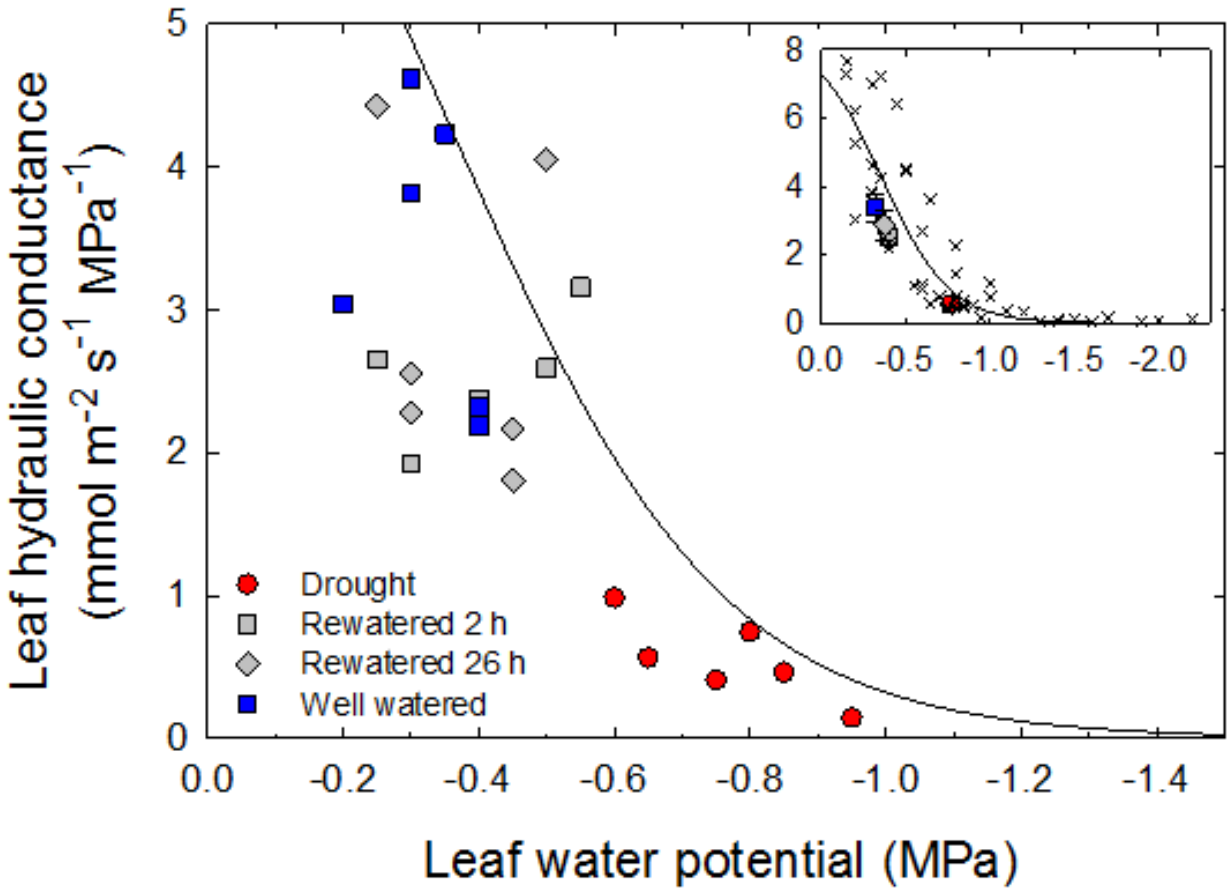

Figure 3-1 Effect of a change in water availability on leaf hydraulic conductance (K_{leaf}) in *Populus trichocarpa* saplings. Kleaf and the associated leaf water potential (leaf) were measured in 6 well-watered control plants (blue squares), 6 drought-stressed plants (red circles), and drought-stressed plants 2 and 26 h after rewatering (grey squares and diamonds, respectively). Each data point represents a single measurement of Kleaf. The solid line shows the previously established vulnerability curve for Kleaf. An overview of the complete vulnerability curve is shown in the upper right corner of the figure. Individual measurements are shown as crosses; the mean values for each group (+SE, n=6) are shown using the same symbols as explained above.

We next tested whether K_{leaf} would recover after a drought treatment when plants were left intact during the dehydration-rehydration episode. In this experiment, leaves of well-watered control plants had a Ψ_{leaf} of -0.33 ±0.03 MPa (±SE, n=6), which was associated with a K_{leaf} of 3.37 ±0.41 mmol m^{-2} s^{-1} MPa^{-1} (±SE, n=6) (Figure 3-1, blue squares). The drought treatment resulted in a drop of Ψ_{leaf} to -0.77 ±0.05 MPa (± SE, n=6) and a six-fold drop of K_{leaf} to 0.55 ±0.12 mmol m^{-2} s^{-1} MPa^{-1} (±SE, n=6) (Figure 3-1, red circles). These values were in good agreement with the previously established vulnerability curve (Figure 3-1, insert). Only 2 h after rewatering (Figure 3-1, grey squares), both Ψ_{leaf} and K_{leaf} reached values that were not statistically different from well-watered control plants (*t* test, $P = 0.083$ for K_{leaf}), indicating that leaves completely recovered their hydraulic function.

c. *AQP* expression in leaves collected from intact plants

To study the role of water channels in the recovery of K_{leaf}, AQP expression was measured in leaves at different stages during the dehydration-rehydration experiment. Three *PIP1*, three *PIP2,* and six *TIP* candidate genes were selected for analysis. Among them, *PtPIP1;1*, *PtPIP1;2*, *PtPIP1;3*; *PtPIP2;4* and *PtTIP2;1* exhibited the highest total number of mRNA molecules in leaves of control plants (Table 3-1). The drought treatment resulted in a significant reduction in the expression of all tested genes (Fig. 3-2). In leaves collected 2 h after rewatering, there were two

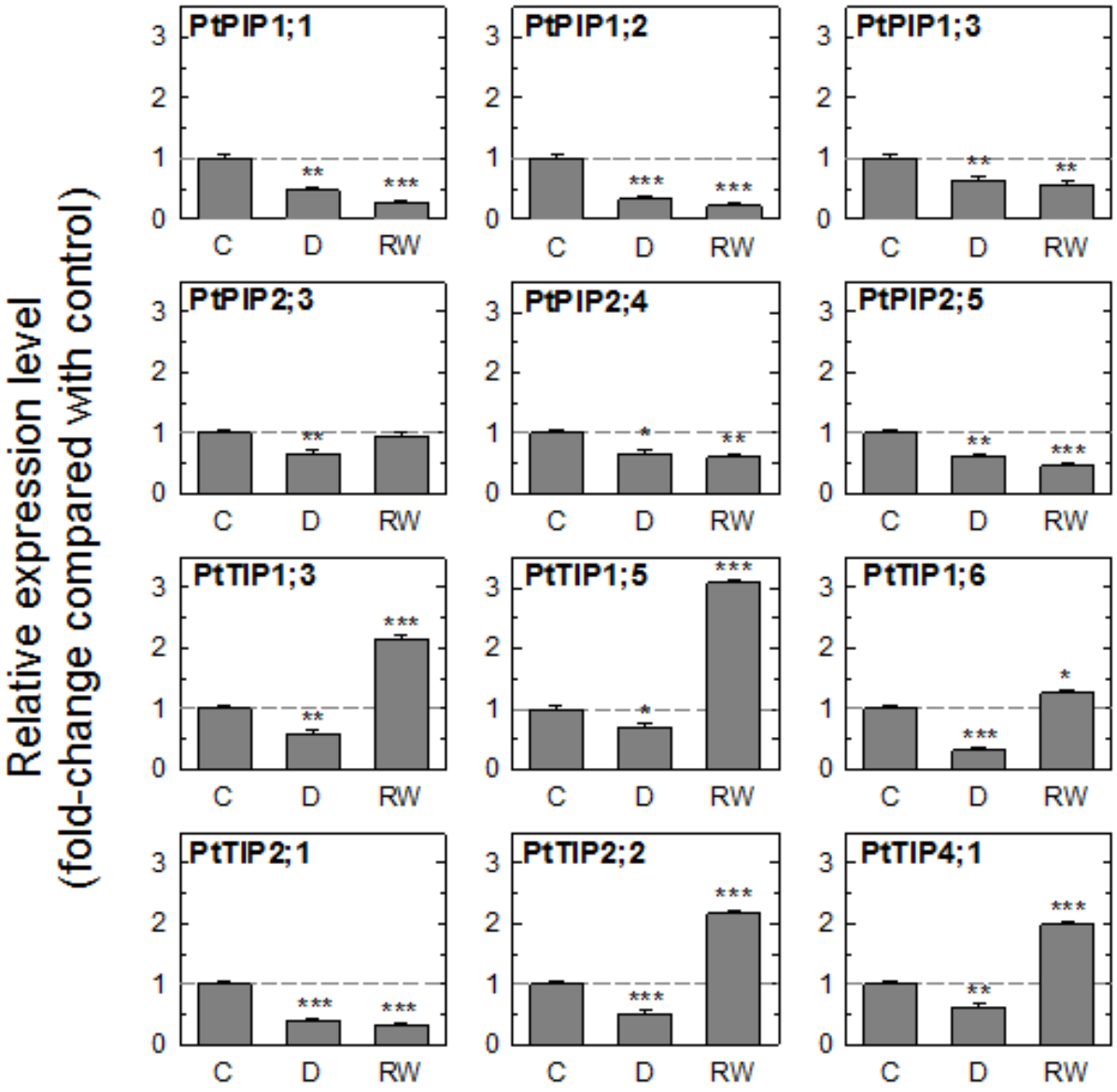

Figure 3-2 Relative expression of aquaporin genes in leaves of plants exposed to a drying-rewatering cycle. Gene expression was measured in leaves of well-watered control plants (C), drought-stressed plants (D), and 3 h after drought-stressed plants were rewatered (RW). The geometric mean of the expression levels of four reference genes (ACT2, CYC063, TIP41-like, UBQ7) was used to normalize the results. Asterisks denote significant differences in expression level compared to control levels (one-way ANOVA, followed by Bonferroni's post test, *P≤0.05; **P≤0.01***P≤0.001). Data are means ±SE of three biological replicates.

Aquaporin name	Expression (copies μg^{-1} of total RNA)
PtPIP1;1	112,960 ±9,067
PtPIP1;2	272,111 ±32,575
PtPIP1;3	229,960 ±44,252
PtPIP2;3	85,667 ±15,402
PtPIP2;4	273,655 ±33,728
PtPIP2;5	11,536 ±1,738
PtTIP1;3	23,105 ±2,540
PtTIP1;5	11,840 ±1,675
PtTIP1;6	2,330 ±121
PtTIP2;1	153,689 ±19,669
PtTIP2;2	24,863 ±3,451
PtTIP4;1	517 ±9

Table 3-1 Transcript abundance of 12 aquaporin genes expressed in leaves of well-watered control plants.
Values are the means ±SE from three biological samples which were tested in triplicate.

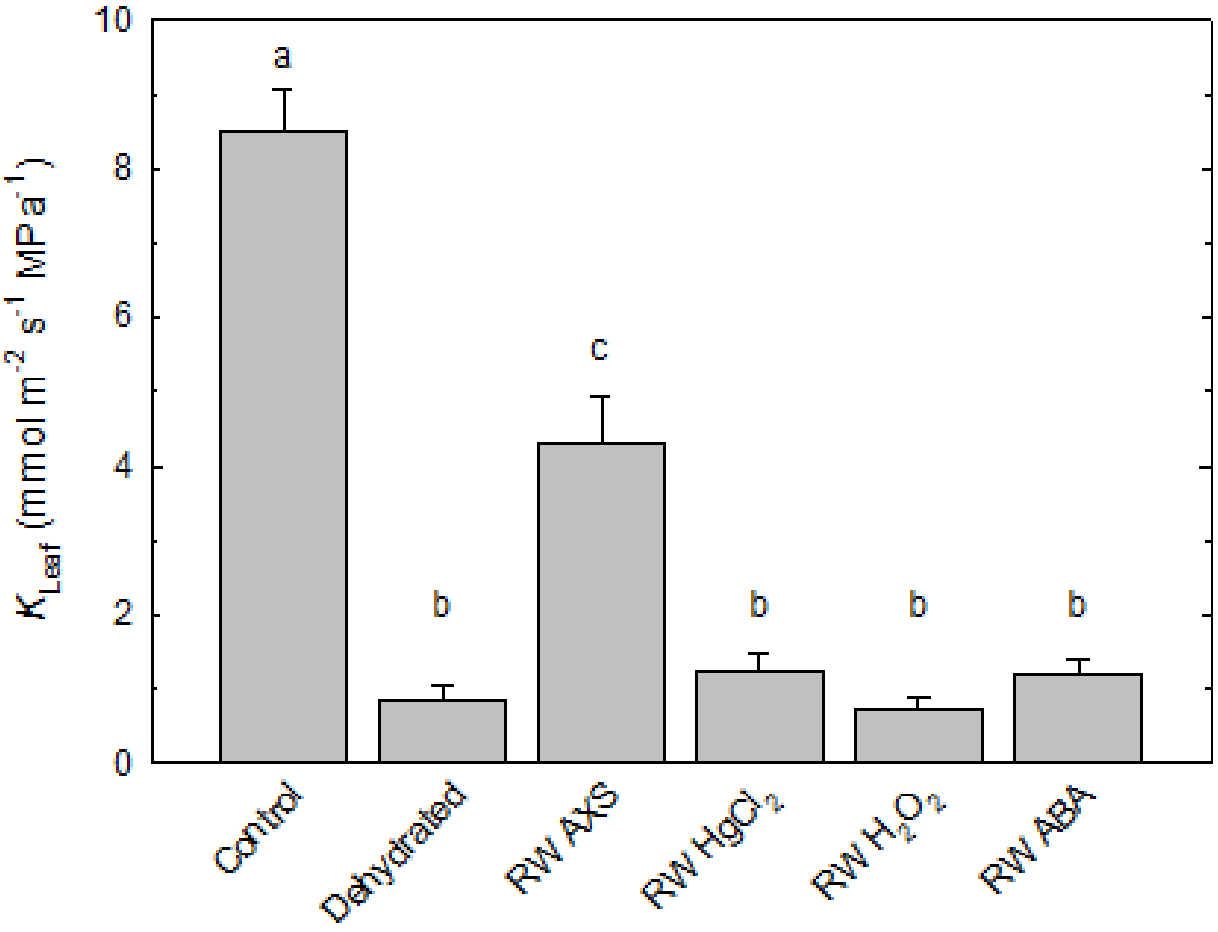

Figure 3-3: Response of leaf hydraulic conductance (K_{leaf}) to different perfusion solutions.

Control conditions refer to the K_{leaf} that was measured after leaves were xylem perfused with filtered (0.2 µm) 20 mM KCl + 1 mM $CaCl_2$ solution (subsequently referred to as 'artificial xylem sap', AXS) for 2 h. K_{leaf} was also measured on leaves that were bench-dried for 1 h (Dehydrated) and on leaves that were bench-dried for 1 h and subsequently perfused for 2 h with AXS (RW AXS), AXS + 0.2 mM $HgCl_2$ (RW $HgCl_2$), AXS + 50 mM H_2O_2 (RW H_2O_2) or AXS + 50 µM ABA (RW ABA). Values are means ±SE (n=6). Different letters denote statistically significant differences by one-way ANOVA with Tukey's test.

patterns of expression between the 12 isoforms. One group of genes (among them all *PIP1s*) remained down-regulated while the expression of a second group of genes matched or exceeded the transcript levels measured in control leaves. With the exception of *PtTIP2;1*, all tested *TIP*s were significantly up-regulated after 2 h. Among the *PIP*s, only the expression level of *PtPIP2;3* increased to match the control level.

d. Recovery of K_{leaf} in detached leaves is impaired by inhibitors

Another set of experiments was conducted on leaves that were excised from the plant prior to the dehydration-rehydration treatment. Working with detached leaves allowed us to study the effect of AQP inhibitors and ABA on the recovery of K_{leaf}. Fully hydrated control leaves exhibited a K_{leaf} of 8.49 ±0.57 mmol m^{-2} s^{-1} MPa^{-1} (±SE, n=6), which is higher than the values shown in Figure 1. One difference between the data shown in Figures 3-1 and 3-3 is that all data in Figure 3-1 was derived from leaves that were excised (petioles were cut under water) from transpiring plants immediately before K_{leaf} was measured while the control leaves in Figure 3-3 were perfused with AXS for 2 h prior to measuring K_{leaf}. Hence, the absolute K_{leaf} values shown in Figures 3-1 and 3-3 may not be readily comparable.

Bench-drying of leaves caused a ~10-fold decline in K_{leaf} relative to fully hydrated control leaves (Figure 3-3). Dehydrated leaves that were subsequently xylem-perfused for 2 h with AXS exhibited a significant recovery to 50% of the hydraulic conductance measured in control leaves. The fact that recovery remained

incomplete in detached leaves is consistent with an involvement of phloem transport in embolism repair (Nardini *et al*., 2011; Christman *et al*., 2012).

Application of commonly used inhibitors allowed us to assess the impact of AQPs on K_{leaf} during leaf rehydration. Leaves fed with $HgCl_2$ and H_2O_2 did not exhibit any recovery of hydraulic conductance, indicating that AQPs were involved in the recovery of K_{leaf} after dehydration. A role of AQPs in embolism repair has also been proposed for other species and plant organs (Martre *et al*., 2002; Secchi and Zwieniecki, 2010; Chitarra *et al*., 2014; Mayr *et al*., 2014; Laur and Hacke, 2014).

We also used the dye uptake experiments in an attempt to study how embolism reversal in leaf veins is impacted by mercury. Nearly all veins of well-watered control leaves were stained and functional (Figure 3-4A). In leaves that were bench-dried and subsequently supplied with ASX + safranin for 2 h, many minor veins exhibited incomplete staining (Figure 3-4B). Staining was even less complete in leaves that were bench-dried and subsequently perfused with ASX + safranin + $HgCl_2$ (Figure 3-4C). These findings suggest that embolism formation in minor veins had a substantial impact on the dynamics of K_{leaf}. Studying water transport in rice leaves, Stiller *et al*. (2003) reported that the leaf xylem experienced high embolism levels, even in watered controls. Nardini *et al*. (2003) found that minor veins of *Cercis siliquastrum* leaves underwent extensive embolism at leaf water potentials <-1.5 MPa, indicating that leaf vein embolism was closely related to K_{leaf} changes. Recently, Johnson *et al*. (2012) provided evidence that reductions in K_{leaf} are directly related to vein embolism.

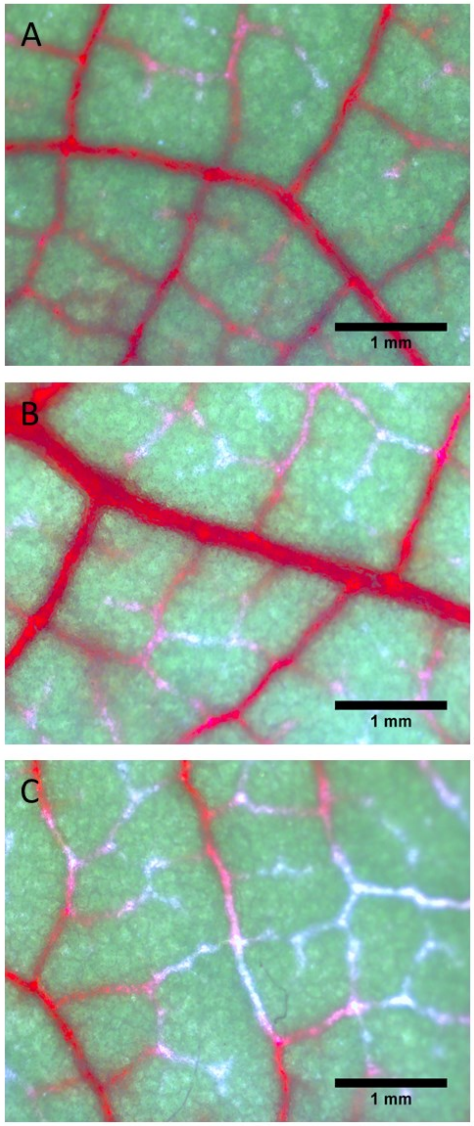

Figure 3-4: Typical images of transpiring *P. trichocarpa* leaves that were allowed to take up safranin solution.

(A) A control leaf was excised from a well-watered plant, and the petiole was immersed for 2 h in safranin solution. Transpiration during dye uptake was promoted by placing the leaf near a fan at ~1,000 μmol m^{-2} s^{-1} photosynthetic active radiation. Most leaf veins were stained indicating minimal xylem embolism. (B) Dye uptake in a bench-dried leaf that was subsequently perfused with safranin solution for 2 h. Minor veins exhibited incomplete staining indicating the presence of embolized xylem conduits in minor veins. (C) Dye uptake of a bench-dried leaf subsequently perfused with safranin + $HgCl_2$ solution for 2 h. Mercury is an aquaporin inhibitor. Staining remained even more incomplete than in (B).

e. *AQP* expression in detached leaves

Aquaporin expression was measured in detached leaves undergoing a dehydration-rehydration cycle (Figure 3-5). Control leaves were perfused with AXS for 2 h before leaf tissue was sampled for the gene expression analysis. As previously seen in intact plants (Figure 3-2), water stress caused down-regulation of all tested *AQP*s (Figure 3-5). This agrees with several previous studies (Alexandersson *et al*., 2005; Laur and Hacke, 2014; Secchi *et al*., 2007).

Notably, very similar degrees of down-regulation were found in bench-dried leaves and in dried leaves that were subsequently xylem-perfused with AXS + ABA (Figure 3-6, r = 0.725, $P < 0.01$). Genes that were strongly down-regulated by dehydration, such as *PtTIP1;6* also exhibited strong down-regulation after perfusion with ABA solution while the expression of other genes, such as *PtPIP2;4*, changed less in response to either of these factors (Figure 3-6). Excluding *PtPIP1;1* from the analysis shown in Figure 3-6 further increased the strength of the linear relationship (r = 0.89, $P < 0.001$).

The lack of recovery in ABA-perfused leaves and down-regulation of *AQP*s in leaves supplied with AXS + ABA is consistent with the model of Shatil-Cohen *et al*. (2011). Working with *Arabidopsis*, these authors also used a 'detached leaf' approach to feed ABA to the xylem via the petiole. Feeding the leaf with ABA decreased K_{leaf} by nearly 50%. In contrast, smearing ABA on the leaf surface, while reducing transpiration, had no effect on K_{leaf}. Shatil-Cohen *et al*. (2011) proposed that the membrane water permeability of bundle sheath cells is controlled by AQPs,

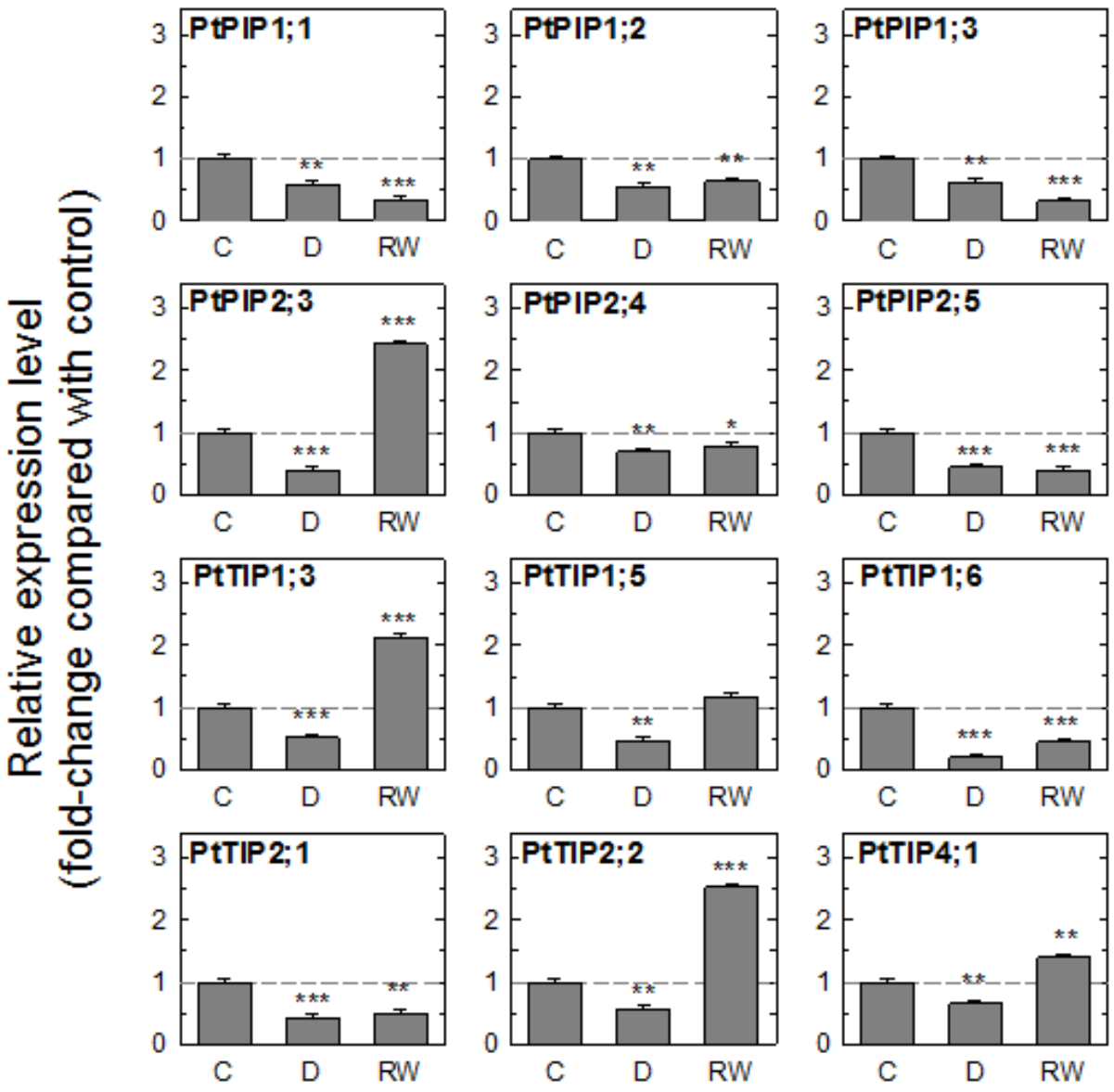

Figure 3-5: Relative expression of aquaporin genes in detached leaves during a dehydration-rehydration experiment.

Data are from control leaves (C) after they were perfused with artificial xylem sap (AXS) for 2 h, leaves that were dehydrated on the bench top for 1 h (D), and leaves that were dehydrated on the bench top for 1 h and then perfused for 2 h with AXS (RW). The geometric mean of the expression levels of four reference genes (*ACT2*, *CYC063*, *TIP41-like*, *UBQ7*) was used to normalize the results. Asterisks denote significant differences in expression level compared to control levels (one-way ANOVA, followed by Bonferroni's post test, **P*≤0.05; ***P*≤0.01****P*≤0.001). Data are means ±SE of three biological replicates.

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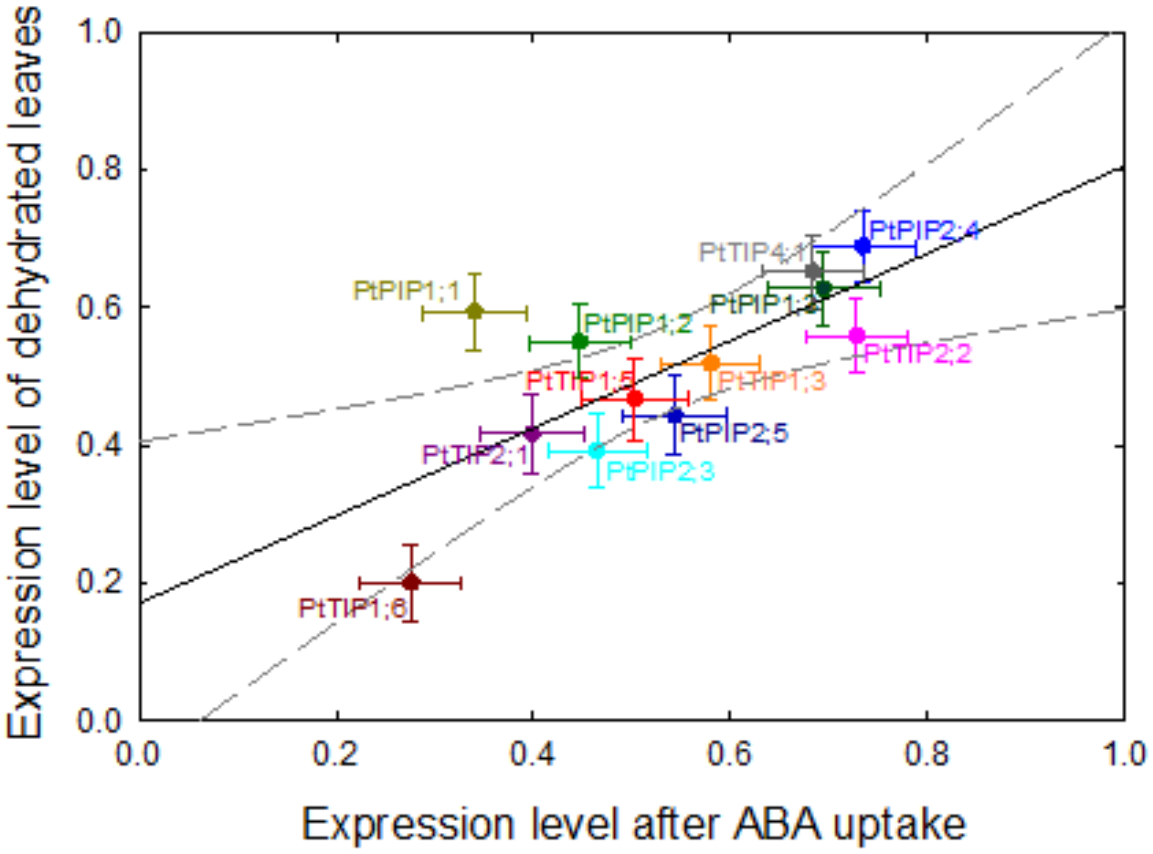

Figure 3-6: Relative expression of aquaporin genes in response to dehydration (y-axis) and dehydration + perfusion with abscisic acid (x-axis).

Detached leaves were either dehydrated on the bench top for 1 h or dehydrated for 1 h and subsequently perfused for 1 h with 50µ M abscisic solution (ABA). Data from fully hydrated detached leaves (perfused for 3 h with 20 mM KCl + 1 mM $CaCl_2$ solution) were used as the control group, and their expression refers to a value of 1. Pearson's r = 0.725; $P \leq 0.01$. Data are means ±SE of three biological replicates

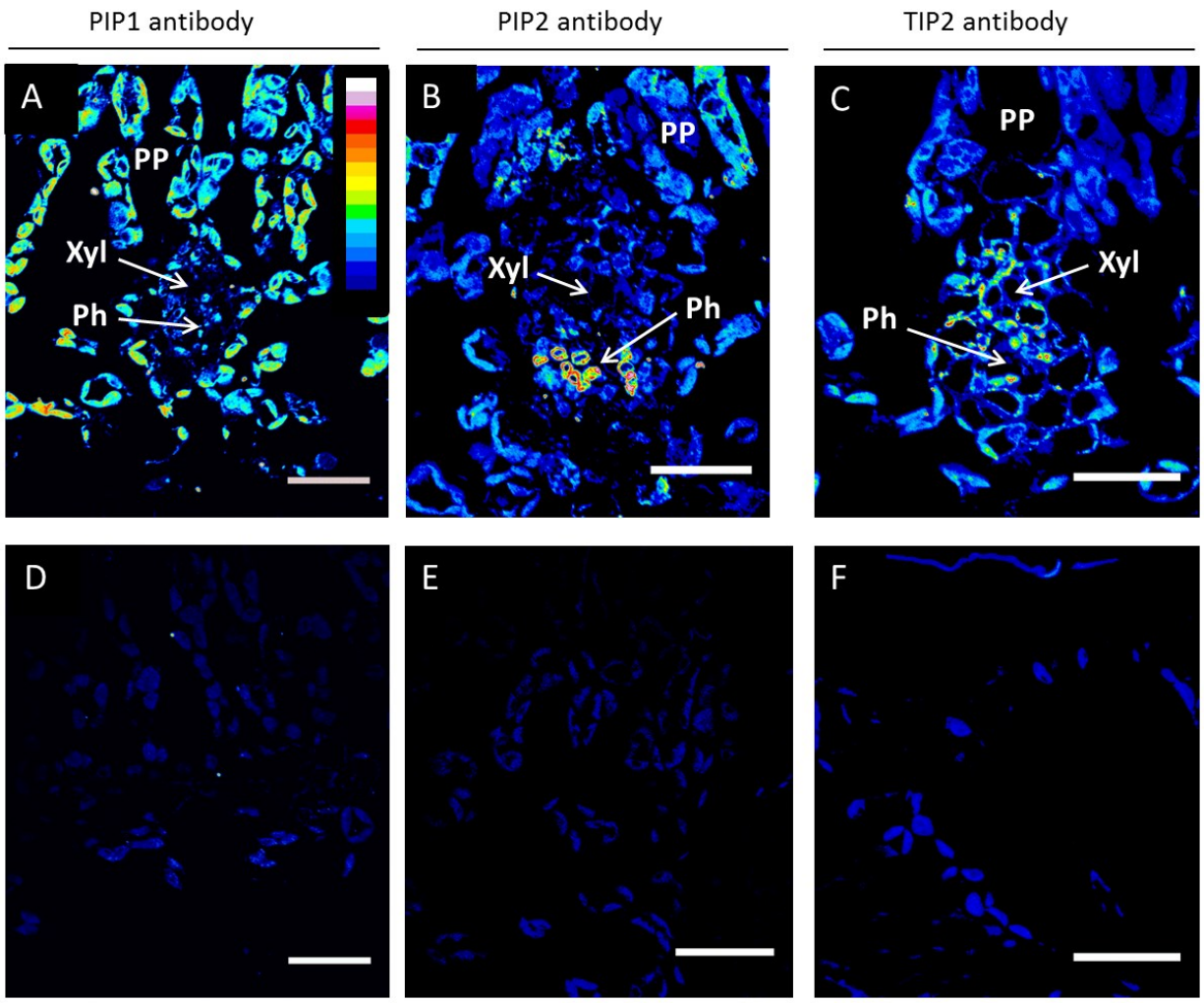

Figure 3-7: Immunolocalization of AQP proteins in leaves of *P. trichocarpa* saplings. Confocal laser scanning micrographs showing the localization of PIP1, PIP2, TIP2 proteins in leaf transverse sections (A, B, C respectively). Controls with no primary antibody indicate minimal background fluorescence (D, E, F respectively). Images were taken at an identical setting and were color-coded with an intensity look-up-table (LUT; displayed in A), in which black was used to encode background, and blue, green, yellow, red and white to encode increasing signal intensities. Ph, phloem; PP, palisade parenchyma; Xyl, xylem. Scale bars = 20 µm.

and that the bundle sheath would act like a control center regulating K_{leaf} in response to signals from the xylem. As the concentration of ABA increases in the xylem, AQP activity in the bundle sheath would be down-regulated, reducing water flow into the leaf mesophyll. Bundle sheath cells, and perhaps xylem parenchyma cells, seem to have a specific responsiveness to ABA, which likely explains the negative effects of this hormone on K_{leaf} (for a recent review see Prado and Maurel, 2013). While our data is consistent with these observations, it is not clear yet which cells may perform the role of a 'control center' in *P. trichocarpa* leaves. While we previously observed prominent PIP1 and PIP2 labeling of the endodermis-like bundle sheath in *Picea glauca* needles (Laur and Hacke, 2014), no such pattern was found in this present study. In rehydrated leaves, four genes showed increased expression levels relative to control leaves. Three of these AQPs (*PtTIP1;3*, *PtTIP2;2*, and *PtTIP4;1*) were TIPs and were also found to be up-regulated when intact plants were rewatered after a drought (compare Figures 3-2 and 3-5). While TIPs have rarely been studied in the context of water flow through tissues and embolism repair, a recent study on grapevine plants found a striking positive correlation between K_{leaf} and the transcript abundance of *VvTIP2;1* (Pou *et al*., 2012). Our immunolocalization experiments indicate that TIP2 protein was present in xylem parenchyma cells (Figure 3-7). This agrees with the expression pattern of *ZmTIP1* in leaves and stems of maize. *In situ* localization revealed that this tonoplast AQP was highly expressed in parenchyma cells surrounding xylem vessels, in phloem companion cells, and between the phloem and the xylem strands (Barrieu *et al*., 1998). Barrieu *et al*. (1998) hypothesized that the high expression of the ZmTIP1

tonoplast AQP in xylem parenchyma cells would allow these cells to control water movement in and out of the xylem vessels. Daniels *et al*. (1996) found that AtTIP2 expression in mature leaves was generally restricted to vascular tissues. In stem xylem of hybrid poplar, a TIP2 AQP was highly expressed in contact cells, suggesting a role in increasing water exchange between vessels and xylem rays (Almeida-Rodriguez and Hacke, 2012).

In this present study, we also determined the cell- and tissue-level localization of PIP1 and PIP2 proteins (Figure 3-7). All sections were taken from leaves of well-watered plants. Strong PIP1 signals were present in the palisade parenchyma (Figure 3-7A). PIP1 antibody was also detected in vein cells, including phloem and xylem parenchyma. This labeling pattern is consistent with a dual role of PIP1s in influencing permeability to water and CO_2 (Secchi and Zwieniecki, 2013). PIP2 was mostly localized in the phloem, which agrees with previous studies (Kirch *et al*., 2000; Yamada and Bohnert, 2000; Vandeleur *et al*., 2009; Almeida-Rodriguez and Hacke, 2012; Laur and Hacke, 2014). Weaker PIP2 labelling was evident in palisade parenchyma cells (Figure 3-7B).

4. Conclusion

We studied how AQPs may be involved in the recovery of water stress-induced declines in K_{leaf}. We examined how K_{leaf} responds to known AQP inhibitors and xylem-fed ABA. We also examined the expression of 12 highly expressed AQP genes

during dehydration-rehydration experiments. Hydraulic measurements and gene expression assays were complemented by dye uptake and immunolocalization experiments. This has revealed that, while *P. trichocarpa* leaves are highly sensitive to dehydration, leaf hydraulic conductance can quickly recover when water becomes available again. Recovery of K_{leaf} was absent when excised leaves were xylem-perfused with AQP inhibitors, suggesting that the recovery of leaf hydraulic function is associated with AQP activity. Among the AQPs tested, several *TIP*s showed large increases in expression in rehydrated leaves, suggesting that TIPs play an important role in reversing drought-induced reductions in K_{leaf}.

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IV. Exploring *Picea glauca* aquaporins in the context of needle water uptake and xylem refilling.

I. Introduction

Water in xylem is usually thought to move unidirectionally from the soil to the leaves. However, a growing body of evidence indicates that many plants take up water from leaf and/or bark surfaces, and that this can result in reverse water flow in stem xylem (Burgess and Dawson, 2004).

The uptake of intercepted water on leaf surfaces into leaves (foliar uptake) has been demonstrated in plants from a range of dew and cloud-affected plant communities, including the redwood forest (Burgess and Dawson, 2004; Limm *et al*., 2009), a mountain pine forest in Tenerife, Spain (Nadezhdina *et al*., 2010), and tropical cloud forests (Eller *et al*., 2013; Goldsmith *et al*., 2013). Dewfall absorption by aerial plant parts has also been reported for the desiccation-tolerant plant *Vellozia flavicans* in the savannas of Brazil (Oliveira *et al*., 2005).

Many reports of foliar uptake come from studies on conifers. Sparks *et al.* (2001) observed increases in stem water content of *Pinus contorta* during the winter, and offered direct water uptake by stems or foliage as a likely explanation. Water may have originated from melting snow (Sparks *et al*., 2001). Foliar absorption of intercepted rainfall was observed in *Juniperus monosperma*, a widely distributed dryland species (Breshears *et al*., 2008). The conclusion that foliar uptake occurred in this species was based on changes in leaf water potential in response to foliar wetting and the use of isotopically labeled water. Moreover, the response to foliar uptake increased with increasing amounts of plant water stress.

Breshears *et al*. (2008) suggested that foliar absorption in *Juniperus monosperma* could play an important role in mitigating water stress and in aiding survival during drought.

Another role for water absorption through the leaves may be to facilitate embolism repair in the xylem of conifers (McCulloh *et al*., 2011; Mayr *et al*., 2014) and other plants (Oliveira *et al*., 2005). If water could be absorbed by leaves, the xylem pressure at the top of tall trees could rise above the pressure predicted on the basis of the height of a tree (McCulloh *et al*., 2011). Apart from mitigating water stress and potentially facilitating embolism reversal, the reduction in leaf water deficit can also result in improved photosynthesis, stomatal conductance, and growth (Boucher *et al*., 1995; Simonin *et al*., 2009; Eller *et al*., 2013). On the basis of all these findings, it appears that foliar uptake is a relatively widespread and potentially important phenomenon, and that it must be considered in ecophysiological and hydrological models (Breshears *et al.*, 2008; Goldsmith, 2013).

Foliar water uptake may occur when water has coalesced on the leaf surface and the leaf is experiencing a water deficit, that is, when leaves have a more negative water potential than the surrounding atmospheric boundary layer (Goldsmith, 2013). Although more work is required to better understand the anatomical pathways for water entry into the leaf, the available evidence suggests that water is taken up via the cuticle and other leaf structures (Burkhardt *et al*., 2012; Eller *et al*., 2013; North *et al*., 2013). In leafy twigs of *Picea abies*, water was taken up through the bark (Katz

et al., 1989). Fluorescent dye movement suggested that water migrated along the rays and parenchyma cells of the bark and the wood.

In the leaf, water can move through the apoplast or from cell to cell. Where lignified or suberized cell walls are present in the bundle sheath, water has to cross cell membranes. Water movement through cell membranes is facilitated and regulated by aquaporins (AQPs). These channel proteins transport water and other molecules and are found in almost all living organisms. According to Heinen *et al*. (2009), there are three ways by which water exchange across cell membranes is regulated by AQPs: (1) their expression level; (2) their trafficking; and (3) their gating, that is, the opening or closing of channels. Expression is one of the most important methods of AQP regulation, and the study of their expression level and localization is highly relevant to a better understanding of their physiological role (Heinen *et al*., 2009).

Plant AQPs form a large family of water channel proteins, with 28 members in *Vitis vinifera* (Fouquet *et al*., 2008), >30 members in *Arabidopsis* and *Oryza sativa*, and >50 members in *Populus trichocarpa* (Maurel *et al*., 2008; Gupta and Sankararamakrishnan, 2009). Plasma membrane intrinsic proteins (PIPs; with two phylogenic subgroups, PIP1 and PIP2) and tonoplast intrinsic proteins (TIPs) are the most abundant AQPs in the plasma membrane and vacuolar membrane, respectively (Maurel *et al*., 2008; Gomes *et al*., 2009). PIPs are thought to represent a major path for cell-to-cell water transport. Their contribution in the cell-to-cell component of root water uptake has been described extensively (Vandeleur *et al*., 2009; Sakurai-Ishikawa *et al*., 2011; Laur and Hacke, 2013). Other AQP

subfamilies include nodulin-26-like intrinsic membrane proteins (NIPs) and small basic intrinsic proteins (SIPs) (Maurel *et al.,* 2008; Gomes *et al.*, 2009).

The role of AQPs in foliar water uptake has been studied in an epiphytic bromeliad (Ohrui *et al*., 2007), but little is known about the role of leaf AQPs in the context of foliar uptake in other plant groups. Mayr *et al*. (2014) reported that conifers at the timberline repaired winter embolism in early spring, at a time when the soil was still frozen. Experimental evidence indicated that water (from melting snow) was taken up through needles and/or bark of stems, and that PIPs were present in the needle endodermis during the refilling period in later winter/early spring.

Here, we studied needle water uptake and the role of AQPs in this process under controlled conditions in clonal *Picea glauca* plants. Plants experienced a moderate drought, and were subsequently exposed to high atmospheric humidity without watering the soil. Physiological, anatomical, and molecular parameters were monitored during the experiment. We were particularly interested in linking foliar water uptake with embolism repair. The following hypotheses were tested: (1) needles are able to take up water; (2) AQPs in needles are involved in this process; and (3) foliar uptake can play a role in embolism repair.

An important objective related to our second hypothesis was to obtain a better understanding of the tissue-level localization of leaf AQPs, both during drought treatment and after plants had been transferred to a high-humidity environment. The endodermis-like bundle sheath of Pinaceae is positioned

between vascular and photosynthetic tissue, and often contains Casparian strips (Liesche *et al*., 2011). Analogous to the situation in many roots, the endodermis in conifer needles may therefore play an important role in modifying radial water flow. Consistent with this idea and based on the findings of Mayr *et al.* (2014), we expected to find a high amount of AQP protein in the endodermis, particularly after plants experienced conditions conducive to foliar water uptake.

Although much has been learned about AQP expression and function in a variety of model plants, very little is known about AQPs in conifers, including spruce. To our knowledge, the AQP family in spruce has not been characterized, although the expression pattern of a few aquaporin homologues has been investigated in the seedlings, mature roots and needles of *Picea abies* (Oliviusson *et al*., 2001; Hakman and Oliviusson, 2002). Therefore, a first step in this study was to comprehensively analyze expressed members of the spruce AQP family in order to identify candidate genes involved in foliar uptake of water.

II. Materials and Methods

a. Plant material and growing conditions

Three-year-old white spruce plants (*Picea glauca* (Moench) Voss, clone EPB-3858) were obtained from the Saint-Modeste Nursery, (Quebec, Canada). Plants were established for 2 months in 3.8-l containers with Sunshine Mix #4 (Sun Gro Horticulture Canada Ltd., Seba Beach, Ab, Canada) under the following conditions:

16h:8 h day:night cycle, 24°C :20°C day:night temperature, c. 50% daytime relative humidity (RH) and photosynthetically active radiation of 350 μmol m^{-2} s^{-1} at plant level. Plants were watered twice a week and fertilized on a weekly basis with 200 ml of 20:20:20 N:P:K fertilizer applied at 0.5 g l^{-1}. One group of plants was well watered (control group); another group of plants was subjected to a drought stress treatment, where water was withheld until the stem water potential (Ψ_{Stem}) was near -3 MPa. This target water potential was associated with *c.* 20% loss of hydraulic conductivity according to the vulnerability curve (for details, see later). To study the ability of shoots to absorb water and to repair xylem embolism after drought treatment, a subset of drought-stressed plants was placed in a humidified box (*c.* 100% RH; high-humidity plants). Pots were completely sealed with plastic bags, using tape and parafilm, to prevent water from reaching the soil. The volumetric soil water content was measured using an EC-5 sensor (Decagon Devices, Pullman, WA, USA). The measurements described below were carried out 2 h, 26 h, and 50 h after plants had been placed in the high-humidity box (exposure to high humidity started at 09:00 h). Another subset of drought-stressed plants was rewatered; these plants were not transferred to the high-humidity environment.

b. Relative water content (RWC)

To evaluate the effects of foliar water absorption on the water relations of needles and twigs, we determined the RWC of needles (five needles per plant) and twigs (one twig per plant) from 6 individual plants. The RWC was calculated as (fresh

weight – dry weight)/(turgid weight – dry weight). To determine turgid weight, needles and twigs were floated on distilled water for 48 h. Dry weight was determined after drying samples at 70°C for 48 h. RWC measurements were made before (control) and after (dehydrated) overnight drying on the bench top. Bench-dried samples were then transferred to a high-humidity environment (*c.* 100% RH[1]) for 16 h (high RH). To prevent water uptake through the part of the needle base, this surface was covered with mineral oil. To assess the role of needles in shoot water uptake, we also measured the RWC of the leaf-less basal part of twigs in control, dehydrated, High-RH conditions. The cut ends of these 3-5-cm-long basal twig segments were covered with parafilm before exposure to high RH.

c. Needle anatomy

An effort was made to study the anatomy and chemical composition of needle tissue as well as possible hydrophilic pathways in needles. Alcian blue (0.5% w/v) was used to stain mucilage, which generally has a high water-binding capacity because of the high concentration of hydroxyl groups (Clifford *et al*., 2002). Hand-cut needle cross-sections were observed using a light microscope (DM3000, Leica, Wetzlar, Germany) and a digital camera (DFC420C, Leica). Fresh tissue was fixed in FAA (10% formaldehyde, 5% acetic acid, 50% ethanol) under vacuum for 1 h, stored in FAA for 16 h at 4°C and embedded in paraffin as described previously (Almeida-Rodriguez *et al*., 2011). The periodic acid-Schiff reaction was also used to identify hydrophilic polysaccharide compounds, such as mucilage, glycolipids, and

[1] 100% humidity was achieved by placing humidifiers in a hermetic cabinet. RH was periodically monitored.

glycoproteins (Eller *et al*., 2013). For detection of lignin, needle cross-sections were stained with 1% (w/v) phloroglucinol in 35% (v/v) HCl. Photographs were taken within 30 min of phloroglucinol-HCl staining.

d. Water potential and stomatal conductance

Water potential was measured after shoots had been sealed in aluminum foil and plastic bags the day before harvesting to ensure water potential equilibration (Begg and Turner, 1970). Stem water potential (Ψ_{Stem}) was measured using a pressure chamber (Model 1000; PMS Instruments, Albany, OR, USA). Stomatal conductance was measured with a steady state porometer (LI-1600, Li-Cor, Lincoln, NE, USA) on at least five plants per group, and normalized by needle surface area (Sigma Scan 5.0, Jandel Scientific, San Rafael, CA, USA).

e. Hydraulic measurements

The percentage loss of hydraulic conductivity (PLC) was measured using a conductivity apparatus (Sperry *et al*., 1988) as described previously (Plavcova and Hacke, 2012). Segments corresponding to the previous year of growth (2012) were used for hydraulic measurements. Segments were gradually trimmed under water to a final length of 14.2 cm. A vulnerability curve was generated using the centrifuge method, as described previously (Schoonmaker *et al*., 2010). Curves were fitted with a Weibull function

f. Analysis of spruce aquaporin sequences

Sequence information from the *Picea glauca* EST database of the NCBI (http://www.ncbi.nlm.nih.gov/) was used for BLASTn, and tBLASTn homology searches (Altschul *et a*l., 1997). The sequences of *Arabidopsis thaliana* (Johanson *et al*., 2001), *Zea mays* (Chaumont *et al*., 2001) and *Physcomitrella patens* (Danielson and Johanson, 2008) were used as queries. Bioinformatics analyses were conducted using the Mobyle web platform (Néron *et al.*, 2009). EST sequence assembly was performed with CAP3 (Huang and Madan, 1999). Concordance of this de novo assembly with previously published *P. glauca* gene catalog (Rigault *et al*., 2011) was assessed manually.

The recent publication of the Picea sp. draft genome (Birol *et al*., 2013; Nystedt *et al*., 2013) allowed us to assess intron positions; when discovered in the *P. abies* 1.0 database, complete coding sequences were included for further analysis (See supplementary Table 4-S2, Fig. 4-S2 & 4-S3). All accession numbers are given in Table 2. Alignment of deduced amino acid sequences (sixpack EMBOSS module; Rice *et al*.) was generated and edited with Clustal Omega 1.1.0 (Sievers *et al*., 2011). The quality of the alignment was assessed by its norMD score (Thompson *et al.*, 2001) (see Supplementary Fig. 4-S1). Phylogenetic analyses were performed using a bootstrapping procedure. The resulting trees including 30 complete aquaporin sequences were displayed using the Figtree program (http://tree.bio.ed.ac.uk/software/figtree) (see Fig. 4-3; Supplementary Fig. 4-S2 and 4-S3). Trans-membrane regions were detected using TopPred II 0.01 (Claros

and von Heijne, 1994). Aromatic/arginine (ar/R) selectivity filters were identified by manual inspection. Subcellular localizations were predicted using Plant-mPLoc (Chou and Shen, 2010) and WoLF PSORT (Horton *et al.*, 2007). The expression profile of each AQP gene was estimated by tallying the tissue distribution of clustering ESTs in non-normalized libraries (Alba *et al*., 2004) and using IDEG6 (Romualdi *et al*., 2003).

g. Gene transcript measurements by quantitative real time PCR

Needles were collected, immediately frozen in liquid nitrogen and stored at -80°C until analyzed. Samples were always collected at the same time of day to minimize any diurnal effect on AQP expression. Total RNA was extracted from needles of 3-4 plants per treatment following the CTAB method of Chang *et al.* (1993) modified by Pavy *et al*. (2008). RNA quality was assessed on an agarose gel and quantified with a spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Wilmington, DE, USA). One microgram of total RNA was treated with Deoxyribonuclease I (Invitrogen, Carlsbad, CA, USA) and used as template for first-strand cDNA synthesis with SuperScript II (Invitrogen) following the manufacturer's instructions. cDNA quality was checked by PCR with intron-spanning actin primers. Putative needle-expressed PIP genes were selected (Table 4-2). Specific primers (Supplementary Table 4-S1a) were designed according to Rutledge and Stewart (2010) using the QuantPrime online tool (Arvidsson *et al*., 2008). PCR efficiency (E) was determined from a five-

point cDNA serial dilution, according to: E=10[-1/slope]. All selected primer pairs showed correlation coefficients of $R^2 > 0.98$ and primer efficiency values ranging between 1.97 and 2.07. Real-time qPCR was performed on a 7900 HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) as described previously (Laur and Hacke, 2013).

h. Gene transcript localization by *in situ* hybridization (ISH)

ISH was performed as described previously (Karlgren *et al*., 2009), with the following adjustments: Protease K digestion was shortened to 10 min at room temperature (1ng/ml), and a carbethoxylation reaction (0.1% DEPC in PBS, 15 min) was included during pre-hybridization (Braissant and Wahli, 1998). Primers were designed (see Supplementary Table 4-S1b) using the QuantPrime online tool. PCR amplicons were ligated (pCRII vector TOPO cloning kit; Invitrogen, Carlsbad, CA, USA) and sequenced to determine orientation. Riboprobes were generated by in vitro transcription and labeled with digoxigenin using Sp6 and T7 RNA polymerase with the DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN, USA) after 5'-overhang linearization of the plasmid with, respectively, EcoR V and BamH I restriction enzymes (Invitrogen). To ensure high specificity and to avoid cross-hybridization between gene family members, the hydrolysis step was not performed as probes were approximately 300bp long. Slides were mounted with a synthetic resin (Permount, Fisher Scientific, Ottawa, Canada). Images were taken using a light microscope as described above.

i. Immunolocalization

Samples were fixed in FAA medium (10% formaldehyde, 5% acetic acid, 50% ethanol) under vacuum for 1 h and stored in FAA for 16 h at 4°C. Next, samples were embedded, sectioned, dewaxed, and rehydrated as described before (Almeida-Rodriguez *et al.,* 2011). Before the first immunoreaction, cross sections were incubated for 45 min with blocking solution (BS; 1.5% glycine, 5% (w/v) bovine serum albumin, 0.1% Tween-20 in PBS) following the protocol of Gong *et al.* (2006). Primary antibodies against the 42 N-terminal amino acids of *AtPIP1;3* (Kammerloher *et al*., 1994) and the conserved 10 amino acids of the C-terminal of PIP2 aquaporins (similar to Daniels *et al.*, 1994) were included (see alignment in Supplementary Figure S4). Secondary antibodies were pre-absorbed with plant tissue extract. DyLight 549-conjugated rabbit anti-chicken secondary antibody (Fisher Scientific, Hampton, NH, USA) and HiLyte Fluor 555-conjugated rabbit anti-mouse secondary antibody (AnaSpec Inc., Fremont, CA, USA) were respectively applied for 2 h at 37°C. Slides were mounted with Permount. Images were taken with a Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

j. Statistical analysis

All statistical analyses were carried out using SigmaPlot 11.0 (Systat, Point Richmond, CA, USA). Differences due to the effect of treatments and growing conditions were analyzed using a one-way ANOVA followed by a Tukey's test. For all

	cDNA clone accession	Amino acids	Number of clones	TMHs	†Tissue specificity	Subcellar location	NPA motifs	Ar/R filters
PgPIP1;1	GQ03401_M18	292	52	6	R N S C	PM	NPA/NPA	F H T R
PgPIP1;2	GQ03610_A06	288	79	6	R N S C	PM	NPA/NPA	F H T R
PgPIP1;3	GQ02828_J14	285	31	6	S N	PM	NPA/NPA	F H T R
PgPIP2;1	GQ03111_E12	282	26	6	R N S C	PM	NPA/NPA	F H T R
PgPIP2;2	GQ02901_B20	282	71	6	R N S C	PM	NPA/NPA	F H T R
PgPIP2;3	GQ03703_H07	282	3	6	R S	PM	NPA/NPA	F H T R
PgPIP2;4	GQ0132_J09	282	5	6	R	PM	NPA/NPA	F H T R
PgPIP2;5	GQ03124_N20	269	5	6	C	PM	NPA/NPA	F H T R
PgPIP2;6	GQ03705_D15	284	49	6	R N S	PM	NPA/NPA	F H T R
PgPIP2;7	GQ02905_E13	282	77	6	R S C	PM	NPA/NPA	F H T R
PgPIP2;8	GQ02902_L14	280	16	6	R N S	PM	NPA/NPA	F H T R
PgPIP2;9	GQ03002_G07	280	6	6	S	PM	NPA/NPA	F H T R
PgPIP2;10	GQ03011_G23	275	15	6	R S	T-PM	NPA/NPA	F Y T R
PgPIP2;11	GQ03010_E09	275	10	6	S	T-PM	NPA/NPA	F Y T R
PgPIP2;12	GQ03001_P18	283	57	6	R S	T-PM	NPA/NPA	F H T R
PgPIP2;13	GQ03216_M18	272	22	6	S	PM	NPA/NPA	F H T R
PgTIP1;1	GQ0197_E19	253	5	6	R	T	NPA/NPA	H I A R
PgTIP1;2	GQ03116_D08	253	12	6	R	C-T	NPA/NPA	H I A R
PgTIP1;3	GQ02908_P24	253	11	6	S	C-T	NPA/NPA	H I A R
PgTIP1;4	GQ03501_N03	255	18	6	R N S	C-T	NPA/NPA	H I A R
PgTIP1;5	GQ0206_N10	253	56	6	R S	T	NPA/NPA	H I A R
PgTIP2;1	GQ03915_M04	250	199	6	R N S C	PM-T	NPA/NPA	H I G R
PgTIP2;2	WS0323_F18	211	3	5	S	T	NPA/NPA	H I G R
PgTIP4;1	GQ0201_M19	248	9	6-7	R S	T	NPA/NPA	H I A R
PgTIP4;2	GQ04012_G01	250	1	6-7	S	T	NPA/NPA	H I A R
	WS02617_N14	115	1	3		T	NPA/NPA	- - A R
PgNIP1;1	GQ03122_A02	280	12	6	S	PM	NPA/NPA	WV AR
	GQ03202_N13	195	2	4	S	PM	NPA/NPA	- V A R
PgNIP2;1	GQ03207_J07	296	6	6	N S	PM	NPA/NPA	A I G R
	GQ03237_P23	42	1	0	S	na	/	- - - -
PgNIP3;1	GQ03810_B10	294	13	6	S	T-PM	NPA/NPA	A I A R
PgNIP3;2	GQ03701_J12	215	1	6	S	PM	NPA/NPA	A I G R
PgSIP1;1	GQ03414_P10	238	29	6	R N S C	T	NPT/NPA	L T P N
	GQ04011_K04	138	4	2	S R	PM-T	NPV/NPA	- K P T

Table 4-1: Features of spruce (*Picea glauca*) major intrinsic proteins (MIPs) cDNA. Gene names; accession number; length of deduced polypeptides; number of cDNA clones included in the assembly; predicted number of trans-membrane helix domains (TMHs); tissue specificity of ESTs; predicted sub-cellular location (C, cytoplasm; PM, plasma membrane; T, tonoplast; na, not available) and and conserved residues (NPA motifs, Ar/R filters) are summarized. † Tissues used for cDNA library preparation are listed: C, reproductive parts; N, needles; R, roots; S, stems.

tests, differences were considered significant at $P < 0.05$.

3. Results

a. Needle water uptake and anatomy

We first asked whether foliar uptake occurred in *P. glauca*, and whether it had a significant impact on needle water status. The RWC of needles of well-watered control plants was 94.5%; bench-dried needles had a RWC of 65.5% (Table 4-1). After needles were exposed to high humidity for 16 h, their RWC recovered to an intermediate level, indicating that water uptake occurred.

RWC measurements were also performed on twigs. After bench drying, the RWC of twigs dropped significantly, but recovered to control levels after twigs had been exposed to high humidity (Table 4-1). By contrast, a significant recovery of RWC did not occur when needles were detached from twigs after bench drying, indicating that water uptake was facilitated by needles.

To study potential anatomical pathways for water uptake, needle sections were prepared for light microscopy and stained. Alcian blue staining indicated that stomata were associated with mucilages (Fig. 4-1A), which generally comprise a mixture of polysaccharides. A high concentration of hydrophilic carbohydrates was detected in the epidermis, hypodermis and other cell types (Fig. 4-1B), which may have facilitated water retention within the tissues. Phloroglucinol-HCl staining revealed the presence of lignified cell walls in bundle sheath cells (especially in

Experimental treatment	Needle RWC[1] (%)	Twig RWC[2] (%)
control	94.49 (2.60) [A]	81.31 (1.19) [A]
dehydrated	65.47 (1.44) [B]	73.40 (0.96) [B]
high RH	78.39 (2.23) [C]	82.27 (2.47) [A]
high RH, detached needles	n.a.	77.28 (1.60) [AB]

Table 4-2 Relative water content (RWC) of white spruce (*Picea glauca*) needles.

[1]RWC of needles was measured before (control) and after (dehydrated) overnight drying on the bench top. Bench-dried needles were then transferred to a high-humidity environment (c. 100% RH) for 16 h (high RH). na, not applicable.

[2]RWC of twigs subjected to the same experimental treatment as needles. To assess the importance of foliage on the water absorption of twigs, basal leaf-less segments of dried twigs were exposed to high RH for 16 h (high RH, no needles). The standard error of the mean is given in parentheses. Different letters indicate significant differences between treatments (n = 6; P≤ 0.05).

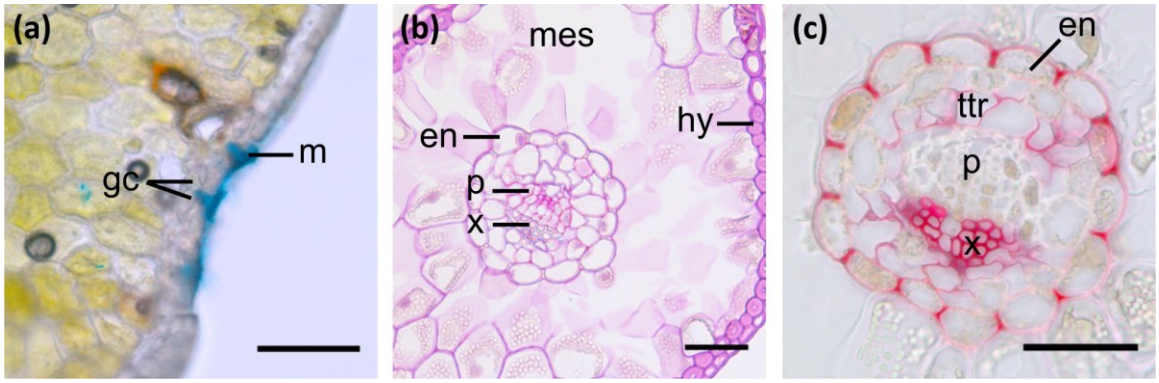

Figure 4-1 Light microscopy images of *Picea glauca* needle cross-sections.

(a) Section showing a stoma (gc, guard cells) covered by mucilage (m). The section was stained with Alcian blue. (b) A cross-section in which polysaccharides were stained with periodic acid-Schiff reagent. A high polysaccharide content (stained pink) was detected in the cell walls of the epidermis, hypodermis (hy), endodermis (en) and phloem (p) cells. mes, mesophyll; x, xylem. (c) Cross- section stained with phloroglucinol–HCl; lignified cell walls are shown in red. Lignin was detected in radial cell walls of the endodermis, in transfusion tracheids (ttr) and in xylem tracheids. Bars, 20 µm.

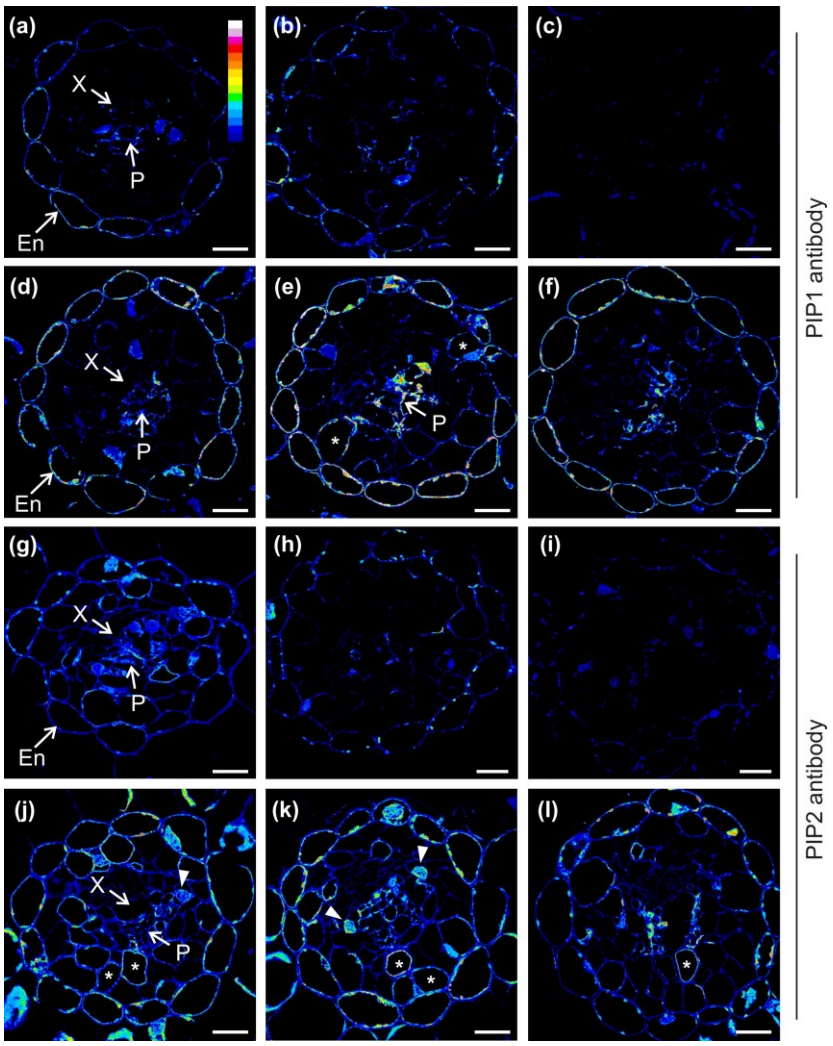

Figure 4-2: Confocal laser scanning micrographs showing the localization of aquaporin proteins in *Picea glauca* needle cross- sections.

Images were taken at an identical setting and were color-coded with an intensity look-up-table (LUT; displayed in a), in which black was used to encode background, and blue, green, yellow, red and white to encode increasing signal intensities. (a–f) PIP1 localization in needles; (g–l) PIP2 localization in needles. Cross- sections of well-watered (a, g) and drought- stressed (b, h) plants. (c, i) Controls with no primary antibody indicate minimal background fluorescence. Sections of previously drought-stressed plants were taken 2 h (d, j), 8 h (e, k) and 26 h (f, l) after plants had been transferred to a high- humidity environment. PIP1 labeling was strongest in the endodermis (En) and in phloem (P). Strong PIP2 signals were detected in the phloem (putative Strasburger cells labeled by arrowheads in j and k) and in transfusion parenchyma (asterisks in j–l). No signal was detected in the xylem (X). Bars, 20 μm.

radial cell walls), transfusion tracheids, and xylem tracheids (Fig. 4-1C).

b. Distribution of PIP1 and PIP2 aquaporins in needle cross sections

To test the hypothesis that AQPs in needles are involved in foliar uptake, we first examined the detailed localization of PIP1 and PIP2 protein using confocal fluorescence microscopy (Fig. 4-2). In well-watered plants, PIP1s were present in the endodermis and in phloem (Fig. 4-2A). Needle cross sections of drought-stressed plants exhibited minimal labeling (Fig. 4-2B). Controls with no primary antibody showed a very weak or no background signal (Fig. 4-2C).

Needle sections taken as soon as 2 h after the increase in relative humidity exhibited strong immunolabeling of the endodermis (Fig. 4-2D). The labeling intensity for PIP1 protein in endodermis, phloem, and transfusion parenchyma cells peaked after 8 h of exposure to high humidity (Fig. 4-2E). After 26 h at high humidity, PIP1 labeling was still evident in the endodermis, but the intensity of the signal in phloem and transfusion parenchyma was reduced (Fig. 4-2F).

A similar trend was observed for PIP2 (Fig. 4-2G-L), although the distribution of PIP1 and PIP2 proteins showed some interesting differences. Under conditions that would be conducive to foliar water uptake (i.e., exposure to high humidity after a drought treatment), PIP1 labeling was more focused in the endodermis than PIP2 labeling suggesting that PIP1s are involved in regulating water movement across the bundle sheath.

PIP2 proteins appeared to be more widely distributed within the central cylinder than PIP1s. While some PIP2 labeling was detected in the endodermis, strong signals were also apparent in the plasma membrane of transfusion parenchyma cells (asterisks in Fig. 4-2J-L) and in the phloem, including in cells that appeared to be Strasburger cells (arrowheads in Fig. 4-2J, K). Labeling also occurred in the mesophyll. PIP2s may therefore facilitate water transport between most, if not all living cells in the central cylinder and mesophyll.

c. Spruce aquaporin family

As a first step in investigating the expression and function of individual AQP genes in spruce, we identified expressed members of the spruce AQP gene family. Information including gene names, accession numbers, length of the deduced polypeptides, and predicted subcellular location is given in Table 4-2. A total of 1,188 ESTs corresponding to putative Major Intrinsic Proteins (MIP) was identified in the NCBI database (http://www.ncbi.nlm.nih.gov/). Based upon sequence overlap, a non-redundant set of 34 contigs was retrieved from the EST assembly (Table 4-2). The 30 putative complete MIP sequences could be grouped into PIP, TIP, NIP, and SIP subfamilies (Fig. 4-3; Supplementary Figure 4-S1).

We also took advantage of the recent sequencing of the *Picea* sp. genome to complete our investigation. Searches of the *Picea abies* 1.0 draft genome at ConGenIE, using the complete set of retrieved *P. glauca* AQPs as well as PpXIPs, PtXIPs, *PpGIP1;1* and *PpHIP1;1* protein sequences, resulted in the identification of

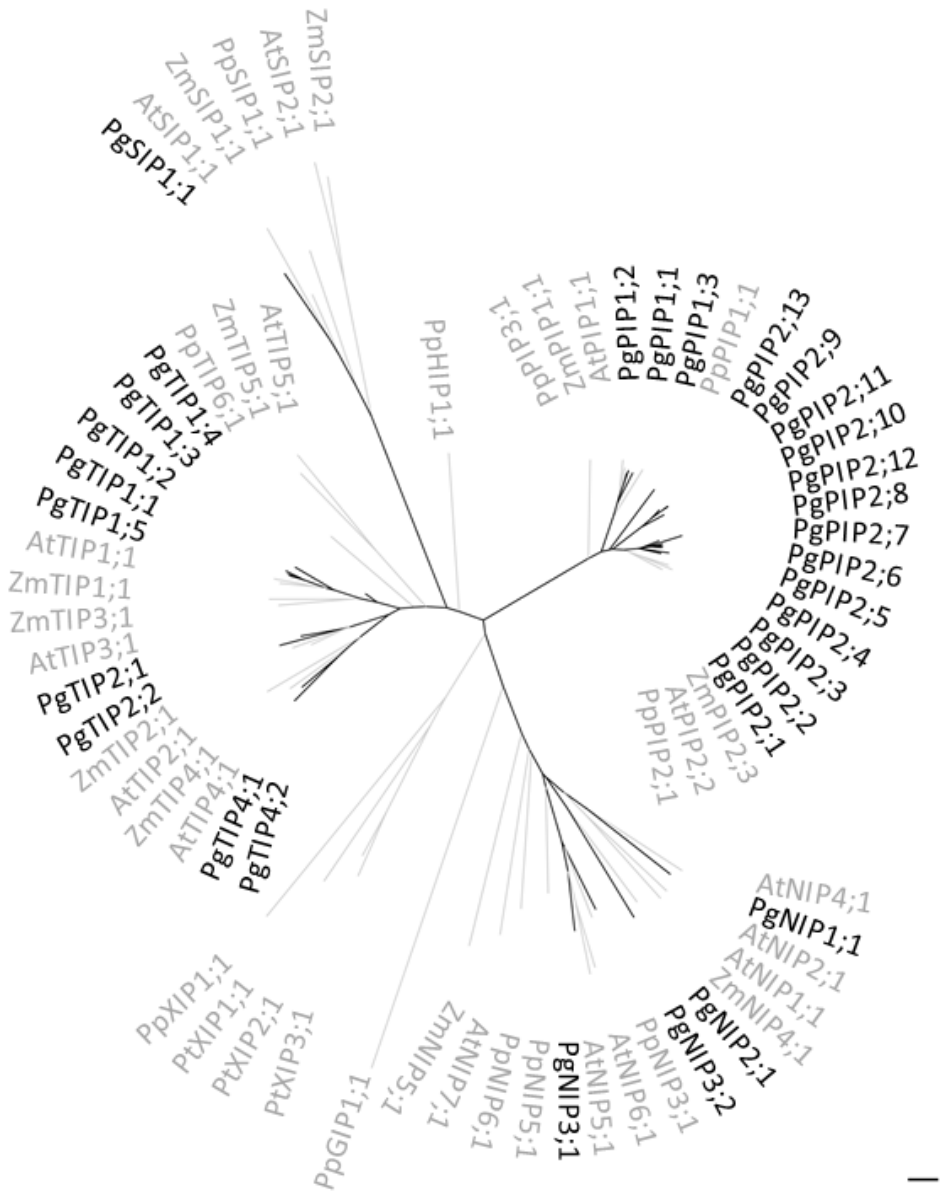

Figure 4-3: Phylogenetic analysis of 30 aquaporins (AQPs) expressed in *Picea glauca*.
The phylogeny was inferred using maximum likelihood. Picea glauca AQPs (PgAQPs) are shown in black type; AQPs from Zea mays (ZmAQPs), Arabidopsis thaliana (AtAQPs) and Physcomitrella patens (PpAQPs) are represented by gray type. In P. glauca, four subfamilies can be identified (plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-26-like intrinsic membrane proteins (NIPs) and small basic intrinsic proteins (SIPs)). Also note the close relationship between PIP subfamily members. The bar indicates the mean distance of 0.1 changes per amino acid residue.

30 complete homolog sequences for *Picea abies* (for the phylogenetic relations of all *Picea* AQP members, see Fig. 4-3, Supplementary Figure 4-S2 & 4-S3). Sequences corresponding to the XIP, GIP, and HIP subfamilies were not retrieved either in *P. abies* or *P. glauca* genomic databases. Compilation of this data allowed us to systematically name PgAQPs (Table 4-2; Fig. 4-3; Supplementary Fig. 4-S1, 4-S2 & 4-S3). In total, 16 PgPIPs, nine PgTIPs, four PgNIPs and one PgSIP full-length sequences were identified from transcriptomic data. PIP subfamily members were further divided into two subgroups with three PIP1s and 13 PIP2s. All PIP genes shared common sequence features (NPA boxes, ar/R residues), except *PIP2;10* and *PIP2;11* where His (H) was substituted by Tyr (Y), indicating a possible difference in substrate specificity. All of the PIP protein sequences were predicted to localize to the plasma membrane (Table 4-2).

d. Expression of selected aquaporin genes in needles

The tissue specificity for each of the P. glauca AQPs was studied using the EST database. The available 28 EST libraries are an unbiased representation of the tissue-specific transcriptome. Two PIP1 (*PgPIP1;1*, *PgPIP1;2*) and two PIP2 (*PgPIP2;1*, PgPIP2;2) candidate genes that have been reported to be expressed in needles were selected for analysis.

All of the four genes showed significant changes in expression during the treatments (Fig. 4-4). Of the genes studied here, *PgPIP1;1* and *PgPIP2;2* ranked first in terms of their proportion to the total number of mRNA molecules (Fig. 4-4A, dark

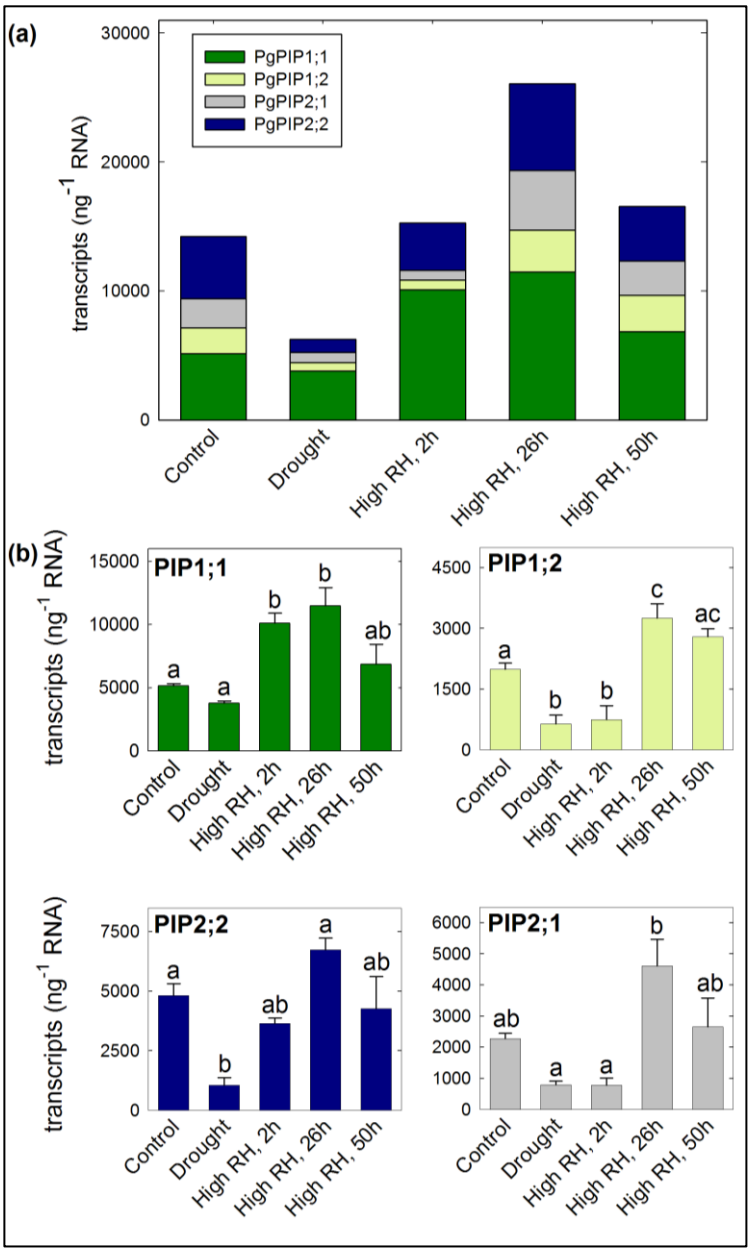

Figure 4-4: Aquaporin transcript amounts in needles of well-watered (Control) and drought-stressed (Drought) white spruce (Picea glauca) plants.

Transcript amounts were also measured 2, 26 and 50 h after drought- stressed plants had been transferred to a high-humidity environment. (a) Cumulative aquaporin transcript amounts in needles. Individual genes are labeled with different colors. Among the different transcripts, PgPIP1;1 ranked first in terms of its proportion to the total number of mRNA molecules. (b) Transcript abundance of PgPIP1;1, PgPIP1;2, PgPIP2;1 and PgPIP2;2. Values are means?SE from three biological samples which were tested in triplicate. Significant differences are indicated by unique letters ($P \leq 0.05$).

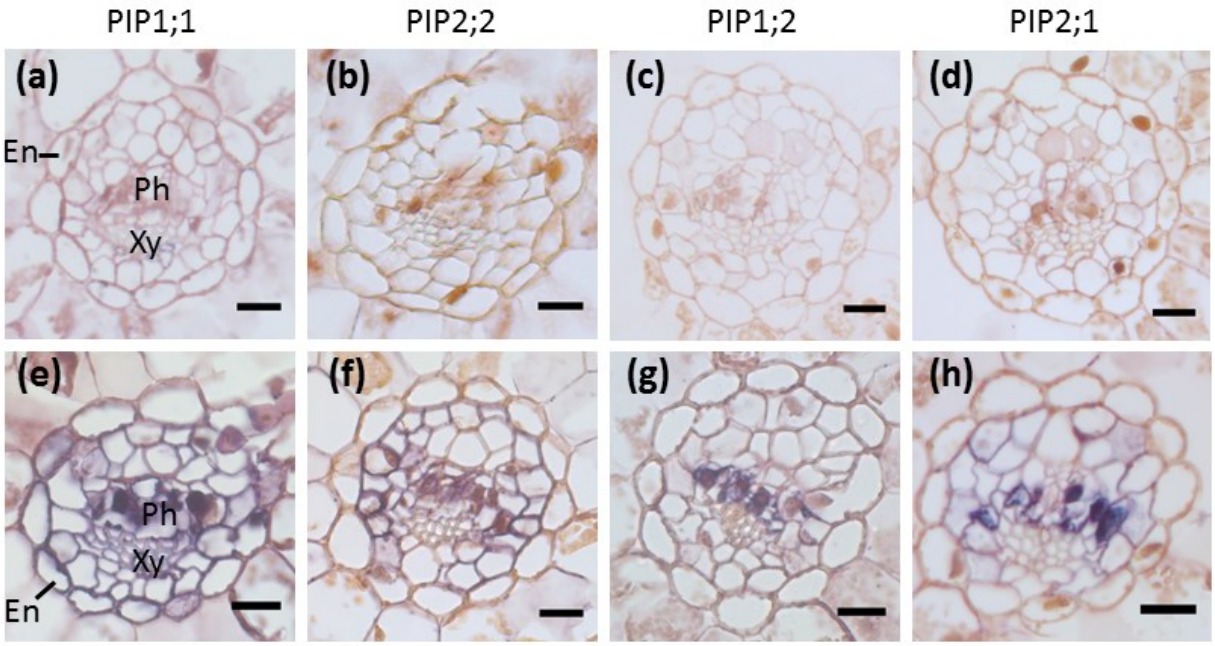

Figure 4-5: *In situ* mRNA hybridization of four aquaporin genes in needle cross-sections of Picea glauca.

(a–d) Negative controls hybridized with digoxigenin (DIG)-labeled sense probes. Sections in (e–h) were hybridized with DIG-labeled antisense PgPIP1;1 (e), PgPIP2;2 (f), PgPIP1;2 (g) and PgPIP2;1 (h) RNA probes. Regions of aquaporin expression are indicated by dark purple staining. PgPIP1;1 and PgPIP2;2 exhibited high expression in the vascular cylinder and in endodermis cells (En). Ph, phloem; Xy, xylem. Bars, 25 μm.

green and dark blue portion of the bars). The drought treatment resulted in more than a 2-fold reduction in the cumulative transcript amount relative to well-watered control plants (Fig. 4-4A). This was mainly driven by reduced expression levels of *PgPIP1;2*, *PgPIP2;2*, and *PgPIP2;1* (Fig. 4-4B). Transcript levels increased rapidly after plants were exposed to high relative humidity. After only 2 h, the cumulative number of AQP mRNA molecules was equivalent to the level found in well-watered control plants and peaked 26 h after the transfer to high humidity. All four genes contributed this peak in transcript levels after 26 h.

An analysis of the expression patterns of individual genes (Fig. 4-4B) reveals that there were two types of responses. Up-regulation of *PgPIP1;1* and *PgPIP2;2* was detected as soon as 2 h after exposure to high humidity. By contrast, expression levels of *PgPIP1;2* and *PgPIP2;1* remained low 2 h after transfer to high humidity, but increased more than 5-fold (relative to drought levels) 26 h after the transfer to high humidity.

e. Tissue localization of expression

In situ hybridization experiments revealed interesting tissue distribution patterns of expression. *PgPIP1;1* and *PgPIP2;2*, which showed the highest transcript levels among the four genes that were studied (Fig. 4-4), were expressed in phloem and transfusion parenchyma cells (Fig. 4-5E, F). In contrast to *PgPIP2;2,* expression of *PgPIP1;1* was also prominent in endodermis cells. The *PgPIP1;2* signal was constrained to phloem cells (Fig. 4-5G). This specific expression pattern is

consistent with the relatively low transcript level of this particular gene (Fig. 4-4). Expression of *PgPIP2;1* was evident in individual phloem cells and in transfusion parenchyma, but not in the endodermis (Fig. 4-5H).

f. Linking foliar uptake with embolism repair in stems

To test whether foliar uptake can play a role in embolism repair, we measured physiological parameters in plants prior to and during the drought treatment, as well as after plants were moved to a high humidity environment. Well-watered control plants had a ψ_{Stem} of -0.6 ± 0.1 MPa, which was associated with minimal xylem embolism in stems (Fig. 4-6). The drought treatment resulted in a drop of ψ_{Stem} to -2.9 ± 0.1 MPa and 16.1 ± 1.8 % loss of hydraulic conductivity. Consistent with a relatively steep increase in embolism levels at xylem pressures more negative than -2 MPa (Fig. 4-7), the drought treatment was associated with stomatal closure (Fig. 4-6B).

Fig. 4-7 shows a more detailed picture of the refilling dynamics of individual plants; each data point represents an individual plant. The amount of xylem embolism measured in drought-stressed plants (Fig. 4-7, red circles) agreed with predictions derived from the centrifuge-generated vulnerability curve measured on similar plant material. The vulnerability curve indicated that stems experienced 50% loss of hydraulic conductivity (P50) at a xylem pressure of -4.2 MPa (Fig. 4-7 insert). Plants that were rewatered after the drought treatment showed partial or complete recovery from xylem embolism within 2 h and 8 h, respectively (Fig. 4-7,

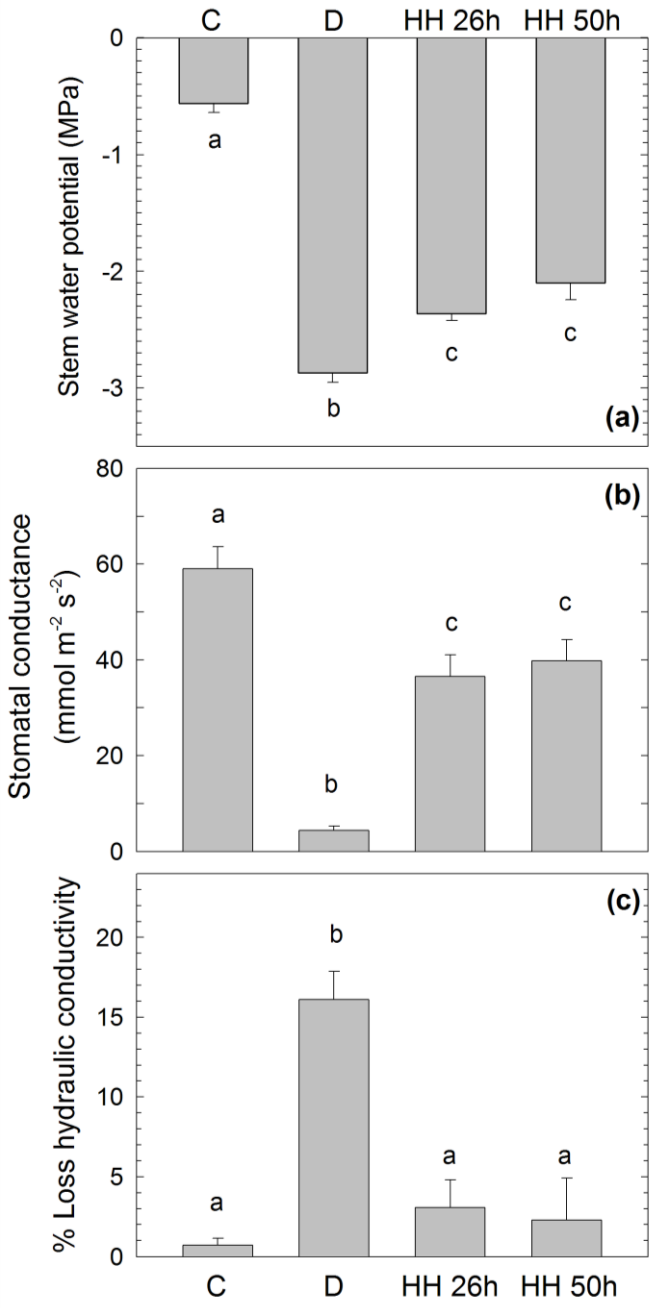

Figure 4-6: Stem water potential (a), stomatal conductance (b) and xylem embolism (expressed as percentage loss of hydraulic conductivity) (c) in white spruce (*Picea glauca*) saplings.

Plants were grown under well- watered (C) or drought (D) conditions. After drought treatment, plants were kept in a high-humidity environment (without watering the pots) for 26 h (HH 26 h) and 50 h (HH 50 h). Values are means±SE (n ≥ 5). Significant differences are indicated by unique letters.

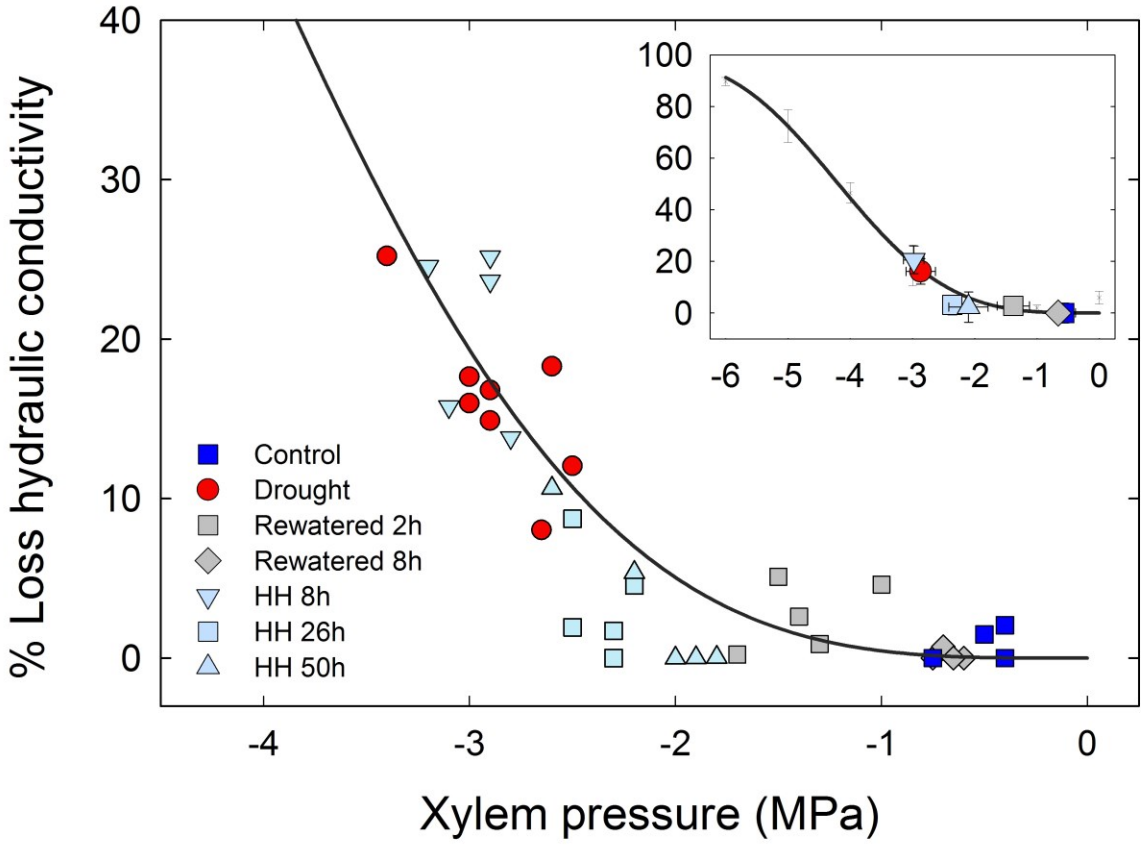

Figure 4-7: Effect of a change in water availability on xylem embolism in white spruce (*Picea glauca*) saplings.

Vulnerability curve (solid line) and native values of percentage loss of conductivity plotted against the native xylem pressure for stem segments of plants grown under well-watered (Control) or drought (Drought) conditions. Xylem embolism and xylem pressure were also measured in drought-stressed plants 2 and 8 h after rewatering, and in previously drought-stressed plants that were exposed to a high- humidity environment for 8 h (HH 8 h), 26 h (HH 26 h) and 50 h (HH 50 h). An overview of the complete vulnerability curve and native values of xylem embolism plotted against the native xylem pressure for each group (mean±SE; n ≥ 5) is shown in the upper right corner of the figure.

grey symbols). Plants that were transferred to the high humidity environment had not repaired embolism after 8 h (Fig. 4-7, HH 8h) but exhibited refilling after 26 h and 50 h (Fig. 4-7, HH 26h and 50h) while xylem pressures were still substantially negative (-2.4 ± 0.1 and -2.1 ± 0.1 MPa, respectively).

4. Discussion

The present study was conducted to gain a better understanding of foliar water uptake in *Picea glauca*, a common species in the boreal forest of North America. We explored the potential role of AQPs in foliar uptake, and impacts on xylem refilling. The remarkably complex anatomy of conifer needles (Fig. 4-1; Liesche *et al*., 2011) and the numerous well-documented cases of needle water uptake (Burgess and Dawson, 2004; Breshears *et al.*, 2008; Limm *et al*., 2009) make conifer needles an interesting model for the investigation of foliar uptake and potential implications for xylem refilling.

Based on the observed increases in RWC in plants exposed to high humidity, we conclude that drought-stressed needles of *P. glauca* are capable of absorbing water. The occurrence of mucilage and the presence of hydrophilic carbohydrates in the epidermis and hypodermis may facilitate water uptake and water retention by needles. Stomata were at least partially opened at high humidity (Fig. 4-6B), and so water uptake via stomata would seem possible (Berkhardt, 2010). Foliar water uptake and subsequent refilling also occurred in timberline trees in late winter

when the soil was still frozen and when trees were still disconnected from soil water (Mayr *et al.*, 2014).

Depending on the water potential gradients, water may refill the plant from two directions (Goldsmith, 2013; his fig. 1b). Our experiment was designed to restrict water uptake to above-ground plant parts. During drought treatment, water was withheld for many days, and so there was sufficient time for soil and plant water potentials to equilibrate. Before the transition to high RH, pots were carefully covered. The soil water content of high-humidity plants remained at the same low level as seen in drought-stressed plants (Supplementary Table 4-S3), indicating that water did not enter the pots. By contrast, soil water content increased quickly when plants were rewatered. Consistent with these data, Fig. 4-7 shows that the recovery of water potentials and hydraulic conductivity was much quicker in plants that were rewatered after the drought treatment than in plants that were transferred to the high-humidity environment without rewatering

Water following a gradient in water potential from the epidermis toward the vascular tissue has to pass the bundle sheath (Fig. 4-1C). Radial cell walls of the bundle sheath were lignified, indicating that water molecules will cross cell membranes. AQPs are likely to play an important role in regulating the hydraulic resistance between vascular and photosynthetic tissue in conifer needles. Immunolocalization and *in situ* hybridization experiments confirmed the presence of AQPs in the endodermis-like bundle sheath of *P. glauca* needles. Although both PIP1 and PIP2 were detected in the bundle sheath, the PIP1 signal was stronger in this cell layer than the PIP2 signal (Fig. 4-2). This was also observed in a study

on Norway spruce (*Picea abies*) trees growing at the timberline (Mayr *et al.* 2014). In agreement with the immunolabeling results, *in situ* hybridization of *PgPIP1;1* antisense probes also showed a strong signal in the endodermis. We therefore suggest that PIP1s, and PgPIP1;1 in particular (Figs. 4-4, 4-5E), play a critical role in mediating water flow through the endodermis.

Figures 4-2 and 4-4 show the down-regulation of AQPs during drought. In leaves of *Arabidopsis*, PIP transcripts were also generally down-regulated in response to drought. The amount of protein was also reduced. Twenty-six hours after rehydration, the expression levels were back at the same level as in control plants (Alexandersson *et al*., 2005). Consistent with these findings, Shatil-Cohen *et al*. (2011) proposed a role for bundle sheath cells as a stress signal-sensing 'control center' in leaves. According to their model, bundle sheath cells sense stress signals in the xylem sap (presumably abscisic acid) and respond by changing their hydraulic conductivity via the down-regulation of AQP activity. Our data are consistent with this idea. In addition, we show that the effect of drought on AQPs can be reversed by the exposure of leaves to high humidity.

PIP labeling was also detected in transfusion parenchyma and phloem cells. AQPs have been previously found in the leaf phloem of angiosperm species (Fraysse *et al*., 2005; Hachez *et al*., 2008) as well as in Picea abies needles (Oliviusson *et al*., 2001), consistent with a role for AQPs in phloem loading and unloading. In the context of foliar water uptake, radial water flow was likely directed toward vascular tissue including phloem. Subsequently, water could have moved from needles to stems via the phloem. Unloading of water and solutes in stems could have

promoted xylem refilling, as has been suggested for angiosperms (Nardini *et al*., 2011). On the way from needles to stems, water may have also moved in the xylem; negative sap flow as a result of foliar absorption has been described in numerous studies (Burgess and Dawson, 2004; Nadezhdina *et al*., 2010; Eller *et al*., 2013; Goldsmith, 2013).

This hypothetical chain of events summarized in Fig. 4-8 provides a theoretical framework that links foliar uptake with AQP function and embolism repair. Regardless of the mechanism, refilling in stem xylem occurred (Figs. 4-6, 4-7), indicating that the uptake of water via needles was physiologically meaningful, and that this water moved from needles to stems. It remains to be tested whether needle water uptake occurs under natural conditions in the boreal forest. Conceivably, foliar water absorption could be beneficial during summer periods when the forest receives small quantities of rain that are not enough to penetrate the soil. Foliar water uptake may also occur on relatively warm days in late winter may be able to absorb water and this could facilitate xylem refilling and offset winter desiccation effects, similar to that which has recently been shown for timberline trees in Austria (Mayr *et al*. 2014).

The amount of xylem embolism during the drought treatment was relatively low although stem water potentials of drought-stressed plants were close to -3 MPa (Fig. 4-6). *P. glauca* stems exhibited no or minimal embolism at water potentials less negative than -2 MPa (Fig. 4-7). The shape of the vulnerability curve and the P50 value measured in this study agree with previously published values for *P. glauca*. Hacke and Jansen (2009) measured a P50 of -4.3 ±0.3 MPa (± SE, n=6), similar to

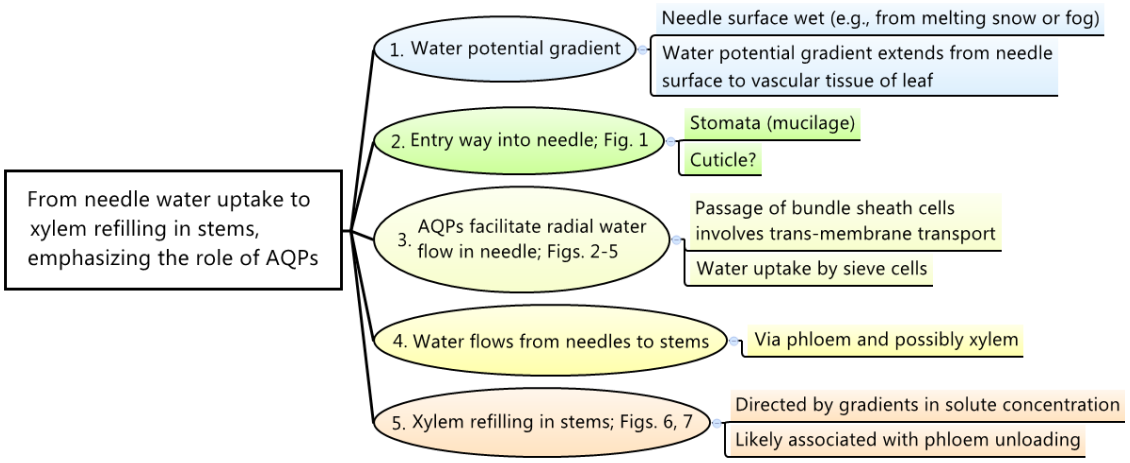

Figure 4-8: Putative chain of events linking needle water uptake to xylem refilling in stems.

Foliar water uptake may occur when a thin film of water has coalesced on the needle surface and the needle is experiencing a water deficit, that is, when the internal leaf tissue has a more negative water potential than the surrounding atmospheric boundary layer. Radial water movement inside the leaf also follows gradients in water potential and is directed from the epidermis towards vascular tissue. The passage of bundle sheath cells involves membrane transport, which is facilitated by aquaporins (AQPs; especially plasma membrane intrinsic proteins 1 (PIP1s)). Water uptake by sieve cells and other phloem cells may also be facilitated by aquaporins (especially PIP2s). Water then flows from needles to stems, where it contributes to embolism repair. Solutes and water are delivered from the phloem to embolized tracheids via rays. In this conceptual model, the direction of water flow is always consistent with gradients in water potential.

the value of -4.6 ±0.1 MPa (± SE, n=6) reported for sun-exposed trees by Schoonmaker *et al*. (2010).

Water potentials were not continuously monitored throughout the experiment, so it is possible that Ψ_{Stem} increased to less negative values during the night. We therefore do not know whether refilling occurred at substantially negative water potentials as reported by others (Sperry *et al.*, 1994; McCulloh *et al*., 2011) or whether it was associated with a nocturnal increase in Ψ_{Stem} that was not captured. However, as pots were not watered, it is unlikely that Ψ_{Stem} reached values close to atmospheric pressure, which would be required for a purely physical dissolution of bubbles.

The present study provides the most comprehensive functional and phylogenetic analysis of spruce AQPs so far. The number of AQP genes in spruce is similar to the total number of MIPs reported for *Arabidopsis* (35, Johanson *et al*., 2001) and maize (33, Chaumont *et al.*, 2001). In *Arabidopsis*, there are 13 PIPs (16 in *P. glauca*), 10 TIPs (nine in *P. glauca*), nine NIPs (four PgNIPs) and three SIPs (one PgSIP). In maize, 14 PIPs, 13 TIPs, five NIPs and three SIPs have been reported (Chaumont *et al*., 2001). Hence, the distribution between the four major subfamilies is similar in these three species. However, both *Arabidopsis* and maize have more PIP1s than *P. glauca*. Consistent with this finding, Chaumont *et al.* (2001) noted that *ZmPIP1;3* and *ZmPIP1;4* are the result of a very recent gene duplication

In conclusion, we report that needles of drought-stressed *P. glauca* plants absorb water when exposed to high RH. AQPs are present in the bundle sheath, in

phloem cells and in transfusion parenchyma of needles. The up-regulation of AQP genes in high RH coincides with embolism repair in stem xylem. Our findings are consistent with the hypothesis that AQPs facilitate radial water movement from the needle epidermis towards the vascular tissue. Water may then move from needles towards stems via phloem and xylem (Fig. 4-8). Refilling in *P. glauca* is apparently not limited to xylem pressures near atmospheric values.

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V. General discussion and conclusions

1. Outcomes of this study

The original aim of my thesis was to provide insights into the possible roles of aquaporins in the fine adjustment of spruce and poplar hydraulics to environmental changes (Figure 5-1). The major outcomes of this work are listed below:

- In a hybrid poplar clone (*Populus trichocarpa* x *deltoides*), root water uptake responds within hours to changes in the above ground environment **(Chapter 2)**. This adjustment is associated with changes in the expression of all tested PIP genes and PIP1 proteins.

- Leaves of *Populus trichocarpa* were sensitive to a moderate drought event; however they recovered within two hours after rewatering **(Chapter 3)**. Leaf hydraulic recovery resulted from aquaporin activity as demonstrated by the use of inhibitors. Several PIP and TIP isoforms were also upregulated at the transcriptional level at the time leaf recovery occurred.

- Foliar water uptake occurred in water-stressed spruce trees (*Picea* sp.) saplings exposed to high relative humidity or melting snow **(Chapter 4)**. Chapter 4 provides the first phylogenetic analysis of the aquaporin family in a conifer species. We observed up-regulation of four PIP genes expressed in the needle vascular bundle by the time xylem refilling occurred downstream in the

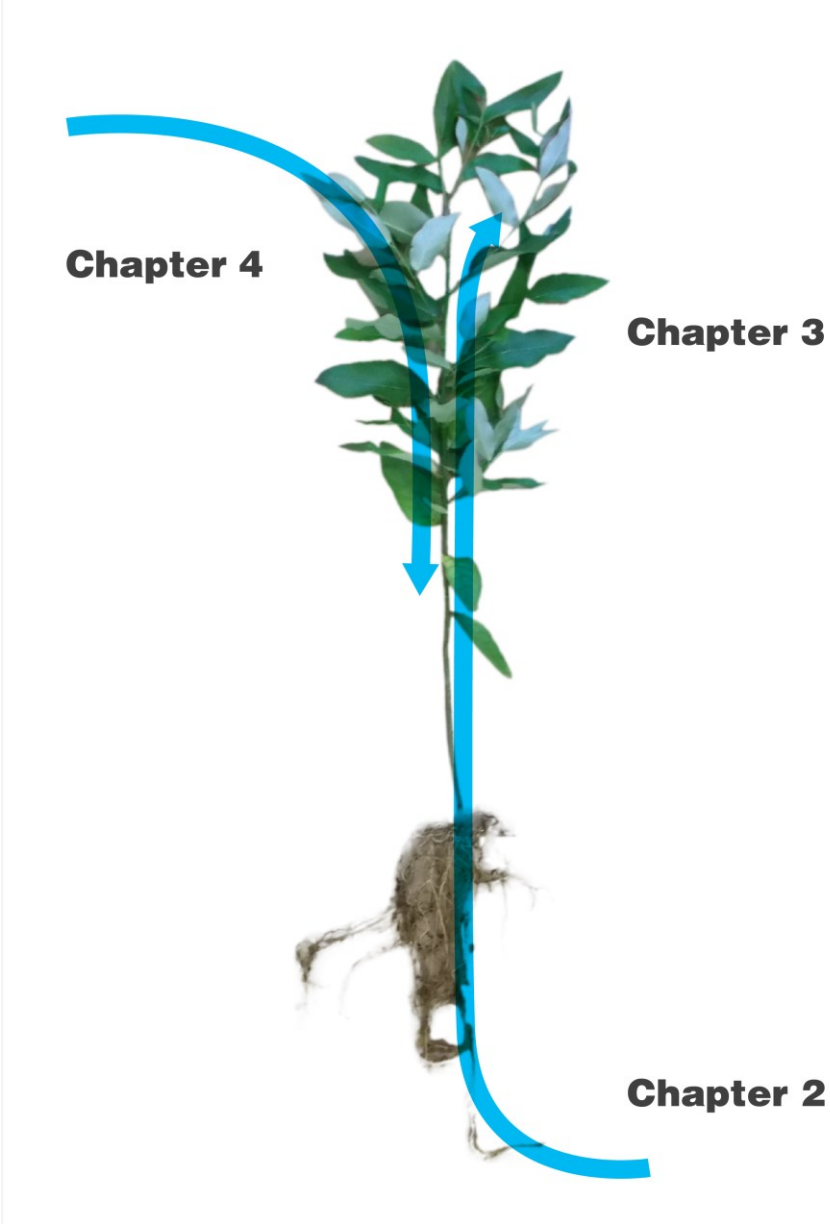

Figure 5-1: thesis outcomes.
In Chapter 2, root water uptake of poplar adjusted within hours to the above-ground demand, this adjustment was associated with the upregulation of PIPs isoforms. Leaves of *Populus trichocarpa* are sensitive to a moderate water stress; their fast recovery was associated with the regulation of TIP isoforms (Chapter 3). Chapter 4: foliar water uptake occurred when water-stressed spruce trees were exposed to high RH, it alleviated stem xylem embolism. To assess the role of AQPs in this phenomenon, we provide the first phylogenetic analysis of AQP family in a conifer species. Four PIP isoforms expressed in the vascular bundle were regulated during foliar water uptake.

stem. This phenomenon may be physiologically significant for plants since it provides alternative water source under otherwise unfavourable conditions.

2. Possible applications and perspectives

Taken together, these results illustrate the importance of aquaporins in the dynamic adjustments of trees to their local environment. The general inability of plants to escape from an adverse habitat is even exacerbated for those long living organisms. Water is the most likely limiting resource for plants worldwide and this is to worsen due to global climate change (FAO, 2008). How will trees manage to maintain the integrity of their hydraulic system?

Along the flow path, the cell membranes where AQP proteins are located could act like control centres regulating the transmembrane movement of water in order to constantly maintain an optimum plant- (and organ-) water balance. AQP function could not only be important for dynamic responses to a changing environment, but also during steady-state conditions. The current findings identified a number of aquaporin isoforms involved in different physiological adjustment to environmental changes.

In **Chapter 3**, the upregulation of *PtTIP1;3*, *PtTIP2;2*, *PtTIP4;*1 in poplar leaves is concomitant with leaf hydraulic conductance recovery from water stress. In a similar experiment, Pou *et al*. (2009) found an interesting correlation between the expression of a TIP isoform and water-related parameters of grapevine leaves.

Thus, the relatively less-studied TIPs (to date there is 11 TIP-related transgenic studies published but none in a woody plant species) can provide several interesting candidate for regulation of leaf hydraulics in woody plants.

Immunolocalization data from **Chapter 3** localized PIP1 proteins in the mesophyll. The well-studied NtAQP1, a PIP1 isoform, acts like a CO_2 transporter in leaves where its expression modulates the CO_2 mesophyll conductance (Flexas et al, 2006; Uehlein et al., 2003). However, mostly expressed in roots, NtAQP1 is also a water channel protein that regulates root hydraulic conductivity (Siefritz et al., 2002). In **Chapter 2**, the upregulation of *PtPIP1;1*, *PtPIP1;2*, and *PtPIP1;3* in poplar roots is correlated with an increase in water uptake in response to transpirational demand. Also significantly induced in our previous experiment (Almeida-Rodriguez et al., 2011), the three PIP1 isoforms may be good gene candidates for regulation of root hydraulics.

The observations made in **Chapter 4** contribute substantially to our comprehension of a phenomenon that was estimated of little impact until recently. Possibly acting to relieve crown water stress (and subsequent photosynthetic carbon starvation), the influence of foliar water uptake in plant ecophysiology is now of interest for the scientific community and the subject of constant work (most recently by Berry et al., 2014). We identified four *Picea* PIP gene candidates (*PgPIP1;1*, *PgPIP1;2*, *PgPIP2;2*, *PgPIP2;1*) regulated in needles at the time of water absorption from a high humidity environment. We took advantage of published ESTs databases and of the recent sequencing of the *Picea* sp. Genome to investigate for the first time the AQP family in a conifer species.

This study has generated a number of AQP gene candidates in two major tree families. To be integrated in future marker assisted selection or genetic engineering programs, these analyses requires additional efforts that could include the use of field-grown material and/or the generation of OE or KO mutants to fully characterize AQP isoforms .

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Appendices

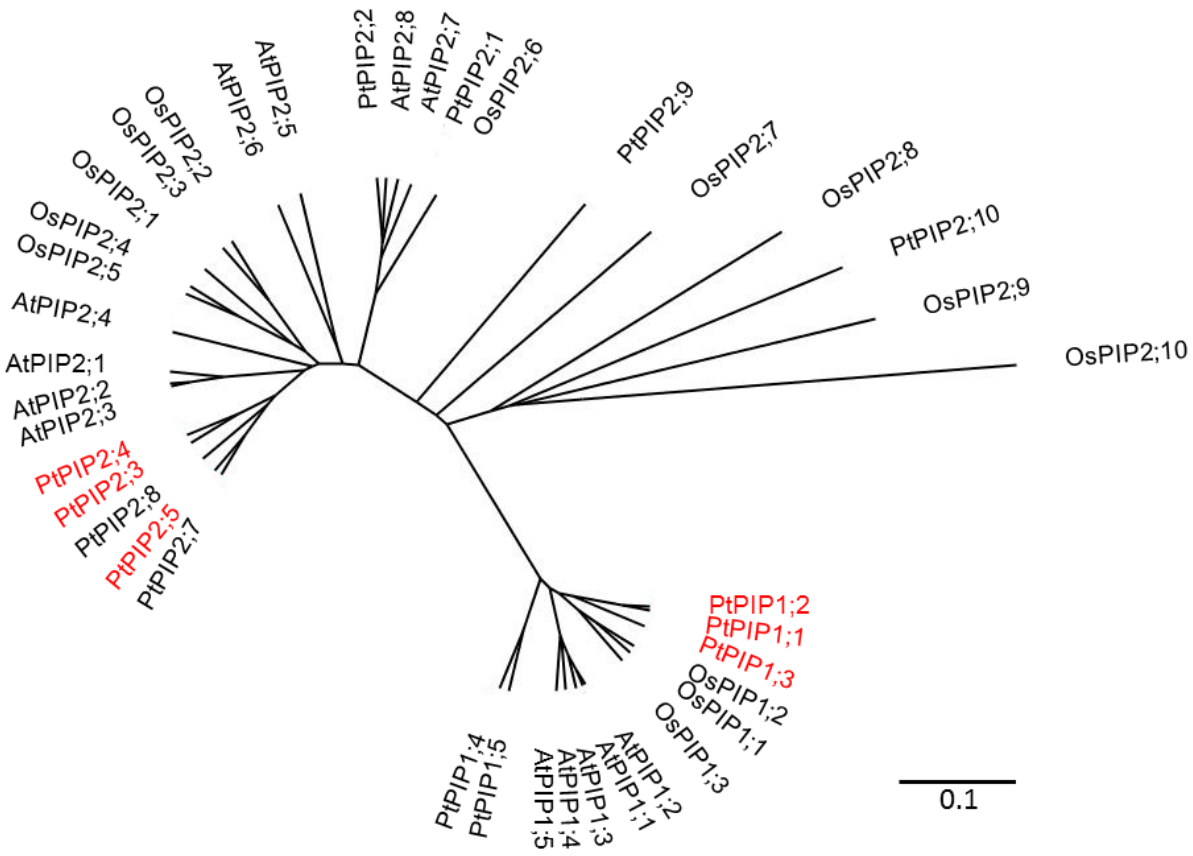

Figure 2-S1: Phylogenetic relationships of plasma membrane intrinsic proteins (PIPs) in *Arabidopsis thaliana*, *Oryza sativa* and *Populus trichocarpa*. The phylogenetic tree was constructed using Genomics Workbench version 5.5 (CLC Bio, Cambridge, MA, USA) and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. The tree was visualized by FigTree (tree.bio.ed.ac.uk/software/figtree/). The scale bar represents the number of amino acid substitutions per site.

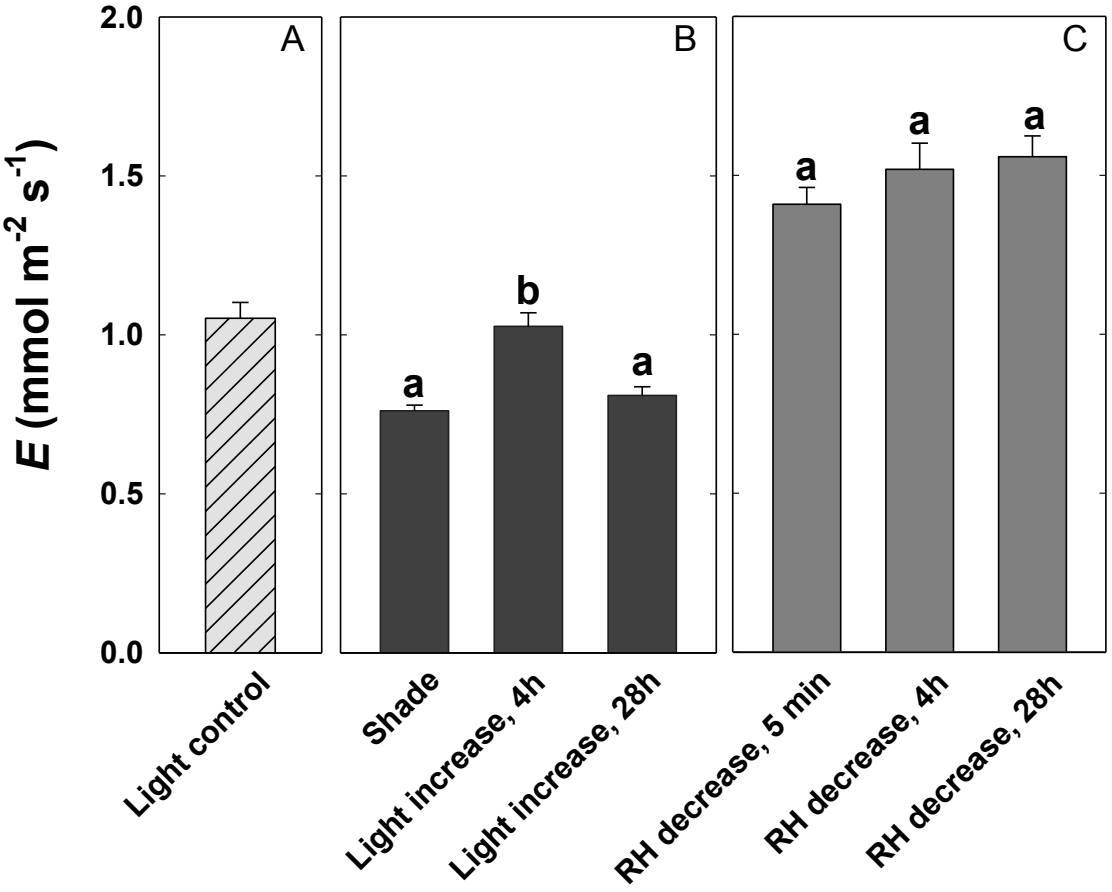

Figure 2-S2: Effect of step changes in light and humidity on transpiration rate (*E*). (A) Transpiration rate of control plants ('Light control'). (B) Transpiration rate of shaded plants ('Shade'), of plants removed from shade after 4 h ('Light increase, 4h'), and of plants removed from shade after 28 h ('Light increase, 28h'). (C) Transpiration rate of plants grown at high relative humidity (RH) after a step change in RH. Transpiration was measured 5 minutes ('RH decrease, 5 min'), 4 h ('RH decrease, 4h'), and 28 h ('RH decrease, 28h') after the decrease in humidity. Data shows means + SE; n = 5 plants. Significant differences are indicated by unique letters ($P < 0.05$).

Table 2-S1: Summary of PIP expression patterns. The poplar gene names follow the nomenclature of Gupta and Sankararamakrishnan (2009). Findings from a previous experiment (Almeida-Rodriguez et al. 2011) are summarized as: * for differentially expressed genes; *nd* for non- differentially expressed genes; blank spaces indicate genes that were not investigated. The *Populus* eFP browser (Wilkins et al. 2009) was used to check the tissue specificity of *PIPs* as well as putative regulation by light in seedlings. Relative transcript abundance of a particular gene is indicated as a fold change ratio between the tissue specific probe signal normalized to the control signal (value=1) as indicated in the eFP Browser. Grey background highlights the genes used in this present study.

P. trichocarpa gene name			Almeida-Rodriguez *et al.* (2011) -------Root tips -------		Poplar eFP Browser - Wilkins *et al.* (2009) -------- Mature tissue --------			-------- Seedlings -------	
Gene name	Phytozome v2.0	Affymetrix probe ID	Acclimation	Dynamic	Leaf	Root	Xylem	Dark grown	+3h light
PtPIP1;1	POPTR_0010s19930	PtpAffx.7686.1.S1_a_at	*		0.24	1.16	0.25	1.15	1.04
PtPIP1;2	POPTR_0008s06580	Ptp.4455.1.S1_s_at	*	*	0.09	2.06	0.14	1.4	1.87
PtPIP1;3	POPTR_0003s12870	PtpAffx.12342.2.S1_s_at			0.13	0.87	1.89	0.74	0.65
PtPIP1;4	POPTR_0006s09920	PtpAffx.54577.1.S1_at	*	*	0.13	1.26	2.09	0.52	0.84
PtPIP1;5	POPTR_0016s12070	PtpAffx.2848.1.S1_a_at	*		0.13	0.8	1.87	0.12	0.24
PtPIP2;1	POPTR_0009s13890	PtpAffx.5465.1.A1_x_at		*	0.18	1.18	1.58	1.13	0.93
PtPIP2;2	POPTR_0004s18240	PtpAffx.5465.2.A1_x_at	*nd*	*nd*	0.05	1.13	4.1	1.05	0.58
PtPIP2;3	POPTR_0010s22950	Ptp.1588.1.S1_s_at	*	*	0.05	0.9	4.42	0.83	0.52
PtPIP2;4	POPTR_0008s03950	PtpAffx.249.108.A1_x_at	*	*	0.2	0.96	0.11	1.04	1.29
PtPIP2;5	POPTR_0006s12980	PtpAffx.7681.3.A1_x_at		*	0.28	12.52	1.68	7.25	6.88
PtPIP2;7	POPTR_0016s09090	Ptp.139.1.S1_at	*nd*	*nd*	0.03	12.46	3.31	0.85	2.96
PtPIP2;8	POPTR_0009s01940	PtpAffx.5992.1.S1_at			0.02	10.31	1.46	1.19	1.25
PtPIP2;9	POPTR_0005s11110	PtpAffx.221954.1.S1_at			0.28	0.04	0.03	1.21	1.1
PtPIP2;10	POPTR_0005s11100	PtpAffx.221953.1.S1_s_at		*	0.08	0.26	0.09	4.85	1.38

Table 2-S2: Primer sequences used for the gene expression study. Primers were designed based on *Populus trichocarpa* reference gene sequences. Primer sequences of the selected candidate genes are represented as well as the specific amplicon length.

P. trichocarpa gene Name		Amplicons		
Gene name	Phytozome v2.0	Forward Primer (5´→3´)	Reverse Primer (5´→3´)	Length (bp)
PtPIP1;1	POPTR_0010s19930	TGCAGAGTTCATGGCCACCTTC	TCGTGTCCTTAAACACGCCCATC	74
PtPIP1;2	POPTR_0008s06580	TGGCCTTGGTGCTGAGATTGTC	GCACTACGCTTGGCATCAGTTG	78
PtPIP1;3	POPTR_0003s12870	AACTGGCATTAACCCGGCAAGG	AATGGGCCAACCCAGAAGATCCAG	96
PtPIP2;3	POPTR_0010s22950	AGTCTGGGAGCCGCTGTTATCTAC	GGGTCCAACCCAGAAGATCCAATG	72
PtPIP2;4	POPTR_0008s03950	GTCATTCAGGAGCAACCCGAATGTC	CCATCATGCACGCACAAGCACTC	81
PtPIP2;5	POPTR_0006s12980	TGTGTTGGCACCACTTCCCATC	GTCATCCCATGCCTTGTCTTCGT	139

Table 3-S1: Primer sequences used in the qRT-PCR assays. Primers were designed based on *Populus trichocarpa* reference genes sequences. Primers sequences of the selected candidate genes (grey) are represented as well as the specific amplicon lenght.

		Amplicons		
		Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Length (bp)
PtPIP1;1	POPTR_0010s 19930	TGCAGAGTTCATGGCCACCTTC	TCGTGTCCTTAAACACGCCCATC	74
PtPIP1;2	POPTR_0008s 06580	TGGCCTTGGTGCTGAGATTGTC	GCACTACGCTTGGCATCAGTTG	78
PtPIP1;3	POPTR_0003s 12870	AACTGGCATTAACCCGGCAAGG	AATGGGCCAACCCAGAAGATCCAG	96
PtPIP2;3	POPTR_0010s 22950	AGTCTGGGAGCCGCTGTTATCTAC	GGGTCCAACCCAGAAGATCCAATG	72
PtPIP2;4	POPTR_0008s 03950	GTCATTCAGGAGCAACCCGAATGTC	CCATCATGCACGCACAAGCACTC	81
PtPIP2;5	POPTR_0006s 12980	TGTGTTGGCACCACTTCCCATC	GTCATCCCATGCCTTGTCTTCGT	139
PtTIP1;3	POPTR_0010s 21700.1	TTCAGGATCTGGCATGGCTTTCAAC	CCAGAAGGAGTAGTCGAAGCATTG TCG	60
PtTIP1;5	POPTR_0016s 10780.1	TCCACTGTCGCTTGCTTGCTTC	ACAGAGCGAAAGCAGAGGTTTCCA AG	67
PtTIP1;6	POPTR_0006s 12350.1	TCCACTGTCGCTTGCTTGCTTCTC	ACAGAGCGAAAGCAGAGGTTTCCA G	67
PtTIP2;1	POPTR_0001s 18730.1	GCCATGGCTTACAATAAGCTGACAGG TG	GGCACCTACAGAAACTGCAACGAA G	111
PtTIP2;2	POPTR_0003s 04930.1	TGGCTTACAATAAGCTGACAGGTGAT GC	ACCAACAGCAACTGCAACAAAGAG C	104
PtTIP4;1	POPTR_0006s 25620.1	TCAAGTATCTCACCGGAGGATTGGC	CCTTGAAGGTAGTCCATCCCACTTG C	70
ACT	POPTR_0001s 31700	TGGAGGATCTATCCTTGCTTCCCTCAG	TACTCACCCTTGGAAATCCACATCTG C	63
CYCL	(POPTR_0005 s26170	ACCAGGTAAGCAAGCGGTTTGGTC	TCGACCGATTTCCATGGAGTGCAAG	72
TIP4	POPTR_0009s 09620.1	AGAGTCATGCCAAGTTGCTGGTTTC	TCGACCGATTTCCATGGAGTGCAAG	60
UBQ	POPTR_0005s 09940	TCCACCTGTGCAACAAAGGC	CACTCCATCAACTCTAAGCCAGAAT CGC	66

Figure 3-S1: Amino acid multiple sequence alignment of the N-terminal region of the *Arabidopsis thaliana* AtPIP1;3 and the *Populus trichocarpa* PtPIP1s (a); of the conserved the C-terminal region of PIP2s (b) and TIP2s (c). Consensus amino acids are underlined in black.

Table 4-S1. Gene names, cDNA clone accession numbers, genbank accession numbers, *Picea glauca* genome accession numbers, and *Picea abies* homolog genomic accession numbers. Complete genomic sequences are shown in bold.

	cDNA clone accession	GenBank accession number	*P. glauca* genome accession number	*P. abies* genome accession number
PgPIP1;1	GQ03401_M18	BT113218.1	ALWZ022680616.1	**MA_3650g0010**
			ALWZ024883929.1	
PgPIP1;2	GQ03610_A06	BT115139.1	**ALWZ026715192.1**	MA_10434016g0010
PgPIP1;3	GQ02828_J14	BT105794.1	**ALWZ024321598.1**	**MA_671655g0010**
			ALWZ021834942.1	
			ALWZ024890198.1	
			ALWZ024827936.1	
PgPIP2;1	GQ03111_E12	BT107672.1	ALWZ026917578.1	MA_10289712g0010
			ALWZ026087674.1	
			ALWZ023460553.1	
PgPIP2;2	GQ02901_B20	BT105999.1	**ALWZ024834522.1**	**MA_72395g0010**
PgPIP2;3	GQ03703_H07	BT115639.1	**ALWZ024834523.1**	**MA_72253g0010**
PgPIP2;4	GQ0132_J09	CO478019.2	**ALWZ026260114.1**	**MA_191627g0010**
			ALWZ023198434.1	
PgPIP2;5	GQ03124_N20	BT108646.1	**ALWZ022229638.1**	**MA_17793g0010**
			ALWZ024541622.1	
PgPIP2;6	GQ03705_D15	BT115731.1	ALWZ026587127.1	**MA_11327g0010**
			ALWZ023471430.1	
PgPIP2;7	GQ02905_E13	BT106222.1	ALWZ025040153.1	**MA_10426681g0010**
			ALWZ025040150.1	
			ALWZ025040147.1	
PgPIP2;8	GQ02902_L14	BT106086.1	ALWZ025361399.1	MA_207341g0010
PgPIP2;9	GQ03002_G07	BT106471.1	ALWZ025471513.1	**MA_68132g0010**
PgPIP2;10	GQ03011_G23	BT106822.1	ALWZ025966231.1	**MA_10177437g0010**
PgPIP2;11	GQ03010_E09	BT106775.1	ALWZ021792796.1	**MA_9821440g0010**
PgPIP2;12	GQ03001_P18	BT106446.1	**ALWZ023919432.1**	MA_93945g0010
PgPIP2;13	GQ03216_M18	BT110135.1	ALWZ021875693.1	**MA_10426909g0020**
			ALWZ022242989.1	**MA_123344g0010**

			ALWZ026052548.1	**MA_41167g0020**
			LWZ023363107.1	MA_629271g0010
PgTIP1;1	GQ0197_E19	BT102589.1	ALWZ024819070.1	**MA_10437001g0010**
			ALWZ024315453.1	
PgTIP1;2	GQ03116_D08	BT108041.1	ALWZ024535225.1	**MA_46360g0010**
PgTIP1;3	GQ02908_P24	BT106406.1	**ALWZ022961470.1**	**MA_112061g0010**
PgTIP1;4	GQ03501_N03	BT113810.1	ALWZ020567052.1	MA_10437001g0040
PgTIP1;5	GQ0206_N10	BT103114.1	**ALWZ025579826.1**	**MA_175978g0010**
PgTIP2;1	GQ03915_M04	BT117884.1	ALWZ022364681.1	MA_18297g0010
PgTIP2;2	WS0323_F18	DR554580.1	**ALWZ024905374.1**	**MA_467865g0010**
PgTIP4;1	GQ0201_M19	BT102857.1	ALWZ026620523.1	**MA_394947g0010**
PgTIP4;2	GQ04012_G01	BT118954.1	ALWZ023733587.1	MA_10426941g0010
	WS02617_N14	DR559801.1		MA_153442g0010
PgNIP1;1	GQ03122_A02	BT108454.1	ALWZ024309972.1	**MA_93825g0010**
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				MA_9571426g0010
				MA_62314g0010
	GQ03202_N13	BT108940.1	ALWZ020198713.1	MA_470542g0010
PgNIP2;1	GQ03207_J07	BT109358.1	ALWZ021437004.1	**MA_10428302g0010**
			ALWZ021141575.1	
			ALWZ025258428.1	
	GQ03237_P23	BT111466.1	ALWZ021717141.1	**MA_158806g0010**
PgNIP3;1	GQ03810_B10	BT116953.1	**ALWZ021012731.1**	**MA_60111g0010**
			ALWZ023644073.1	
			ALWZ021905214.1	
PgNIP3;2	GQ03701_J12	BT115558.1	ALWZ021193474.1	**MA_158586g0010**
				MA_7702134g0010
PgSIP1;1	GQ03414_P10	BT113612.1	ALWZ022969142.1	**MA_78511g0010**
			ALWZ025225505.1	
			ALWZ024581523.1	
	GQ04011_K04	BT118897.1	ALWZ026845950.1	**MA_938669g0010**

Table 4-S2. Primer sequences used for the gene expression study.

Primer sequences of the selected candidate genes are shown as well as the specific amplicon lengths for (a) RTqPCR analysis, (b*) in situ* hybridization.

	Amplicons		
	Forward Primer (5´→3´)	Reverse Primer (5´→3´)	Length (bp)
(a)			
PgPIP1;1	TGCAACAATTCCCATCACCGGAAC	TGATGGCAGCTCCCAAACTTCGAG	62
PgPIP1;2	TCCTAGAAACAGCCCAGCGTATCG	ACACATGCGCTAACAGACCTCAGC	66
PgPIP1;3	TCATCAGCTCATCATCCGAGCCATAC	AACAGCCCAAACGAGAAGAGACTGA	80
PgPIP2;1	TAGGCAGCAGCTAATGCAGCTCC	GCCACAAACAATCCTGGGATGCC	76
PgPIP2;2	AGGGTAGCTTCTCTCCGAACCTTGA	AAACATCCATCGCCCTCTCTGACG	76
PgPIP2;6	CCATGTTCCCGTATTAGCACCTCTGC	CAGTTATAGGGATGGTGGCCAAATGTACC	78
PgPIP2;8	TGCTGCGATTGCATCAGCCTAC	AACACTGCGGAAAGAACCCAAGG	79
(b)			
PgPIP1;1	TGCCAGGGACTCTCACGTTCCTCTAC	TCATCTACCACGTAGCCCATACATAA	340
PgPIP1;2	CATGCGCTAACAGACCTCAGCC	TTCTCAGCCACCGATGCCAAAC	342
PgPIP2;1	TGATGCCGGTCCCAGTGATAGG	GGAGGTGGAGCTAACTACGTGC	220
PgPIP2;2	AGCAGCTAACGCAGCTCCAATG	GTGCACCCTGGATACACCAAAGG	309

Table 4-S3. Soil water content measurements.

Soil water content was measured during the experiment using an EC-5 sensor (Decagon Devices, Pullman, WA, USA). Values are the means ± SE from 5 to 6 biological replicates.

Experimental treatment	SWC (%)
Control	22.3 ± 1.8
Drought	6.3 ± 1.2
High RH, 2h	5.5 ± 1.4
High RH, 26h	6.0 ± 1.2
High RH, 50h	7.3 ±0.9
Rewatered, 2h	18.2 ± 3.1
Rewatered, 8h	19.9 ± 2.1

Figure 4-S1. Protein sequence alignment of *Picea glauca* MIPs.

Alignment of the predicted amino acid sequences for PgMIPs, PpMIPs, AtMIPs, and ZmMIPs. The NorMD score of the alignement is > 0.68 (Thompson *et al.*, 2001). Shading is indicating the degree of conservation of an amino acid at a position within each monophyletic subfamily. Black lines above sequences are indicative of transmembrane (TMHs) regions. The two NPA motifs are outlined with a red box and the AEF motif with a blue box. Residues determining the ar/R filters are indicated in green.

```
PpPIP1_1   ------------------------------------------------MNQD-------KDDD
PpPIP1_2   ------------------------------------------------MQQD-------KDDD
PpPIP1_3   -------------------------------------------------MAD-------RGDD
AtPIP1_1   -------------------------------------------------MEG-------KEED
AtPIP1_2   -------------------------------------------------MEG-------KEED
AtPIP1_3   -------------------------------------------------MEG-------KEED
AtPIP1_4   -------------------------------------------------MEG-------KEED
AtPIP1_5   -------------------------------------------------MEG-------KEED
ZmPIP1_1   -------------------------------------------------MEG-------KEED
ZmPIP1_2   -------------------------------------------------MEG-------KEED
ZmPIP1_3   -------------------------------------------------MEG-------KEED
ZmPIP1_5   -------------------------------------------------MEG-------KEED
ZmPIP1_6   -------------------------------------------------MAGGTLQDRSEEED
PgPIP1_1   -------------------------------------------------MEG-------KEED
PgPIP1_2   -------------------------------------------------MEG-------KEED
PgPIP1_3   ------------------------------------------------------------MED
PpPIP2_1   ---------------------------------------------------------------
PpPIP2_2   ---------------------------------------------------------------
PpPIP2_3   ---------------------------------------------------------------
PpPIP2_4   ---------------------------------------------------------------
AtPIP2_1   ---------------------------------------------------------------
AtPIP2_2   ---------------------------------------------------------------
AtPIP2_3   ---------------------------------------------------------------
AtPIP2_4   ---------------------------------------------------------------
AtPIP2_5   ---------------------------------------------------------------
AtPIP2_6   ---------------------------------------------------------------
AtPIP2_7   ---------------------------------------------------------------
AtPIP2_8   ---------------------------------------------------------------
ZmPIP2_1   ---------------------------------------------------------------
ZmPIP2_2   ---------------------------------------------------------------
ZmPIP2_3   ---------------------------------------------------------------
ZmPIP2_4   ---------------------------------------------------------------
```

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PgPIP2_10	- -
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PpTIP6_3	- -
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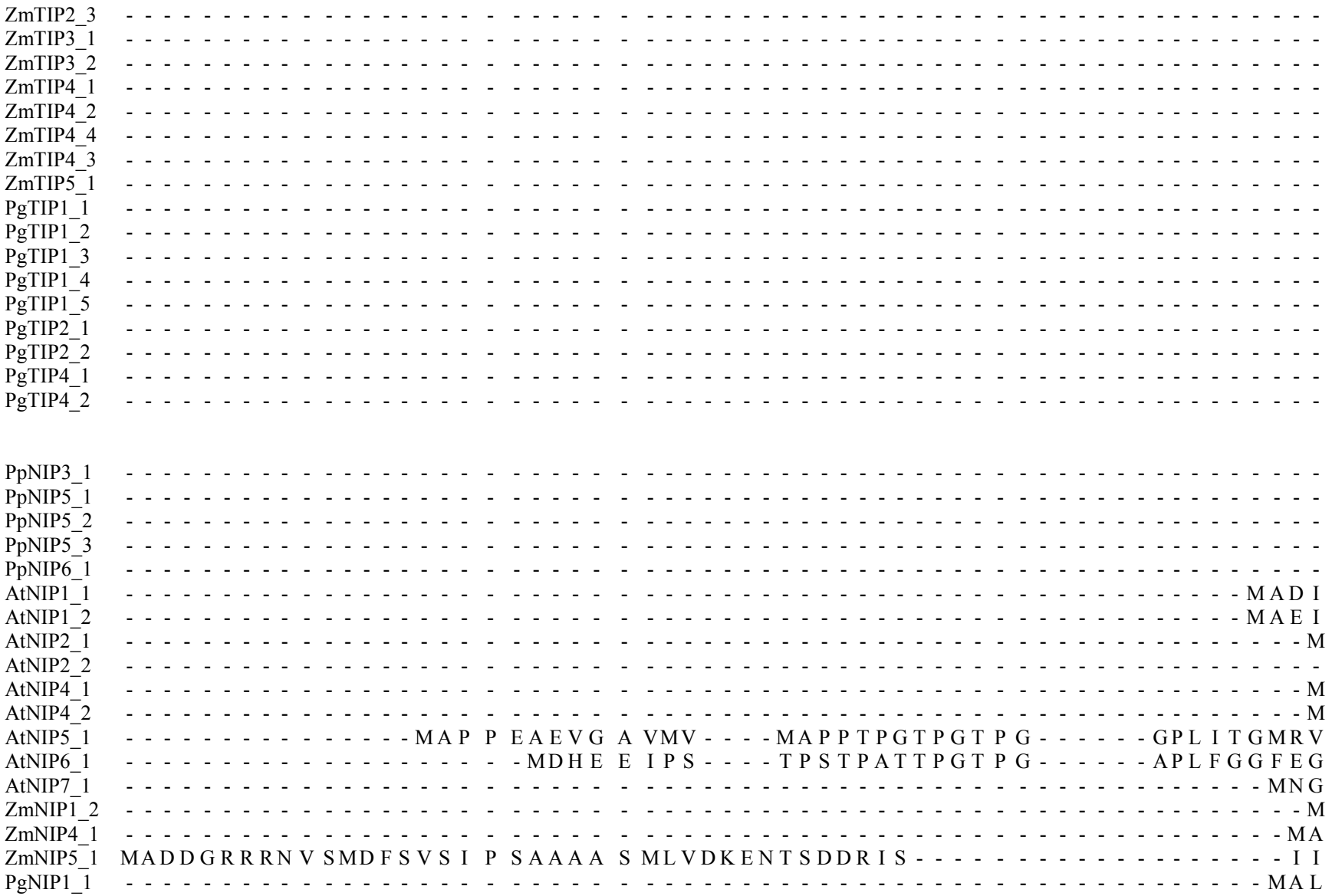
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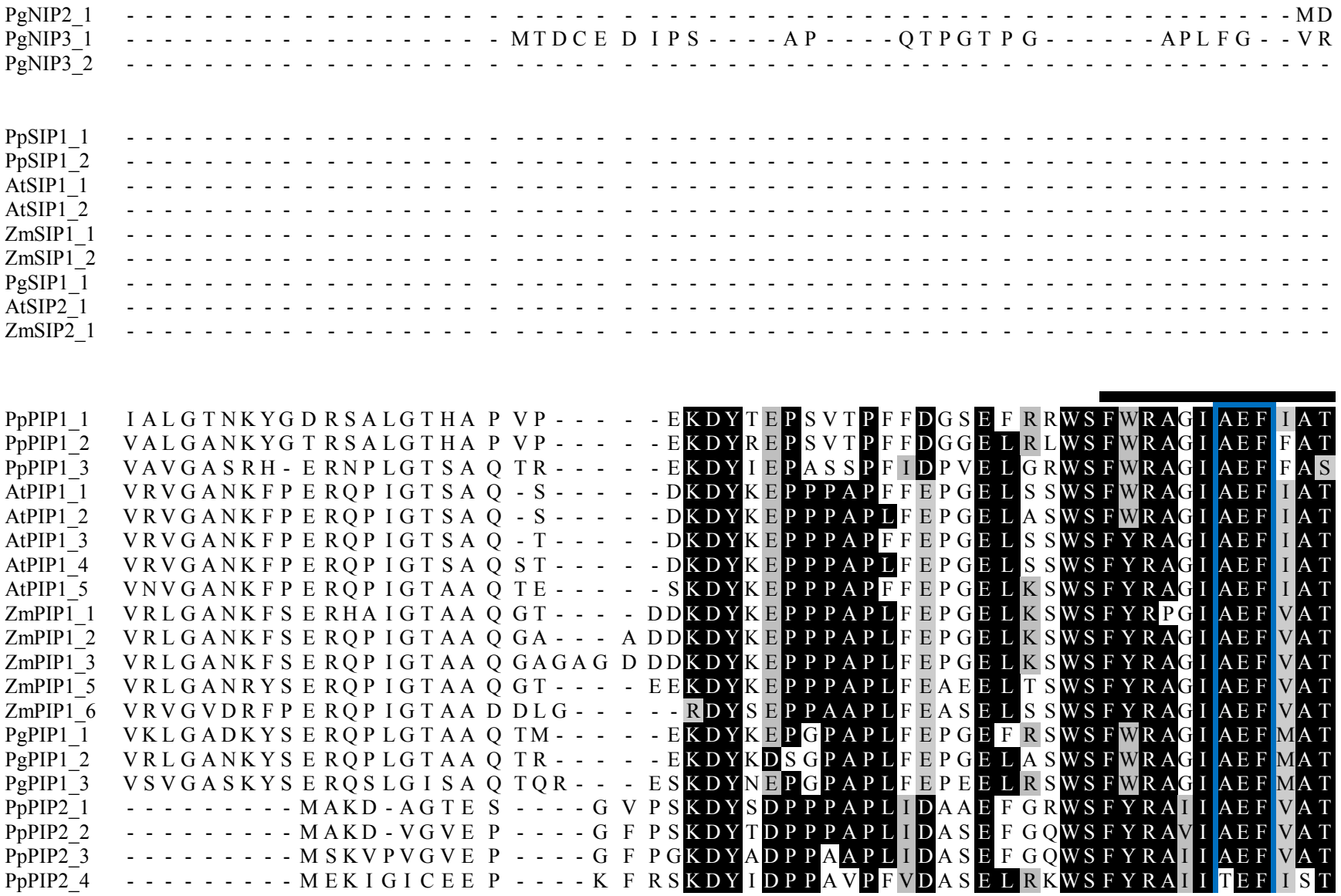
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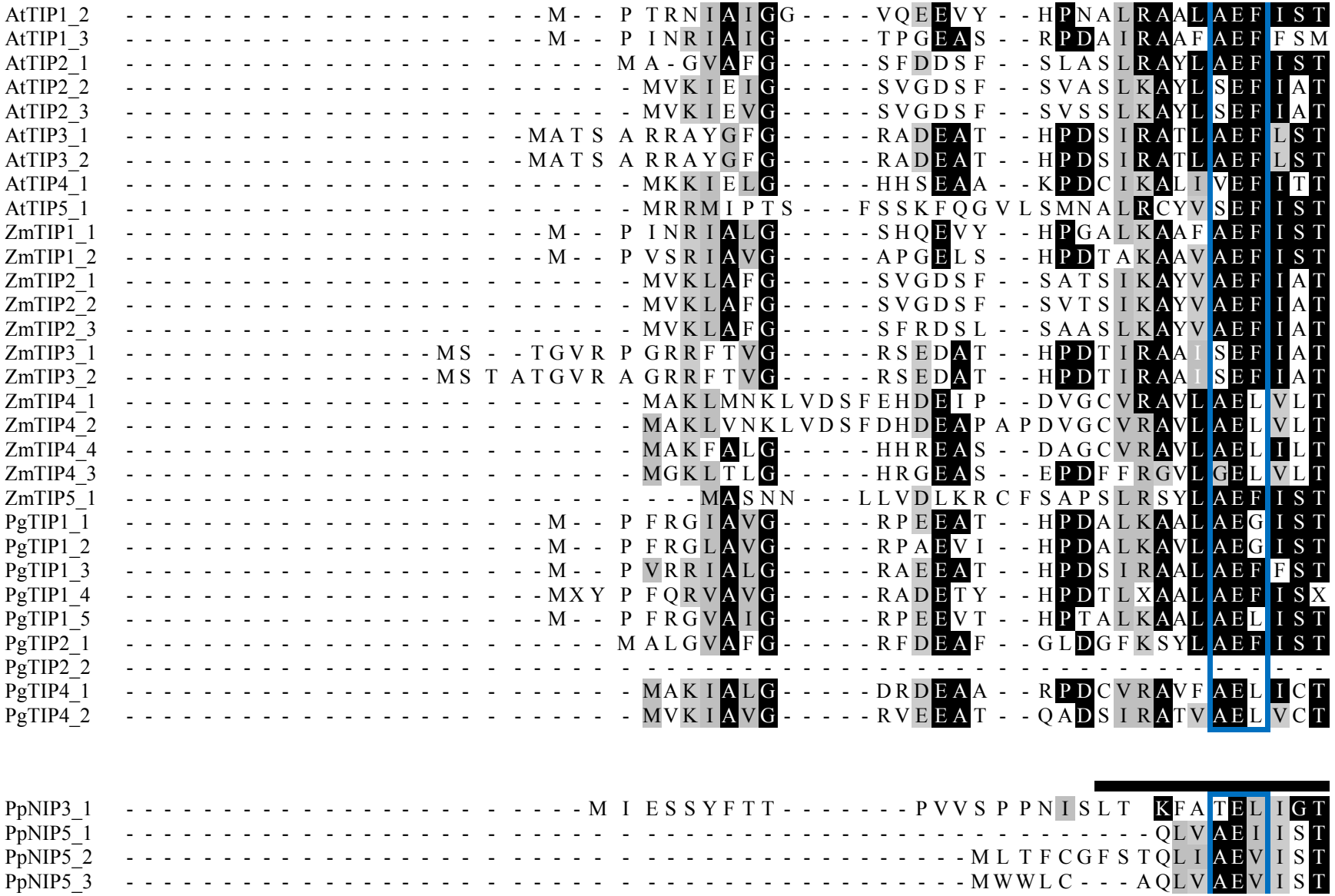
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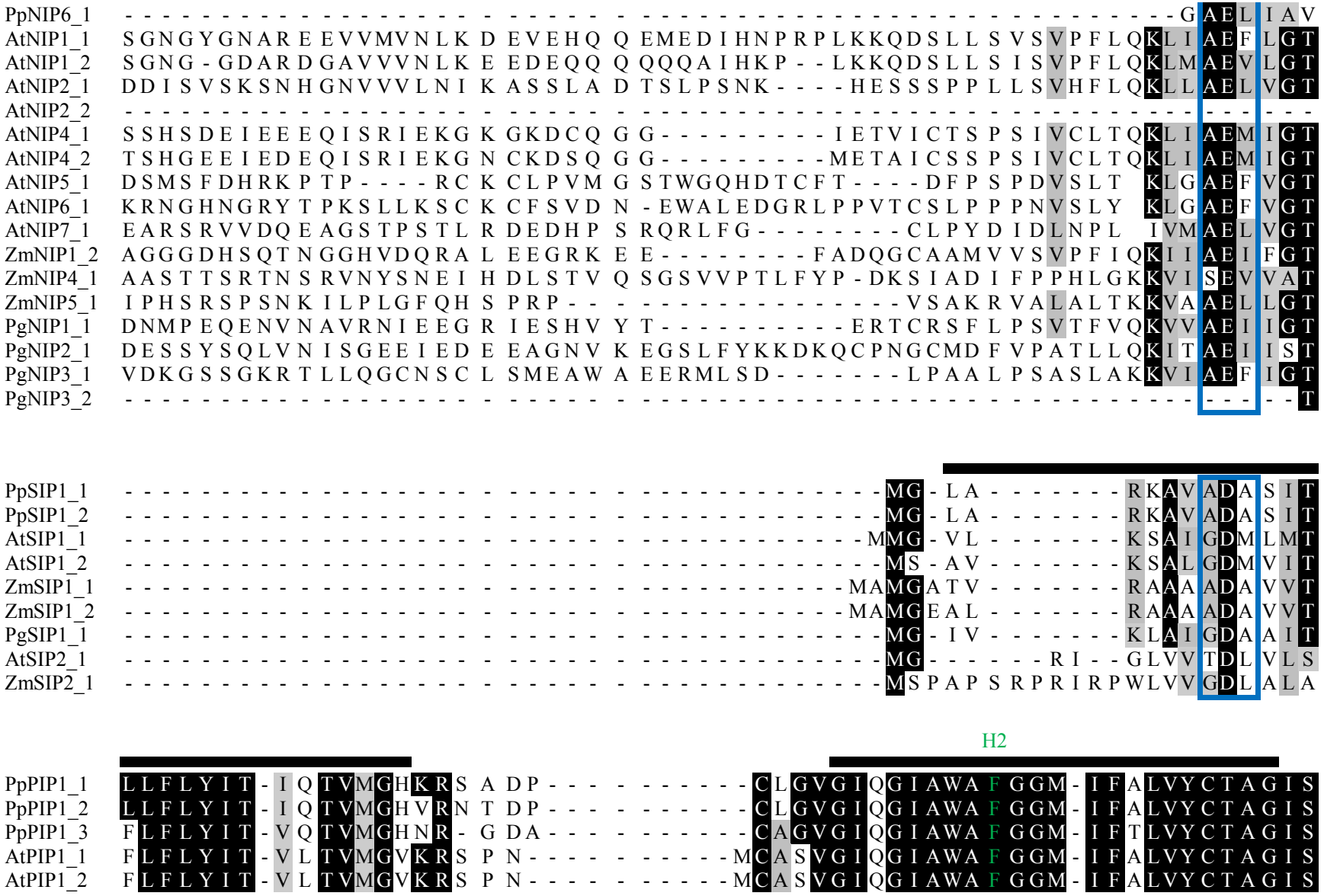
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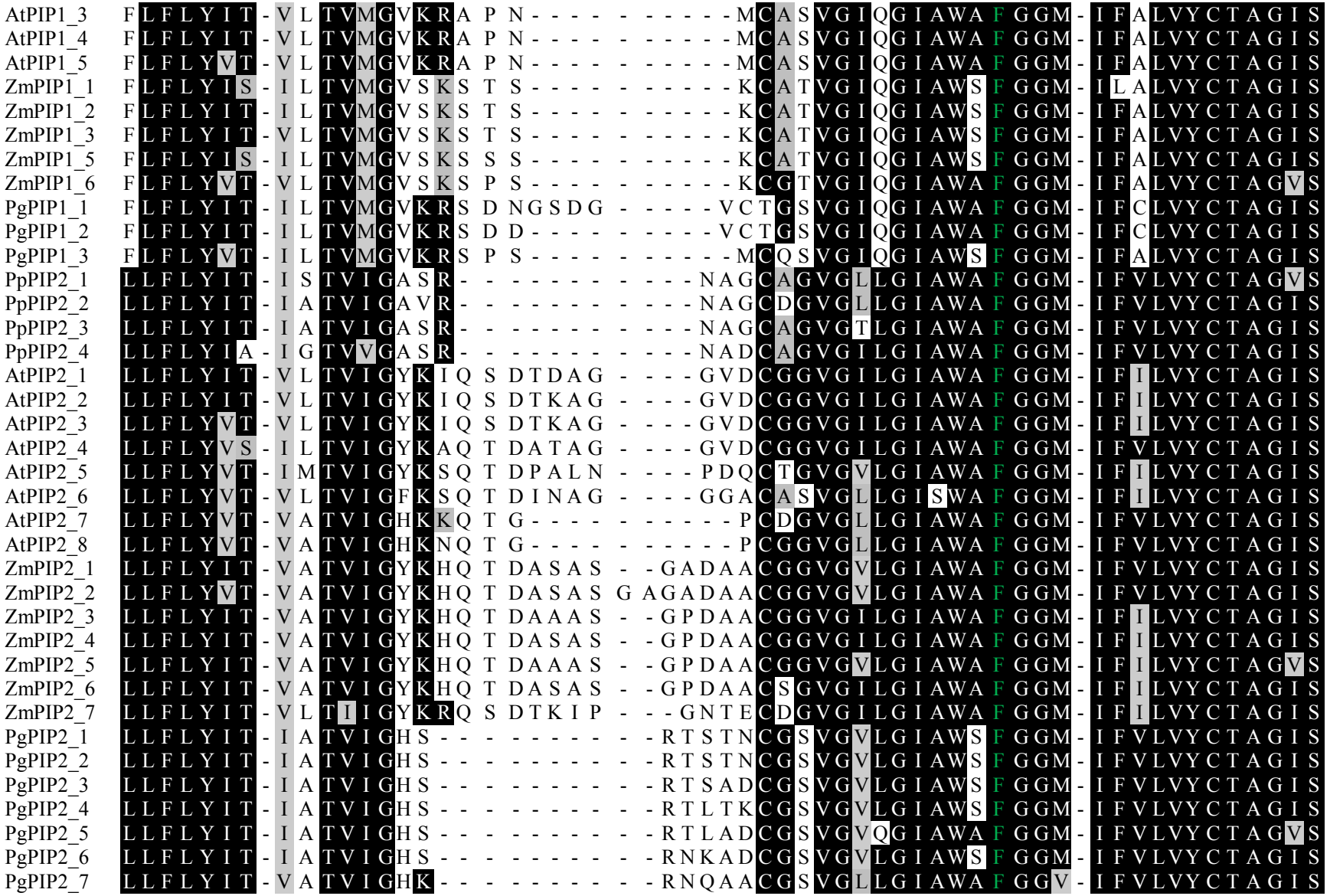
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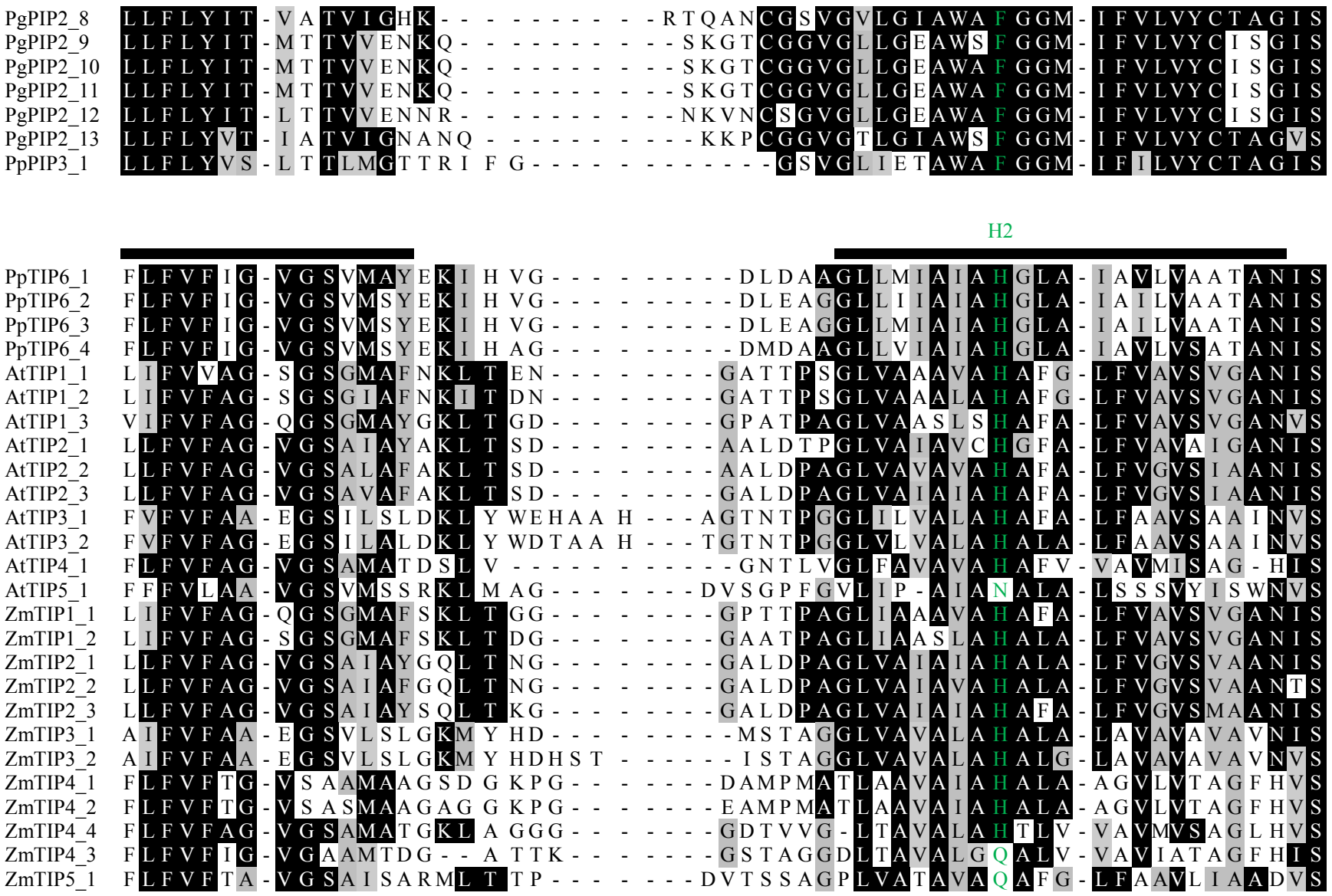
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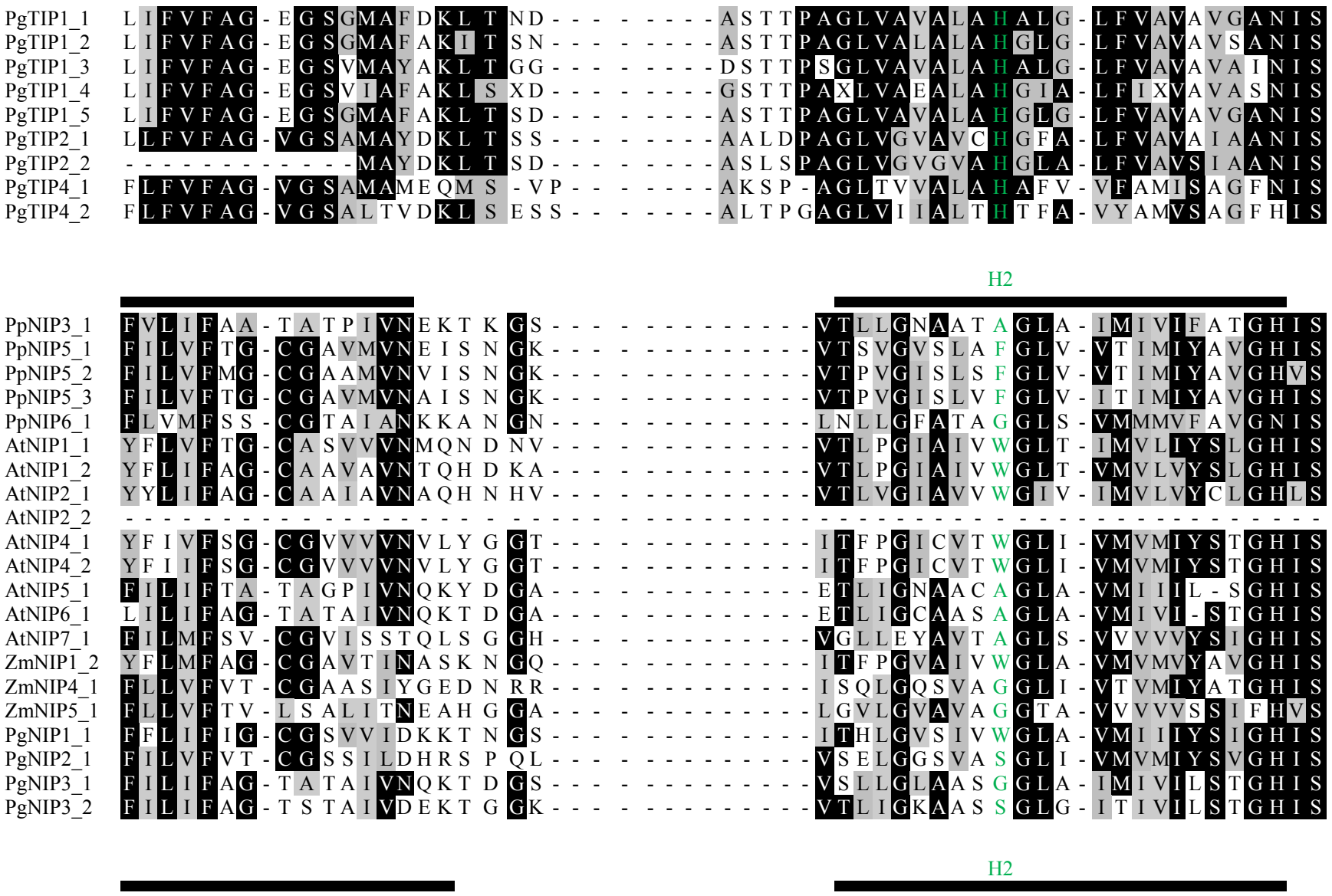
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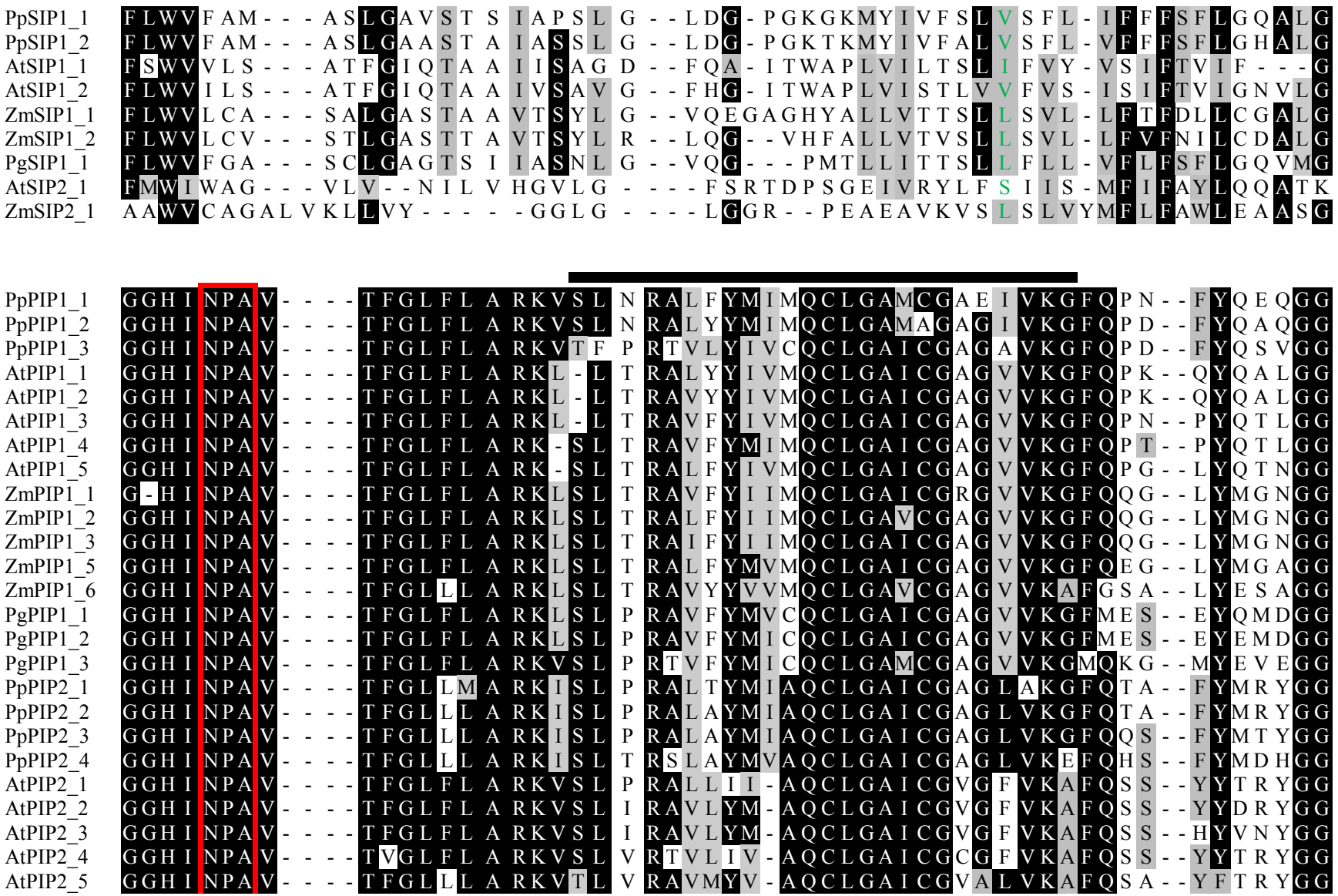
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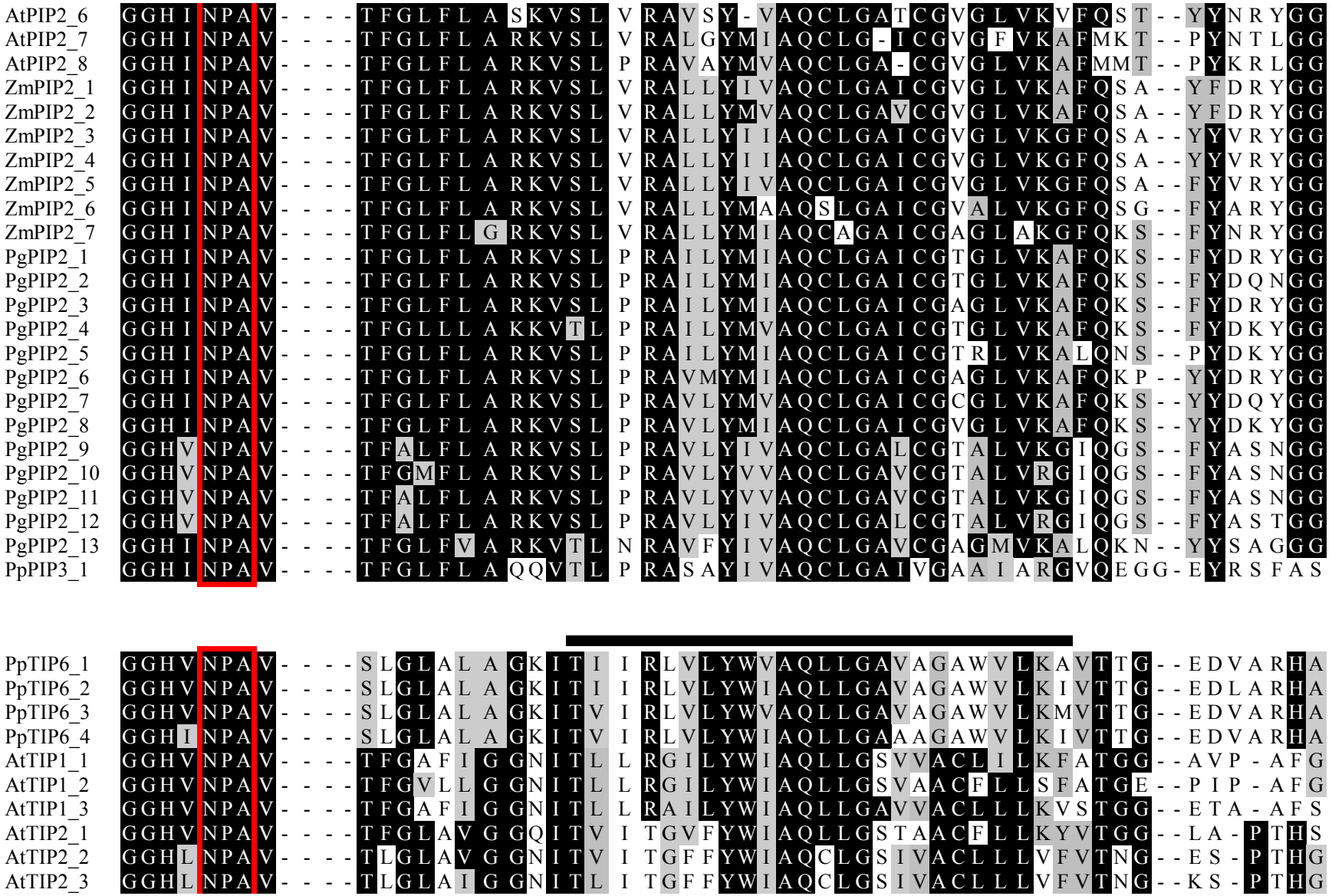
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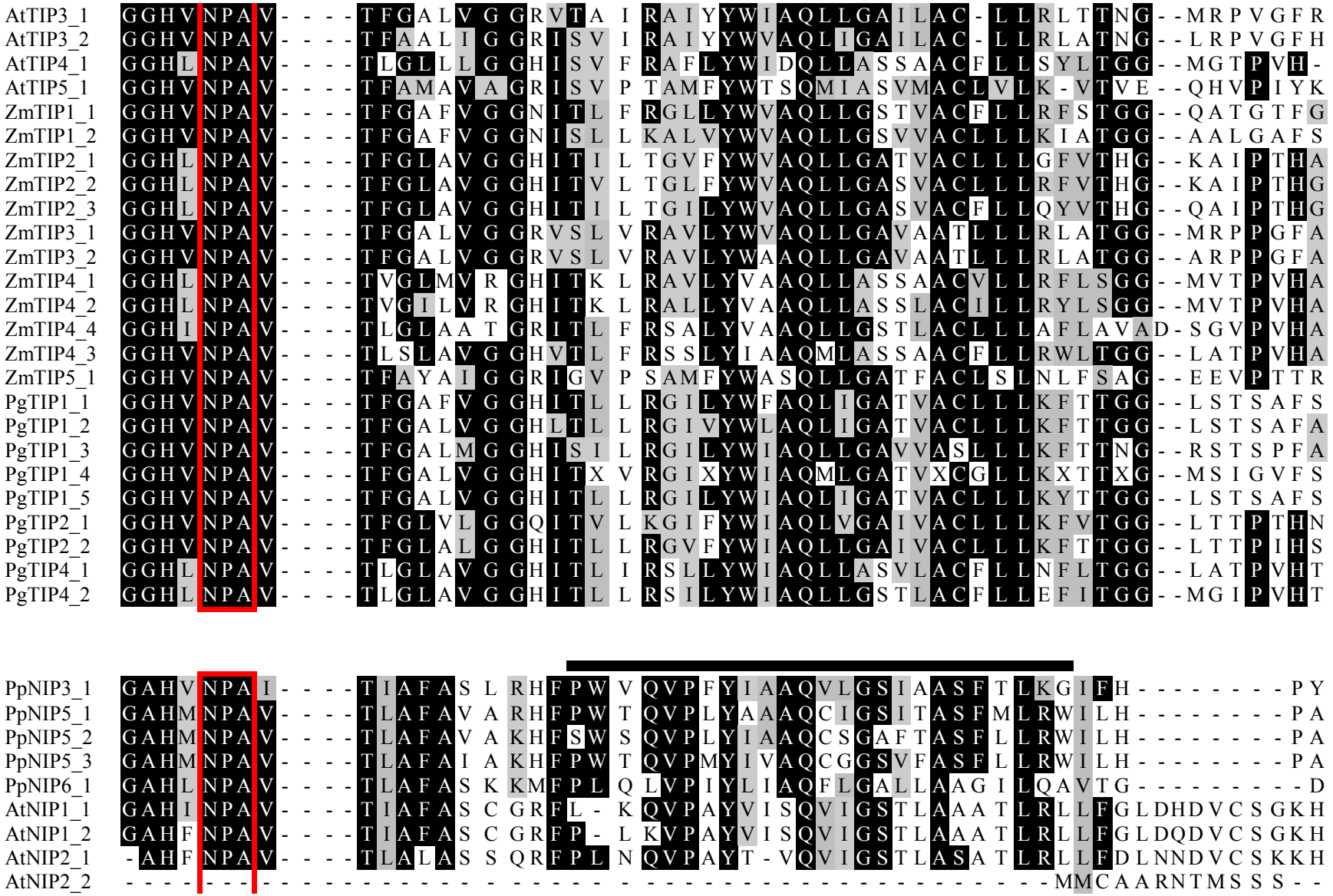
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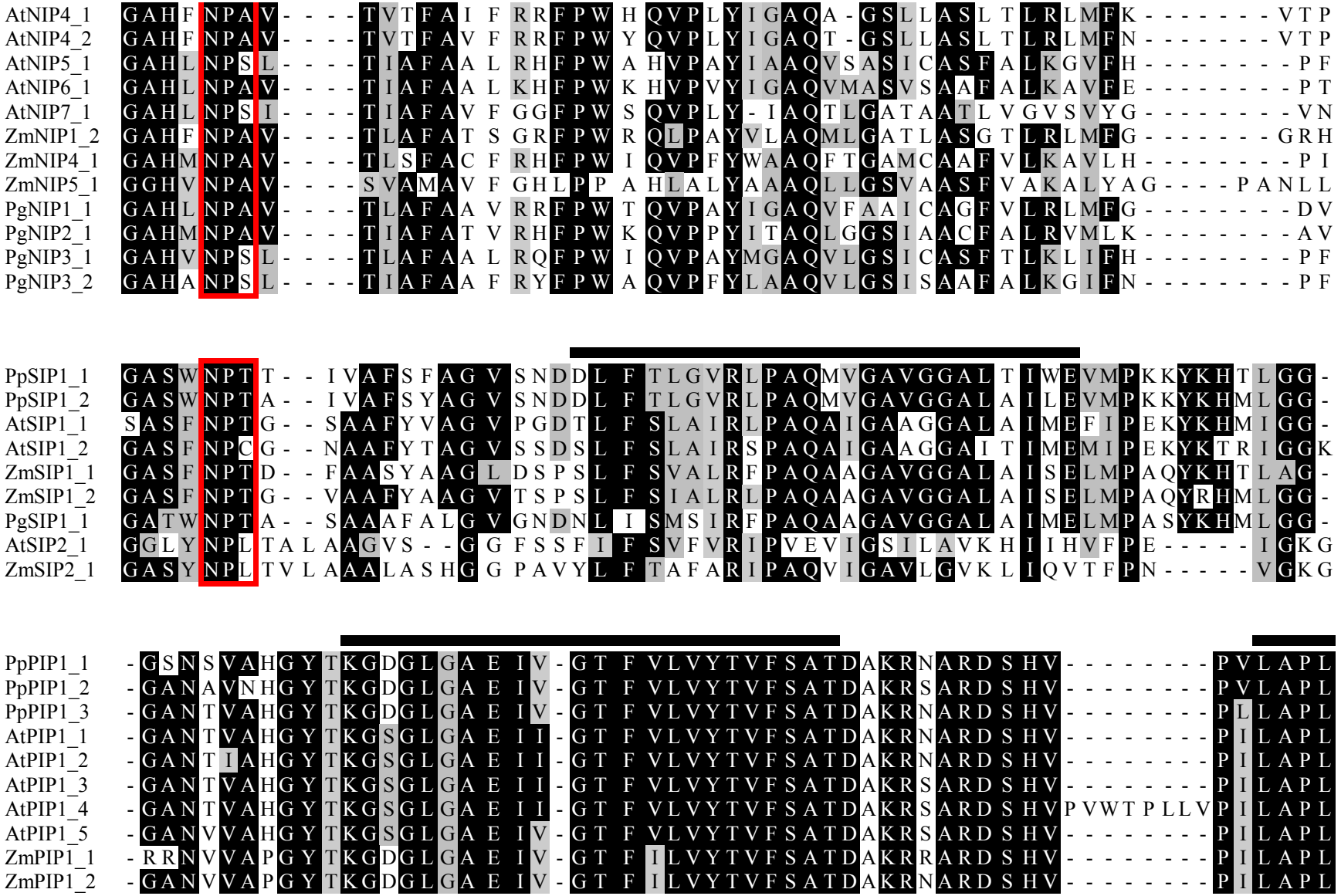
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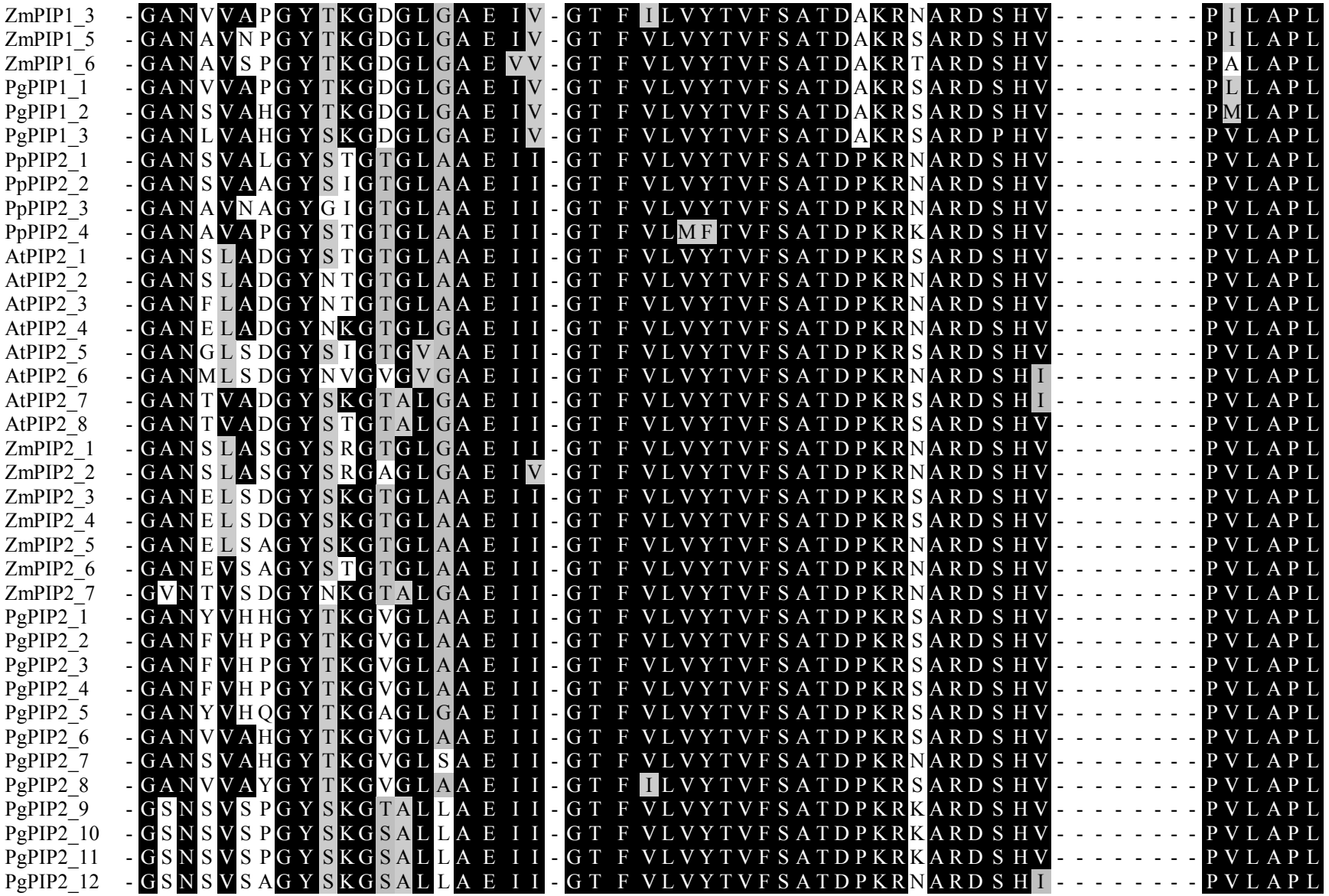
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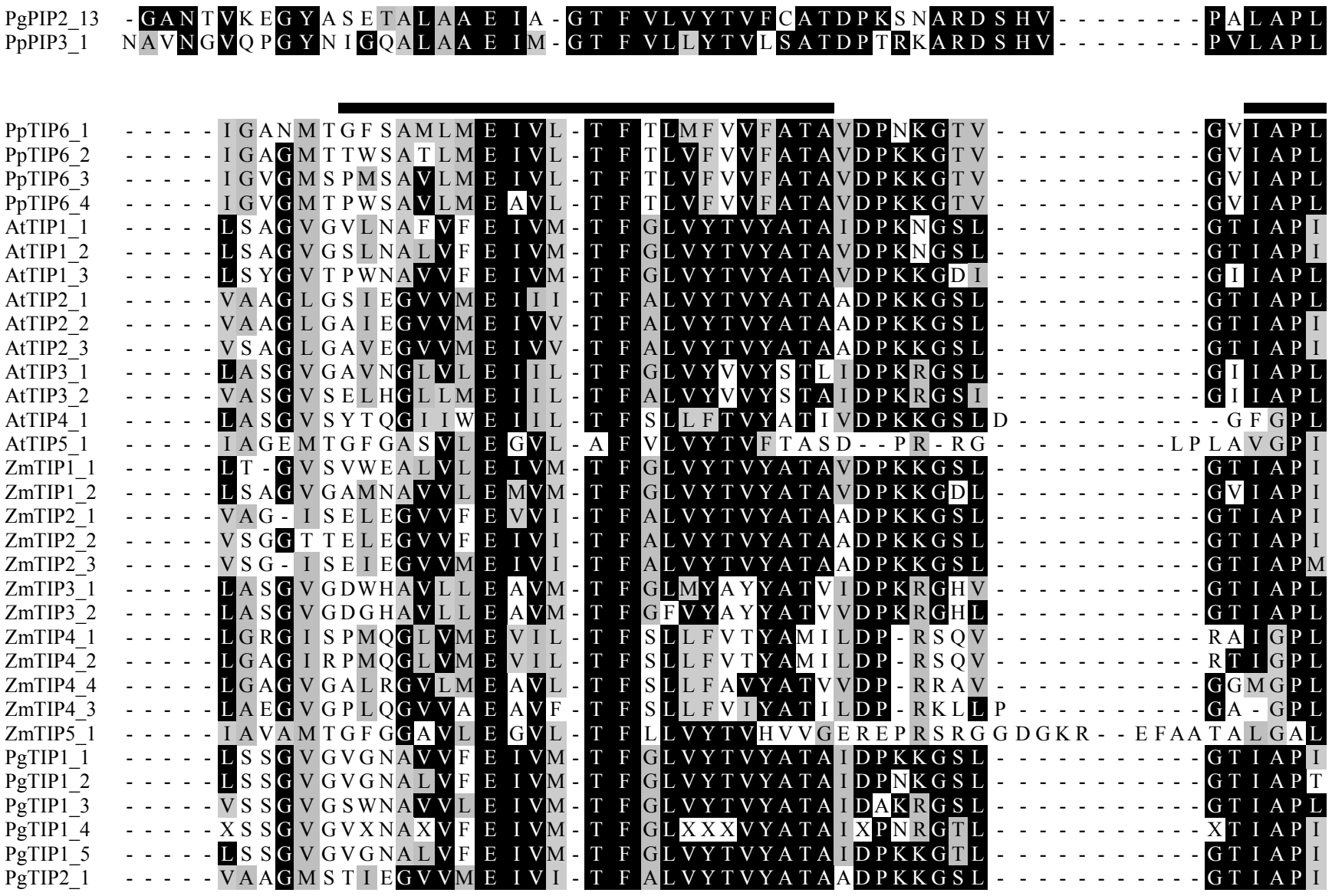
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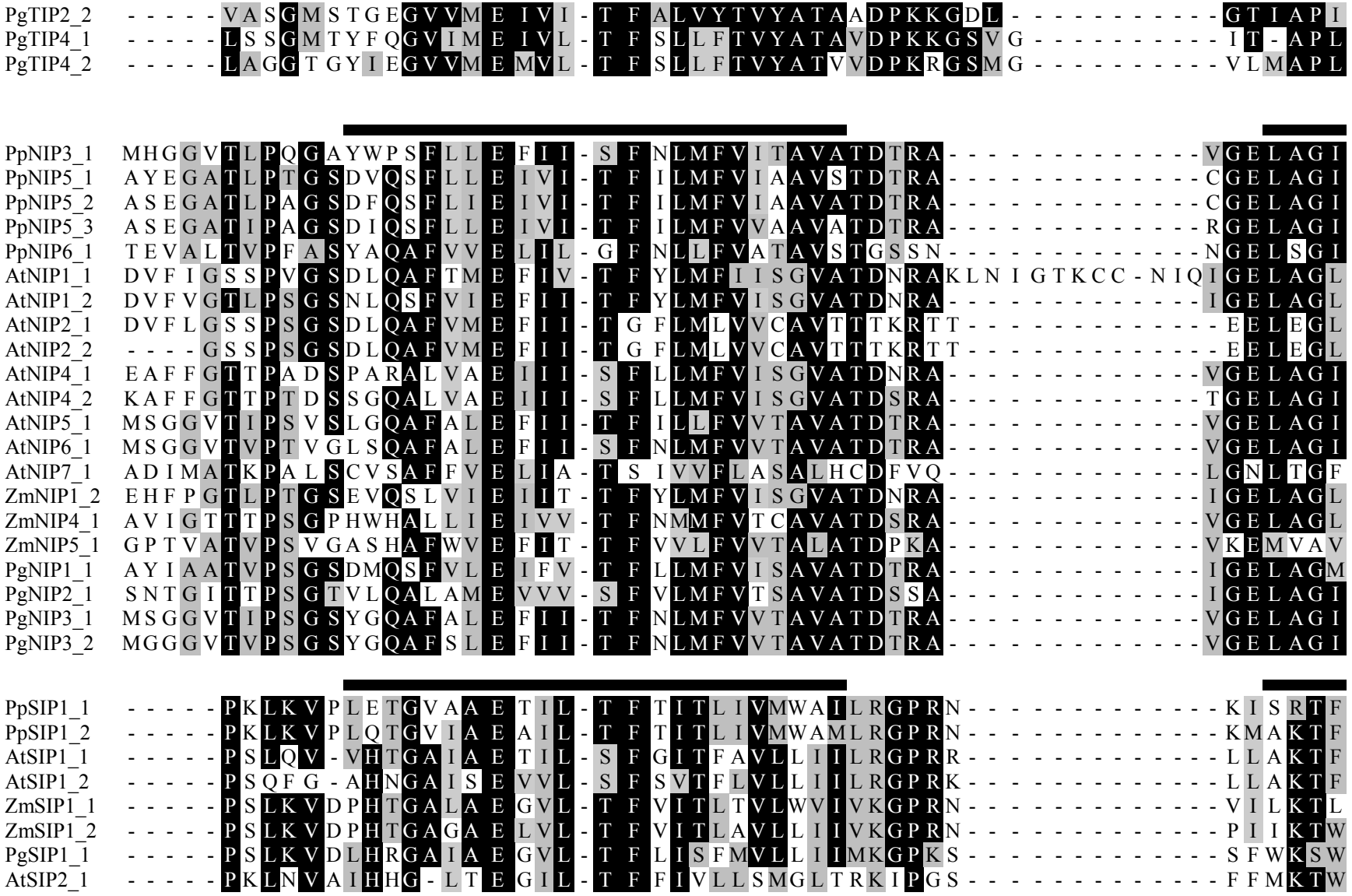
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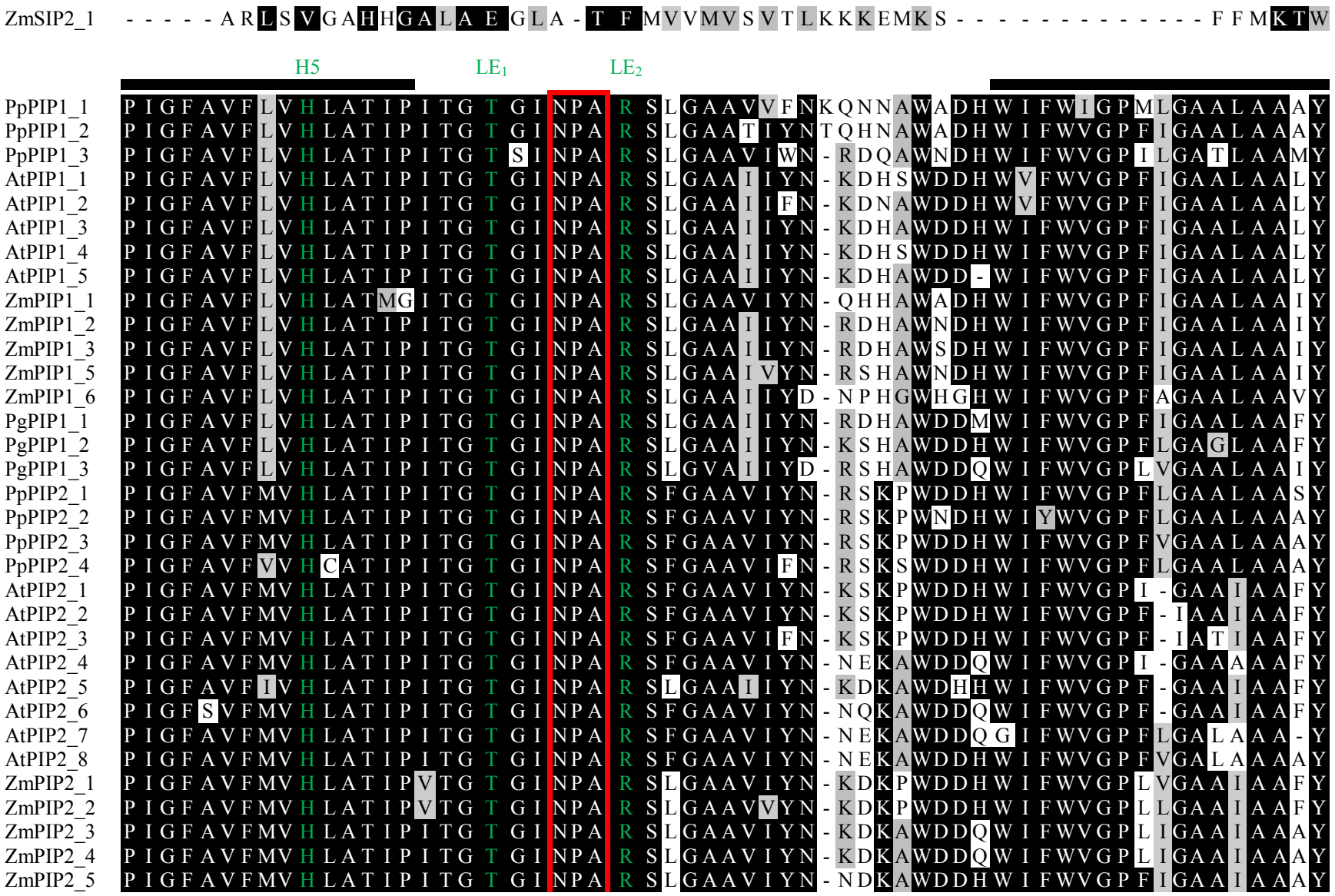
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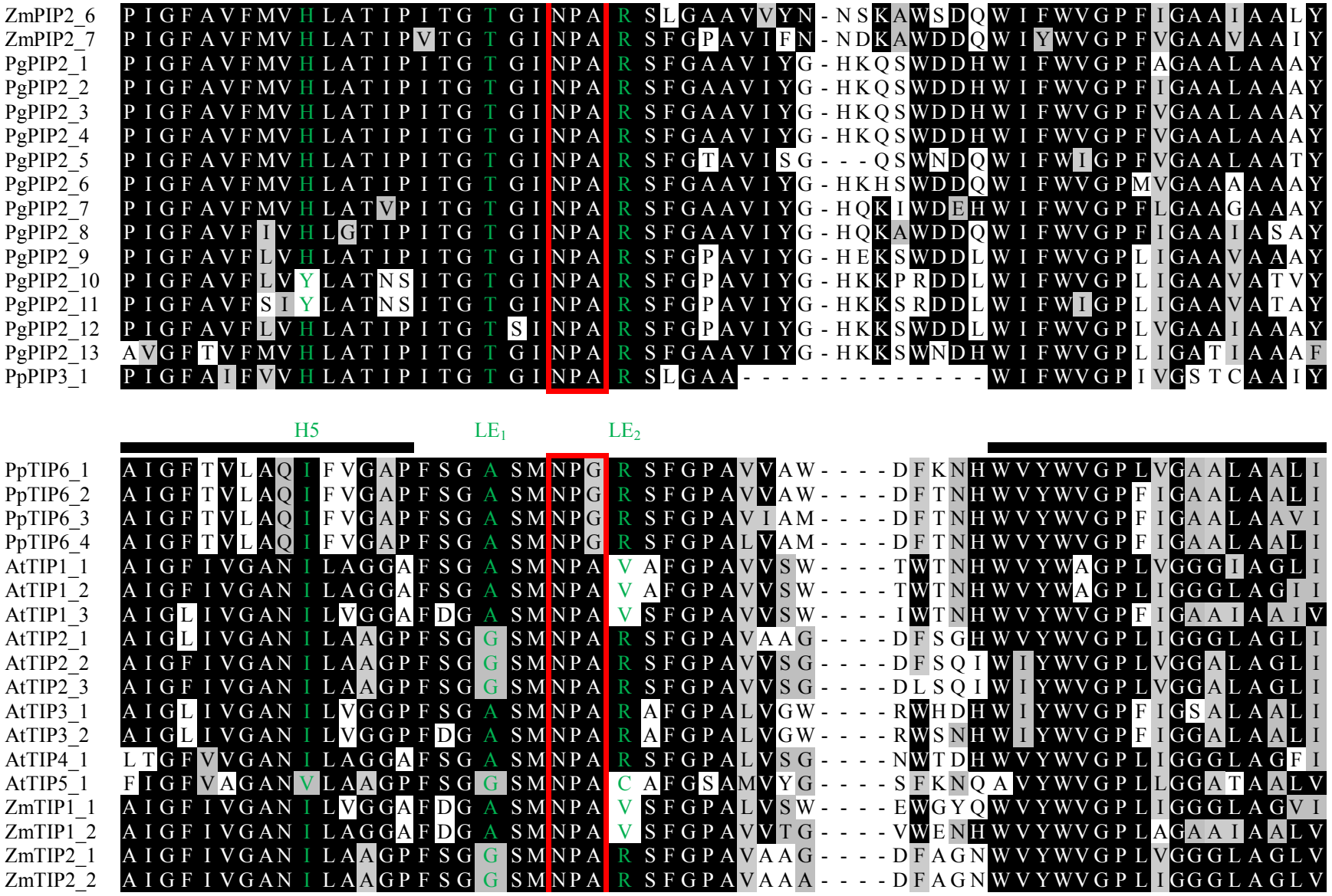
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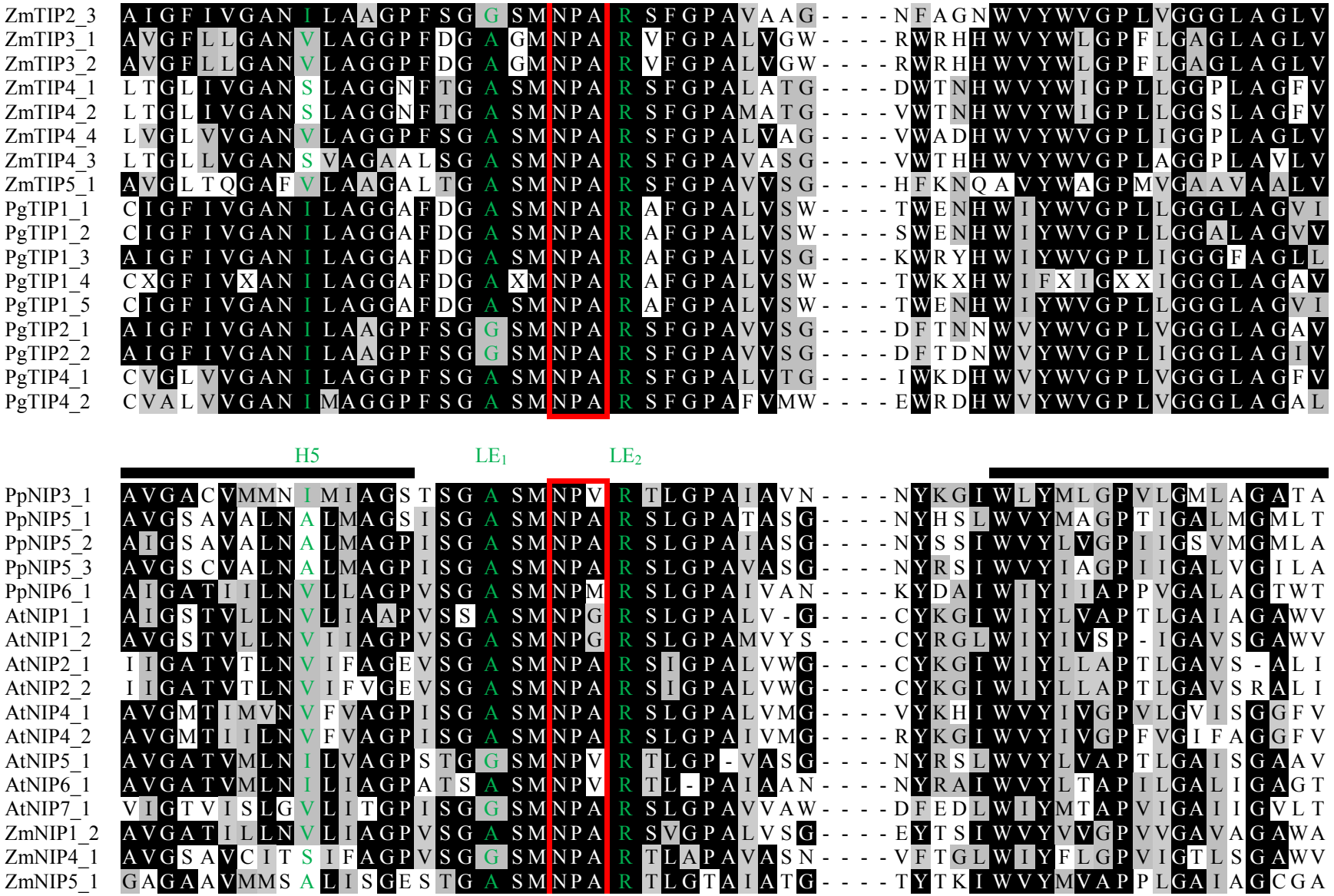
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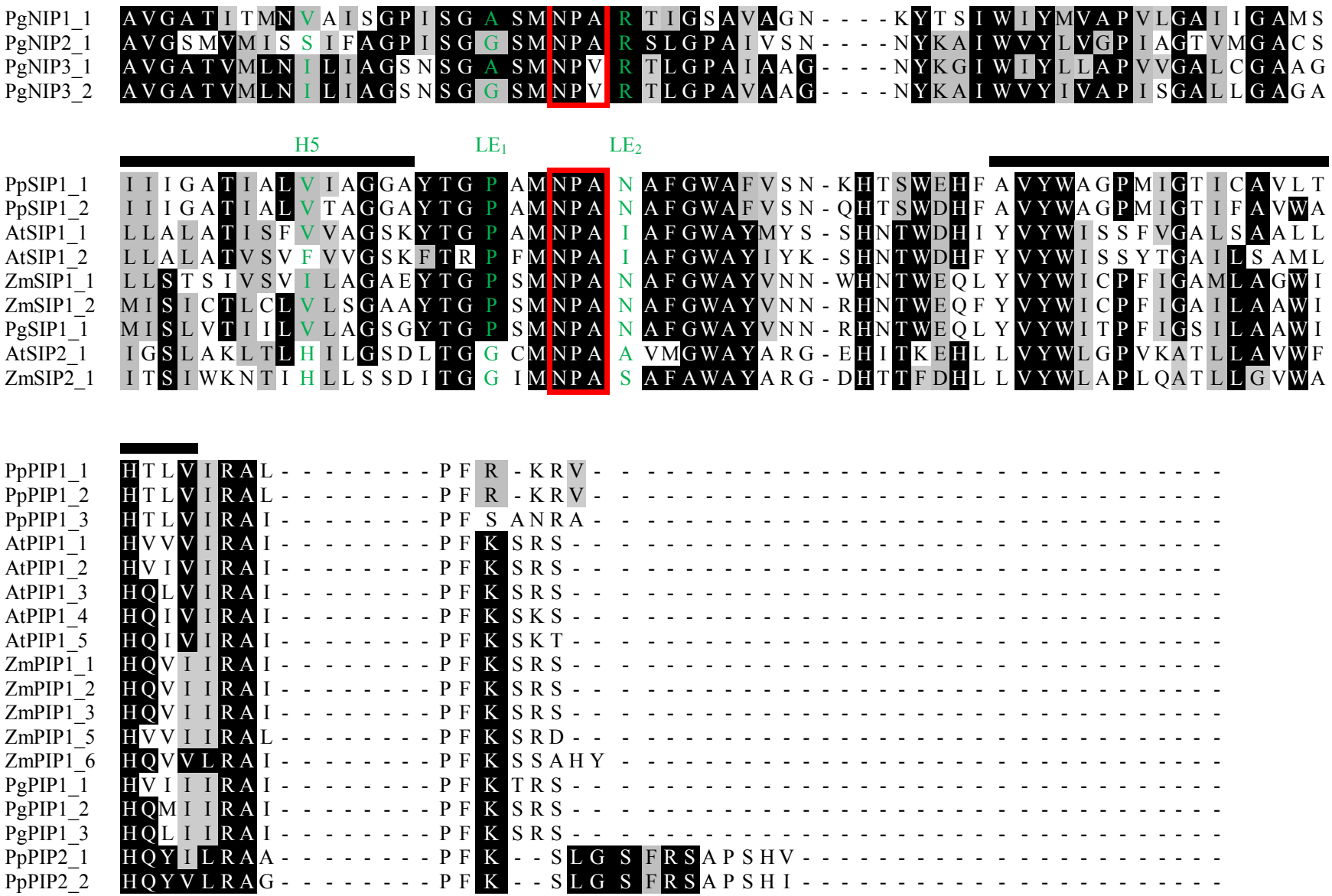
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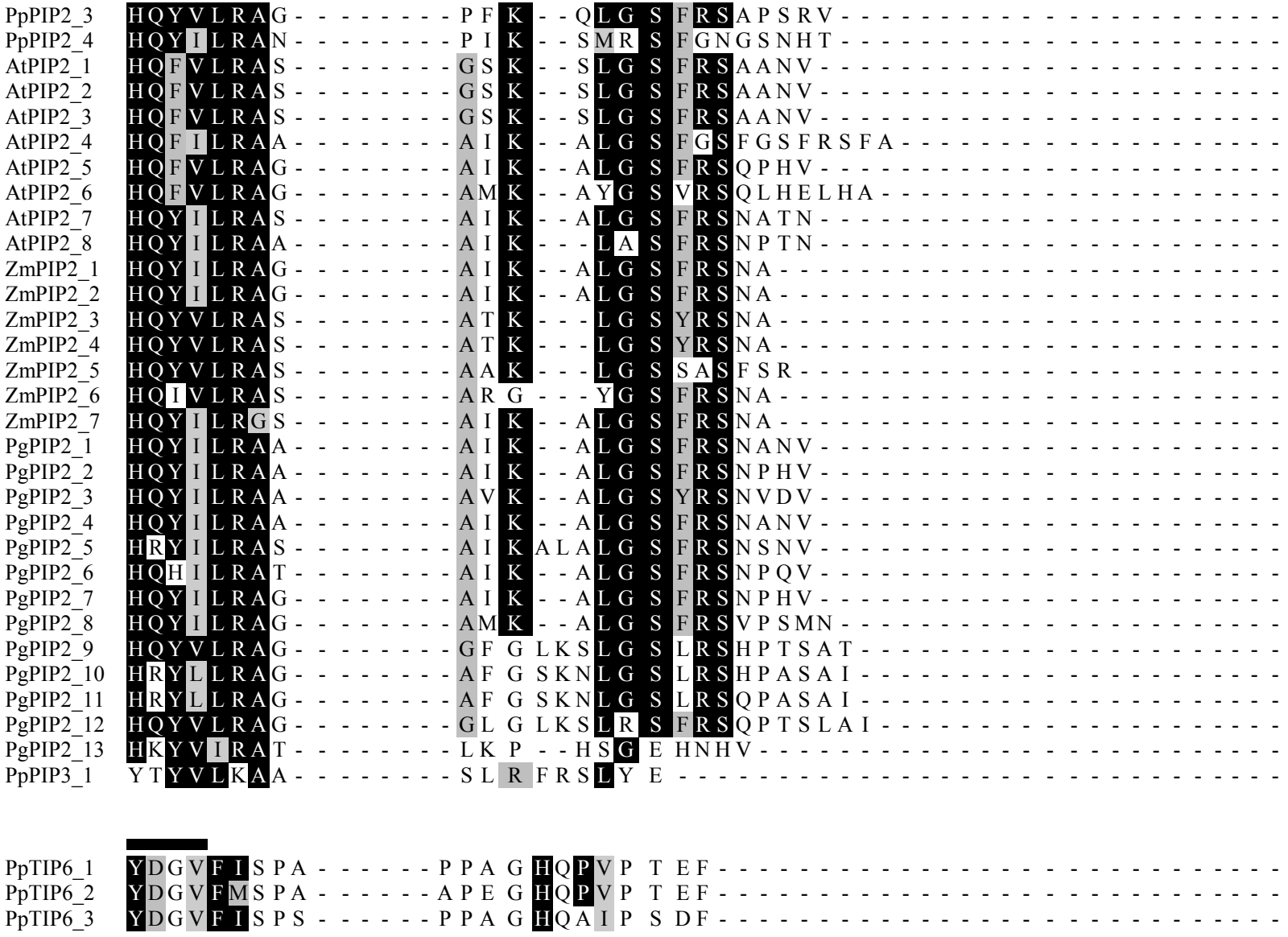
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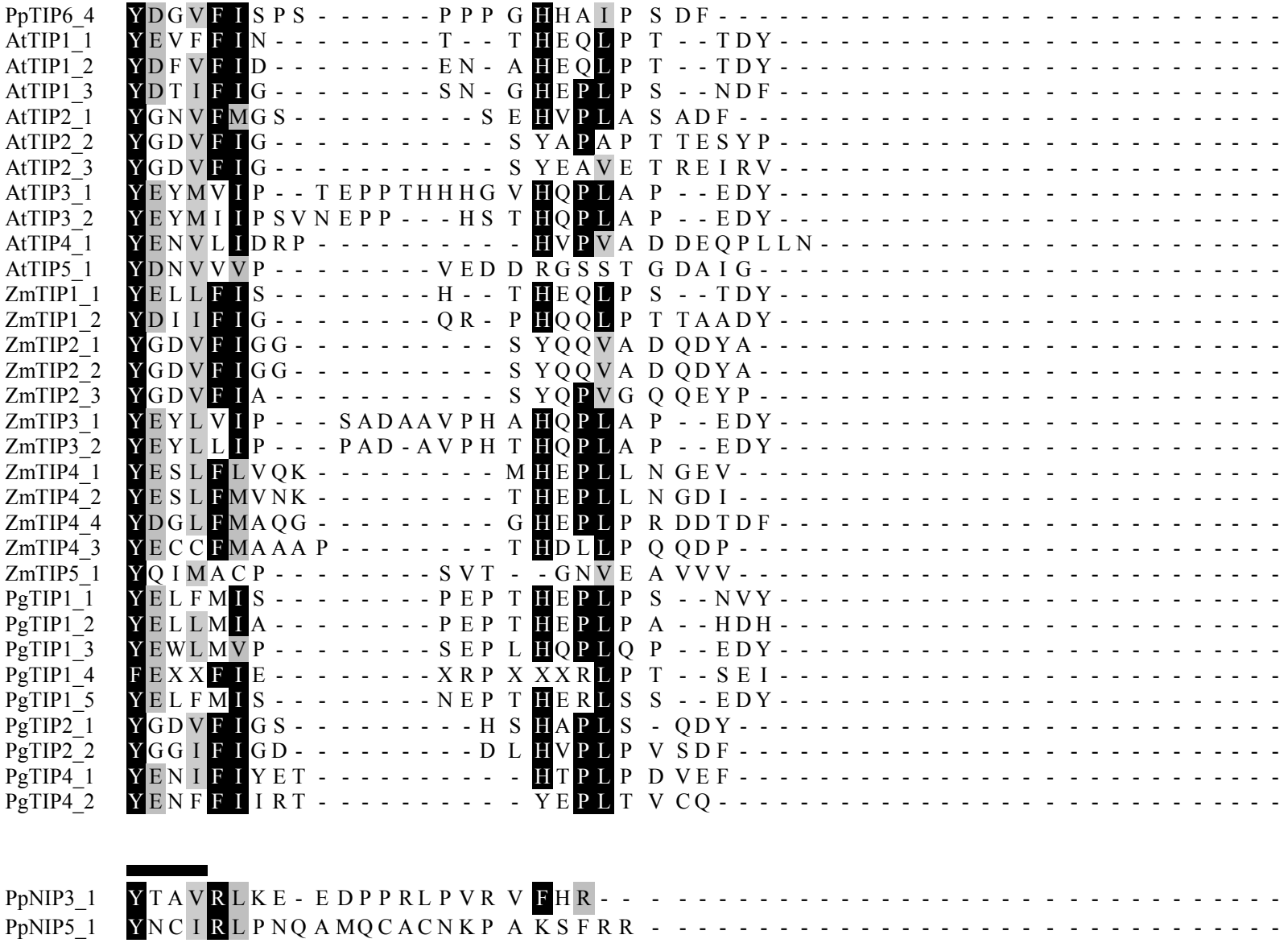
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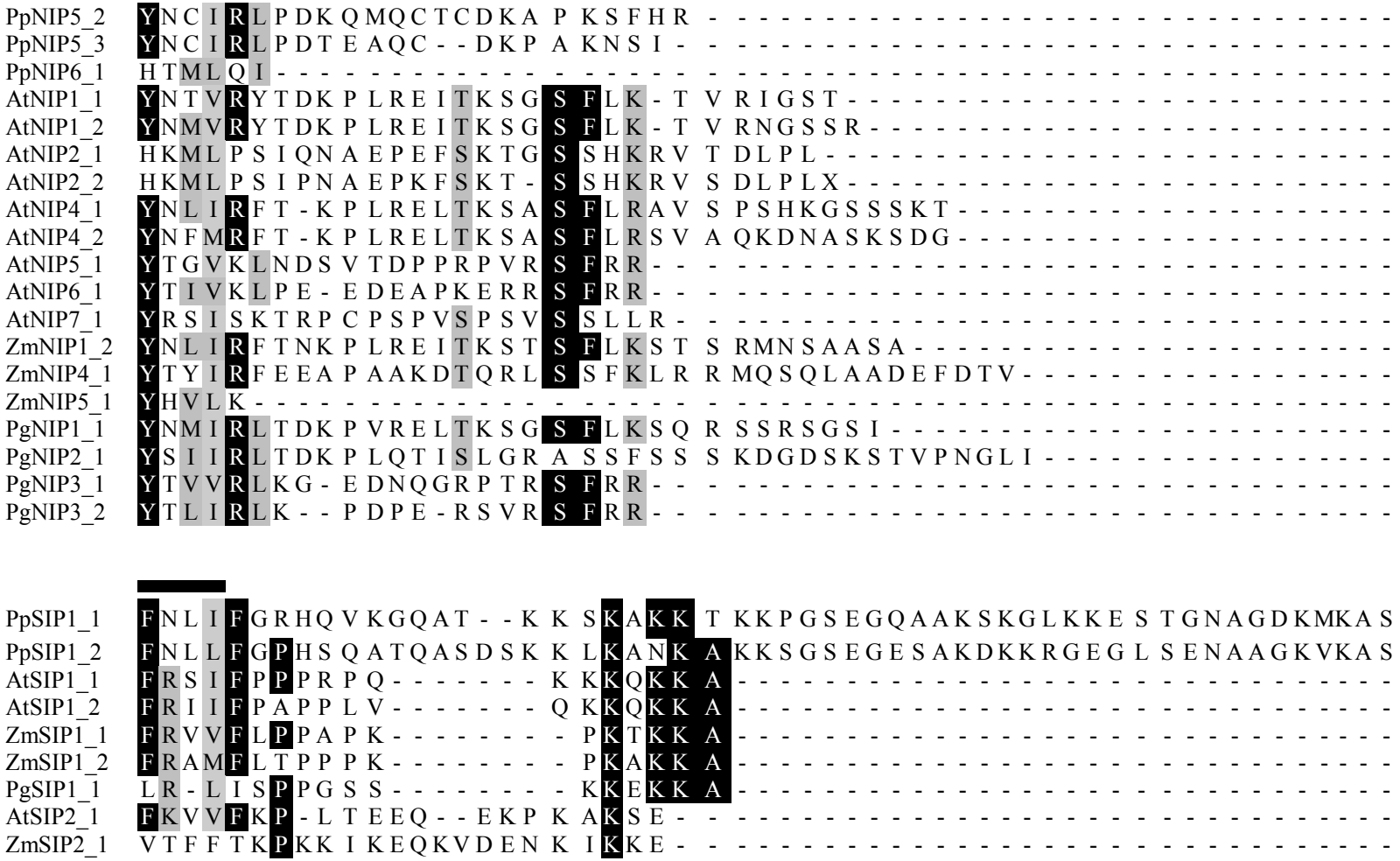
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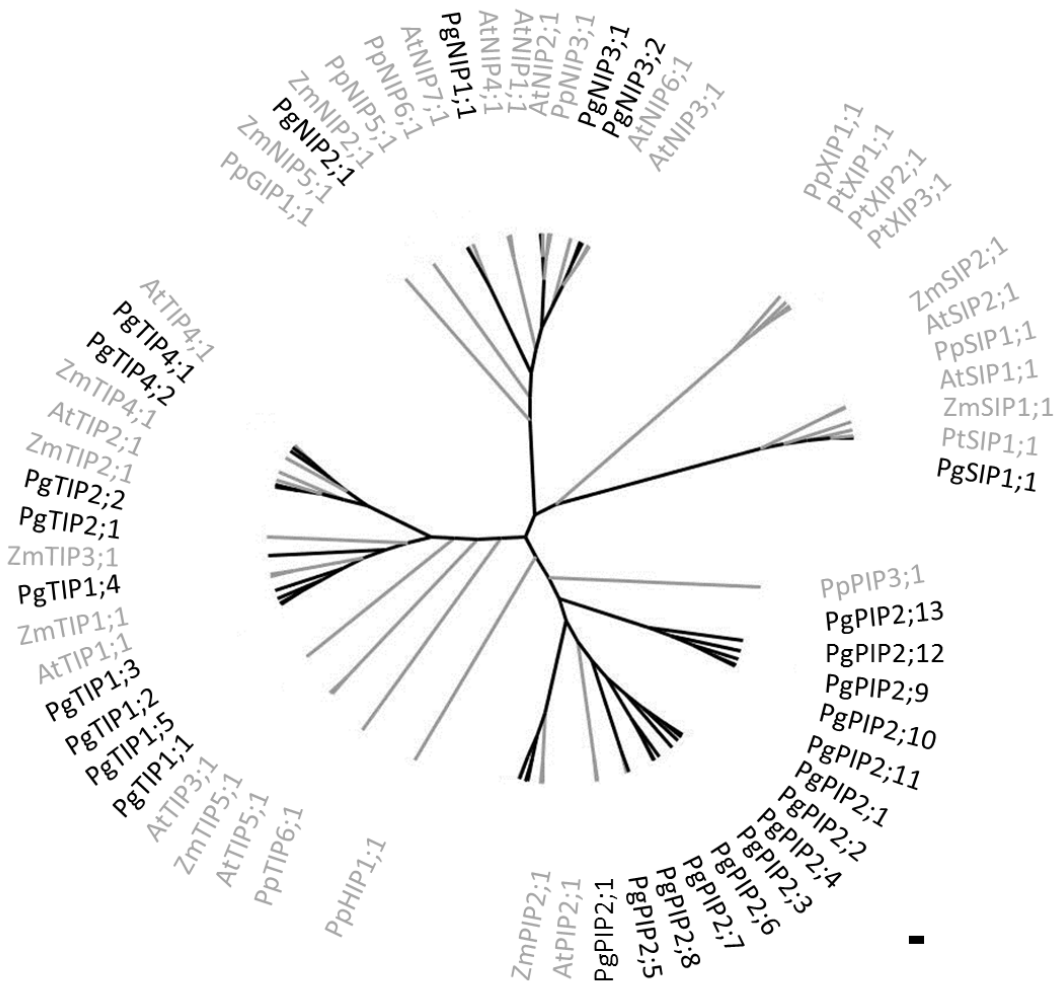

Figure 4-S2. Neighbor-joining phylogeny of *Picea glauca* MIP proteins.

An unrooted neighbour-joining tree showing the phylogenetic relationship of the complete set of different MIP sequences from *Picea sp.* in black and representative MIPs from *A. thaliana* (At), *Z. mays* (Zm), *P. patens* (Pp) and *P. trichocarpa* (Pt) in gray. Seven subfamilies are present, but note that the XIP, HIP and GIP subfamilies have not been found in *Picea sp*. The bar indicates the mean distance of 0.1 changes per amino acid residue.

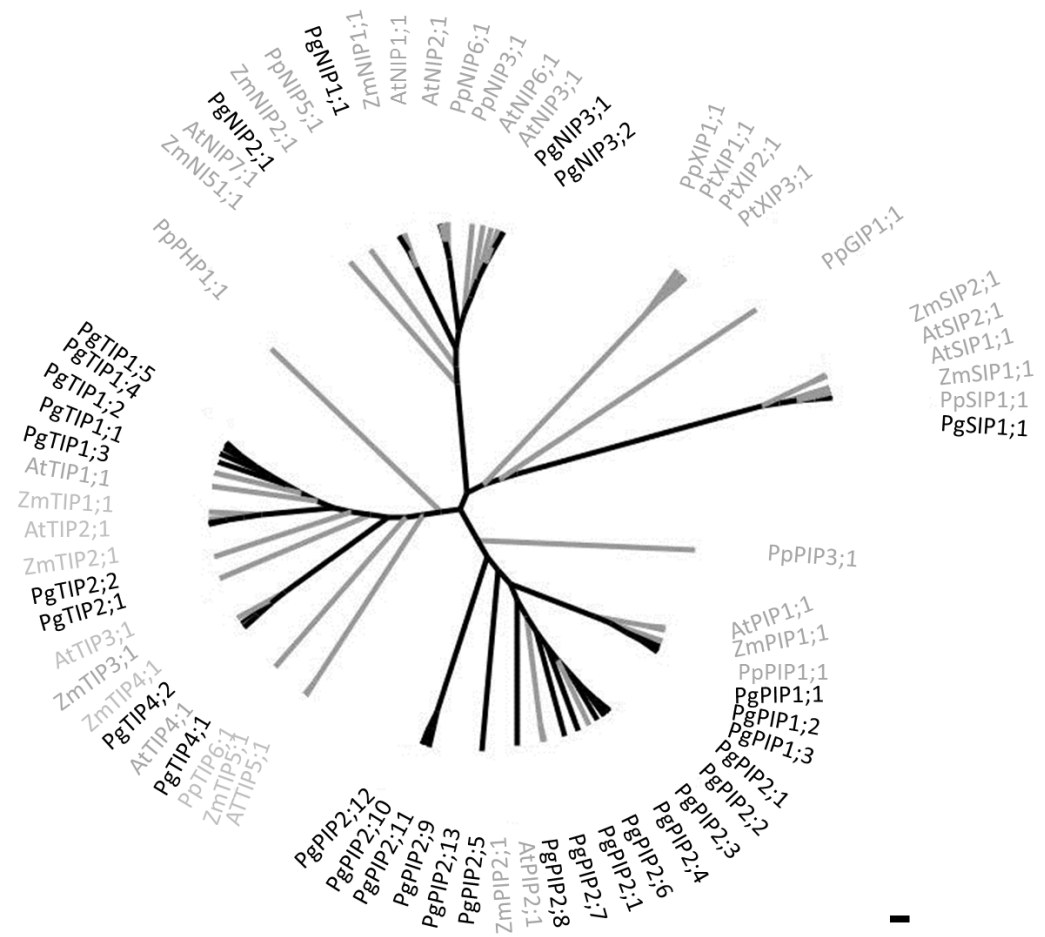

Figure 4-S3. UPGMA phylogeny of *Picea glauca* MIP proteins.

An unrooted UPGMA tree showing the phylogenetic relationship of the complete set of different MIP sequences from *Picea sp.* in black and representative MIPs from *A. thaliana* (At), *Z. mays* (Zm), *P. patens* (Pp) and *P. trichocarpa* (Pt) in gray. Seven subfamilies are present, but note that the XIP, HIP and GIP subfamilies have not been found in *Picea sp*. The bar indicates the mean distance of 0.1 changes per amino acid residue.

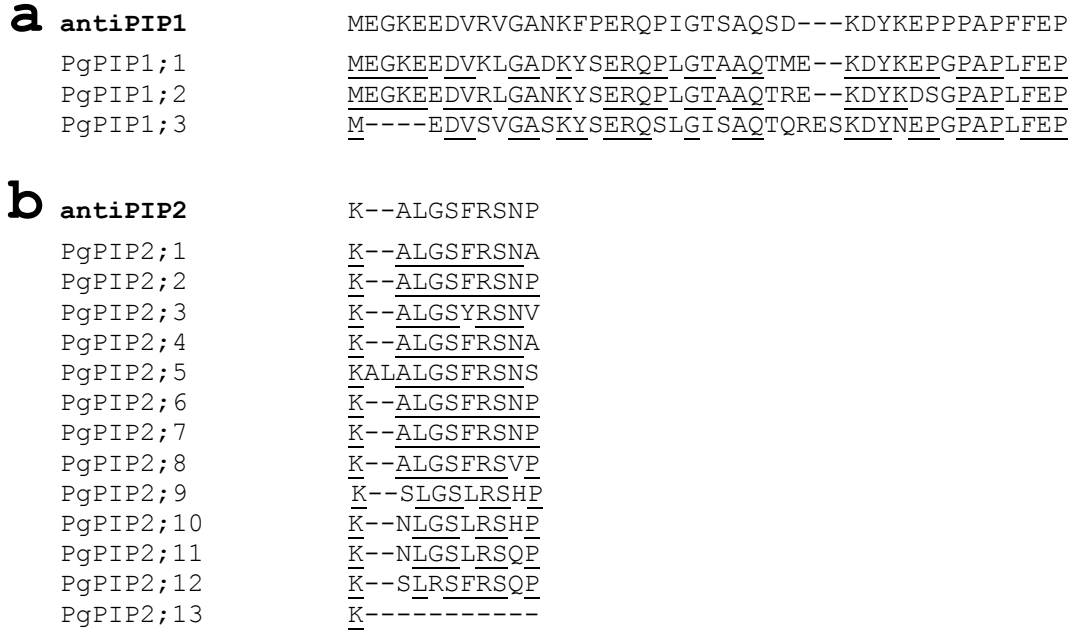

Figure 4-S4. Amino acid multiple sequence alignment of the N-terminal region of *Arabidopsis thaliana* AtPIP1;3 and the *Picea glauca* PgPIP1s (a) and of the highly conserved 10 amino acids of the C-terminal region of PIP2s (b). Consensus amino acids are underlined in black.