Role of Aquaporins in Plant Responses to Oxygen Deprivation

by

Xiangfeng Tan

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Abstract

Oxygen deprivation (hypoxia and anoxia) is an environmental stress affecting plants worldwide in many natural and man-made ecosystems. Although abundant literature exists describing plant responses to root hypoxia, little is known about the functional significance of aquaporins in plants suffering from low O_2 conditions. Aquaporins exhibit multiple physiological functions in challenging environments. The central role of aquaporins in plant water relations has been widely acknowledged, but the role of aquaporins under O_2 deficiency remains elusive. Growing evidence has shown that aquaporins are involved in pore-mediated gas trans-membrane movement. In this thesis, *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum* L.) and trembling aspen (*Populus tremuloides* Michx.) seedlings were studied to shed more light on the responses of aquaporins to root hypoxia. Oxygen transport through aquaporins was also examined in yeast heterologously expressing aquaporins from plant, human and fungal organisms.

Formation of adventitious roots in all plant species is a common response to hypoxia caused by flooding. In tobacco, after one week of root hypoxia treatment, plants produced twice as many adventitious roots as the aerated plants, but their maximum length was reduced. Hypoxia severely reduced net photosynthesis, transpiration rates, and photosynthetic light responses. Relative transcript abundance

of the examined aquaporins in lateral roots was reduced by hypoxia, but in adventitious roots it remained unchanged. This apparent lack of an effect of root hypoxia on the aquaporin expression likely contributed to maintenance of high hydraulic conductance in adventitious roots. Lateral roots had lower porosity compared with adventitious roots and the expression of the *ACS* (*1-aminocyclopropane-1-carboxylate synthase*) gene was induced in hypoxic lateral roots, but not in adventitious roots, providing additional evidence that lateral roots were more affected by hypoxia compared with adventitious roots. ATP concentrations were markedly lower in both hypoxic lateral and adventitious roots compared with aerated roots, while the expression of fermentation-related genes, *ADH1* (*alcohol dehydrogenase 1*) and *PDC1* (*pyruvate decarboxylase 1*), was higher in lateral roots compared with adventitious roots. Since root porosity was greater in adventitious compared with lateral roots, the results suggest that the improved O_2 delivery and stable root aquaporin expression in adventitious roots were likely the key factors helping flooded tobacco plants maintain high rates of root hydraulic conductance and, consequently, shoot gas exchange.

Effects of ethylene on hypoxia tolerance in plants are little understood. In this thesis research, I examined the physiology and gene expression of several aquaporins in aspen (*Populus tremuloides*) seedlings exposed to root hypoxia and treated with exogenous ethylene. Ethylene enhanced net photosynthesis, light saturation level for net photosynthesis, transpiration rates, and root hydraulic conductance in hypoxic

plants. Root O_2 concentrations increased in the hypoxic plants treated with ethylene compared to the hypoxic plants that were not subjected to ethylene treatment. The effect of ethylene on root O_2 content could be either due to the enhanced net photosynthesis or facilitated O_2 transport from shoots. Ethylene triggered the expression of *PIP2;4* aquaporin in hypoxic aspen which could potentially help facilitate root water transport. The enhanced root water transport by ethylene could be also partly responsible for the increase in net photosynthesis of the hypoxic plants which, in turn, could enhance root O_2 supply. A possibility that this effect may involve an enhancement of aquaporin-mediated O_2 transport from shoots to roots by ethylene deserves further attention.

Arabidopsis seedlings were subjected to waterlogging and compared with well-aerated plants. Photosynthesis and transpiration were inhibited by waterlogging. Waterlogging increased the relative transcript abundance of *ADH*, *PDC-1*, and *NIP2;1* in plants. After 24 and 48 hours of waterlogging, relative transcript abundance of most of the *PIP*s decreased, while *PIP1;1* and *PIP1;4* in waterlogged roots exhibited significantly higher relative transcript abundance compared with aerated roots suggesting that they may play a role in plant resistance mechanisms under hypoxic conditions.

O_2 permeability of various human, plant, and fungal aquaporins was examined by co-expressing heterologous aquaporin and myoglobin in yeast. Two of the most promising O_2-transporters (*Homo sapiens* AQP1 and *N. tabacum* PIP1;3) were

confirmed to facilitate O_2 transport in further spectrophotometric assay using yeast protoplasts. The over-expression of *Nt*PIP1;3 in yeasts significantly increased their O_2 uptake rates in suspension culture. In *N. tabacum* roots subjected to hypoxic conditions in hydroponic culture, it demonstrated a sharp increase in the relative transcript abundance of the O_2-transporting aquaporin *Nt*PIP1;3 after seven-day hypoxia treatment, accompanied by the increase in root ATP levels, particularly in the apical root segments. The results show that the functional significance of aquaporin-mediated O_2 transport should be considered in further studies and that the possibility of controlling the rate of transmembrane O_2 transport should be further explored.

Keywords: Adventitious roots, aquaporins, ethylene, flooding, hydraulic conductance, hypoxia, hypoxia responsive genes, oxygen transport, yeast expression system

Preface

This thesis is an original work by Xiangfeng Tan. The work presented in this thesis is part of the research project of Dr. Janusz Zwiazek on Functions of aquaporins in plants. With the exceptions explained below, all experiments were carried out and the data analyzed by Xiangfeng Tan under the supervison of Dr. Zwiazek.

Chapter 2, 3 and 4 are intended for publication. I designed all the experiments with the guidance of Dr. Zwiazek. The data collection and analysis were completed by myself. Dr. Feng Xu and Wenqing Zhang provided me with training on root hydraulic conductance measurements.

Chapter 5 of this thesis has been published as "Zwiazek JJ, Xu H, Tan X, Navarro-Ródenas A, Morte A (2017) Significance of oxygen transport through aquaporins. Sci Rep 7: 40411". All authors designed the experiments. The yeast strains used in this chapter were transformed by Dr. Hao Xu. I performed the experiments with the guidance of Dr. Zwiazek and with the help of Dr. Hao Xu.

Figure 6.1 in Chapter 6 was reproduced from "Tan X, Xu H, Khan S, Equiza MA, Lee SH, Vaziriyeganeh M, Zwiazek JJ (2018) Plant water transport and aquaporins in oxygen-deprived environments. J Plant Physiol 227: 20–30" with the permission of authors.

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ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
ACO	1-aminocyclopropane-1-carboxylate oxidase
ACS	1-aminocyclopropane-1-carboxylate synthase
ADH	alcohol dehydrogenase
AR	adventitious root
Chl	chlorophyll
E	transpiration
ERF	ethylene response factor
FAA	formaldehyde-acetic acid- ethanol
GA_3	gibberellic acid
GIP	GlpF-like intrinsic protein
g_s	stomatal conductance
HIF	hypoxia-inducible transcription factor
HIP	hybrid intrinsic protein
HPFM	high–pressure flow meter
HRE	hypoxia responsive ethylene response factor
IAA	indole-3-acetic acid
I_c	light compensation point
I_m	light saturation point
K_r	root hydraulic conductance
LDH	lactate dehydrogenase
Lp_r	root hydraulic conductivity
LR	lateral root
MIP	major intrinsic protein
NAA	1-naphthaleneacetic acid

NIP	nodulin 26-like intrinsic protein
NPQ	non-photochemical quenching
O_2	oxygen
PDC	pyruvate decarboxylase
PIP	plasma membrane intrinsic protein
P_m	light saturated P_n
P_n	net photosynthetic
PPFD	photosynthetic photon flux density
PSII	photosystem II
ROS	reactive oxygen species
SIP	small intrinsic protein
TIP	tonoplast membrane intrinsic protein
XIP	uncategorized intrinsic protein
Φ_{PSII}	effective quantum yield

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1 Introduction

1.1 General introduction

Oxygen acts as the final electron receptor in aerobic respiration, which drives the process of ATP synthesis in oxidative phosphorylation that releases energy stored in organic molecules. Plants require continuous access to O_2 to supply sufficient energy for maintenance and growth processes. However, supply of O_2 to roots can be restricted by excess water, soil compaction and other conditions which decrease O_2 diffusion rate into plants (van Dongen and Licausi, 2014). Low levels of O_2 reduce water and nutrient uptake, and growth (Bailey-Serres and Voesenek, 2008). Some plants have developed adaptations to improve O_2 delivery or help them cope with O_2 deficiency. Molecular, metabolic and growth adjustments help plants survive under O_2 deficiency for a certain period of time, while prolonged O_2 deficiency can result in severe irreversible damage to plants (Drew, 1997). Since an inhibition of water uptake and transpiration are among the earliest observed processes in sensitive plants subjected to flooding stress (Bailey-Serres and Voesenek, 2008), plant water transport properties may provide some clues concerning hypoxia resistance mechanisms in plants.

Aquaporins are a group of membrane proteins with multiple biological functions. In addition to acting as water transmembrane transporters and playing a central role in plant water relations, aquaporins are involved in the transport of other small molecules (Maurel et al., 2015). O_2 deficiency leads to cellular acidosis and energy deprivation which affect aquaporins in terms of transcription, posttranslational modification and gating properties (Tournaire-Roux et al., 2003; Maurel et al., 2015). Transmembrane transport of H_2O_2 and lactic acid, which are associated with metabolism under O_2 deficiency, has been demonstrated to be mediated by specific

aquaporins (Bienert et al., 2007; Choi and Roberts, 2007). However, little is known about the responses of aquaporins to hypoxia and the significance of these responses in hypoxia tolerance.

Interestingly, some aquaporin homologs have also been found to mediate the transmembrane transport of gas molecules such as CO_2 (Nakhoul et al., 1998; Uehlein et al., 2003), NH_3 (Jahn et al., 2004) and NO (Herrera et al., 2006). These discoveries suggest the possibility that other gases such as O_2 may also be transported through aquaporins (Echevarría et al., 2007). Due to the fundamental role of O_2 in physiology, the possibility of pore-mediated O_2 transport is exiting. However, compared to other gas molecules, reliable detection methods to measure cellular O_2 fluxes are not available, which limits transmembrane transport assays for O_2.

The studies in this thesis are based on the central hypothesis that aquaporin-mediated O_2 transport plays important roles in plants. Earlier studies have established that regulation of aquaporins plays an important role in response to low O_2. Since low O_2 induced transcription includes an aquaporin homolog (*Arabidopsis thaliana At*NIP2;1) (Liu et al., 2005), it can be hypothesized that through examining the transcription responses of the studied species, hypoxia insensitive genes can be screened for further functional assays. The main objectives of this thesis research were to: (1) examine the effects of hypoxia on aquaporin transcription and screen the hypoxia induced aquaporin genes in the studied species, (2) Eeamine the effects of adventitious root formation and exogenous ethylene on aquaporin transcription under hypoxia, and (3) construct yeast strains heterologously expressing aquaporins from different species and perform functional assays of O_2 transport. Thus, plant physiology and gene expression of tobacco (*Nicotiana tabacum* L.), *Arabidopsis* and trembling aspen (*Populus tremuloides* Michx.) were studied in the Chapters 2, 3, and 4. In the Chapter 5, aquaporins, including the identified hypoxia-insensitive isoforms from tobacco and *Arabidopsis*, were expressed in yeast (*Saccharomyces cerevisiae*) for functional assays of O_2 transport.

1.2 O_2 deficiency (hypoxia and anoxia) in plants

1.2.1 Physiological role of O_2 in plants

Before the presence of oxygenic photosynthesis, the content of O_2 in the atmosphere was negligible (Crowe et al., 2013). In plants and other photosynthetic organisms, O_2 is produced through the photolysis of water in photosynthesis. In the light-dependent reactions, chlorophyll accepts photons and loses electrons, which are passed downstream through the electron transport chain for the reduction from $NADP^+$ to NADPH (Trebst, 1974). The electrons lost from chlorophyll are compensated by the photo-induced oxidation of water molecules in an O_2-evolving center, where electrons and protons are derived from water, and O_2 are produced as a by-product (Ferreira, 2004).

Access to O_2 is required for all aerobic organisms. Oxygen is the main substrate in respiration and acts as the final electron acceptor in respiratory energy conversions. All plants need sufficient amount of O_2 to release stored energy and maintain normal metabolism. Oxygen is also involved in many other biochemical reactions (Drew, 1997). Molecular O_2 serves as a substrate in reactive oxygen species (ROS) production. ROS are important signaling molecules in plants, but can also cause damage to cells (Mittler et al., 2004). Other biochemical reactions which involve molecular O_2 include desaturation of fatty acids and synthesis of phytohormones such as ethylene (van Dongen and Licausi, 2014).

1.2.2 O_2 deficiency in plants

In natural environments, excess water through waterlogging, flooding or submergence as well as soil compaction may restrict O_2 availability. Even under normoxic (high O_2 levels) conditions, some tissues or organs, such as the root apex, can still be O_2 deficient because of high respirational O_2 consumption (Whipple, 2012). Hypoxic conditions are characterized by the reduced production of ATP by oxidative

phosphorylation. This condition occurs in plants more frequently compared with anoxia in which ATP is generated only by glycolysis in the absence of O_2 (Drew, 1997). In natural environments, plants usually experience long-term hypoxia before the onset of anoxia.

O_2 deprivation is the main detrimental consequence of flooding (Bailey-Serres et al., 2012). The number of flooding events around the world has continuously increased in the last several decades (Bailey-Serres et al., 2012), and flooding events are predicted to rise globally in the 21st Century compared to the last century likely because of the climate change (Hirabayashi et al., 2013). The diffusion rate of O_2 in water is extremely low compared with that in air. Although flooding may result in the increased availability of resources such as phosphate and nitrate (Wright et al., 2015), the depletion of free O_2 in water leads to severe injury to plants. Flooding has a profound influence on the distribution and physiology of plants (Kozlowski, 1984; Wright et al., 2015). Most terrestrial plant species cannot survive under long-term O_2 deficiency caused by flooding, while wetlands species develop adaptive traits and strategies to survive under low O_2 conditions (Bailey-Serres and Voesenek, 2008).

Steep O_2 gradients are commonly observed in plant tissues (Ho et al., 2010; Whipple, 2012). Since free diffusion is still commonly considered to be the main process of passive O_2 transport in plants, the length of the transport pathway, the gas conductivity of tissues and the O_2 consumption rate of cells determine the O_2 status in a specific tissue (van Dongen and Licausi, 2014). The tissues with high O_2 consumption rate, such as root tips, can be O_2 deficient even when the O_2 concentrations are relatively high in the soil (Whipple, 2012).

1.2.3 Injury mechanisms of O_2 deficiency

Plants have to cope with numerous environmental stresses. All plant cells can survive some period of O_2 deficiency, but the injury will eventually occur and lead to irreversible damage. A common consequence of O_2 deficiency is cell death during the

depletion of O_2, during which cells experience a transition from aerobic metabolism to anaerobic metabolism (Drew, 1997). The accumulation of toxic end products of anaerobic metabolism, lower energy status and lack of respirational substrates contribute to the cellular injury and death (Drew, 1997). The cytosolic pH drop, which is associated with the transition from aerobic metabolism to anaerobic metabolism, is attributed to the accumulation of metabolic end products in the cytoplasm (Roberts et al., 1984a; Roberts et al., 1984b). In maize, lactic fermentation is initiated by anoxia, which causes the early decrease of cytoplasmic pH (Roberts et al., 1984b). Further decrease of cytoplasmic pH is attributed to the switch form lactic fermentation to ethanol fermentation. The decrease of pH has been closely associated with cell death (Crawford and Braendle, 1996; Drew, 1997).

Water transport is often affected by different stresses as a result of imbalanced root water uptake and shoot water loss through transpiration. In flooded plants, decreased transpiration is commonly observed as a result of increased stomatal closure (Islam et al., 2003; Calvo-Polanco et al., 2012). Flooding stress in tobacco plants has drawn attention for a long time because of the economic value of tobacco. Effects of aeration and soil temperature on wilting of flooded tobacco plants was reported as early as 1950s (Kramer and Jackson, 1954). Decreased root hydraulic conductivity (Lp_r) under O_2 deficiency has been commonly reported (Else et al., 1995b; Kamaluddin and Zwiazek, 2002; Tournaire-Roux et al., 2003; Aroca et al., 2012). Decreased Lp_r under O_2 deficiency is associated with cytosolic acidosis and inhibited respiration (Kamaluddin and Zwiazek, 2001; Tournaire-Roux et al., 2003). O_2 deficiency results in the transition from respiration to fermentation causing cell acidosis, which could further inhibit aquaporin gating (Tournaire-Roux et al., 2003; Törnroth-Horsefield et al., 2006) and, consequently, inhibit aquaporin-mediated transmembrane water transport (Gerbeau et al., 2002; Alleva et al., 2006). Aerenchyma and radical O_2 loss barriers are often induced under flooding. However, the enlarged air space and O_2 loss barriers in roots may negatively affect water flow in

roots, and that can be considered as a trade-off under O_2 deficiency.

O_2 deficiency and re-aeration after hypoxia can lead to increased production of ROS. Through Fenton reaction, which is catalyzed by iron, highly reactive and damaging OH- is produced from H_2O_2 (Luo et al., 1996). H_2O_2 accumulates in *Triticum aestivum* under re-aeration after hypoxia (Biemelt et al., 2000). ROS can cause oxidative stress in plant tissues. H_2O_2 is an reversible inhibitor of water transport through the plasma-membrane (Bramley and Tyerman, 2010).

1.2.4 Acclimation to O_2 deficiency

1.2.4.1 Metabolic adjustments under O_2 deficiency

The primary effect of O_2 deficiency on cells is decreased energy metabolism because O_2 acts as the respiratory electron acceptor. Lack of O_2 effectively blocks ATP synthesis in the mitochondria. In the absence of an adaptive response, ATP in the flooded root cells would be rapidly consumed (Drew, 1997). Metabolic adjustments are reversible and an economic ways to cope with low- O_2 conditions instead of irreversible morphological changes (Bailey-Serres and Voesenek, 2008). In order to produce ATP, plants have both aerobic respiration and anaerobic energy metabolic pathways (glycolysis/fermentation). Aerobic respiration produces ATP through oxidative phosphorylation and 36 units of ATP are produced per hexose unit, compared to only 2 ATP units produced in anaerobic fermentation. The depletion of O_2 results in the transition from respiration to substrate level energy production (Drew, 1997). Since this is a low efficiency process compared to respiratory energy metabolism, substrate level energy metabolism is sharply induced under O_2 deficiency to increase energy production (Zabalza et al., 2008). Many of the hypoxia responsive genes and the proteins they encode are associated with sugar metabolism, glycolysis and fermentation (Liu et al., 2005; Mustroph et al., 2010). For example, expression and activities of pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH, for lactate pathway) or alcohol dehydrogenase (ADH, for alcohol pathway) were reported

to be induced under O_2 deficiency (Roberts et al., 1989; Mustroph et al., 2010; Mugnai et al., 2011), catalyzing the conversion from pyruvate to alcohol or lactate using NADH as one of the substrates. Activities of PDC, LDH and ADH are considered to be essential for cell survival (Gibbs and Greenway, 2003). Through fermentation, plant cells recycle NAD^+ from NADH under low O_2 conditions to maintain the function of glycolysis (van Dongen and Licausi, 2014). The futile nitric oxide cycle can also recycle NAD^+ to compensate its consumption in fermentation (Limami et al., 2014). The function of nitrite reductase under anoxia was also studied in tobacco (Stoimenova et al., 2003). Plants that were engineered to have reduced nitrite reductase showed higher ATP content and enhanced fermentation, which is attributed to the effects of nitrite reduction on the cytosolic acidification.

Due to the low efficiency of ATP production in fermentation, plant cells commonly experience reversible curtailment of energy consuming processes under low O_2 conditions in addition to the adjustment of energy production (Bailey-Serres et al., 2012). The *SUB1A* gene in rice (*Oryza sativa*) mediates a 'quiescence strategy' that can save energy consumption (Xu et al., 2006; Fukao and Bailey-Serres, 2008). DNA synthesis, cell division and rRNA synthesis are inhibited under low O_2 conditions (Fennoy et al., 1998; Gibbs and Greenway, 2003). Similarly, storage compounds, such as lipids and proteins, are reduced under low O_2 conditions (Bailey-Serres et al., 2012). Selective mRNA translation also occurs in plants as a consequence of post-transcriptional regulation to save energy consumption (Bailey-Serres, 1999).

1.2.4.2 Transcriptional responses and low O_2 sensing

Plant cells need to reprogramme transcription under O_2 deficiency. Numerous hypoxia responsive genes have been identified with the application of proteomic approach and microarrays (Sachs et al., 1980; Chang et al., 2000; Gibbs et al., 2011a; Lee et al., 2011). These findings shed lights on the low O_2 sensing and regulation network in

plant cells under low O_2 conditions. Glycolysis and fermentation related genes are most commonly observed to be upregulated in O_2 deficiency, such as ADH and PDC (Liu et al., 2005; Mustroph et al., 2010). Genes encoding 1-Aminocyclopropane-1-Carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO) are also induced under O_2 deficiency (Mekhedov and Kende, 1996; Rieu et al., 2005). ACS and ACO catalyze regulatory steps in endogenous ethylene synthesis which is a major biochemical process in response to O_2 deficiency (Voesenek and Sasidharan, 2013). Transcripts involved in ROS signaling are induced under low O_2, some of which, including ROS scavenging enzymes and lipid peroxidation inhibitors, contribute to the amelioration of ROS damage (Mustroph et al., 2010; Chang et al., 2012). Interestingly, a group of genes related to photosynthetic processes also showed increased expression in flooded tobacco plants (Lee et al., 2007), which may be be a shock effect of early flooding stress.

The exact mechanisms of low O_2 sensing in plants still remain elusive (van Dongen and Licausi, 2014). In animals, O_2-dependent prolyl hydroxylases directly sense the decline of O_2. Under normoxia, prolyl hydroxylases promotes the degradation of hypoxia-inducible transcription factor (HIF), and the decreasing activities of prolyl hydroxylases under low O_2 conditions are paralleled by the elevating HIF, which mediates downstream responses in animal cells (Kaelin and Ratcliffe, 2008). However, a direct O_2 sensor in plant cells is still missing. A role of Group-VII ethylene response factors (ERFs) has been well demonstrated in the O_2-sensing of plants (Nakano et al., 2006; Fukao and Bailey-Serres, 2008; Licausi et al., 2011). *Arabidopsis* has five members of ERF-VII named Related to AP (RAP) 2.12, RAP 2.2, RAP2.3, Hypoxia Responsive ERF (HRE) 1 and HRE2 (Nakano et al., 2006). Under normoxia, RAP2.12 is present in the plasma membrane, while it is detached under hypoxia and accumulates in the nucleus, which subsequently triggers responsive gene transcription (Licausi et al., 2011). Transcripts of ERF-VII are associated with the adaption of plants under low O_2 by escape strategies or adaption

(Xu et al., 2006; Fukao and Bailey-Serres, 2008; Shahzad et al., 2016). The N-end rule pathway regulates the abundance of most of the ERF-VII transcription factors, which contain specific amino sequence (MCGGAI) in the N-terminus. This sequence can be recognized by specific aminopeptidases and the terminal methionine (M) is removed. Thus, the cysteine residue is exposed in the N-terminus and can be oxidized by the plant cysteine oxidase with O_2 acting as a substrate (van Dongen and Licausi, 2014).

1.2.4.3 Adaptive traits and strategies under O_2 deficiency

Resistance to low O_2 is species-specific in plants. Plant species have developed adaptive traits and strategies to cope with low O_2 challenges, including escape strategies, quiescence scheme and changes in plant architecture.

Low O_2 escape strategies are often seen in flooding-resistant species. *Rumex maritimus*, a flooding tolerant species, exhibits fast shoot elongation to escape from submergence (Van der Sman et al., 1991). Through the fast elongation, shoots could be more likely to obtain O_2 and CO_2 from the air as the gases are extremely limited during flooding. Though no active O_2 transport mechanism has been confirmed in plants, anatomical changes, such as the formation of aerenchyma, facilitate the diffusion and distribution of O_2 in plants (Chen et al., 2002; Herzog et al., 2016). Aerenchyma can supply flooded tissues with O_2 and escape from O_2 deficiency (Colmer, 2003).

Low O_2 escape strategy is an efficient way in some cases, while it can also be risky, especially with the possibility that plants could deplete energy before gaining access to O_2 (Bailey-Serres and Voesenek, 2008). Low O_2 quiescence syndrome is an alternative strategy in O_2 deficiency (Bailey-Serres et al., 2012). The research of flooded rice plants provides a good example of the mechanism of quiescence under low O_2. The *SUB1* in some cultivars encodes two ethylene responsive factors (ERF-VII TFs) and plays a key role in regulating quiescence traits (Fukao and

Bailey-Serres, 2008; Gibbs et al., 2011b). *SUB1* is also involved in the regulation of hormonal responses other than ethylene (Xu et al., 2006). Sub1A restricts ethylene-promoted gibberellic acid (GA_3) responses by increasing GA_3 signaling repressors, which inhibited the elongation promoted by GA_3 (Fukao and Bailey-Serres, 2008).

Formation of adventitious roots (ARs) has been highlighted as one of the most important adaptive traits under flooding in numerous species (Bailey-Serres et al., 2012). ARs, which are highly aerenchymatous roots with lower barrier for gas diffusion, are often induced in flooded plants (Calvo-Polanco et al., 2012; Ayi et al., 2016; Herzog et al., 2016). ARs promote the internal diffusion of O_2 from shoots to roots and elevate ATP production in roots (Bailey-serres, 2015). Lateral roots (LRs) originate from pericycle cells of existing roots. In contrast, ARs develop from different tissues (Bellini et al., 2014). Different kinds of biotic or abiotic stresses other than flooding can also induce AR formation including wounding (Ahkami et al., 2009) and exogenous hormones (Pagnussat et al., 2002). Ethylene and other phytohormones are involved in the regulation network of AR formation (McDonald and Visser, 2003; Steffens et al., 2006). However, it still remains elusive how plants could manage a sharing regulation network in AR formation (Bellini et al., 2014).

1.2.4.4 Hormone synthesis

Phytohormones, ethylene, abscisic acid (ABA) and gibberellins are closely associated with the induction of low O_2 survival strategies (Bailey-Serres et al., 2012). Ethylene production increases under hypoxia (Van der Sman et al., 1991; Voesenek et al., 2003). ACC is the immediate precursor of ethylene (Yang and Hoffman, 1984). It has been proven that the synthesis of ACC does not require O_2, and in fact, ACC synthase activity is stimulated under waterlogging conditions (Sairam et al., 2008). Hypoxic roots have higher concentrations of the ethylene precursor ACC, and display a greater activity of ACC synthase and ACC oxidase (Grichko and Glick, 2001). The synthesis

of ethylene by roots is strongly promoted by hypoxia but blocked by anoxia because of the requirement for free O_2 for the conversion of ACC to ethylene. Ethylene is required in the anoxia induced cell lysis and aerenchyma formation (He et al., 1996). Ethylene is thought to be a signal for flooding adaptive traits (Sasidharan and Voesenek, 2015). Due to the low diffusion rate in water under flooding, ethylene synthesized in flooded roots accumulates in the root tissues. Hypoxia lead to a decreased stomatal conductance and Lp_r in trembling aspen and this effect was partly reversed by ehtylene (Kamaluddin and Zwiazek, 2002). The effects of ethylene were in turn reversed by $HgCl_2$, which pointed to the involevement of aquaporins in this response (Kamaluddin and Zwiazek, 2002).

In the early responses to flooding, ABA level of plants declines (Voesenek et al., 2003). Stomatal closure under flooding is induced by ABA and impaired stomatal responses are found in ABA-deficient mutants of pea (*Pisum sativum*) (Jackson, 2002). Cross-talk of the phytohormones is important and highly complex in the responses of plants to low O_2. ABA and GA_3, which act antagonistically, regulate ethylene–induced elongation. In the deepwater rice, the inhibited growth of ethylene-treated internodes by ABA was counteracted by GA_3 (Hoffmann-Benning and Kende, 1992). Flooded tomato plants with decreased ABA delivery, which takes place soon after flooding, are more sensitive to ethylene and this may be important to the rapid response to flooding (Else et al., 1995a). Auxins also play a role in the responses to low O_2, especially in the elongation of shoots. Upregulation of AUX/IAA genes in petioles was observed in *Rumex palustris* (van Veen et al., 2013).The removal of leaf blade in *R. palustris* led to decreased indole-3-acetic acid (IAA) concentration and therefore lagged petiole elongation under flooding, which could be reversed by adding exogenous NAA (1-Naphthaleneacetic acid, an analog of IAA) (Cox et al., 2006). The interaction of phytohormones also affect the resistance of tobacco to low O_2 in terms of the formation of adventitious roots (McDonald and Visser, 2003).

1.3 Aquaporins

1.3.1 Classification of aquaporins

Aquaporins are membrane proteins that belong to the ancient Major Intrinsic Protein (MIP) family. Aquaporin-1 of *Homo sapiens* (Agre et al. 1993) and TIP1;1 (tonoplast intrinsic protein) of *Arabidopsis* were firstly confirmed to have water channel function (Maurel et al., 1993). These findings greatly expanded our knowledge of water transmembrane movement at the cell level. Aquaporins exhibit high diversity in higher plants. Genome-wide analyses have identified more than 30 aquaporin homologs in each of the examined plant species (Maurel et al., 2015). The number of aquaporin homologs is 33 in rice (Sakurai et al., 2005), 35 in *Arabidopsis* (Johanson et al., 2001), and 66 in *Glycine max* (Zhang et al., 2013). Based on the homology of amino acid sequence, subcellular localization and structural characteristics, five subfamilies of aquaporin have been categorized, including PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast membrane intrinsic proteins), NIPs (nodulin 26-like intrinsic proteins), SIPs (small intrinsic proteins) and XIPs (uncategorized intrinsic proteins). Aquaporins are localized in the plasma and intracellular membranes and have diverse functions. Most of the PIPs and TIPs exhibit water channel ability. The subdivisions of PIPs (PIP1s and PIP2s) are quite conserved in all higher plants, which may indicate their early evolutionary origin (Maurel 2015). Some NIPs facilitate the diffusion of both water other small neutral molecules. Lower plants, algae and mosses have two more subfamilies of aquaporins, which are the hybrid intrinsic proteins (HIPs) and GlpF-like intrinsic proteins (GIPs). Based on sequence similarity and function assays, mammalian MIPs were classified into orthodox aquaporins (water transporters) and aquaglyceroporins (permeable to water and other small soluetes) (Krane et al., 2007). In fungi, 229 fungal MIPs from 88 species were classified into orthodox aquaporins, aquaglyceroporins, facultative aquaporins, and XIPs (Xu et al., 2013).

1.3.2 Functions of plant aquaporins

Heterologous expression systems have been used to characterize aquaporin functions. The expression system of *Xenopus* oocytes was first used and is still frequently used in functional analyses of aquaporins (Agre et al., 1993; Perrone et al., 2012; Xu et al., 2015). Cell volume, intercellular pH was measured as an indicator in transport of molecules such as water, glycerol and CO_2 through aquaporins (Maurel et al., 2015). Yeast cells have been used in transport assays of H_2O_2, ROS, NH_3, and boric acid (Maurel et al., 2015). Yeast growth assay can also be used to indicate the physiological role of expressed aquaporins (Bienert et al., 2008).

PIPs are localized in plasma membrane and are crucial in facilitating water transport in cell-to-cell pathway (Chaumont and Tyerman, 2014). Most PIPs show water permeability. However, expression of PIP1s in *Xenopus* oocytes failed to be functional unless coexpressed with PIP2s (Fetter, 2004), suggesting that PIP2s may be essential in PIP1 trafficking (Maurel et al., 2008; Chaumont and Tyerman, 2014). PIPs play a regulatory role in plant water relations, especially in the hydraulic-limiting tissues, such as the root endodermis and leaf bundle sheath cells (Chaumont and Tyerman, 2014; Maurel et al., 2015). Contribution of PIPs to root hydraulic conductivity has been examined in several species and PIP isoforms (Siefritz et al., 2002; Javot, 2003; Péret et al., 2012). PIP expression is also well correlated with leaf hydraulic conductance and PIPs play a role in hydraulic adjustment of leaf veins (Postaire et al., 2010; Prado et al., 2013). The preferential expression of PIPs in some tissues such as the endodermis, xylem parenchyma and phloem-associated cells, indicates the essential role of PIPs in regulating transmembrane water transport in these tissues (Maurel et al., 2008). PIPs also contribute to embolism recovery. Affected embolism refilling by manipulated expression of PIPs suggests that aquaporins contribute to this process (Sakr et al., 2003; Lovisolo and Schubert, 2006). In addition to water transport, PIPs also show multiple functions. Tobacco AQP1 showed CO_2 and glycerol permeability when

expressed in *Xenopus* oocytes (Biela et al., 1999; Uehlein et al., 2003). *Arabidopsis* PIP2;1 showed H_2O_2 permeability when expressed in yeast cells (Dynowski et al., 2008). The role of PIPs in tissue expansion and plant growth is apparent. Cell expansion is driven by the positive pressure (turgor) and is restricted by the cell wall. PIPs affect cell expansion by regulating water uptake rate and strong PIP expression was observed in growing tissues of barley (Ludevid et al., 1992; Eisenbarth and Weig, 2005; Hachez et al., 2008).

TIPs are localized in the tonoplast membrane of vacuoles, which are the largest organelles in plant cells and have important functions in turgor regulation and storage of inorganic and organic molecules. TIPs are known to be expressed in roots, leaves and seeds (Maurel et al., 2015). Most TIPs show water permeability. By expressing *Arabidopsis* TIP1;1 in *Xenopus* oocytes, plant aquaporins were first confirmed to facilitate water transmembrane transport (Maurel et al., 1993). TIPs also transport urea, glycerol, H_2O_2, and NH_3 transport (Maurel et al., 2008). They play an important role in cell expansion and, consequently, plant growth enhancement (Ludevid et al., 1992; Phillips and Huttly, 1994; Lin et al., 2007). A *Hydrangea macrophylla* TIP was also shown to facilitate aluminum transport and contributed to plant resistance of soil acidification (Negishi et al., 2012).

Some NIPs are expressed in the plasma membrane. Preferential expression of NIPs in roots has been observed. NIPs often show reduced water permeability compared to PIPs and TIPs, and may be permeable to nutrients or toxic molecules, such as B, Si, As, Ge and NH_3 (Maurel et al., 2015). Two *Arabidopsis* NIP homologs have shown to be involved in the uptake and distribution of B in plants (Tanaka et al., 2008; Li et al., 2011). Similarly, NIP homologs of rice have been implicated in Si uptake (Ma et al., 2006; Yamaji and Ma, 2009). Transcription of *Arabidopsis NIP2;1* was induced by low O_2 conditions (Liu et al., 2005), and shown to be the lactic acid transporter facilitating the export of lactic acid out of cells and regulating cellular pH during hypoxia (Choi and Roberts, 2007).

In contrast to the other three subfamilies of aquaporins, SIPs and XIPs have been less studied. SIPs are localized in the endoplasmic reticulum membranes. *Vitis vinifera* SIP1 showed water permeability when expressed in yeast (Noronha et al., 2014). XIPs are localized in the plasma membrane. *Nt*XIP1;1 and *Pt*XIP2;1 have been to transport H_2O_2 and water, respectively (Bienert et al., 2011).

1.3.3 Structure and regulation of plant aquaporins

Plant aquaporin structures have been investigated by the application of cryoelectron microscopy and X-ray crystallography. Highly conserved structure of aquaporins has been identified in plants, animals and other organisms (Daniels et al., 1999; Törnroth-Horsefield et al., 2006; Gibbs et al., 2015). Limited number of studies on plant aquaporins have revealed that plant PIPs and TIPs are tetramers (Fig 1.1), but the structures of other plant aquaporins remain obscure. Each monomer has six transmembrane domains connected by five loops (A-E). Loops B and D are localized on the cytoplasmic side and the other three loops (A, C and E) are on the outer membrane side. Each monomer independently forms a pore. Two highly conserved NPA motifs (Asn-Pro-Ala) are localized in loop B and E. The NPA motifs and another aromatic/Arg site form the narrowest pore constriction functioning as a selective filter in the pore.

Both transcriptional and post-transcriptional regulation affect the abundance and activities of aquaporins. Gating of aquaporins is affected by various biotic and abiotic factors such as cytosolic pH, Ca^{2+} and ROS (Tournaire-Roux et al., 2003; Törnroth-Horsefield et al., 2006; Maurel et al., 2015). Gating mechanism of plant aquaporins was revealed mostly using the model of spinach (*Spinacia oleracea*) SoPIP2;1 (Törnroth-Horsefield et al., 2006). X-ray crystallography showed the open and closed conformations of SoPIP2;1. The protonation of a conserved histidine residue of SoPIP2;1 during flooding and dephosphorylation during drought lead to the closure of the channel. The phosphorylation of loop B of SoPIP2;1 unlocks loop D

and results in the opening of the aquaporin conformation. The regulation mechanism of aquaporin gating by protonation links the aquaporin gating with cytosolic pH drop caused by environmental factors. Phosphorylation sites have also been identified in other aquaporins, and these findings have laid the foundation for the the most widely accepted aquaporin gating mechanisms (Lee et al., 1995; Maurel et al., 1995; Johansson et al., 1998).

1.3.4 Relationships between plant aquaporins and O_2 deficiency

Localized in cell membranes, aquaporins participate in responses to different environmental changes, such as drought, flooding and salinity (Maurel et al., 2015). Regulation of aquaporins is thought to be an important factor for hydraulic adjustment. Hydraulic conductivity can be affected by the location, abundance and gating of aquaporins. Under O_2 deficiency, the closure of aquaporins is seen to be the reason for decreased water uptake in roots (Tournaire-Roux et al., 2003).

O_2 deficiency caused by flooding, waterlogging or submergence greatly affects plant physiology and aquaporin activities. Aquaporin-mediated water transport was down-regulated in O_2 deficient maize (*Zea mays*) and tamarack (*Larix laricina*) (Ehlert et al., 2009; Calvo-Polanco et al., 2012). PIP expression was also down-regulated by prolonged flooding in *Carrizo citrange* (Rodríguez-Gamir et al., 2011). A drop of cytosolic pH under O_2 deficiency is an early phenomenon in flooding because of the accumulation of end-products of anaerobic metabolism. Water permeability of isolated plasma membrane of *Arabidopsis* and *Beta vulgaris* storage was inhibited by decreased pH (Gerbeau et al., 2002; Alleva et al., 2006). Under flooding, it was reported that the inhibition of aquaporin gating resulted in the decreased cell and root hydraulic conductivites of *Arabidopsis*, and was regulated by cellular acidosis caused by the shift from aerobic respiration to fermentation (Tournaire-Roux et al., 2003). X-ray structure confirmed the protonation of a conserved histidine residue under low pH resulted in the closed conformation of the

spinach SoPIP2;1 (Törnroth-Horsefield et al., 2006).

In addition to aquaporin gating analysis, expression patterns can also provide information concerning the responses of aquaporins to various biotic or abiotic stresses (Liu et al., 2005; Mustroph et al., 2010; Reeksting et al., 2016). Although gene transcripts may not be translated under certain conditions and proteins are affected by the posttranslational regulation. Transcriptional responses can be evaluated in light of the translational potential of cells experiencing O_2 deficiency (van Dongen and Licausi, 2014). Microarray analysis showed that aquaporin expression was downregulated in O_2-deficient *Arabidopsis* (Liu et al., 2005) and *Persea americana* (Reeksting et al., 2016). However, transcription of *Arabidopsis* aquaporin *At*NIP2;1 was induced under flooding (Liu et al., 2005). *At*NIP2;1 belongs to the nodulin 26 intrinsic proteins (NIPs) and is a root-specific and highly conserved transport protein with structural and functional homology to the soybean (*Glycine max*) nodulin 26. *At*NIP2;1 is mainly localized in the endoplasmic reticulum membrane and shows root-specific accumulation (Mizutani et al., 2006). A dramatically 300-fold increase in *AtNIP2;1* transcript was reported in anoxic *Arabidopsis*. *At*NIP2;1 shows low water transport capacity. Instead, this protein is mainly responsible for lactic acid transport, which is an end product of lactic fermentation (Choi and Roberts, 2007). The efflux of lactic acid may reduce cell damage and contribute to preventing the cytosolic pH drop.

1.3.5 Responses of plant aquaporins to other abiotic and biotic factors

Due to the essential role in plant water relations, responses of aquaporins to water stress have been extensively studied. Drought and salinity cause water deficit stress in plants and lead to the inhibition of aquaporin activities (Sutka et al., 2011; Perrone et al., 2012; Hachez et al., 2014). Aquaporins play an important role during the recovery from water deficit stress (Martre et al., 2002). Efforts have been made to overexpress aquaporins in to improve stress resistance, but the results have been mixed. In some

species, the overexpression of aquaporins increased plant resistance to water stresses (Yu et al., 2005; Guo et al., 2006; Zhou et al., 2012), while in some other studies, overexpression of aquaporins led to increased sensitivity to water and salinity stress (Aharon, 2003; Katsuhara et al., 2003).

Low temperature stress leads to leaf dehydration and decreases root water uptake, largely due to the inhibition of aquaporin-mediated water transport. Decreased cellular pH during low temperature, similarly to hypoxic stress, contributes to the closure of aquaporins (Maurel et al., 2008). Transcription of aquaporins in some species decreases markedly under low temperature (Jang et al., 2004; Herman et al., 2005; Sakurai et al., 2005). However, in some other species, the change of PIP transcripts and the abundance of aquaporin proteins are not always correlated, which indicates that post-transcriptional regulation also plays an important role (Herman et al., 2005; Yu et al., 2006; Lin et al., 2007). Low root temperature decreased the Lp_c of root cortical cells in *Cucumis sativus* through closing the water channels, and this inhibition could be ameliorated by ABA (Lee et al., 2005). The overexpression of aquaporins in rice and poplar improved chilling tolerance in both plant species (Matsumoto et al., 2009; Ranganathan et al., 2016).

Aquaporins also play a role in the biotic interactions of plants. In *Rhizobium*-legume symbiosis, the formation of nodules required the re-localization of a TIP1 homolog from the plant tonoplast membrane to the symbiosome membrane (Gavrin et al., 2014). In the symbiosome membrane, NIPs serve in both water and NH_3 transmembrane transport (Guenther and Roberts, 2000; Hwang et al., 2010). Mycorrizhae is another common symbiotic interaction between the plants and fungi that optimizes water and nutrient availability to both symbiotic partners. The aquaporin homologs in fungi have been identified by genome sequencing and are well linked with water relations and nutrient availability (Maurel et al., 2015). A recent study showed that the overexpression of a fungal aquaporin homolog in *Laccaria bicolor* altered the expression pattern of aquaporins in the host *Picea glauca* and

enhanced Lp_r in the host plant (Xu et al., 2015). However, trembling aspen colonized with the same *Laccaria bicolor* strain as in the study with *P. glauca*, resulted in a decreased Lp_r indicating that the effect of gene manipulation of the fungi aquaporin varies between the mycorrhizal partners (Xu et al., 2016).

1.4 Gas transmembrane transport and the role of aquaporins

It has been accepted for a long time that gases enter and exit cells by free diffusion. However, this has been challenged by the fact that the gas permeability of some membranes is quite low (Ivanov et al., 2004; Wang et al., 2007). Thus, facilitated diffusion has recently attracted research interest (Herrera and Garvin, 2011; Somersalo et al., 2012).

1.4.1 Transmembrane transport of CO_2, NH_3 and NO

It was first demonstrated in leaves that the diffusion of CO_2 into the chloroplast can be blocked with the treatment of mercury (Terashima and Ono, 2002), which is one of the few effective aquaporin blockers. Subsequent studies showed that the expression of *Nt*AQP1 from tobacco, which is highly homologous with human AQP1, markedly increased transmembrane CO_2 transport (Uehlein et al., 2003). Efficient t CO_2 transport into the mesophyll cells is of great importance to plant. As the main substrate in photosynthesis, CO_2 serves as the carbon source for plants and is often a limiting factor to photosynthesis. Evidence has shown that aquaporins are involved in the regulation of stomatal conductance (Uehlein et al., 2003; Siefritz et al., 2004; Flexas et al., 2006). More research is needed to confirm whether the role of aquaporins in stomatal regulation is associated with the regulation of stomatal movement or with cell membrane permeability to CO_2.

NH_3 and the conjugated ion (NH_4^+) are the substrates for amino acids synthesis in plants. The first link between aquaporins and nitrogen compounds comes from the observation of the dependence of aquaporin expression on N compounds (Gaspar et

al., 2003). Further evidence showed that tobacco aquaporin TIP4;1 could transport urea (Gerbeau et al., 1999), which confirms the association between aquaporins and nitrogen assimilation. Growth assay of yeast expressing heterologous aquaporin has demonstrated that TIPs facilitate the diffusion of NH_3 (Jahn et al., 2004). *Xenopus* oocytes expressing *Arabidopsis AtTIP2;1* and *AtTIP2;3* are shown to facilitate NH_3 transmembrane transport (Loque et al., 2005). However, these transport assays performed in yeast or *Xenopus* oocyte lack functional significance in plants. For example, transgenic plants overexpressing *AtTIP2;1* do not show increased NH_3 uptake (Loque et al., 2005). Thus, further research is needed to demonstrate the physiological effects of NH_3 transport through aquaporins.

NO plays important roles in human physiology (Garthwaite and Boulton, 1995; Calabrese et al., 2007). Endothelial cells produce NO and release it into adjacent vascular smooth muscle cells (Herrera et al., 2006). In plants, progress has been made to understand the signaling and physiological roles of NO (Delledonne, 2005; Santolini et al., 2017). Functioning as a general signal, NO is involved in germination, root growth, stomatal closure and responses to biotic and abiotic stresses (Besson-Bard et al., 2008). Human AQP1 facilitates the transmembrane flux of NO (Herrera et al., 2006). By transfecting animal cells with human AQP1 subcloned plasmid, NO flux can be detected with fluorescence. Until now, no evidence for NO transport through aquaporins exists for plants, but the similarity of sequences and structures may hint on a potential function of plant aquaporins as NO transporters.

1.4.2 Evidence for facilitated O_2 transport

There appears to be no direct evidence for the presence of O_2 channels in cell membranes, which may be due to the lack of solid detection techniques for subcellular O_2 (van Dongen and Licausi, 2014). Cellular CO_2 and NH_3 can be detected by the pH change across the membrane (Uehlein et al., 2003; Hwang et al., 2010), and the detection of NO includes a fluorescent reporter, which shows dynamic changes in NO

concentrations by the changes in fluorescence (Herrera et al., 2006). The detection of O_2 in cells is difficult. Efforts have been made to construct subcellular O_2 reporters based on the fluorescence fusion protein, while problems exist in recording dynamic changes in the intracellular O_2 concentrations (Potzkei et al., 2012; Dongen et al., 2014).

Evidence has shown that, O_2 permeability of lipid monolayers is much lower than previously thought (Ivanov et al., 2004), and the presence of O_2 channel(s) in cell membranes has been proposed. Aquaporins are good candidates as potential O_2 channels. With the overexpressed human AQP1, mammalian cells show higher hypoxia-inducible transcription factors, which act as O_2 sensors in mammalian cells (Echevarría et al., 2007). This may suggest that AQP1 facilitates the transmembrane diffusion of O_2. However, this research is also hampered by the absence of intracellular O_2 detection technique and no direct evidence has been provided. In this thesis, a new technique for intracellular O_2 detection is proposed based on the spectrophotometry of O_2 binding to myoglobin.

1.5 Studied species

1.5.1 Aquaporin research in trembling aspen (Populus tremuloides)

Trembling aspen (*Populus tremuloides* Michx.) is a deciduous tree that is native to North America and is the most widely spread tree species in North America. Seven PIPs genes have been identified in *Populus tremula* × *tremuloides* including two *PIP1*s (*PIP1;1* and *PIP1;2*) and four *PIP2*s (*PIP2;1*, *PIP2;2*, *PIP2;3*, *PIP2;4* and *PIP2;5*) (Marjanović et al., 2005). Functional analysis of aspen PIPs in *Xenopus* oocytes has shown that aspen PIP2;1 and PIP2;2 have low water permeability, while PIP2;3 and PIP2;5 show high water permeability (Marjanović et al., 2005). Adefoliation study in aspen demonstrated changes in Lp_r and leaf transpiration rates that were accompanied by changes in *PIP* expression, suggesting that aquaporins were involved in the adjustment of root and leaf hydraulic properties (Liu et al., 2014).

Trembling aspen plants colonized by the mycorrhizal fungus *Laccaria bicolor* showed decreased *PIP1;2*, *PIP2;1* and *PIP2;2* expression in the absence of changes in root hydraulic conductivity (Xu et al., 2016). This finding strengthens the notion of the importance of aquaporins in regulation of root water transport and emphasizes the functional complexity of fungal-root interactions.

1.5.2 Aquaporin research in tobacco (Nicotiana tabacum)

Common tobacco is one the most important economic crops in the world. Tobacco is widely used as a model plant for studying fundamental biological processes and disease susceptibility (Sierro et al., 2014). The tobacco cell line BY-2 acts as a key tool in plant molecular studies (Nagata et al., 1992). BY-2 cells have been effectively used as a transgenic system for the analysis of aquaporin function. Expression of *Nt*TIP homolog in BY-2 cells promotes cell expansion and growth (Okubo-Kurihara et al., 2009). As a model organism, tobacco is also widely used as a host plant heterologously expressing aquaporins from other organisms, such as *Arabidopsis* (Lee et al., 2009), *Zea mays* (Chaumont et al., 2000), *Lilium longifloru* (Ding et al., 2004), *Solanum tuberosum* (Wu et al., 2009) and *Brassica napus* (Yu et al., 2005). Dozens of aquaporin-like sequences have been identified in the tobacco genome sequence (Sierro et al., 2014), while the research of aquaporins concentrates on a few analogues, especially *Nt*PIP1;2 (*Nt*AQP1). *Nt*AQP1 plays a role in the whole-plant water relations. Antisense plants of *NtAQP1* show decreased Lp_r and lower water stress resistance (Siefritz et al., 2002). The function of *Nt*AQP1 in plant water relations has profound influence. The expression of *NtAQP1* is associated with the unfolding movement of leaves (Siefritz et al., 2004). Except for water transport, *Nt*AQP1 is also involved in the permeability of other molecules, such as glycerol and CO_2 (Biela et al., 1999; Uehlein et al., 2003). Transmembrane transport of CO_2 through *Nt*AQP1 has profound physiological significance, and has been introduced in §1.4.1 of this thesis. The finding of pore-mediated CO_2 transmembrane transport through aquaporins is

astonishing, as it overturns the concept of free diffusion of gas molecules across biomembranes. This finding also inspired the research of other gas molecule transporters in cell membranes, and brought out the concept of gas channel (Boron, 2010). Other tobacco aquaporin homologs have been also linked with fundamental physiological processes. *Nt*PIP2s are required for tobacco anther development, and silencing their expression delays anther dehiscence (Bots et al., 2005b). In functional studies of *Xenopus* oocytes, *Nt*PIP1;1 showed low capacity for water transport, while *Nt*PIP2;1 showed high permeability to water (Bots et al., 2005a). *Nt*XIP1;1, which is localized in the plasma membrane, has been shown to facilitate the transmembrane transport of urea and glycerol (Bienert et al., 2011).

1.5.3 Aquaporin research in Arabidopsis thaliana

Arabidopsis is one of the most studied model plant species. Thirty-five aquaporin homologs have been identified in *Arabidopsis* (Johanson et al., 2001), including 13 PIPs, 10 TIPs, 9 NIPs and 3 SIPs. The 3 SIPs were shown to reside mainly in the endoplasmic reticulum (Ishikawa et al., 2005). As a model plant, the functions of different aquaporins in *Arabidopsis* have been well studied. Functional assays have revealed the boron permeability of *Arabidopsis* NIP6;1 and NIP7;1, the H_2O_2 permeability of PIP2;1 and TIP1;2 (Bienert et al., 2007; Dynowski et al., 2008), the arsenite permeability of NIP5;1 (Mitani-Ueno et al., 2011), and the NH_3 permeability of TIP2;3 (Loqué et al., 2005). The studies of *Arabidopsis* provide a good example of the regulation of aquaporin gating. The *Arabidopsis* TIP homolog was shown to be mercury-insensitive (Daniels et al., 1994). The water transport measurements in plasma membrane vesicles purified from *Arabidopsis* suspension cells suggest a regulatory role of protons and divalent cations in aquaporin gating (Gerbeau et al., 2002). Reactive oxygen species can inhibit the opening of *Arabidopsis* aquaporins. The antisense inhibition of aquaporins reduced Lp_r by about 50% in *Arabidopsis*

(Martre et al., 2002). Aquaporins of *Arabidopsis* have been shown to play important roles in several developmental processes including cell enlargement in roots, leaves, hypocotyls, and flower stems (Ludevid et al., 1992). The responses of *Arabidopsis* aquaporins to abiotic stresses were also widely studied. The transcription of *Arabidopsis* PIP2;5 was up-regulated by low temperature treatment (Jang et al., 2004). Salt and oxidative stresses can affect the phosphorylation of *Arabidopsis* PIP2;1 (Park et al., 2008; Pietro et al., 2013). Transgenic *Arabidopsis* with antisense inhibition of PIP1 and PIP2 expression showed significantly delayed rehydration after drought, which suggests an important role of aquaporins in drought recovery (Martre et al., 2002).

1.5.4 Yeast (Saccharomyces cerevisiae) as an expression system in aquaporin research

Yeast is one of the most commonly used expression systems in the functional assays of aquaporin transport capacities. By detecting the rapid change of scattered light intensity of yeast protoplasts, stopped-flow technique has been successfully applied in examining water permeability of expressed aquaporins (Fischer and Kaldenhoff, 2008). Yeast was also used to study gas transport through aquaporins. CO_2 permeability through aquaporins is detected by the change of pH across the membrane through fluorescence signal with a stopped-flow spectrometer (Prasad et al., 1998; Navarro-Ródenas et al., 2015). Growth assay of yeast expressing heterologous aquaporin showed that TIP facilitate the diffusion of NH_3 (Jahn et al., 2004). Yeast has also been used as an expression system in determining the transport capacity of toxic compounds. With the use of graphite furnace atomic absorption spectrometer technique, a group of NIPs were proven to facilitate the transport of arsenite (Bienert et al., 2008).

1.6 Reference

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Table 1.1 Representative aquaporins involved in gas transport. The abbreviations of species names are *Hs* - *Homo sapiens*, *Nt* - *Nicotiana tabacum*, *Ta* - *Triticum aestivum*, *At* - *Arabidopsis thaliana*, *Bj* - B*radyrhizobium japonicum*, *Lb* - *Laccaria bicolor*.

Isoform	Substrate	Expression System	Assay	References
*Hs*AQP1	CO_2	*Xenopus* oocyte	Cellular pH	(Nakhoul et al., 1998)
*Nt*AQP1	CO_2	*Xenopus* oocyte	Cellular pH	(Uehlein et al., 2003)
*Lb*AQP1	CO_2	Yeast	Fluorescence dye	(Navarro-Ródenas et al., 2015)
*Ta*TIP2	NH_3	Yeast	Growth assay	(Jahn et al., 2004)
*At*TIP2;1	NH_3	*Xenopus* oocyte	Radiolabeling	(Loque et al., 2005)
*At*TIP2;3	NH_3	*Xenopus* oocyte	Radiolabeling	(Loque et al., 2005)
*Bj*NOD26	NH_3	Proteoliposome	Internal pH	(Hwang et al., 2010)
*Hs*AQP1	NO	CHO-K1 cells	Fluorescence labeling	(Herrera et al., 2006)
*Hs*AQP1	O_2	PC12 cells	HIF factors	(Echevarría et al., 2007)
*Lb*AQP1	NO	Yeast	Fluorescence dye	(Navarro-Ródenas et al., 2015)

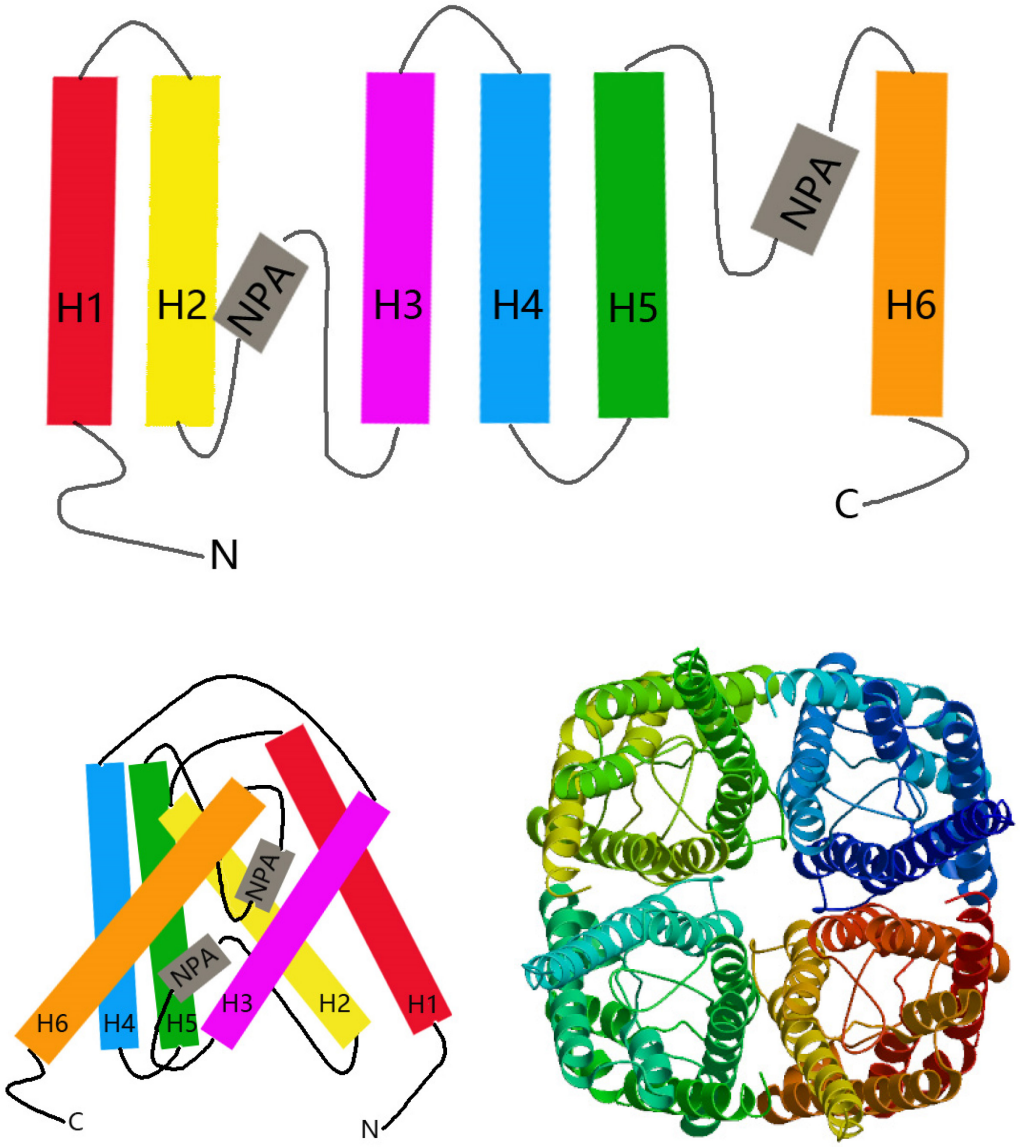

Figure 1.1 (A) Schematic diagram of a representative aquaporin structure. Six helices (H1-H6) are connected by five loops and two conserved NPA motifs are shown. (B) Schematic diagram of folding aquaporin structure. Two NPA motifs form the selectivity filter in the pore of aquaporin monomer. The schematic diagrams of representative aquaporin structure was drawn based on the published article (Huber et al., 2012). (C) Top view of *Nicotiana tabacum Nt*AQP1 tetramer simulated with the SWISS-MODEL online server (Arnold et al., 2006).

2 Stable Expression of Aquaporins and Hypoxia-Responsive Genes in Adventitious Roots Are Linked to Maintaining Hydraulic Conductance in Tobacco (*Nicotiana tabacum*) Exposed to Root Hypoxia

2.1 Introduction

Frequency of floods is predicted to increase globally due to the climate changes (Hirabayashi et al., 2013). As O_2 diffusion rate is extremely low in water compared to air, prolonged flooding can lead to O_2 deprivation and reductions of growth and survival in terrestrial plant species (Bailey-Serres and Voesenek, 2008). Root O_2 deficiency (hypoxia) limits respirational ATP synthesis and results in an energy crisis and toxicity due to a transition to glycolysis and fermentation (Drew, 1997).

Faced with hypoxia, plants need to reprogram transcription (Gibbs and Greenway, 2003), and curtail energy-consuming processes such as DNA and protein synthesis and cell division (Bailey-Serres and Voesenek, 2008). Numerous hypoxia-responsive proteins and genes that have been identified by the application of proteomic approach and microarrays are associated with sugar metabolism, glycolysis, fermentation and hormonal regulation (Sachs et al., 1980; Chang et al., 2000; Gibbs et al., 2011; Lee et al., 2011). Alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) are the two key enzymes in fermentation and are commonly induced by hypoxia and anoxia in plants and fungi (Lee et al., 2007; Mustroph et al., 2010; Mugnai et al., 2011). High activities of ADH and PDC are associated with improved survival of plants under low O_2 conditions, mainly due to their involvement in alleviating energy crisis. (Drew, 1997). However, the increase in fermentation may also lead to the accumulation of toxic end products, such as ethanol and acetaldehyde (Gibbs and Greenway, 2003).

Genes encoding 1-Aminocyclopropane-1-Carboxylate (ACC) synthase (ACS) are also induced under O_2 deficiency (Mekhedov and Kende, 1996; Rieu et al., 2005). ACS catalyzes a regulatory step in endogenous ethylene synthesis, which is a major biochemical process in response to hypoxia (Voesenek and Sasidharan, 2013).

Hydraulic adjustments are among the early responses of plants to flooding (Tan et al., 2018). This is often manifested as wilting due to the loss of balance between water loss and uptake (Kamaluddin and Zwiazek, 2002; Islam et al., 2003). Aquaporins, including plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins, nodulin26-like intrinsic proteins, small basic intrinsic proteins, and the uncategorized intrinsic proteins, are membrane intrinsic proteins that can rapidly respond to various abiotic and biotic stresses including O_2 deprivation (Maurel et al., 2015; Tan et al., 2018). The inhibition of aquaporin gating and root hydraulic conductivity in flooded plants may be regulated by cellular acidosis caused by the shift from respiration to fermentation (Tournaire-Roux et al., 2003), and depletion of ATP required for the phosphorylation of some aquaporins (Kamalludin and Zwiazek, 2002). X-ray structure confirmed that the protonation of a conserved histidine residue under low pH during flooding resulted in the closed conformation of aquaporins (Törnroth-Horsefield et al., 2006). In addition to regulating plant hydraulics, aquaporins are involved in the transport of other small molecules including CO_2 (Uehlein et al., 2003; Navarro-Ródenas et al., 2015), NH_3 (Jahn et al., 2004) and O_2 (Zwiazek et al., 2017) . Under flooding, transcription of *Arabidopsis* aquaporin NIP2;1 was induced (Liu et al., 2005), and shown to be a transporter of lactic acid that is produced by fermentation (Choi and Roberts, 2007).

Plants vary in their flooding tolerance and adopt different survival strategies. These strategies include fast shoot elongation to escape from submergence (Van der Sman et al., 1991) and development of suberized barriers in roots to reduce radial O_2 loss (De Simone et al., 2003; Abiko et al. 2012). Adventitious roots (ARs), are often induced in flooded plants. They may be placed close to the surface in some plants and

usually have low gas diffusive resistance (Calvo-Polanco et al., 2012; Ayi et al., 2016; Herzog et al., 2016). ARs promote internal diffusion of O_2 from shoots to roots and elevate respiratory ATP production in roots (Bailey-Serres, 2015). Different types of biotic and abiotic stresses other than flooding can induce AR formation including wounding (Ahkami et al., 2009) and exogenous hormonal treatments (Pagnussat et al., 2002). Ethylene and other phytohormones have been implicated in the regulation of AR formation (Islam et al., 2003; McDonald and Visser, 2003; Steffens et al., 2006). Different hormones may interact with each other in AR formation, but it still remains elusive how plants manage this complex regulation network in AR formation (Bellini et al., 2014).

Formation of ARs has been highlighted as one of the most important adaptive traits under flooding in numerous species (Bailey-Serres et al., 2012). However, more evidence is still needed to evaluate the functional traits of ARs contributing to hypoxia tolerance. It was previously demonstrated that ARs had higher hydraulic conductivity than similarly-sized lateral roots (LRs) in flooded tamarack (*Larix laricina*) (Islam and Macdonald, 2004). However, it remains unclear whether the high hydraulic conductivity of adventitious root is associated with aquaporin activities. In the present study, hydroponically grown tobacco plants were subjected to root hypoxia to shed more light on the processes in hypoxia-induced ARs that facilitate water transport. It was hypothesized that root hydraulics and ATP production under hypoxia can be enhanced by the formation of ARs. Root porosity, ATP contents and transcript profiles of hypoxia-responsive genes were compared between ARs and existing LRs. Transcription profiling of PIPs of ARs was also compared to LRs to examine potential significance of various PIPs in the responses of roots to hypoxia.

2.2 Materials and Methods

2.2.1 Growth conditions and hypoxia treatment

Tobacco seeds were germinated and seedlings were grown in horticultural soil a

controlled growth room with 18 h photoperiod, 22/18ºC (day/night) temperature, 400 μmol m^{-2} s^{-1} photosynthetic photon flux density and 50% relative humidity. After 3 weeks of growth, seedlings were transferred to 40-L plastic tubs (~ 60×40×20 cm) containing 50% strength modified aerated Hoagland's solution (Epstein, 1972). Thirty-two seedlings were randomly selected and grown in four tubs (8 plants in each tub). After one week, 16 plants in two tubs were subjected to hypoxia by flushing nitrogen gas (99.998%, Praxair, Danbury, CT, USA) through the solution to reach a dissolved O_2 level of ~ 2 mg L^{-1} and then leaving the solution stagnant. The other 16 plants in two tubs were well-aerated with air pumps and served as control (dissolved O_2 concentration of ~ 8 mg L^{-1}).

2.2.2 Gas exchange and photosynthetic light responses

After two days and one week of treatments, gas exchange was measured with the Li-Cor LI-6400XT portable photosynthesis system equipped with the 2×3 cm^2 red-blue light chamber (Li-Cor, Lincoln, NE, USA). Six plants in each treatment and three middle-position fully expanded leaves on each plant were randomly selected. Net photosynthesis (P_n), transpiration (E) and stomatal conductance (g_s) were measured. Air flow rate was set to 400 μmol s^{-1}, photosynthetic photon flux density (PPFD) was 400 μmol m^{-2} s^{-1}, and reference CO_2 concentration was 400 μmol mol^{-1}. An automated program of LI-6400XT was used to determine photosynthetic light responses starting at PPFD of 1500 μmol m^{-2} s^{-1}, followed by 1200, 1000, 800, 500, 300, 200, 100, 50, 20 and 0 μmol m^{-2} s^{-1}. Three plants in each treatment were randomly selected and net photosynthesis was auto-logged when it reached a steady rate. A modified rectangular hyperbole model was employed to estimate light saturated P_n (P_m), light saturation point (I_m) and light compensation point (I_c) (Ye, 2007).

2.2.3 Root hydraulic conductance (K_r)

A high–pressure flow meter (HPFM, Dynamax Incorporated, Houston, TX, USA) was

used to measure tobacco root K_r as previously described (Tyree et al., 1995; Lee et al., 2010). Shoots of six plants in each treatment were excised about 2 cm above the root collar, and the roots were connected to the HPFM. Roots were kept in treatment solutions during the measurements. Water was forced into roots at increasing pressures (0 to 0.5 MPa) and linear regression between applied pressure and flow rate was used to obtain a slope of the relationship which represented K_r.

2.2.4 Number, dry mass and maximum length of ARs

After one week of treatment, number of ARs was counted and the maximum length of ARs of six plants in each treatment was measured with a ruler. Dry mass of ARs was determined after drying the roots in an oven at 85°C.

2.2.5 Root porosity

Root porosity in lateral (LR) and adventitious (AR) roots of four to six plants in each treatment was estimated with the pycnometer method based on Archimedes' principle (Jensen et al., 1969) after one week of treatment using the following equation:

$$\text{Porosity (\%)} = 100 \times (M_h - M_{r+w})/(M_w + M_r - M_{r+w})$$

where M_w is mass of the water filled pycnometer, M_r is mass of roots, M_{r+w} is mass of pycnometer with roots and water and M_h is mass of pycnometer with homogenized roots (Jensen et al., 1969).

2.2.6 Root ATP concentration

LRs and ARs of three plants in each treatment were sampled and ground in liquid nitrogen after two days and one week of treatment, respectively. ATP concentration was determined with the ENLITEN ATP Assay Kit (Promega, Madison, WI, USA) by measuring bioluminescence and quantified with a standard curve using ATP standard (Promega). Luminescence signal was detected using a microplate reader (Fluostar

Optima, BMG Labtech, Ortenberg, Germany) as previously described (Zwiazek et al., 2017).

2.2.7 RNA transcription profiling

After two days and one week of treatment, LRs and ARs from 6 plants in each treatment were sampled. The samples were quickly frozen and kept in liquid nitrogen before being transferred to the –80°C freezer. The samples were ground with a mortar and pestle in liquid nitrogen. Total RNA was extracted with a Plant RNeasy extraction kit (Qiagen, Valencia, CA USA). First strand of cDNA was synthesized from 1µg total RNA using a Reverse Transcription Kit (Qiagen). Quantitative RT-PCR was employed to analyze relative RNA expression using the $2^{-\Delta Ct}$ method. The relative transcript abundance of *PIP*s was normalized against geometric mean of the CT value of two reference genes, *NtEF1-α* (AF120093) and *L25* (L18908) (Schmidt and Delaney, 2010). Transcripts levels of *NtPIP1;1* (AF440271), *PIP1;2* (= *AQP1*, AF024511), *PIP1;3* (U62280), *PIP1;4* (DQ914525) and *PIP2;1* (AF440272) were analyzed. *NtADH1* (*alcohol dehydrogenase 1*, X81853.1) and *PDC1* (*pyruvate decarboxylase 1*, X81854.1) were selected as hypoxic indicators. Relative transcript abundance of *ACS* (X65982.1) was also determined since it encodes an enzyme catalyzing a rate-limiting step in ethylene synthesis (Yang and Hoffman 1984). Gene-specific primers are described in Appendix 1.

2.2.8 Statistical analysis

Means (n = 3-6) and standard errors (SE) were calculated. Paired t-test was performed to compare AR formation and K_r between aerated and hypoxic roots ($\alpha = 0.05$). In all other comparisons, one-way ANOVA followed by Tukey's test was performed to compare means ($\alpha = 0.05$). Three out of four replications of *PDC1* relative transcript abundance of aerated LRs were too low to be detected and consequently only the relative transcript abundance of *PDC1* of aerated ARs, hypoxic LRs and hypoxic ARs

were compared in Tukey's test.

2.3 Results

2.3.1 Leaf gas exchange and photosynthetic light responses.

P_n of hypoxic plants decreased by over 50% and 70% compared with aerated plants after two days and one week of treatment, respectively (Fig 2.1a). Hypoxic plants also showed a decrease of more than 70% in E and g_s on the two treatment days (Fig 2.1b and c).

Hypoxia treatment profoundly affected photosynthetic light responses (Fig 2.1d). Both two days and one week of hypoxia treatments resulted in significant declines of I_c and I_m (Table 2.1). P_m of hypoxic plants also showed a significant decrease compared with aerated plants after one week of treatment (Table 2.1).

2.3.2 AR formation and root porosity

Formation of ARs was induced by hypoxia (Table 2.2). Hypoxic plants had over two-fold higher AR number compared with aerated plants. However, the maximum length of ARs in hypoxic plants was significantly lower compared with aerated plants (Table 2.2). Hypoxia treatment did not affect the total dry mass of ARs (Table 2.2).

Root air space was estimated by the root porosity test after one week of treatment. Hypoxia lead to a 13% increase of porosity in ARs and a slight decrease of porosity in LRs (Fig 2.2a). The porosity of hypoxic ARs was higher by over 50% compared with hypoxic LRs. The porosity of aerated ARs was higher by over 25% compared with aerated LRs.

2.3.3 K_r and root ATP concentration

There was no significant difference in K_r between hypoxic roots and aerated roots after one week of treatment (Fig 2.2b).

After two days of treatment, hypoxia resulted in a significant decrease of ATP concentration in both ARs and LRs (Fig 2.2c). ATP concentration of hypoxic LRs and ARs decreased by about 60% and 40%, respectively, compared with aerated roots (Fig 2.2c). After one week of treatment, no significant difference in ATP concentration between hypoxic and aerated roots was detected.

2.3.4 RNA expression profiling in roots

Hypoxia resulted in significant decreases of *PIP1;1* and *PIP1;3* relative transcript abundance in LRs after two days of treatment (Fig 2.3a). However, relative transcript abundance in ARs remained unchanged (Fig 2.3a). Relative transcript abundance of *ACS*, *ADH1* and *PDC1* in LRs were sharply induced by hypoxia (Fig 2.3b). Hypoxic ARs also showed significantly higher *ADH1* and *PDC1* relative transcript abundance compared with aerated ARs, however, *ACS* remained unchanged (Fig 2.3b).

After one week of treatment, relative transcript abundance of *PIP1;2*, *PIP1;4* and *PIP2;1* in hypoxic LRs were significantly lower compared with the aerated LRs, whereas relative transcript abundance of *PIP1;1* and *PIP1;3* showed no change (Fig 2.3c). In contrast, hypoxia did not result in significant changes of PIP relative transcript abundance in ARs (Fig 2.3c). Relative transcript abundance of *ACS*, *ADH1* and *PDC1* exhibited similar trends as on day two (Fig 2.3d). A sharp increase of *ACS*, *ADH1* and *PDC1* expression was triggered in LRs by hypoxia (Fig 2.3d). Hypoxic ARs showed no changes in relative transcript abundance of the *ACS*, *ADH1* and *PDC1* compared with aerated ARs (Fig 2.3d).

2.4 Discussion

O_2 deficiency is a challenging environmental factor that produces complex responses in plants. Following two days and one week of treatment, gas exchange in tobacco was sharply reduced by hypoxia. Hypoxia also increased the number of ARs but with a similar biomass as in aerated plants. ARs showed different response patterns to

hypoxia compared with the LRs in terms of the transcript profiles of *PIP*s and hypoxia-responsive genes, which may partially contribute to maintaining K_r of hydroponically-grown tobacco plants.

Stomatal conductance, which is regulated by hydraulic and (or) chemical signals, is the main limiting factor for leaf carbon assimilation (Farquhar and Sharkey, 1982). The decline in P_n after two days and one week of hypoxia was likely due to the stomatal closure, which was reflected by the decreased *E*. The decrease in stomatal opening, as demonstrated by lower g_s, was likely responsible for the reductions in photosynthetic light responses, which showed that P_n of hypoxic plants was saturated at a significantly lower light intensity. Reduced g_s of hypoxic leaves limits internal CO_2 concentration used for carbon assimilation. Root hypoxia also reduced I_c, the light intensity at which the rate of total photosynthesis is balanced by the rate of respiration suggeststing that root hypoxia inhibited leaf respiration in addition to photosynthesis. However, hypoxic plants maintained positive P_n at similar rates on days two and seven, which demonstrates relative tolerance of tobacco plants to hypoxic conditions.

One week of root hypoxia did not influence K_r in hydroponically grown tobacco. Effects of O_2 deprivation on root hydraulics vary between plant species and experimental conditions. While some studies reported reduced K_r in response to O_2 deficiency (Else et al., 2001; Araki, 2006), others showed no effect (Jackson et al., 1996; Islam and Macdonald, 2004). In contrast to K_r, hypoxia resulted in a significant decrease of *E* in the present study. *E* and K_r are frequently strongly linked in plants (Liu et al., 2014), but this relationship may also be affected by other factors including leaf to root ratio (Rodríguez-Gamir et al., 2010). Root growth is typically reduced more than leaf growth under hypoxic contidions (Kozlowski, 1997). Thus, even though K_r remained unchanged under hypoxic conditions, the increasing transpiration demand, as a result of leaf growth, may still lead to decreased *E* in the present study. The diurnal changes of root hydraulic conductivity were also found to be independent

of transpiration in flooded *Zea mays* (Else et al., 1995). More likely, the signal triggering hydraulic adjustment either does not originate in the stomata or is impaired by the secondary changes caused by hypoxia.

It appears that the number of ARs, not its overall length and dry mass, was the overriding factor in maintaining root K_r in hypoxic tobacco plants. The formation of ARs is an important adaptation to low O_2 conditions in some plants (Bailey-Serres and Voesenek, 2008; Calvo-Polanco et al., 2012). In the present study, hypoxia induced the formation of over twice as many ARs compared with aerated tobacco plants, but their length was reduced by hypoxia. Similar results were previously reported in tobacco and the authors concluded that the formation of short ARs cannot functionally replace the primary root system which contributed to the relative intolerance of tobacco to O_2 deficiency (McDonald and Visser, 2003). However, in the present study, K_r in hypoxic plants showed no change compared with aerated plants. Additionally, P_n and E of hypoxic plants showed no further decrease after one week of hypoxia compared with two days of treatment. These results indicate that the ARs were effective in maintaining root water transport. The roles of hypoxia-induced ARs are not limited to replacing existing roots. ARs induced by hypoxia had higher porosity than the LRs and could conduct more air from shoots to roots and the rhizosphere (radial O_2 loss, ROL). ROL of ARs may have a profound influence on rhizosphere aeration and nutrient availability in plants under O_2 deficiency (Colmer, 2003). Thus, the numerous short ARs induced by hypoxia in this study could potentially affect tobacco rhizosphere aeration and contribute to the survival and functioning of the root system under hypoxia.

Decreased root hydraulic conductivity of anoxic *Arabidopsis* has been linked to aquaporin closure (Tournaire-Roux et al., 2003), and a structure-based protonation mechanism under O_2 deprivation conditions has been demonstrated (Tournaire-Roux et al., 2003; Törnroth-Horsefield et al., 2006). In addition to aquaporin gating analysis, aquaporin gene expression patterns can reveal the importance of various aquaporins in

plant responses to O_2 deficiency (Tan et al., 2018). Although transcripts may not be translated under certain conditions and posttranslational regulation can modify the function of aquaporins, transcriptional responses can shed light on the translational potential of cells experiencing O_2 deficiency (van Dongen and Licausi, 2014). Microarray analysis showed that aquaporin expression was downregulated in O_2 deficient *Arabidopsis* (Liu et al., 2005) and *Persea americana* (Reeksting et al., 2016). Here, I compared the relative transcript abundance of *PIP*s in ARs with LRs. After two days of hypoxia, relative transcript abundance of *PIP1;1* and *PIP1;3* decreased in LRs but showed no change in ARs. One week of hypoxia resulted in decreased relative transcript abundance of *PIP*s in LRs except *PIP1;1* and *PIP1;3*, while hypoxic ARs showed unchanged expression of *PIP*s on day seven. These results indicate that the relative transcript abundance of *PIP*s in ARs was less affected by hypoxia, which may contribute to hydraulic adjustment. Interestingly, expression of *PIP1;1* and *PIP1;3* in LRs showed different responsive patterns compared with the other examined *PIP* genes under hypoxia. Further research is needed to shed more light on the biological roles of PIP1;1 and PIP1;3 in tobacco.

Tobacco PIP1;3 has been shown to be potentially involved in the O_2 transmembrane transport (Chapter 5). Although the experimental set-up in this present study is similar to the tobacco root hypoxia experiment in Chapter 5, the objective of this study was mainly comparing the differences between ARs and LRs under hypoxic conditions. However, unlike the present study, relative transcript abundance of tobacco *PIP1;3* was upregulated by root hypoxia (Chapter 5). The reason for the difference may be that in the present study LRs and newly formed ARs were sampled saparately rather than the whole root. Additionally, gene expression patterns in response to abiotic stresses may vary between different developmental stages (Skirycz et al., 2010). Tobacco plants were exposed to root hypoxia three weeks after germination in the present study while plants were subjected to root hypoxia two weeks after germination in Chapter 5, which may also contribute to the differences in

results. Since ARs were less affected by hypoxia than LRs in terms of *PIP* transcription, ARs can likely more actively participate in root water uptake under hypoxic conditions. In fact, ARs induced by hypoxia in *Larix laricina* were shown to have higher hydraulic conductivity than the existing roots (Islam and Macdonald, 2004).

Phytohormones, especially ethylene, are involved in the regulation network in response to O_2 deficiency, including the initiation and regulation of ARs (Vidoz et al., 2010; Voesenek and Bailey-Serres, 2013). In this study, transcript profiling showed that more *ACS* transcripts were induced in hypoxic LRs than ARs, which could result in the accumulation of ACC (an ethylene synthesis precursor) in hypoxic LRs. ACC is induced in flooded roots while the conversion of ACC to ethylene needs the presence of O_2 (Bradford and Yang, 1980). The transport of ACC from stressed roots to other tissues serves as a signal and causes the formation of ARs in aerated shoots (Yang and Hoffman, 1984). Both *ADH* and *PDC* were frequently reported to be up-regulated in O_2 deficient tissues (Mugnai et al., 2011; Reeksting et al., 2016). In this study, transcripts of tobacco *ADH1* and *PDC1* were sharply up-regulated in hypoxic LRs, while ARs showed much lower expression. Despite the inefficiency of ethanolic fermentation, the activities of ADH and PDC are essential to plants under low O_2 conditions to meet the energy demand (Drew, 1997). However, the accumulation of end products of fermentation is toxic (Drew, 1997). In this present study, hypoxic ARs maintained markedly lower levels of *ADH* and *PDC* transcripts compared with LRs. This suggests that the better aeration of ARs reduced their dependence on inefficient and toxic fermentation compared with LRs under low O_2 conditions.

In conclusion, the formation of short ARs was induced by hypoxia in hydroponically grown tobacco plants. Although, the length of ARs was reduced in hypoxic plants, it appears that they were likely more effective in facilitating O_2 diffusion into the roots and maintaining low level of fermentation and high K_r. *PIP* expression patterns likely reflect the metabolic status in roots and may also be related

to gas transport. Stable *PIP* transcripts of ARs under hypoxic conditions may be indicative of an active role of ARs in root water transport under hypoxic conditions.

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Table 2.1 Comparison of estimated light-saturated photosynthesis (P_m, μmol CO_2 m^{-2} s^{-1}), light saturation point (I_m, μmol m^{-2} s^{-1}) and light compensation point (I_c, μmol m^{-2} s^{-1}) in photosynthetic light responses of tobacco (*Nicotiana tabacum*) plants subjected to aeration and hypoxia treatment for two days and one week. Means ± SE (n = 3) are shown. Different letters in the same row indicate statistically significant differences determined by the Tukey's test after one-way ANOVA ($P \leq 0.05$)

	Aerated DAY2	Hypoxia DAY2	Aerated DAY7	Hypoxia DAY7
P_m	11.47 ± 0.82 a	7.39 ± 0.79 a	17.85 ± 0.87 b	8.76 ± 1.2 a
I_m	1045.99 ± 84.98 a	683.54 ± 40.39 b	1129.38 ± 52.12 a	551.66 ± 103.49 b
I_c	28.69 ± 2.55 a	19.7 ± 0.11 b	26.34 ± 1.94 a	14.524 ± 3.68 b

Table 2.2 Number, maximum length, and dry mass of adventitious roots of tobacco (*Nicotiana tabacum*) plants in aerated and hypoxia treatment for one week. Means ± SE are shown (n = 6 for number and maximum length, and n = 8 for dry mass of adventitious roots). Asterisks indicate significance between aerated and hypoxic ARs determined by the t-test ($P \leq 0.05$).

	adventitious roots	
	Aerated	Hypoxic
Number	14 ± 1.51	28.17 ± 2.27*
Maximum length	5.77 ± 0.46*	3.53 ± 0.18
Dry mass	0.059 ± 0.012	0.038 ± 0.007

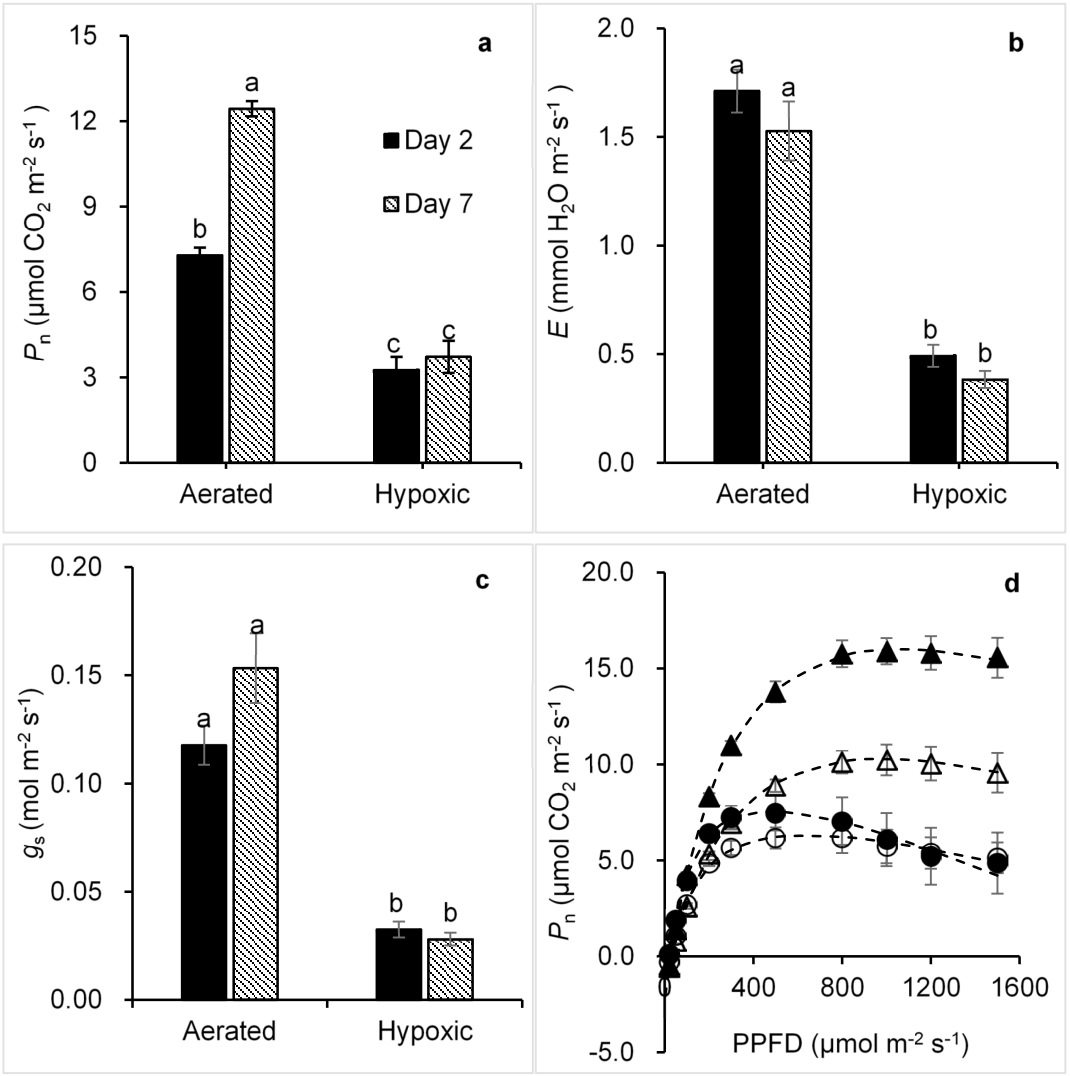

Figure 2.1 Net photosynthesis (P_n, a), transpiration rates (E, b), stomatal conductance (g_s, c) and photosynthetic light responses (d) of well-aerated tobacco (*Nicotiana tabacum*) plants and plants subjected to root oxygen deprivation (hypoxia) treatment for two days and one week. Means ± SE (n = 5 or 6 for P_n, E and g_s. and n = 3 for photosynthetic light responses) are shown. Different letters above the bars indicate statistically significant differences determined by the Tukey's test after one-way ANOVA ($P \leq 0.05$). Aerated after two days (△), hypoxia after two days (○), aerated after one week (▲), and hypoxia after one week (●).

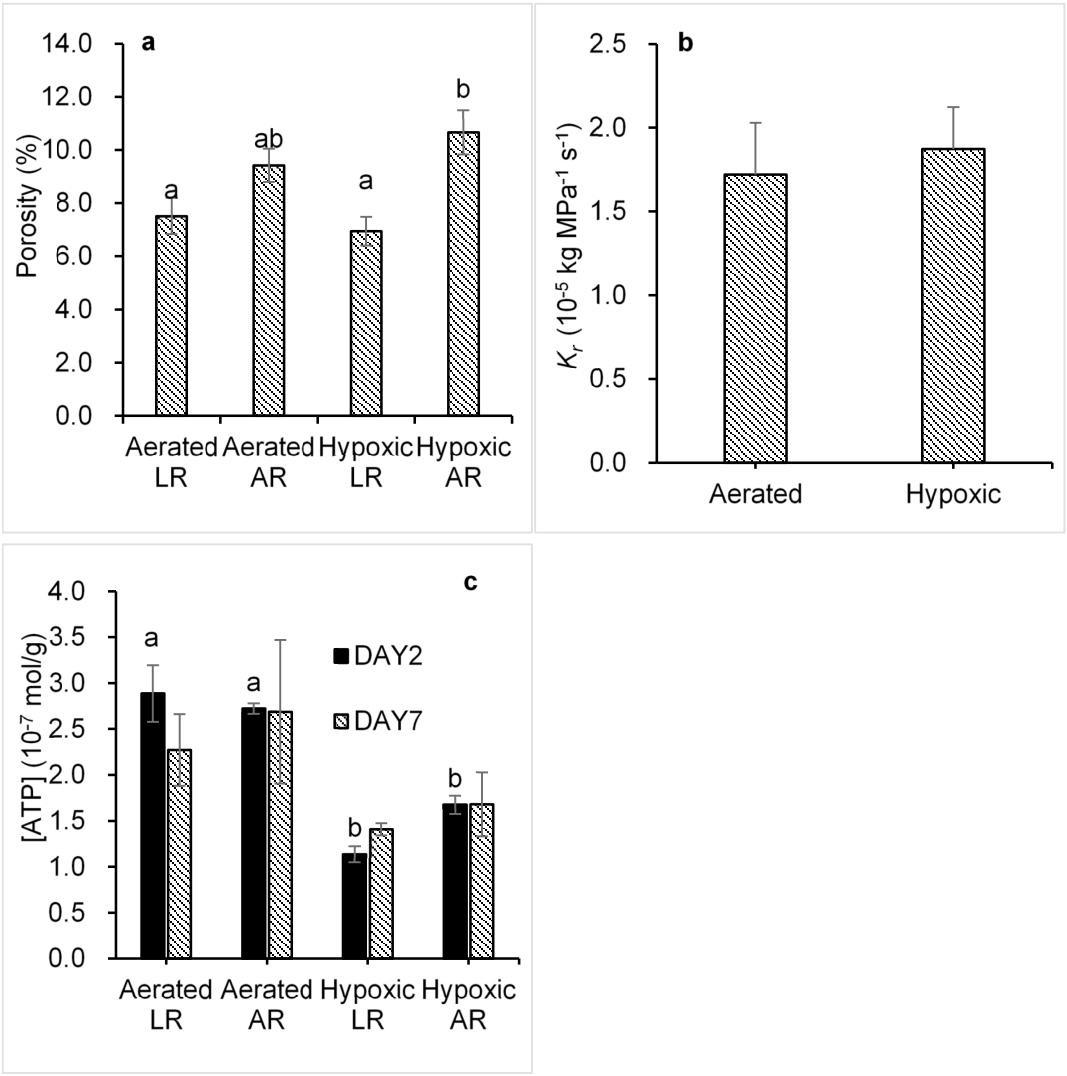

Figure 2.2 Root porosity of adventitious (ARs) and lateral roots (LRs) (a) and hydraulic conductance (K_r, b) of tobacco (*Nicotiana tabacum*) plants subjected to aerated or root oxygen deprivation (hypoxia) treatments for one week, and ATP concentration of ARs and LRs (c) after two days and one week of treatment. Means ± SE (n = 4-6 for porosity test, n = 6 for K_r and n = 3 for ATP assay) are shown. Different letters above the bars indicate statistically significant differences determined by the Tukey's test after one-way ANOVA ($P \leq 0.05$).

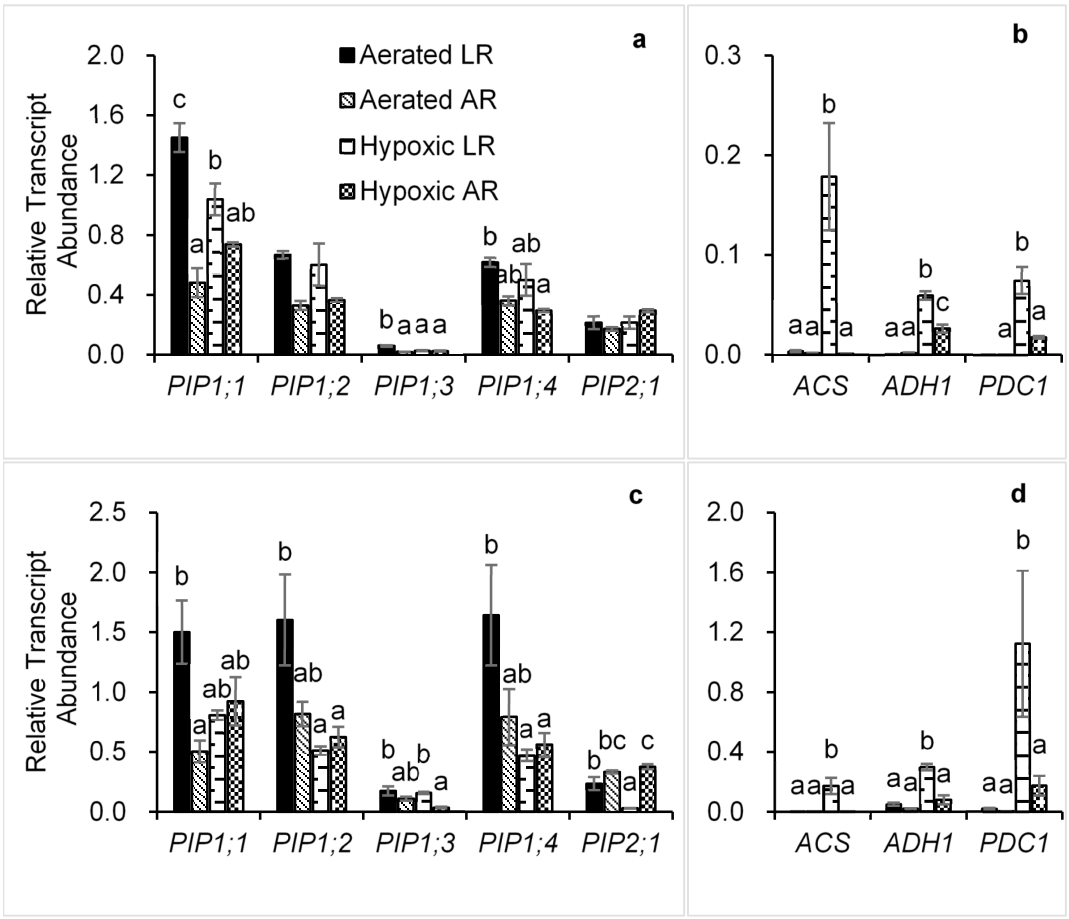

Figure 2.3 Relative transcript abundance of well-aerated tobacco (*Nicotiana tabacum*) plants and plants subjected to root oxygen deprivation (hypoxia) treatment for two days (a and b) and one week (c and d). Relative transcript abundance of plasma membrane intrinsic proteins (*PIP*s, a and c), *1-aminocyclopropane-1-carboxylate synthase* (*ACS*), *alcohol dehydrogenase 1* (*ADH1*) and *pyruvate decarboxylase 1* (*PDC1*) (b and d) is shown. Means ± SE are shown (n = 4-6). *PDC1* expression of LRs aerated after two days is not shown because of un-determined values. Different letters above the bars indicate statistically significant differences within each gene determined by the Tukey's test after one-way ANOVA ($P \leq 0.05$).

3 Aquaporins Are Involved in Ethylene-Induced Hypoxia Resistance of Trembling Aspen (*Populus tremuloides*)

3.1 Introduction

Plants require sufficient amount of O_2 to support growth and maintain respiration as well as for other biochemical reactions (van Dongen and Licausi 2014). In some cases, including flooding (Herzog et al. 2016; Shahzad et al. 2016) and soil compaction (Calvo Polanco et al. 2008), plants are confronted with O_2 deficiency (hypoxia). Even in a normoxic environment, O_2 gradients exist across tissues due to the differences in metabolic rates (Ho et al. 2010; Whipple 2012). Hypoxic conditions are characterized by the reduced ATP synthesis from oxidative phosphorylation. This condition occurs in plants more frequently compared with anoxia in which ATP is generated only by glycolysis in the absence of O_2 (Drew 1997). Most terrestrial plant species cannot survive long-term O_2 deficiency and widely vary in their ability to cope with a short-term disruption of metabolism and the resulting toxicity (Voesenek and Bailey-Serres 2013).

Root hypoxia has been often reported to induce wilting and alter water relations in sensitive plants. This effect was attributed to the inhibition of aquaporin-mediated root water transport and the resulting decrease of root hydraulic conductivity (Tournaire-Roux et al. 2003; Törnroth-Horsefield et al. 2006). Aquaporins play a central role in plant water relations, especially in the radial water flow in roots (Maurel et al. 2015) and may transport other small molecules including CO_2 (Uehlein et al. 2003; Navarro-Ródenas et al. 2015), NH_3 (Jahn et al. 2004) and O_2 (Zwiazek et al. 2017). Plant aquaporins show high diversity and the number of aquaporin isoforms that is higher than 30 in all of the examined species (Maurel et al. 2015). In higher plants, five subfamilies of aquaporins have been categorized including the PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast membrane intrinsic proteins), NIPs (nodulin 26-like intrinsic proteins), SIPs (small intrinsic proteins) and XIPs (uncategorized intrinsic proteins). Aquaporins mediate root hydraulic adjustments in response to environmental factors (Parent et al. 2009; Maurel et al. 2015). The closure of aquaporins under

hypoxia is thought to be the reason for decreased water uptake in the roots through intracellular acidosis (Tournaire-Roux et al. 2003) and energy deprivation (Kamaluddin and Zwiazek 2001, 2002) that can both result in the down-regulation of aquaporin gating. Recently, a potassium-dependent O_2 sensing pathway was proposed in *Arabidopsis* (Shahzad et al. 2016), which may regulate the aquaporin activity downstream. Although the aquaporin activities and expression were suggested to be inhibited under O_2 deficiency (Kamaluddin and Zwiazek 2002; Liu et al. 2005), the expression of *Arabidopsis NIP2;1* was highly induced by hypoxia (Liu et al. 2005), and proven to act as a transporter of lactic acid (Choi and Roberts 2007).

Ethylene may be induced by various biotic and abiotic stress, including drought (Sobeih et al. 2004), wounding (Reymond et al. 2000), and flooding stresses (Hahn et al. 1920). Endogenous ethylene is produced through the Yang cycle (Yang and Hoffman 1984), and can be synthesized by almost all kinds of plant cells. Ethylene production is induced under hypoxia but blocked by anoxia as free O_2 is needed for the conversion from 1-amino cyclopropane 1-carboxylic acid (ACC) to ethylene (Yang and Hoffman 1984). The gene expression of ACC synthase (ACS) and ACC oxidase (ACO) enzymes, which catalyze the regulatory steps in ethylene synthesis, was reported to be up-regulated by hypoxia (Mekhedov and Kende 1996; Rieu et al. 2005). Ethylene can improve plant hypoxia tolerance through the stimulation of adventitious roots and aerenchymatous tissue formation (Voesenek and Sasidharan 2013). Only a few studies have focused on the relationship between ethylene and aquaporins. Aquaporins were reported to be involved in the ethylene regulated ripening of grape berry and latex yield of rubber tree (Chervin et al. 2008; Tungngoen et al. 2009). A recent study revealed that ethylene increased the water transport rate in *Arabidopsis* cells through the enhanced C-Terminal phosphorylation of *Arabidopsis* aquaporin PIP2;1 (Qing et al. 2016). These findings built the links between ethylene and aquaporin transcripts profiling as well as posttranslational regulation, while more evidence is still needed for comprehensive understanding of the underlying processes. Exogenous ethylene promoted the water transport of hypoxic aspen (*Populus tremuloides*) seedlings, and the enhanced aquaporin activity in response to ethylene under hypoxia was speculated (Kamaluddin and Zwiazek 2002). In the present study, I examined the effects of hypoxia and exogenous ethylene on aquaporin expression in aspen seedlings to shed more light on the regulation of aquaporin-mediated water transport by O_2 deficiency and hormones. It was hypothesized that ethylene can affect root hydraulics in hypoxic conditions by

affecting aquaporin gene expression.

3.2 Materials and Methods

3.2.1 Growth conditions and hypoxia treatment

Populus tremuloides seeds were germinated and the seedlings were grown in horticultural soil in a controlled growth room with 18 h photoperiod, 22/18ºC (day/night) temperature, 400 μmol m^{-2} s^{-1} photosynthetic photon flux density and 50% relative humidity. After five weeks of growth, the plants were transferred to 40-L polyethylene containers (~ 60×40×20 cm) filled with a quarter-strength aerated modified Hoagland's solution (Epstein, 1972).

After one week, 24 plants were transferred into individual 1 L amber plastic flasks. Of these plants, 12 were randomly selected and subjected to aeration treatment by flushing the solution in the flasks with air pumps to maintain a dissolved O_2 concentration of about 7.5 mg L^{-1}, while the other 12 plants were subjected to the hypoxic treatment by flushing N_2 gas (99.998%, Praxair, Danbury, CT, USA) through the solution to reach an O_2 level of 2 mg L^{-1} and then leaving it stagnant. Dissolved O_2 concentration, which was monitored during the experiment with the YSI-5000 (YSI Incorporated, Yellow Springs, OH, USA) O_2 electrode, declined to less than 1 mg L^{-1} within one week.

3.2.2 Ethylene treatment

One-half of the aerated control and hypoxia-treated plants (n = 6) were treated with exogenous ethylene at the beginning of the hypoxia treatment. Ethylene was supplied to the hydroponic solution from the compressed gas cylinder through a tube stretched to the bottom of plastic bottles (Kamaluddin and Zwiazek 2002). Dissolved ethylene concentration was determined with the headspace analysis method (Kampbell and Vandegrift 1998). Briefly, 5 ml sample of hydroponic solution was collected into a gas-tight vacutainer. The headspace (10%) was prepared by replacing water with N_2, and the vacutainers were shaken at 1400 rpm for 5 minutes. From the headspace, 100 μl gas samples were collected and injected into the Varian 430-GC gas chromatograph (Varian, Palo Alto, CA, USA) equipped with a HP Plot-Q column (30 m×0.53 mm×40 μm, Agilent, Santa Clara, CA, USA). The concentration of dissolved ethylene was

calculated based on the Henry's Law and a constant of 4.9×10^{-3} M atm^{-1} was used (Kampbell and Vandegrift 1998; Sander 2015). The ethylene treatment resulted in the average dissolved ethylene concentration of 3.85 mmol L^{-1}.

3.2.3 Gas exchange, chlorophyll fluorescence and leaf chlorophyll concentrations

One fully extended leaf was randomly selected from each of the six plants per treatment to measure net photosynthesis rate (P_n) and transpiration rate (E) using a Li-Cor LI-6400XT Portable Photosynthesis System with a 2×3 cm^2 red-blue light chamber (Li-Cor, Lincoln, NE, USA). Light responses of P_n and g_s were established by setting an automatic program with a starting photosynthetic photon flux density (PPFD) of 1500 μmol m^{-2} s^{-1}, followed by 1200, 1000, 800, 500, 300, 200, 100, 50, 20 and 0 μmol m^{-2} s^{-1}. Parameters were auto-logged when P_n reached steady state. Light saturated P_n (P_m) and light saturation (I_m) were estimated by fitting the data to a modified rectangular hyperbole model (Ye 2007).

Leaf chlorophyll fluorescence of six plants in each treatment was measured with Li-Cor LI-6400XT with a leaf chamber fluorometer. The maximum quantum yield of photosystem II (PSII) (F_v/F_m) was measured with dark-adapted leaves, and F_s, F_o' and F_m' was subsequently measured by turning on actinic light and equilibrating. Effective quantum yield (Φ_{PSII}) and non-photochemical quenching (NPQ) were calculated using the following equations:

$$\Phi_{PS\,II} = \frac{F_m' - F_s}{F_m'}$$

$$NPQ = \frac{F_m - F_m'}{F_m'}$$

Leaf chlorophyll (Chl) of three plants in each treatment was extracted with 10ml dimethyl sulfoxide for 2 h at 65ºC from three fully expanded leaves of each treatment (Parry et al. 2014). The leaf fresh weight (M) was recorded. Light absorbance of the chlorophyll extract was measured at 649.1 nm and 665.1 nm using a Genesys-10 UV/Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Chlorophyll concentrations were expressed on a mass basis and calculated using the following equations (Wellburn 1994):

$$\text{Chl a} = \frac{(12.47 \times A_{665.1} - 3.62 \times A_{649.1}) \times 0.01\ \text{L}}{M}$$

$$\text{Chl b} = \frac{(25.06 \times A_{649.1} - 6.5 \times A_{665.1}) \times 0.01\text{L}}{M}$$

$$\text{Chl a/b} = \text{Chl a}/\text{Chl b}$$

3.2.4 Root hydraulic conductance and root O_2 concentration

Root hydraulic conductance (K_r) was measured with a high-pressure flow meter (HPFM, Dynamax Incorporated, Houston, TX, USA, Tyree et al. 1995). During the measurements of six plants in each treatment, roots were kept in the treatment solution. Linear regression between supplied pressure and flow rate was obtained by applying increasing pressure to the roots, and the slope was calculated as K_r (Xu et al. 2015).

Root O_2 concentration was measured in six plants of each treatment with an O_2 sensor (OX50, Unisense, Aarhus, Denmark) with a tip diameter of 50 μm connected to an OXY-Meter (Unisense). The O_2 sensor was inserted ~ 200 μm into root cortex 3–5 cm from the root tip with a micromanipulator (Leica Leitz, Wetzlar, Germany). The insertion depth of O_2 sensor was determined by sectioning the roots and oberserving under micrpscope.

3.2.5 Root porosity

Root porosity was measured in six plants of each treatment with the pycnometer (Jensen et al. 1969). A 25 ml pycnometer filled with degassed water was weighed (M_w). Roots were cut into segments and placed in the pycnometer. The pycnometer with roots was weighed again (M_{r+w}). The roots were removed from the bottle, blotted dry with paper towel, and weighed (M_r). The roots were then homogenized, and the foam were eliminated by repeated vacuum. Homogenate was poured back into the pycnometer, toped up with degassed water and the weight was recorded (M_h). The porosity was calculated from the following equation (Jensen et al. 1969):

$$\%\ \text{porosity} = \frac{100 \times (\text{Mh} - \text{Mr+w})}{\text{Mw} + \text{Mr} - \text{Mr+w}}$$

3.2.6 mRNA expression profiling

After one week of treatment, primary roots of six plants in each treatment were collected and kept in liquid nitrogen before being transferred to -80ºC freezer. Samples were ground with mortar and pestle in liquid nitrogen. Total RNA was extracted with a Plant RNeasy extraction kit (Qiagen, Valencia, CA, USA). First strand of cDNA was synthesized from 1µg total RNA using a Reverse Transcription Kit (Qiagen). Quantitative RT-PCR was employed to analyze RNA expression using *JIP1* (AJ407583.1) as a reference gene, which did not change significantly across all tested samples (P = 0.36). mRNA sequences of seven PIPs were obtained by searching the database. The transcript abundance of *PIP1;1* (AJ849323.1), *PIP1;2* (AJ849322.1), *PIP2;1* (AJ849324.1), *PIP2;2* (AJ849325.1), *PIP2;3* (AJ849326.1), *PIP2;4* (AJ849327.1) and *PIP2;5* (AJ849328.1) were determined. These PIPs have been proven to be functional and stress responsive in poplar plants (Marjanovic et al. 2005; Liu et al. 2014). The transcript abundance of five ethylene responsive factors (*ERF*s), *ERF17*, *ERF18*, *ERF35*, *ERF71* and *ERF76*, was determined since these genes were reported to be induced by ethylene in *P. tremula* × *tremuloides* (Vahala et al. 2013). The transcript abundance of three genes involved in ethylene biosynthesis, *ACO1* (AY167040.1), *ACS1* (AF518326.1) and *ACS2* (AF518327.1), were also determined (Andersson-Gunnerås et al. 2003; Vahala et al. 2003). The relative transcript abundance was calculated using the $2^{-\Delta Ct}$ method. The primers for aspen PIPs (Liu et al. 2014), and ERFs (Vahala et al. 2013) were designed as reported in the corresponding references (Appendix 1). The primers for *ACO1*, *ACS1* and *ACS2* were designed with the PrimerQuest Tool (IDT DNA Tech., Coralville, IA, USA) (Appendix 1).

3.2.7 SDS-PAGE and immunoblotting

SDS gel electrophoresis was performed to separate proteins (10 µg) extracted from roots after one week of treatment. Gel was transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) by electroblotting at 100 V for 1 h. The membrane was blocked in 1% BSA in TBST buffer (20 mM Tris pH 7.5, 150 mM NaCl and 0.1% Tween 20) for 3 h and then incubated with anti-PIP1 (1:500) or -PIP2 (1:1000) primary antibody overnight at 4ºC. The PIP antibodies, provided by Dr. François Chaumont from the Catholique University of Louvain, were raised in rabbit against maize (*Zea mays*) PIPs (Calvo-Polanco et al. 2012). The membrane was rinsed 5 times for 5 min with TBST

and then incubated in GAR IgG antibody (1:2000) (Sigma-Aldrich, Oakville, ON, Canada) for 1h at room temperature. After rinsing the membrane with TBST 5 times for 5 min, alkaline phosphatase (Bio-Rad) was conjugated to detect signals.

3.2.8 Statistical Analysis

The means and standard errors of aerated plants (A), aerated plants treated with ethylene (AE), hypoxic plants (H), and hypoxic plants treated with ethylene (HE) were calculated for each of the measured parameters. Two-way ANOVA was used to examine the effects of oxygen (aeration or hypoxia) and ethylene treatments (with or without ethylene). To examine the effects of exogenous ethylene on the determined parameters under aeration or hypoxia separately, contrasts between A and AE treatments, and between H and HE treatments were also performed, respectively (α = 0.05).

3.3 Results

3.3.1 Leaf gas exchange and light responses

Hypoxia treatments significantly reduced leaf P_n, g_s and E, but ethylene treatments only affected P_n and E (Table 3.1, Fig 3.1). Ethylene-treated plants showed significantly higher P_n compared with plants without ethylene treatment under aerated conditions (P = 0.03), but not under hypoxic conditions (P = 0.074) (Fig 3.1a). E of ethylene-treated plants showed significantly higher values compared with plants without ethylene treatment under both aerated (P = 0.002) and hypoxic (P = 0.012) conditions (Fig 3.1c). However, g_s showed no change with ethylene treatment (Fig 3.1b).

Hypoxia treatments significantly decreased I_m and P_m (Table 3.1, Fig 3.2a), indicating that root hypoxia comprehensively inhibited light repsonses of leaf. Ethylene treatments signigicantly affected P_m but not I_m (Table 3.1). When exposed to hypoxia, ethylene treated plants showed significantly higher P_m (than that of plants without ethylene treatments (P = 0.006) (Table 3.2). Light response of g_s showed that g_s gradually increased with increasing light intensity under aerated conditions, but did not change under hypoxic conditions (Fig 3.2b).

3.3.2 Leaf chlorophyll fluorescence and chlorophyll concentrations

Dark-adapted leaves were used to measure F_o and F_m. F_v/F_m showed similar values of approximately 0.8 for all treatments (Fig 3.3a). Hypoxia significantly decreased Φ_{PSII} and increased *NPQ* values (Table 3.1), but no significant effects of ethylene on these parameters were detected (Fig 3.3 b and c).

Hypoxia and ethylene treatments did not affect leaf Chl concentrations and Chl a/b ratios (Fig 3.3d).

3.3.3 Root hydraulic conductance, O_2 concentration and porosity

Hypoxia significantly decreased both K_r and root O_2 concentration (Table 3.1) and resulted in a drastic decrease of K_r (Fig 3.4a). Exogenous ethylene did not affect K_r in well-aerated plants (P = 0.702), but in the plants subjected to hypoxia, ethylene treatment significantly increased K_r by about 20% compared with the hypoxic plants that were not treated with ethylene (P = 0.021) (Fig 3.4a).

Root O_2 concentration sharply decreased in the hypoxic plants to less than 10% of the concentration measured in the well-aerated plants (Fig 3.4b). Ethylene treatment significantly increased the root O_2 concentration in the HE plants by almost five-fold compared with the plants of H treatment (P = 0.021) but had no effect on O_2 concentrations in well-aerated roots (P = 0.834) (Fig 3.4b).

Hypoxia and ethylene treatments did not affect root porosity (Table3.1, Fig 3.4c).

3.3.4 mRNA expression profiling

After one week of treatments, mRNA expression profiling was carried out by qRT-PCR. The relative transcript abundance of *ACO1*, *ACS1* and *ACS2*, three genes encoding key proteins involved in ethylene synthesis, was not changed by either hypoxia or ethylene treatments (Table 3.1, Fig 3.5a). Hypoxia treatment significantly increased the relative transcript abundance of five *ERFs* (Table 3.1). Ethylene treatments increased the relative transcript abundance of *ERF35* (Table 3.1) under hypoxic conditions (P = 0.006) but not under aerated conditions (P = 0.12) (Fig 3.5b). However, the relative transcript abundance of the other four *ERFs* showed no change compared with the plants not exposed to ethylene (Table 3.1).

Hypoxia significantly decreased the relative transcript abundance of *PIP1;2* and

PIP2;2, but significantly increased the relative transcript abundance of *PIP2;1* and *PIP2;5* (Table 3.1). Ethylene treated plants exhibited significantly higher *PIP2;4* transcripts than non-treated plants (Table 3.1) under hypoxic conditions (P = 0.044) but not under aerated conditions (P = 0.599) (Fig 3.5c).

3.3.5 Immunodetection of PIPs

Antibodies against PIP1 and PIP2 were used in the immunodetection of PIPs. Signals at ~ 30 kDa were detected (Fig 3.6). Both the antibodies detected weaker signals in the roots of hypoxia treatments compared with the aeration treatments. Under hypoxia, ethylene treatments the immunoreactive bands of PIP2 showed an increase in intensty, but only slight effects on PIP1 were detected.

3.4 Discussion

The results demonstrate that the application of exogenous ethylene to roots partly alleviated the effects of hypoxia on root hydraulic conductance and gas exchange. The effects of ethylene on *PIP* transcript profiles under hypoxic conditions suggest that aquaporins were likely involved in these responses.

A decrease of root hydraulic conductance under O_2 deficiency has been commonly reported (Else et al. 1995; Tournaire-Roux et al. 2003; Aroca et al. 2012). Phosphorylation plays a central role in aquaporin gating (Maurel et al. 2015). However, the uptake of phosphate, which acts a substrate of phosphorylation, is inhibited under flooding (Zhang and Davies 1986) since energy is needed in its transmembrane transport (Schachtman et al. 1998). O_2 deficiency results in the transition from aerobic respiration to fermentation causing cell acidosis, which could further inhibit aquaporin gating (Tournaire-Roux et al. 2003; Törnroth-Horsefield et al. 2006) and, consequently, inhibit aquaporin-mediated water transport (Gerbeau et al. 2002; Alleva et al. 2006). Under hypoxia, aspen plants treated with exogenous ethylene showed significantly higher root hydraulic conductance compared with the plants that did not receive ethylene treatment. A similar response has been previously reported in aspen and aquaporins were proposed to participate in this response (Kamaluddin and Zwiazek 2002). Several reports demonstrated that ethylene affected aquaporin transcripts during leaf petal expansion and fruit ripening (Ma et al. 2008; Tungngoen et al. 2009). Post-transcriptional regulation of aquaporins also plays an

important role in plant water fluxes. A C-terminal phosphorylation mechanism of PIP2;1 was proposed to participate in water transport regulation by ethylene in *Arabidopsis* cells (Qing et al. 2016). In the present study, the relative transcript abundance of several *PIP*s was determined, which responded differently to hypoxia and ethylene treatments. *PIP1;2* and *PIP2;2* transcripts were down-regulated while *PIP2;1* and *PIP2;5* transcripts were significantly up-regulated by hypoxia. The gene expression of another PIP aquaporin, *PIP2;4,* was not changed by the hypoxia treatment. However, ethylene treatment significantly increased the relative transcript abundance of *PIP2;4* under hypoxic conditions. Western blot analyses confirmed that hypoxia decreased the protein abundance of both PIP1s and PIP2s. However, ethylene treatments increased the abundance of PIP2 under hypoxia, suggesting that PIP2 is the main target of the effects of ethylene on aquaporins. Given that aquaporins alter root hydraulic properties and regulate root water transport (Maggio and Joly 1995; Javot 2003), and that PIP2s are the main group of aquaporins involved in root water transport (Chrispeels et al. 2001), including PIP2;5 in *Populus* (Marjanović et al.2005*)*, it is plausible that the increase in the abundance of PIP2s played a role in the amelioration of root hydraulics caused by ethylene. However, the possibility that the increase in the abundance of PIP2s may also affect the transport of other molecules that alter responses to hypoxia should not be excluded since some of the aquaporins may also be involved in the transport of lactic acid (Choi and Roberts 2007) and O_2 (Zwiazek et al. 2017).

Photosynthetic responses to root hypoxia differ between plant species and are often used as indicators of hypoxia tolerance. In hypoxia-sensitive maize, net photosynthesis gradually decreased in response to flooding (Bragina et al. 2002), but was maintained nearly unchanged in hypoxia-tolerant tamarack (*Larix laricina*) subjected to prolonged flooding (Calvo-Polanco et al. 2012). The tolerance mechanisms that are responsible for this genetic diversity are little known (Bailey-Serres and Voesenek 2008). In the present study, the P_n of aspen showed a dramatic decrease in response to root hypoxia, but ethylene treatment did not change the P_n in hypoxic plants. A decrease in Φ_{PSII} and an increase in *NPQ* in hypoxic, compared to well-aerated, plants reflects lower photochemical efficiency for CO_2 assimilation and energy transduction in PSII that are frequently observed in plants subjected to abiotic stresses (Baker 2008). Ethylene treatments of hypoxic aspen seedlings had little effect on Φ_{PSII}, *NPQ* and leaf chlorophyll concentrations. However, ethylene treatments significantly increased *E*

suggesting that the main targets of ethylene treatments was water flux. It is well acknowledged that root hydraulics is the main factor restricting water delivery to leaves and is tightly linked to transpiration rates and photosynthesis (Rodríguez-Gamir et al. 2010; Liu et al. 2014). Given that root hydraulic conductance was enhanced by the ethylene treatment in hypoxic plants, amelioration of root hydraulics was a likely contributor to the increase of leaf transpiration.

The changes of root hydraulic conductivity and leaf transpiration rates were accompanied by major changes in PIP expression in roots and leaves of partly defoliated aspen indicating that aquaporins are likely key in the adjustment and synchronization of root and leaf hydraulics (Liu et al. 2014). Similarly, in our study, enhanced root hydraulic conductance and leaf transpiration by the ethylene treatment in hypoxic plants were accompanied by increased PIP2 expression suggesting that the responses of aquaporins to ethylene may contribute to the amelioration of plant hydraulics under hypoxia.

Hypoxic treatment showed much lower light-saturated photosynthesis. However, ethylene treatment markedly increased the P_m in hypoxic plants but did not change I_m. The decrease in P_m was also observed in flooded *Genipa americana* seedlings (Mielke et al. 2003). The change of light-saturated photosynthesis in plants has been often reported to be due to electron transport saturation and is reflected by the changes in chlorophyll fluorescence parameters (Mielke et al. 2003; Behrenfeld et al. 2004). However, in our study, the effects of ethylene on chlorophyll fluorescence were small and could not explain profound effects on photosynthetic responses of hypoxic plants. Additionally, light responses of g_s showed that hypoxia greatly hampered the light responses of g_s. Therefore, the effects of ethylene in hypoxic plants were likely due to other factors, including stomatal constrains, likely caused by changes in the aquaporin-mediated root water transport.

Since the function of aquaporins and root hydraulic conductance is strongly linked to metabolism (Kamaluddin and Zwiazek 2001; Tournaire-Roux et al. 2003), the effect of ethylene on root O_2 concentration and root porosity was also examined. The effects of ethylene on root aeration under hypoxia have been often attributed to the formation of aerenchyma and adventitious roots (Drew et al. 1979). In the present study, aspen seedlings did not produce adventitious roots after one week of hypoxia treatment and root porosities did not significantly differ between the roots in different treatments. However, root O_2 concentrations increased in hypoxic plants as a result of ethylene

treatment. O_2 can be taken up by the stomata and transported to roots. It was also reported that the internal aeration of submerged *Oryza sativa* relied on underwater photosynthetic O_2 in daytime (Winkel et al. 2013), and photosynthetic O_2 was demonstrated to be transported to roots through diffusion in submerged *Arabidopsis* (Lee et al. 2011). O_2 transport through plant tissues may be facilitated by some aquaporins (Zwiazek et al. 2017). Considering higher photosynthetic rates in ethylene-treated hypoxic plants and greater transpiration rate, the notion that higher expression of O_2-transporting aquaporins could be linked to higher root O_2 concentrations in ethylene-treated hypoxic plants should be further explored.

In conclusion, the present study showed that exogenous ethylene affected aquaporin abundance, root hydraulics, gas exchange and root internal aeration. The study points to the aquaporin-mediated water transport and, possibly, O_2 transport, as the likely targets of ethylene that contribute to the improved root aeration and increased hydraulic conductance in hypoxic plants.

3.5 References

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Table 3.1 Effects of oxygen and ethylene on the parameters determined in trembling aspen (*Populus tremuloides*) in this study. *P*-values of two-way ANOVA are shown (n = 3–6). P_n net photosynthesis rate, g_s stomatal conductance, E transpiration rate, P_m estimated light-saturated photosynthesis, I_m light saturation point, F_v/F_m maximum quantum yield of photosystem II, Φ_{PSII} effective quantum yield, *NPQ* non-photochemical quenching, K_r root hydraulic conductance

	Variable	*P*-value		
		Oxygen	Ethylene	Oxygen × Ethylene
Physiological Measurements	P_n	**<0.001*****	**0.008****	0.502
	g_s	**<0.001*****	0.338	0.181
	E	**<0.001*****	**<0.001*****	0.554
	P_m	**<0.001*****	**0.014***	0.067
	I_m	**0.001*****	0.532	0.218
	F_v/F_m	0.527	0.174	0.176
	Φ_{PSII}	**<0.001*****	0.399	0.621
	NPQ	**<0.001*****	0.629	0.369
	K_r	**<0.001*****	0.452	0.169
	Root Oxygen	**<0.001*****	0.269	0.142
	Root Porosity	0.86	0.641	0.127
Relative Transcript Abundance	*PtACO1*	0.828	0.478	0.317
	PtACS1	0.238	0.403	0.339
	PtACS2	0.13	0.202	0.2
	ERF17	**0.014***	0.16	0.16
	ERF18	**0.019***	0.084	0.072
	ERF35	**0.001****	**<0.001*****	**0.002***
	ERF71	**<0.001*****	0.052	**0.044***
	ERF76	**0.002****	0.684	0.671
	PtPIP1;1	0.279	0.298	0.07
	PtPIP1;2	**0.007****	0.056	0.305
	PtPIP2;1	**0.023***	0.389	0.467
	PtPIP2;2	**0.009****	0.604	0.491
	PtPIP2;3	0.807	0.738	0.087
	PtPIP2;4	0.103	**0.034***	0.054
	PtPIP2;5	**0.004****	0.239	0.376

* $P \leq 0.05$
** $P \leq 0.01$
*** $P \leq 0.001$

Table 3.2 Comparison of estimated light-saturated photosynthesis (P_m) and light saturation point (I_m) of aerated (A), aerated treated with ethylene (AE), hypoxic (H) and hypoxic treated with ethylene (HE) aspen (*Populus tremuloides*) plants for one week with a modified rectangular hyperbole model (Ye 2007). Means ± SE (n = 3) are shown. Asterisks indicate statistically significant differences between H and HE treatments determined by planned contrast ($P \leq 0.05$)

	A	AE	H	HE
P_m (μmol CO_2 m^{-2} s^{-1})	17.52 ± 0.93	18.71 ± 1.02	3.65 ± 1.88	9.83 ± 0.38**
I_m (μmol m^{-2} s^{-1})	1481.16 ± 101.89	1402.99 ± 43.43	747.67 ± 163.74	975.33 ± 115.59

** $P \leq 0.01$

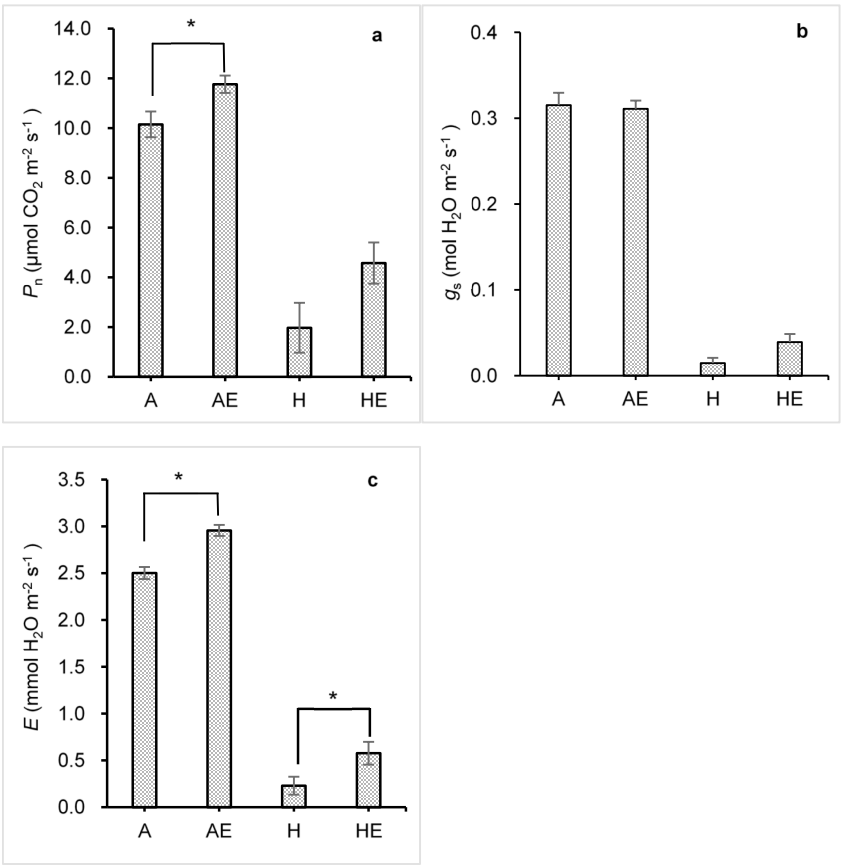

Figure 3.1 Net photosynthesis (P_n, a), stomatal conductance (g_s, b) and transpiration rates (E, c) and light response of net photosynthesis (c) in well-aerated aspen (*Populus tremuloides*) plants (A), well-aerated plants treated with ethylene (AE), plants exposed to hypoxia (H) and subjected to ethylene treatment (HE) for one week. Means ± SE (n = 6) are shown. Asterisks indicate statistically significant differences between A and AE treatments, or between H and HE treatments determined by contrast ($P \leq 0.05$).

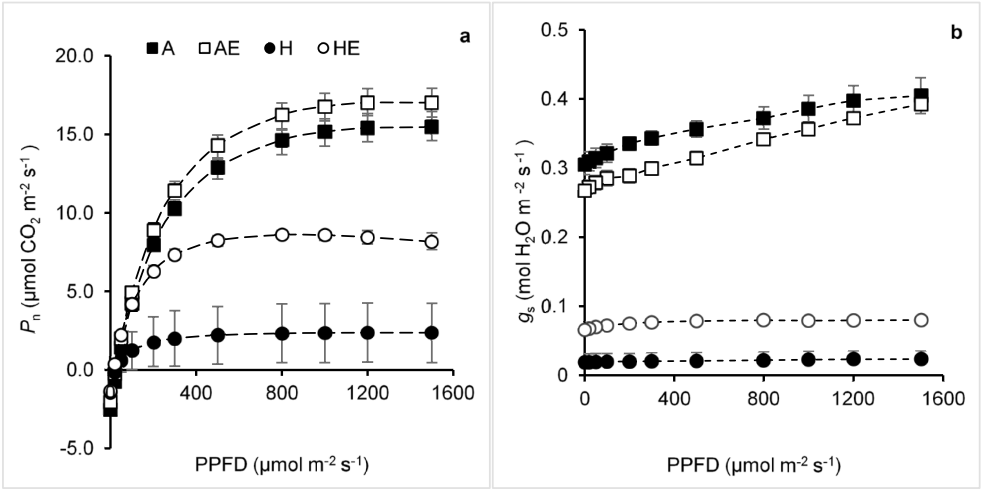

Figure 3.2 Light response of net photosynthesis (P_n, a) and stomatal conductance (g_s, b) in well-aerated aspen (*Populus tremuloides*) plants (A), well-aerated plants treated with ethylene (AE), plants exposed to hypoxia (H) and subjected to ethylene treatment (HE) for one week. Means ± SE (n = 3) are shown

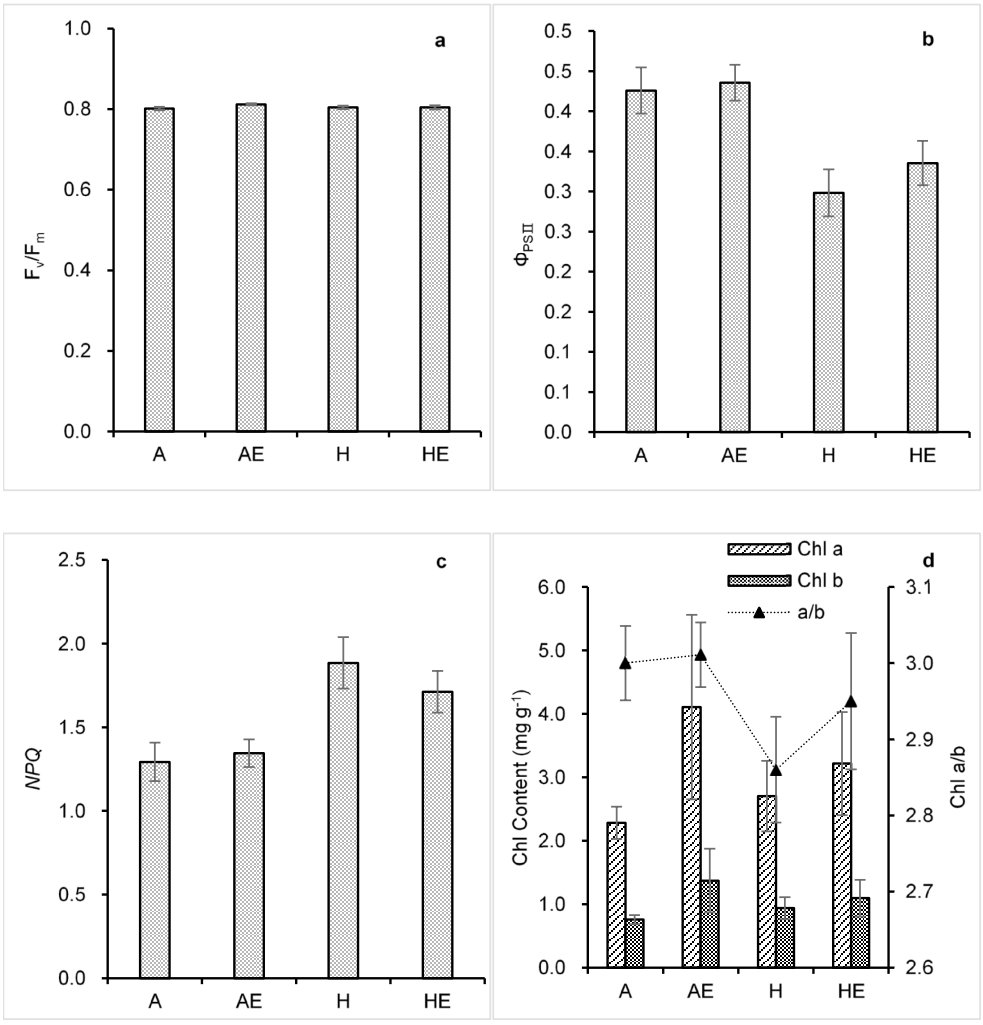

Figure 3.3 Maximum quantum yield of photosystem II (F_v/F_m, a), effective quantum yield (Φ_{PSII}, b), non-photochemical quenqing (*NPQ*, c) and leaf chlorophyll content (Chl, d) after one week of treatments in well-aerated aspen (*Populus tremuloides*) plants (A), well-aerated plants treated with ethylene (AE), hypoxic plants (H) and hypoxic plants treated with ethylene (HE). Means ± SE (n = 6 for chlorophyll fluorescence and n = 3 for chlorophyll concentration) are shown

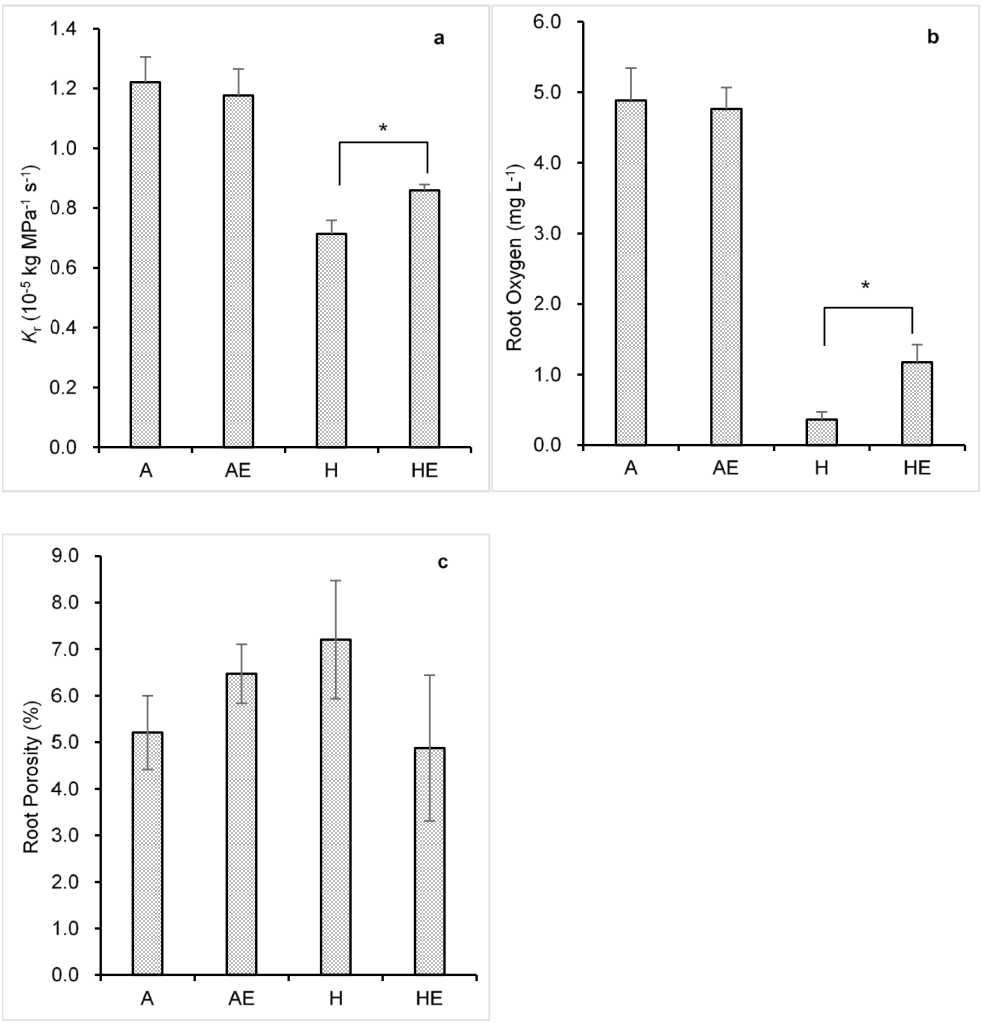

Figure 3.4 Root hydraulic conductance (a), root oxygen concentration (b) and root porosity (c) after one week of hypoxia and ethylene treatments in well-aerated plants (A), well-aerated aspen (*Populus tremuloides*) plants treated with ethylene (AE), hypoxic plants (H) and hypoxic plants treated with ethylene (HE). Means ± SE (n = 6) are shown. Asterisks indicate statistically significant differences between H and HE treatments determined by contrast ($P \leq 0.05$)

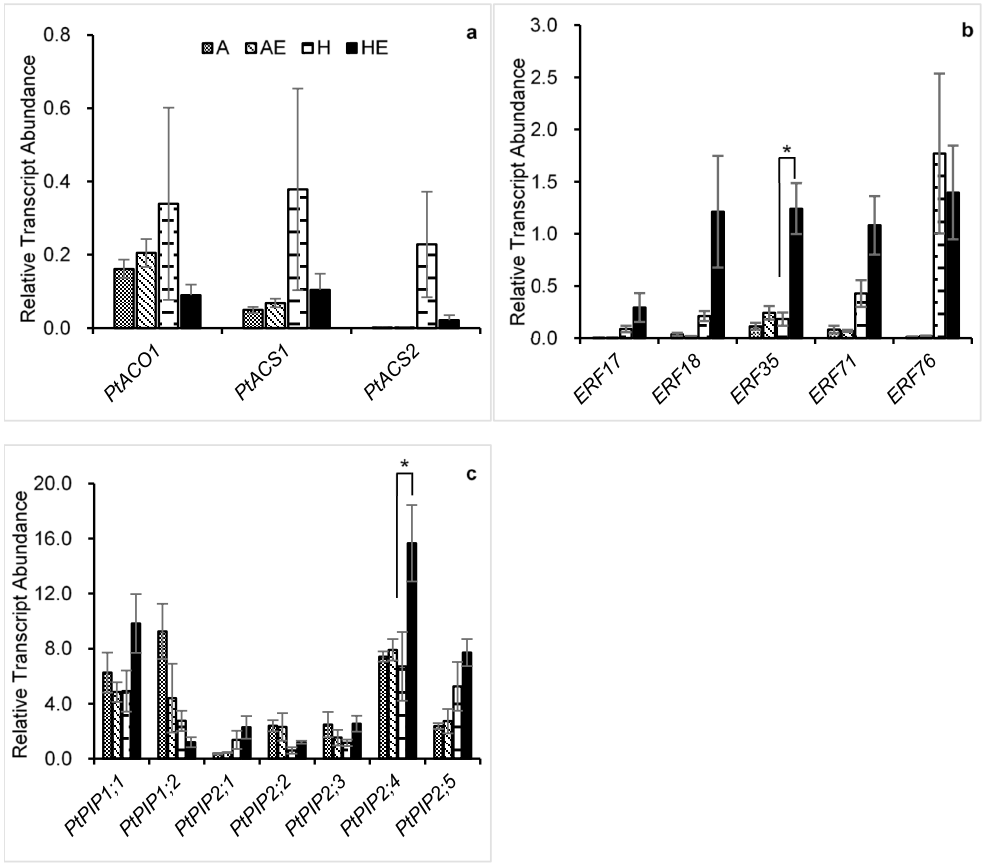

Figure 3.5 Transcript abundance of aspen (*Populus tremuloides*) endo-ethylene synthesis related genes (*ACO1*, *ACS1* and *ACS2*) (a), ethylene responsive factors (*ERF*s) and plasma membrane intrinsic proteins (*PIP*s, c) after one week of hypoxia and ethylene treatments in well-aerated plants (A), well-aerated plants treated with ethylene (AE), hypoxic plants (H) and hypoxic plants treated with ethylene (HE). Means ± SE (n = 6) are shown. Asterisks indicate statistically significant differences between H and HE treatments determined by contrast ($P \leq 0.05$)

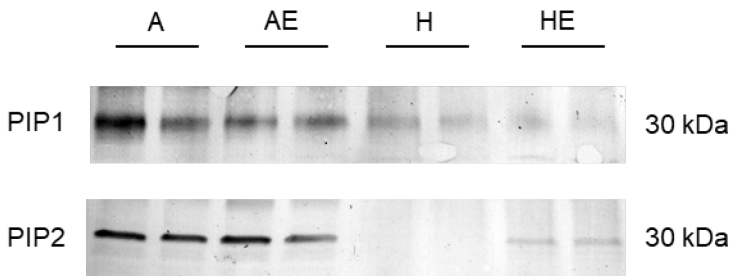

Figure 3.6 Western-blot analyses of aspen (*Populus tremuloides*) plasma membrane intrinsic proteins (*PIP*s) after one week of hypoxia and ethylene treatments in the roots of well-aerated plants (A), well-aerated plants treated with ethylene (AE), hypoxic plants (H) and hypoxic plants treated with ethylene (HE). Results from two independent biological replicates of each treatment are shown in the figure. Antibodies against *Zea mays* PIP1 and PIP2 were used in the immunodetection of PIPs

4 Aquaporin Gene Expression and Gas Exchange in Waterlogged *Arabidopsis thaliana*

4.1 Introduction

Waterlogging impedes the uptake of O_2 by plant roots since O_2 diffusion rate in water is quite low. Responses of *Arabidopsis* to O_2 deficiency (hypoxia) have been studied in terms of morphologically-adaptive strategies (Bailey-Serres and Voesenek, 2008; Voesenek and Bailey-Serres, 2013), signaling pathways (Bailey-Serres et al., 2012; Dongen et al., 2014), and molecular responses (Mustroph et al., 2010; Lee et al., 2011). The application of high-throughput technology has helped identify numerous *Arabidopsis* genes playing important roles in response to hypoxia, some of which have shown upregulated orthologs in other plant species (Mustroph et al., 2010; Lee et al., 2011). A group of core hypoxia-responsive genes was identified in hypoxic *Arabidopsis* plants (Mustroph et al., 2009). Genes associated with photosynthesis were downregulated while genes involved in ABA synthesis were upregulated in hypoxic *Arabidopsis* plants (Hsu et al., 2011). Two protein kinases were recognized to be essential in adjusting energy homeostasis during hypoxia (Baena-González et al., 2007). The Ethylene Responsive Factor (ERF) gene family of *Arabidopsis* encodes transcriptional regulator proteins, which are involved in low O_2 sensing and responses to ethylene (Nakano et al., 2006; Bailey-Serres et al., 2012).

Plant water uptake is hampered in most plants by low O_2 conditions (Maurel et al., 2015). The closure of aquaporins caused by cytosolic acidosis contributes to the downregulation of hydraulic conductivity under O_2 deficiency (Tournaire-Roux et al., 2003). Hypoxia also alters root water transport through changes at the transcriptional level including the *Hydraulic Conductivity of Root 1* gene in *Arabidopsis*, which regulates root hydraulic conductivity under low O_2 conditions through the control of RAP2.12 transcriptional regulator (Shahzad et al., 2016). Transcription of several aquaporin homologs in *Arabidopsis* are also downregulated under hypoxia (Liu et al., 2005). However, transcription of *Arabidopsis nodulin-26 like intrinsic protein 2;1* (*NIP2;1*) was shown to be induced under hypoxia and proven to be a lactic acid transporter (Liu et al., 2005; Choi and Roberts, 2007).

Cross-kingdom comparison of transcriptomic profiles highlights plant specific

responsive genes under low O_2 (Mustroph et al., 2010). Effects of hypoxia on transcription profiles have been studied in several plant species including *Arabidopsis* (Branco-Price et al., 2005; Liu et al., 2005), cotton (*Gossypium hirsutum*) (Christianson et al., 2010) and poplar (*Populus* × *canescens*) (Kreuzwieser et al., 2009). Genes encoding proteins involved in glycolysis, fermentation, and reactive oxygen species amelioration were identified as core genes in response to low O_2 conditions (Bailey-Serres et al., 2012; Dongen et al., 2014. Aqauporin transcripts show diverse responses to low O_2 conditions (Liu et al., 2005; Calvo-Polanco et al., 2014; Reeksting et al., 2016). However, these changes are poorly understood due to the diverse functions and regulating mechanisms of aquaporins (Tan et al., 2018). In this study, I examined the effects of waterlogging on gene transcription and leaf gas exchange. I tested the hypothesis that waterlogging can regulate the transcription of aquaporins and affect leaf gas exchange. The study was carried out to provide evidence of transcriptional responses of aquaporins to waterlogging.

4.2 Materials and Methods

4.2.1 Growth conditions and waterlogging treatment

Arabidopsis thaliana seeds were germinated in horticultural soil in 0.25 L pots. Plants were placed in a controlled growth room with an 18 h photoperiod, 22/18 ºC (day/night) temperature, 400 μmol m^{-2} s^{-1} photosynthetic photon flux density and 50% relative humidity. Plants were regularly watered and fertilized weekly with the full-strength modified Hoagland's solution (Epstein, 1972). After 3 weeks of growth, 16 plants were randomly selected, and the pots were immersed in water to the rosette level. The water used in the waterlogging treatment had been previously flushed with nitrogen gas (99.998%, Praxair, Danbury, CT, USA) to reach a dissolved O_2 level of ~ 2 mg L^{-1} and then left stagnant. Dissolved O_2 concentration was monitored with the YSI-5000 O_2 meter (YSI Incorporated, Yellow Springs, OH, USA). A second group of 16 plants was not flooded and served as a control.

4.2.2 Gas exchange and photosynthetic light responses

After 24h and 48h of treatment, gas exchange was measured with a Li-Cor LI-6400XT portable photosynthesis system equipped with a 2×3 cm^2 red-blue light

chamber (Li-Cor, Lincoln, NE, USA). Six flooded and six control plants were randomly selected, and gas exchange was measured in detached leaves. Net photosynthesis (P_n), transpiration (E) and stomatal conductance (g_s) rates were measured. Air flow rate was set to 400 μmol s^{-1} and relative humidity was the same as in the growth chamber (50%). Photosynthetic photon flux density (PPFD) was 400 μmol m^{-2} s^{-1} and reference CO_2 concentration was 400 μmol mol^{-1}. Gas exchange was normalized to leaf areas calculated by SigmaScan Pro 5.0 software (Systat Software, San Jose, CA, USA) after scanning the leaves with a desk-top scanner.

4.2.3 RNA expression profiling

After 24h and 48h of flooding treatment, roots from 6 treated and 6 control plants were sampled. The samples were frozen in liquid nitrogen before being transferred to –80°C freezer. Total RNA was extracted with a Plant RNeasy extraction kit (Qiagen, Valencia, CA, USA) from the samples ground with a pestle and mortar in liquid nitrogen. First strand of cDNA was synthesized from 1μg total RNA using a Reverse Transcription Kit (Qiagen). Quantitative RT-PCR was employed to analyze RNA expression with standard curve method. The transcript abundance of *PIP*s was normalized against the CT value of the reference gene, *ACTIN 2/8*, which showed constitutive expression in all vegetative tissues of *Arabidopsis* (An et al., 1996). *ADH1* (*alcohol dehydrogenase 1*, AT1G77120) and *PDC1* (*pyruvate decarboxylase 1*, At4g33070) were selected as hypoxic indicators (Liu et al., 2005). Gene-specific primers were described in Appendix 1.

4.2.4 Statistical analysis

Means and standard errors (SE) were calculated. Means of all determined parameters were compared by t-test between well-aerated and waterlogged plants at $\alpha = 0.05$ after 24 h and 48 h of treatment, respectively.

4.3 Results

4.3.1 Net photosynthesis (P_n) and transpiration rates (E)

Leaf gas exchange sharply decreased after 24 h of waterlogging (Fig 4.1). P_n of waterlogged plants decreased from over 12 μmol CO_2 m^{-2} s^{-1} in control to less than 4

μmol CO_2 m^{-2} s^{-1} in waterlogged plants (Fig 4.1a). *E* of waterlogged plants decreased from over 4 μmol H_2O m^{-2} s^{-1} in control to less than 1 μmol H_2O m^{-2} s^{-1} (Fig 4.1b) and g_s of waterlogged plants decreased from about 0.3 mol m^{-2} s^{-1} in control to less than 0.03 mol m^{-2} s^{-1} (Fig 4.1c).

Similarly to the 24-h waterlogging treatment, 48 h of waterlogging resulted in drastic decreases of P_n, *E* and g_s compared with well-aerated plants (Fig 4.1).

4.3.2 RNA expression profiling in roots

ADH1 and *PDC1* exhibited consistently higher expression in waterlogged *A. thaliana* after both 24 h and 48 h of treatment (Fig 4.2a and c). Twenty-four hours of waterlogging resulted in sharp increases of *ADH1* and *PDC1* expression by more than 10- and 20-fold, respectively. Forty-eight hours of waterlogging lead to the sharp increases of *ADH1* and *PDC1* expression by more than 140-fold and 58-fold, respectively.

Most *PIP*s showed decreased expression after 24 h of waterlogging (Fig 4.2b). However, after 48 h of waterlogging, *PIP1;1* and *PIP1;4* in the waterlogged treatment showed significantly higher expression compared with control plants (Fig 4.2d). Expression of *NIP2;1* was sharply induced by both 24 h and 48 h of waterlogging (Fig 4.2).

4.4 Discussion

The highly induced expression of *ADH1* and *PDC1* reflected the sustaining energy crisis in both 24h- and 48h-waterlogged *Arabidopsis*. Induced transcription of *ADH* and *PDC* is commonly observed in hypoxic plants (Liu et al., 2005; Mustroph et al., 2010; Lee et al., 2011). These two genes encode proteins that are involved in the anaerobic energy metabolism in response to hypoxia. Most of the *PIP*s showed decreased expression after 24 h of waterlogging. However, after 48 h of waterlogging, *PIP1;1* and *PIP1;4* showed significantly higher expression levels compared with control plants. In a recent study, transcription of PIPs was also inhibited by the boron treatment after 24 h but not 48 h (Macho-Rivero et al., 2018), which indicates that the transcriptional regulation of PIP1;1 may actively play a role in responses to abiotic stress. Transcription of PIP1;4 increased in plants exposed to low temperature, and overexpression of this gene in *Arabidopsis* roots alleviated the inhibition of short-term

low root temperature on root hydraulic conductivity (Lee et al., 2012). Functional assays showed that *Arabidopsis PIP1;4* can facilitate the diffusion of H_2O_2 (Tian et al., 2016), which acts as an important signaling molecule in response to abiotic stress (Choudhury et al., 2013). *Arabidopsis NIP2;1* was up-regulated under low O_2 conditions, which is consistent with other studies (Liu et al., 2005; Mizutani et al., 2006). The function of *Arabidopsis* NIP2;1 has been linked to lactic acid transmembrane transport under hypoxia (Choi and Roberts, 2007). Under low O_2 conditions, anaerobic energy metabolism is activated, and lactic acid is a final product of lactic acid fermentation. Lactic acid fermentation exists in the early stage of fermentation under low O_2 conditions, which leads to a dramatic drop of cytosolic pH (Drew, 1997).

RNA expression profiling can provide some clues concerning stress-induced responses of plants, however, it should be viewed in the light of transcription potential. Previous studies showed that gene transcription in anoxic plants may be different compared with anoxic plants acclimated to hypoxia, which points to the existence of a shock effect by anoxia (Andrews et al., 1994). Our results showed that the patterns of PIP expression may change along with the treatment time, which needs to be considered in the interpretation of responses of genes to stresses. Additional studies are needed to demonstrate the translation of transcripts as well as the posttranscriptional regulation. The downregulation of aquaporins (Matsuo et al., 2012; Kadam et al., 2017) is generally associated with reduced water uptake ability of roots in response to O_2 deprivation (Schildwacht, 1989; Else et al., 1995; Else et al., 2001). However, in waterlogged *Quercus robur*, *PIP1;3*, *TIP2;1* and *TIP2;2* showed higher expression levels in root tips (North et al., 2004). It remains to be determined whether these changes in gene expression are functionally significant. The analysis of gene expression must be interpreted with caution. Several studies have shown the lack of correlation between mRNA and protein abundance (Maier et al., 2009; Nagaraj et al., 2011; Payne, 2015; Vogel and Marcotte, 2012). O_2 deficiency may affect both transcriptional and post-transcriptional regulation of aquaporins.

Physiological responses in *Arabidopsis* plants also changed with the duration of low O_2 treatment. The increase of *E* after 48 h compared with 24 h of treatment suggests that the plants developed some resistance to waterlogging through the increased water delivery to leaves. The earliest responses to O_2 deprivation include hydraulic adjustments. Of the three pathways of root water transport (Steudle and

Peterson, 1998), transmembrane pathway is the most sensitive to low O_2 conditions and can be regulated through the abundance and activities of aquaporins. In leaves, stomatal closure in plants deprived of O_2 (Islam et al., 2003; Blanke and Cooke, 2004; Atkinson et al., 2008) reduces leaf transpiration rates that is commonly observed in flooded plants (Mielke et al., 2003; Rodríguez-Gamir et al., 2011; Shao et al., 2013; Herzog et al., 2016). The transcriptional changes of *PIP*s in the present study during that time suggest that they could be linked to this process, Future studies should investigate the links between fluctuations in *PIP* profiling and physiological changes under waterlogging stress.

In conclusion, the results have demonstrated that the expression of *PIP*s in *Arabidopsis* changed depending on the duration of waterlogging. Waterlogging also reduced the gas exchange in leaves, which was likely due to the impaired root water uptake. Future studies should examine how the changes of *PIP*s are translated into physiological responses of *Arabidopsis* in response to waterlogging.

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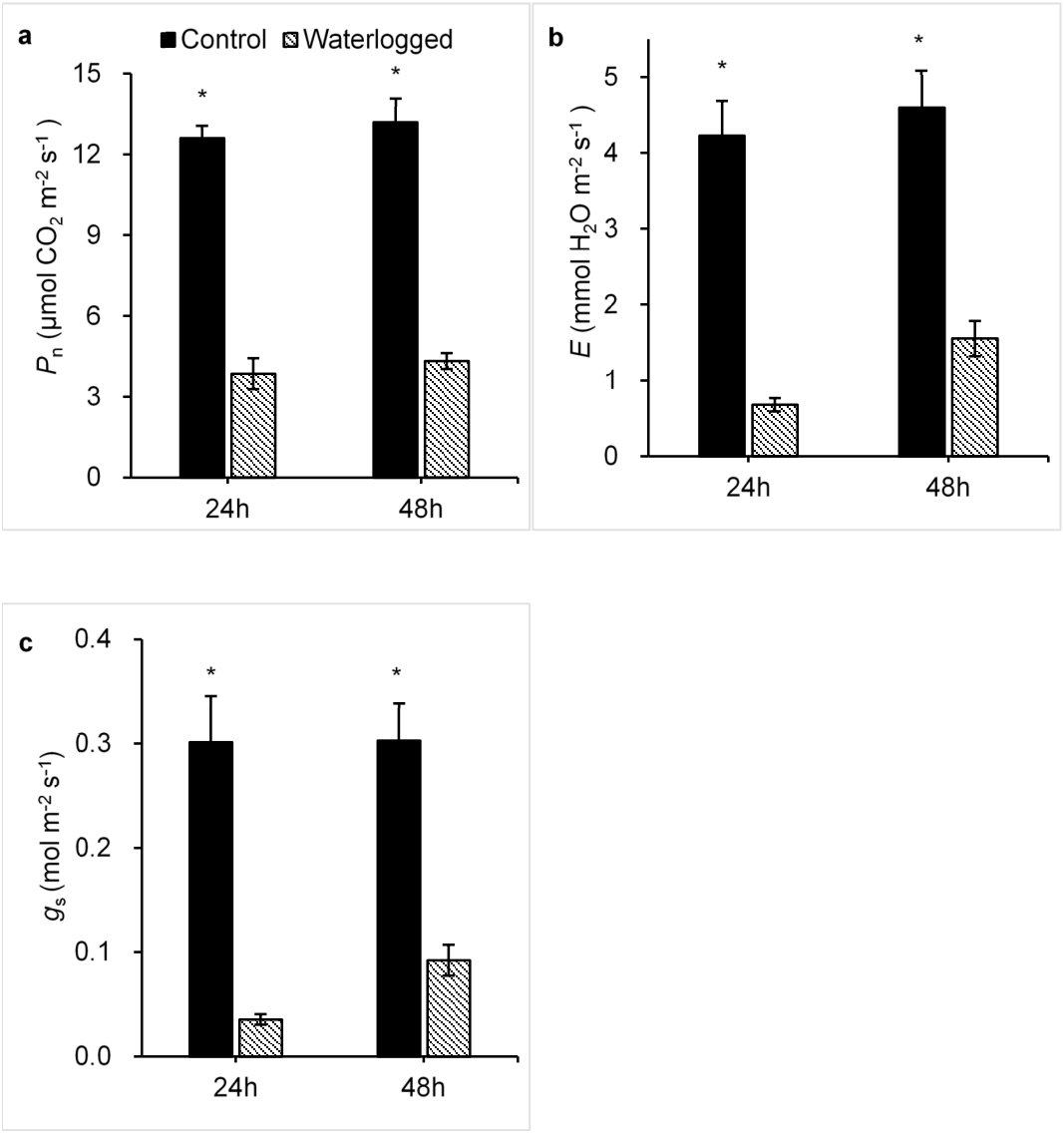

Figure 4.1 Net photosynthesis (P_n), transpiration rates (E) and stomatal conductance (g_s) of *Arabidopsis thaliana* seedlings after exposure to well-aerated and waterlogged conditions for 24 h and 48 h. Asterisks indicate significant difference between well-aerated and waterlogged plants (t-test, $P \leq 0.05$). Means ($n = 6$) ± SE are shown.

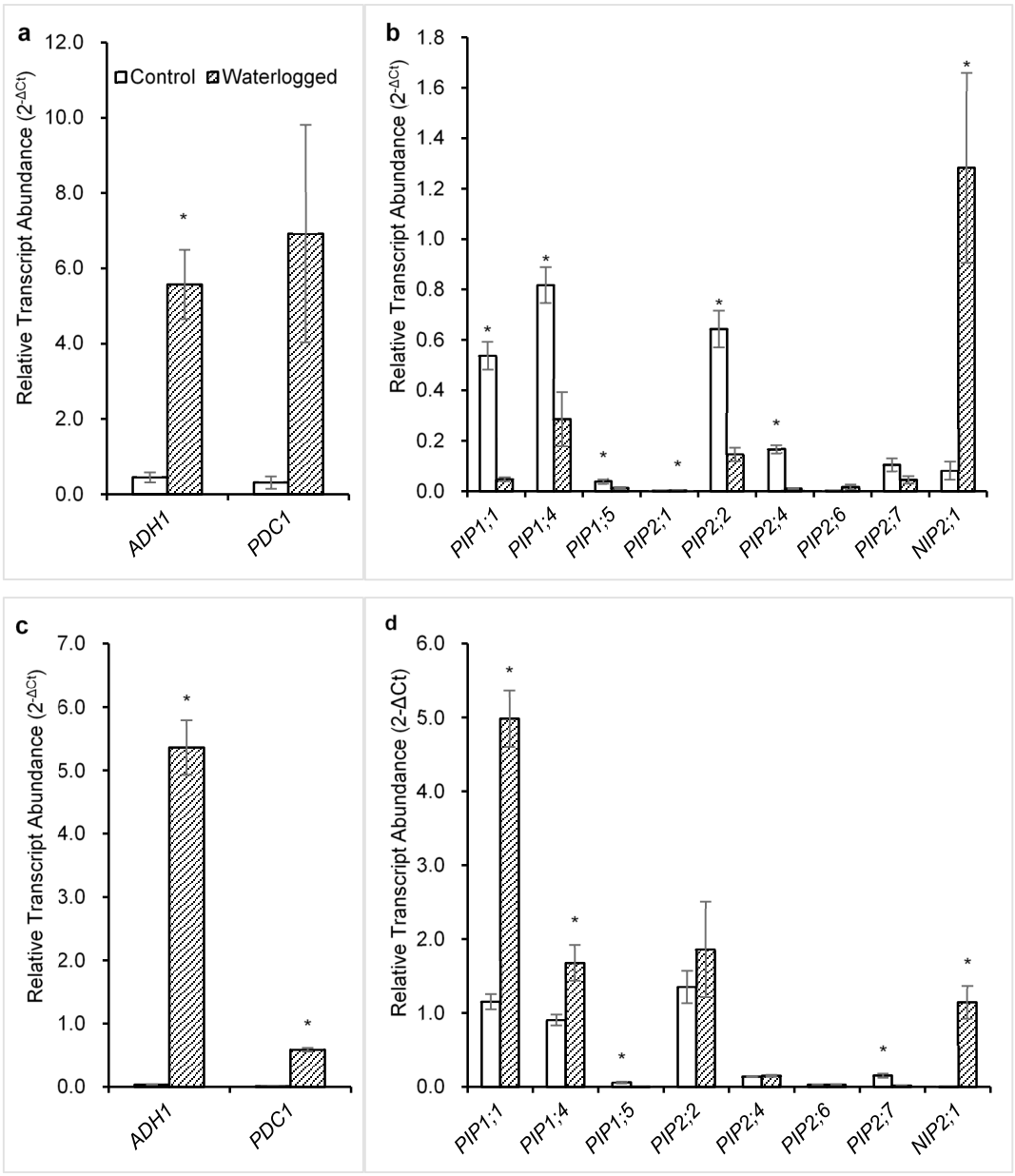

Figure 4.2 Transcript abundance of *Arabidopsis thaliana ADH1* (*alcohol dehydrogenase 1*), *PDC1* (*pyruvate decarboxylase 1*), and plasma membrane intrinsic proteins (*PIP*s) after exposure to waterlogged and control conditions for 24 h (A and B) and 48 h (C and D). Means (n = 6) ± SE are shown. Asterisks indicate significant difference between well-aerated and waterlogged plants (t-test, $P \leq 0.05$).

5 Significance of Oxygen Transport through Aquaporins

5.1 Introduction

Since the discoveries that membrane intrinsic proteins (MIPs) are involved in transmembrane water transport (Benga et al., 1986; Agre et al., 1993), evidence has been growing that links different members of the aquaporin family to transport processes of other small neutral molecules, including CO_2 (Uehlein et al., 2003; Navarro-Ródenas et al., 2012; Uehlein et al., 2012). The transport of these molecules has been associated with fundamental physiological processes (Uehlein et al., 2003; Navarro-Ródenas et al., 2015). Similarly to the long-prevailing views of water transport, a possible significance of pore-mediated transport for CO_2 and O_2 has been sometimes downplayed due to theoretical and experimental evidence suggesting rapid diffusion of these gases through the lipid bilayer (Verkman, 2002; Missner and Pohl, 2009). While functional significance of aquaporin-mediated CO_2 transport has been demonstrated for photosynthesis and cell signaling processes (Uehlein et al., 2003; Navarro-Ródenas et al., 2015), the importance of pore-mediated O_2 transport to transcellular O_2 fluxes and cell function remains elusive (Verkman, 2011; Echevarría et al., 2007; Bramley and Tyerman, 2010)

In the present study, I used the yeast cell system (*Saccharomyces cerevisiae* INVSc1, Invitrogen) to co-express sperm whale (*Physeter macrocephalus*) myoglobin (Springer and Sligar, 1987) in the yeast expression vector pAG425GAL-ccdB together with one of the 20 different aquaporins from human, plants, or fungi (Appendices 2 and 3) in the vector pAG426GAL-ccdB, to evaluate the impact of heterologous aquaporin expression on myoglobin oxygenation as an indicator for O_2 permeability of the yeast plasma membrane. I also examined the transcript abundance of plasma membrane intrinsic proteins (PIPs) in relation to ATP levels in the roots of *Nicotiana tabacum* under hypoxia in hydroponic culture in order to evaluate possible functional significance of the O_2-transporting aquaporins.

5.2 Materials and Methods

5.2.1 Expression of myoglobin and aquaporins in yeast

The complete ORF of sperm whale (*Physeter macrocephalus*) myoglobin (NCBI accession number *J03566.1*) was sub-cloned from pMB413 (Springer and Sligar, 1987) into the yeast expression vector pAG425GAL-ccdB (http://www.addgene.org/yeast-gateway/), by the Gateway technology (Invitrogen, Carlsbad, CA, USA). The complete ORFs of the 20 aquaporin genes of interest were sub-cloned from pGEM-T Easy into the yeast expression vector pAG426GAL-ccdB (http://www.addgene.org/yeast-gateway), by the same method, respectively. These genes include three animal aquaporins from *Homo sapiens* - *HsAQP1* (*DQ895575*), *HsAQP2* (*CR542024*) and *HsAQP3* (*CR541991*), 12 plant aquaporins - *NtPIP1;1* (*AF440271*), *NtPIP1;2* (*AF024511*), *NtPIP1;3* (*U62280*), *NtPIP1;4* (*DQ914525*), *NtPIP2;1* (*AF440272*) and *NtXIP1;1* (*HM475294*) from *N. tabacum*, and *AtPIP1;1* (*AT3G61430*), *AtPIP1;2* (*AT2G45960*), *AtPIP1;3* (*AF348574*), *AtPIP1;4* (*AT4G00430*), *AtPIP1;5* (*AT4G23400*) and *AtNIP2;1* (*AT2G34390*) from *Arabidopsis thaliana*, and five fungal aquaporins from *Laccaria bicolor – LbAQP1* (*JQ585592*), *LbAQP3* (*JQ585593*), *LbAQP5* (*JQ585594*), *LbAQP6* (*JQ585595*) and *LbAQP7* (*JQ585596*). The constructs were verified using primer GAL1 (AATATACCTCTATACTTTAACGTC) in Sanger sequencing. *Saccharomyces cerevisiae* strain INVSc1 (MATa his3D1 leu2 trp1-289 ura3-52; Invitrogen) was double-transformed with pAG425GAL-ccdB+myoglobin vector and one of the PAG426GAL-ccdB+aquaporin vectors, following the protocol of small-scale yeast transformation (Invitrogen). For mock control, INVSc1 was transformed with pAG425GAL-ccdB+myoglobin vector and empty PAG426GAL-ccdB vector.

Selection was based on ura3 and leu2 complementation. Transformed yeasts were cultured in glucose containing synthetic complete medium without Ura/Leu (United States Biological) (2 g of yeast nitrogen base, 2 g of dropout amino acids, 5 g of $(NH_4)_2SO_4$, 30 g of glucose in 1 L of SD-L-U + glucose medium, pH = 6) for 24 h at 1.2 × *g* and 30 °C. Cultures were diluted to an optical density of OD_{600} = 0.6. Heterologous protein expression was induced by changing the carbon source of the medium from glucose to galactose (30 g in 1 L of SD-L-U + galactose medium) and growing yeast cells for 24 h (1.2 × *g*, 30°C), with 25 mg L^{-1} $FeSO_4$ as iron source to

promote the formation of myoglobin-iron binding structure (Govindarajan and Kotula, 1977), validated by transcript abundance assay of quantitative RT-PCR (Xu et al., 2015; Crankorur-Cetinkaya et al., 2012), immunoblotting and indirect immunofluorescence detection.

5.2.2 Quantitative RT-PCR in yeast

After 24-h induction in SD-L-U galactose solution, five milliliters of cell suspension at OD_{600} = 1 were harvested at 3000 × g for 5 min and frozen in liquid N_2 for cell lysis and RNA extraction by Trizol method (Thermofisher, manufacturer's protocol) (n = 3). The first strand cDNA was synthesized from 1 µg total RNA using QuantiTect Reverse Transcription Kit (Qiagen), and used for SYBR Green quantitative real-time polymerase chain reaction (qRT-PCR) with standard curve method. Each 10 µL of the reaction consisted of 2.5 µL of 1.6 µM primer, 2.5 µL of 10 ng/µL cDNA and 5 µL of qPCR Mastermix (Xu et al., 2015). The transcript abundance of myoglobin, *HsAQP1*, *NtPIP1;3* and *AtPIP1;2* (Appendix 1) in the transformed strains was normalized to the yeast reference gene *ScFBA1* (YKL060C) (Crankorur-Cetinkaya et al., 2012), by relative quantification of standard curve method with correction for amplification efficiency.

5.2.3 SDS-PAGE and immunoblotting

After induction, 20 mL of *S. cerevisiae* culture (OD_{600} = 2.5) was centrifuged at 600 × *g* for 5 min to yield 120 mg fresh weight yeast cells for total protein extraction (Kushnirov 2000). The pellet was suspended in 500 µL of distilled water and 500 µL of 0.2 M NaOH was added followed by incubation for 5 min at room temperature. Following centrifugation, the resulting pellet was, re-suspended in 250 µL of SDS sample buffer, boiled for 3 min and pelleted again. The protein-containing supernatant was stored at -20°C for future use.

About 15 µg of extracted total protein in sample buffer was loaded in each sample lane of the SDS-PAGE precast gel (Mini-PROTEAN TGX Precast Gel, Bio-Rad, Berkeley, CA, USA), including the mock strain that expressed whale myoglobin but no heterologous aquaporin, yeast strains that expressed whale myoglobin and aquaporin *Hs*AQP1, *Nt*PIP1;3 or *At*PIP1;2, respectively, and untransformed yeast strain INVSc1. Three microliters of protein standard (Precision

Plus Protein DualColor Standards, Bio-Rad) was loaded in the ladder lane. Two micrograms of 17-kDa purified horse myoglobin (Sigma-Aldrich, St Louis, MO, USA) was used as the positive control. The gels were stained with Commassie Blue or transferred onto PVDF membrane, blocked in 3% BSA in TBST at 4°C for 12 h, and incubated in the solution of 1:500 diluted primary polyclonal anti-myoglobin antibody produced in rabbit (Sigma-Aldrich) overnight at 4°C. The western blots were then incubated in 1:3000 diluted secondary antibody of GAR-alkaline phosphatase (Bio-Rad) at room temperature for 1 h with gentle agitation. Color was developed for 2 min using alkaline phosphatase conjugate substrate kit (Bio-Rad).

For aquaporin detection, the blocked membrane was incubated in the solution of 1:2000 diluted primary anti-human aquaporin 1 monoclonal antibody produced in mouse (OriGene) at 4°C overnight, followed by incubation in the solution of 1:10000 diluted secondary antibody of GAM IgG-alkaline phosphatase (Sigma-Aldrich) at room temperature for 1 h with gentle agitation. Color was developed for 1 min using alkaline phosphatase conjugate substrate kit (Bio-Rad).

5.2.4 Indirect immunofluorescence

One milliliter of yeast suspension at OD_{600} = 1 was centrifuged at 600 × *g* for 5 min. The pellets were re-suspended in 500 µL of heated 3% agarose. The solidified agarose-yeast plugs were kept in formaldehyde-acetic acid- ethanol (FAA) for fixation in vacuum for 24 h. The samples were embedded in paraffin and sliced using a microtome. The sections were kept at 37°C overnight, de-waxed in toluene, rehydrated, and circled out using an ImmEdge® hydrophobic barrier pen, before blocking and antibody incubation in a humidity chamber. The cells in the circle were blocked by adding a drop of PBS buffer with 2% of BSA and 0.1% of Tween-20 at room temperature for 5 min. The blocked yeasts were incubated in the solution of 1:100 diluted primary anti-aquaporin 1 monoclonal antibody produced in mouse (OriGene) at 4°C overnight, followed by incubation in the solution of 1:50 diluted secondary antibody of Fluorescein (FITC-isomer 1; A max = 492 nm, E max = 520 nm)-conjugated AffiniPure rat anti-mouse IgG (H+L) (Jackson ImmunoResearch) at room temperature in dark for 2 h. The yeast cells of mock strain that had not been treated with the primary antibody were the negative control to show the background of autofluorescence. The cells were washed in blocking buffer for 3 times. A drop of

water was added onto the samples before mounting the slide cover. The green fluorescence was viewed under 40× or 100× objective lens of a Leica HC fluorescence microscope with blue light excitation. The images were captured using Q-Capture Pro 7, with exposure time as 2650 ms for 40× objective lens and 100 ms for 100× objective lens. Subcellular localization of the aquaporins was also predicted using TargetP1.1 (Emanuelsson et al., 2007), based on the predicted presence of any of the N-terminal presequences, mitochondrial targeting peptide or secretory pathway signal peptide in eukaryotic cells.

5.2.5 O_2 Transport Assay

The yeasts were washed in KH_2PO_4 buffer (0.1M, pH 6) twice, and then suspended in N_2-bubbled KH_2PO_4 buffer. The yeast suspension was bubbled with N_2 for 30 s and vacuumed for 30 min, to minimize the soluble O_2 in yeast suspension. This step is crucial to maintain the state of deoxymyoglobin, as the minimal O_2 solubility reduces the possibility of deoxymyoglobin oxygenation by Fe^{2+}-O_2 binding that leads to the formation of oxymyoglobin, and the low pressure during vacuuming helps to prevent Fe^{2+} ions from being oxidized into Fe^{3+} ions, which converts deoxymyoglobin to the highly stable metmyoglobin (Zhao et al., 1995; Schenkman et al., 1997). The spectrum between 500 nm and 600 nm of yeast suspension was scanned after O_2 depletion and re-aeration. Compared to the spectrum of purified myoglobin (Schenkman et al., 1997), the spectrum of yeast suspension suggests that the state of metmyoglobin was likely dominant over deoxymyoglobin and oxymyoglobin. In addition, the absorbance spectrum of yeast cell suspension is expectably more complex than purified myoglobin proteins. Despite these limitations, the change in A_{541} after re-aeration was noticeable, which was in the range of 541-543 nm, i.e., the absorption peak of purified oxymyoglobin in the study of Zhao et al. (Zhao et al., 1995) as well as in our observation. The increase in A_{541} reflects the conversion of deoxymyoglobin into the oxygenated state upon Fe^{2+} - O_2 binding, which can be attributed to O_2 influx. The rate of increase in A_{541} (ΔA_{541} s^{-1}) can reflect the capacity of O_2 uptake by the yeast strains expressing different aquaporins. Therefore, absorbance of 1 mL yeast suspension of each strain was recorded at 541 nm for 2 min at 1 s interval immediately after the addition of 1 mL of air-saturated KH_2PO_4 buffer or N_2-saturated KH_2PO_4 as negative control, respectively, using a spectrophotometer

(Thermo Genesys 10S V4.002, ThermoFisher Scientific). All measurements were carried out at 22ºC. The mean and standard error were calculated based on six biological replications. CM-H_2DCFDA (Eruslanov and Kusmartsev, 2010) was used to indicate oxidative state of selected yeast strains due to such pretreatment and to determine H_2O_2 transport capacity of selected aquaporins (Bienert et al., 2007).

After screening the strains that expressed putative O_2-transporting aquaporins by measuring the increase in A_{541} over 60 s with the spectrophotometer, yeast protoplasts were prepared for the selected ones for the refined O_2 transport assay, as yeast protoplast was expected to have less interference on reading the absorbance of intracellular myoglobin due to the removal of cell walls from whole cells. After induction, 4 mL of yeast culture at OD_{600} = 2 of each strain was harvested, pre-incubated, washed and treated with zymolyase (Yeast lyticase 100T, United States Biological) at 37°C, 50 rpm (MAXQ4000 bench-top orbital shaker, Thermofisher) for 2 hr according to manufacturer's protocol. Yeast protoplasts were re-suspended in 10 mL of enzyme buffer (1.2 M sorbitol, 50 mM magnesium acetate, 10mM $CaCl_2$ in autoclaved deionized distilled water). For the initial O_2-depleted state, the absorbance spectrum was scanned from 300 nm to 650 nm immediately after mixing 500 µL of protoplast suspension with 500 µL of isosmotic sodium ascorbate buffer (0.6 M sodium ascorbate, 50 mM magnesium acetate, 10 mM $CaCl_2$ in autoclaved deionized distilled water). Sequential scanning was conducted after each 30 s of direct aeration at time points of 30 s, 90 s, 150 s and every minute up to the 10^{th} min. At 541-543 nm, myoglobin of the oxygenated state has a pronounced absorbance peak (Schenkman et al., 1997). At 319-330 nm, both myoglobin and myoglobin-expressing yeast protoplasts showed a second, much more pronounced absorbance peak in the oxygenated state, which was absent in untransformed yeast strain. The value of A_{319}/A_{341} increased dramatically along with multiple aeration, suggesting $\Delta A_{319}/\Delta A_{341}$ be a good indicator for myoglobin oxygenation. Therefore, both $\Delta A_{541}/\Delta_{A600}$ and $\Delta A_{319}/\Delta A_{341}$ were calculated to present the change in absorbance due to myoglobin oxygenation. ANOVA was used to analyze the statistically significant difference between $\Delta A_{541}/\Delta A_{600}$ at 90 s of the aquaporin-expressing strains and that of the mock strain, as well as across all the strains (Tukey test, $P \leq 0.05$, $n = 19$; P values shown in the table of Fig. 7). Statistically significant difference across all the strains was analyzed in $\Delta A_{319}/\Delta A_{341}$ of yeast protoplasts after 5 min with 5 times of 30s aeration (Tukey test, $P \leq 0.05$, $n = 6$; P values shown in the table of Fig. 8).

For spectrum scanning of myoglobin, purified horse myoglobin protein at 1 mg/mL was mixed with 1 volume of 10% sodium ascorbate to generate the deoxygenated state, followed by the above-mentioned series of aeration to achieve the state of oxymyoglobin.

5.2.6 O_2 uptake by yeast

To evaluate the impact of the expression of O_2-transporting aquaporins on O_2 consumption capacity of yeast, the O_2 uptake rates in yeast suspension culture were continuously monitored over 40 min in the over-expression and mock strains. The time required for the yeast suspension cultures to deplete O_2 from the solution was also measured, with glucose as a carbon source. *S. cerevisiae* strains INVSc1 for the expression of *Hs*AQP1, or *Nt*PIP1;3, or *At*PIP1;2, and the mock control strain, were cultured in SD-L-U + glucose medium for 24 h (1.2 × *g*, 30°C). Cultures were diluted to an optical density of OD_{600} = 1. Heterologous protein expression was induced in SD-L-U + galactose medium for 24 h (1.2 × *g*, 30°C). The yeasts were washed in KH_2PO_4 buffer (0.1M, pH 6) twice, and then suspended in 15 mL of N_2-bubbled SD-L-U + glucose medium in 50 mL Falcon tubes to reach OD_{600} = 5. Air was supplied into the yeast suspension until its concentration of soluble O_2 reached about 235 μmol L^{-1}, the stable saturation level of the still medium at 25°C. Starting from this point, the decrease of O_2 concentration in yeast suspension was monitored and logged per second using an O_2 microsensor with tip diameter of 50 μm (OX-50) connected to the OXY-Meter, a compact O_2 microsensor amplifier (Unisense, Aarhus, Denmark). Parafilm was used to seal and minimize free air diffusion to the Falcon tubes. The slopes of the decline in O_2 concentration during the initial 0-1000 s were calculated by linear regression, in which absolute values represented the rates of O_2 consumption by different yeast strains during the corresponding intervals. O_2 depletion time of each yeast suspension was recorded. The means and standard errors were calculated based on six biological replications.

5.2.7 Oxidative state and H_2O_2 Transport Assay

CM-H_2DCFDA, the derivative of H_2DCFDA, one of the most widely used methods for directly measuring the redox state in cells (Eruslanov and Kusmartsev, 2010), was used to indicate the redox state and the oxidative stress in selected yeast strains that

had been pretreated for O_2 transport assay. *Nt*PIP1;2 strain was used as a positive control since this aquaporin (i.e., *Nt*AQP1) was previously demonstrated to transport H_2O_2 (Uehlein et al., 2003; Navarro-Ródenas et al., 2015). Heterologous protein expression was induced in the SD-L-U + galactose medium, for 24 h in dark (1.2 × *g*, 30°C), with 25 mg L-1 of $FeSO_4$ as iron source and 1 µM of CM-H_2DCFDA as the ROS indicator. The yeasts were washed twice with KH_2PO_4 buffer (0.1 M, pH 6) and then suspended in N_2-bubbled KH_2PO_4 buffer. The yeast suspension was bubbled with N_2 for 30 s and vacuum was applied for 30 min. Fluorescence generated from each 200 µL sample was recorded using a microplate reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany) with excitation wavelength of 485 nm and emission wavelength of 520 nm at 27 s intervals for 10 min (n = 6).

Strain of *S. cerevisiae* INVSc1 (MATa his3D1 leu2 trp1-289 ura3-52; Invitrogen) containing the empty pAG425GAL-ccdB vector was transformed with the vector pAG426GAL-ccdB expressing *Homo sapiens HsAQP1* (P29972), *N. tabacum NtPIP1;2 (NtAQP1*, AJ001416) and *NtPIP1;3* (U62280), *Arabidopsis thaliana AtPIP1;2* (AT2G45960) or the empty vector pAG426GAL-ccdB as the mock control, respectively. Transformed yeasts were cultured in glucose containing synthetic complete medium without Ura/Leu for 24 h (1.2 × *g*, 30°C). Cultures were diluted to OD_{600} = 0.6, and heterologous protein expression was induced by changing the carbon source of the medium from glucose to galactose. CM-H_2DCFDA [5-(&-6)-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate, acetylester mixed isomers] (Life Technologies; Carlsbad, CA, USA) was used for detecting intracellular H_2O_2 and as an indicator to measure the effect of aquaporin expression on H_2O_2 permeability of yeast cells (Bienert et al., 2007). Greater increase in fluorescence over time indicates higher intracellular concentration of the examined molecule H_2O_2 being transported.

Yeasts were grown and dyed for 24 h in dark (1.2 × *g*, 30°C) in galactose synthetic medium with 1 µM of CM-H_2DCFDA, and then were washed with 20 mM HEPES buffer (pH = 7) for five times and re-suspended in HEPES to reach OD_{600} = 1.4 (Navarro-Ródenas et al., 2015). Non-dye mock yeasts were examined to show the background fluorescence. In H_2O_2 treatment, 100 µM H_2O_2 was used as H_2O_2 source. In H_2O_2 + $AgNO_3$ treatment, yeasts were incubated in 15 µm of the aquaporin inhibitor $AgNO_3$ for one hour in dark before H_2O_2 was added. In HEPES treatment,

neither H_2O_2 nor $AgNO_3$ was applied. Fluorescence generated from each 200 µL reaction per well of a 96-well microplate was recorded using a microplate reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany) with excitation wavelength of 485 nm and emission wavelength of 520 nm at 27 s intervals for 30 min (n = 6).

5.2.8 Tobacco Root Hypoxia Study: Growth Conditions and Treatment

Tobacco *(Nicotiana tabacum L.)* seeds were germinated in soil and plants grown for two weeks in a controlled-environment growth room maintained at 22/18°C (day/night) temperatures, 60±10% relative humidity, and 18-h photoperiod with photosynthetic photon flux density of approximately 350 µmol m^{-2} s^{-1}. After two weeks of growth, plants were transferred to containers with half-strength modified Hoagland's solution aerated with aquarium pumps (dissolved O_2 of approximately 7.5 mg L^{-1}). Thirty-six plants were randomly placed in six containers. After one week, the plants in three containers were subjected to hypoxia by flushing water with nitrogen gas to reach a dissolved O_2 level of ~ 2 mg L^{-1}, and then left stagnant. The plants in the other three containers continued to be aerated.

5.2.9 Quantitative RT-PCR in tobacco

Roots and leaves were sampled after two and seven days of hypoxia treatment (n = 6). The tissue samples were frozen and homogenized in liquid nitrogen using mortar and pestle. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The cDNA synthesis and qPCR were conducted as described in §5.2.2. The transcript abundance of *NtPIP1;1, PIP1;2, PIP1;3, PIP1;4* and *PIP2;1* was normalized against geometric mean of that of the two reference genes, *EF1-α* and ribosomal protein *L25*. Gene-specific primers were designed using Primer Express 3.0 (Applied Biosystems, Life Technologies) (Appendix 1).

5.2.10 Determination of ATP Concentration

ATP concentrations were measured in leaves and apical, central and basal root segments after 2 and 7 days of hypoxia and in well-aerated plants. Roots and leaves of tobacco were sampled after two and seven days of treatments. The roots were divided into the basal, apical and central segments. Tissue samples were ground, and 50 mg ground samples were placed in 600 µL of ice-cold 5% Trichloroacetic acid (TCA)

(Cole and Ross, 1966) in 2 mL centrifuge tubes. The samples were vigorously vortexed for 20 s, left on ice for 10 min and centrifuged at 10,000 × *g*, 4°C for 10 min. Each 400 µL of supernatant was collected and added to 400 µL of ice-cold Tris-acetate buffer (pH = 7.75, 1M). For the ATP assay, 4 µL of the mixture was pipetted into 96 µL of ATP-free water into a well of the 96-well plate (Costar 96 well plate with flat bottom). To quantify ATP, 50 µL of rLuciferase/Luciferin reagent from ENLITEN ATP Assay Kit (Promega, Madison, WI, USA) was added into each well, and the standard curve was prepared following the manufacturer's protocol. Bioluminescence signal (McElroy and DeLuca, 1983) was detected using a microplate reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany).

5.2.11 Statistical Analysis

The means and standard errors were calculated based on the biological replications in each assay by descriptive statistics. Statistical difference was analyzed using one-way ANOVA (Tukey's test, $P \leq 0.05$).

5.3 Results

5.3.1 Protein expression and transcript abundance of myoglobin and aquaporins

Immunoblotting with anti-myoglobin antibody demonstrated the presence of myoglobin in the selected yeast strains that were constructed to express myoglobin, but not in INVSc1 (Fig 5.1a). Quantitative RT-PCR showed that transcript abundance of myoglobin was similar in the transformed yeast strains (Fig 5.2). Immunoblotting with anti-human aquaporin 1 antibody demonstrated that the antibody recognized the expressed heterologous aquaporins *Homo sapiens Hs*AQP1, *N. tabacum Nt*PIP1;3, and *Arabidopsis thaliana At*PIP1;2 in the respective strains, and also, weakly, the yeast homologous aquaporins in the mock strain constructed to express myoglobin only (Fig 5.1b). The qRT-PCR assay with higher specificity than immunoblotting showed that the transcript abundance of the heterologously-expressed aquaporin genes *HsAQP1*, *NtPIP1;3* and *AtPIP1;2* was negligible in the mock strain, but significantly high in each corresponding strain (Fig 5.2).

Following formaldehyde fixation (Pemberton, 2014), paraffin embedding and preparation of sectioned yeast cells for immunodetection with the anti-human

aquaporin 1 antibody, strong immunofluorescence was detected in the outer edges of the protoplast of the *Hs*AQP1 strain (Fig 5.3), pointing to the plasma membrane as the likely localization site. The subcellular localization prediction by TargetP (Emanuelsson et al., 2007) also indicated the absence of mitochondrial targeting peptide in these expressed aquaporins and the secretory pathway was the most likely location of *Hs*AQP1 in yeast (Table 5.1).

5.3.2 O_2 transport

Of the yeast strains that were examined, those expressing *Hs*AQP1, *Nt*PIP1;3 *Nt*PIP1;4, *Nt*PIP2;1, and *Nt*XIP1;1 showed statistically significant increases in O_2 permeability with preliminary spectrophotometric measurements as evidenced by higher rates of change in myoglobin A_{541} absorbance (Fig 5.4 and 5a) compared with mock control (Fig 5.5b). Over-expression of the *A. thaliana* (*At*PIP1;1, *At*PIP1;2, *At*PIP 1;3, *At*PIP1;4, and *At*PIP2;1) and *Laccaria bicolor* (*Lb*AQP1, *Lb*AQP3, *Lb*AQP5, *Lb*AQP6, and *Lb*AQP7) aquaporins did not alter A_{541} absorbance (Fig 5.5b), indicating no significant effect on O_2 permeability.

Two of the most promising O_2-transporters (*Hs*APQ1 and *Nt*PIP1;3) and one that did not show O_2-transporting properties in preliminary experiments (*At*PIP1;2), as well as the mock strain were further analyzed in yeast protoplast assay. Based on the spectrum scanning on purified myoglobin (Fig 5.4) and yeast protoplasts (Fig 5.6), $\Delta A_{541}/\Delta A_{600}$ and $\Delta A_{319}/\Delta A_{341}$ (Fig 5.7) were chosen to indicate myoglobin oxygenation. $\Delta A_{541}/\Delta A_{600}$ at 90 s showed the same trend across the strains with the preliminary assay: the strain expressing *Nt*PIP1;3 had the highest value, followed by *Hs*AQP1, mock and *At*PIP1;2 in order (Fig 5.8). $\Delta A_{319}/\Delta A_{341}$ after 5 min with 5 times of 30s aeration demonstrated more distinct statistical difference between *Hs*AQP1 and mock, and between all of the myoglobin-expressing strains and untransformed strain INVSc1 (Fig 5.9).

Since the conversion of deoxymyoglobin to oxymyoglobin is iron-dependent, and may be affected by the cell redox status, the redox state of selected strains after being pretreated for O_2 transport assay was measured using CM-H_2DCFDA. Fluorescence intensity generated by CM-H_2DCFDA showed no significant difference between yeast strains after the pretreatment of O_2 transport assay (Fig 5.10). This suggested that the cell redox state in the mock, *Hs*AQP1, *Nt*PIP1;3 and *At*PIP1;2

strains was similar prior to the O_2 transport assay. Similarly to the earlier report (Navarro-Ródenas et al., 2015), increased H_2O_2 permeability was detected in *Nt*PIP1;2 strain (Fig 5.11). However, no increase in H_2O_2 permeability was measured in *Nt*PIP1;3, *Hs*AQP1 or mock strains, whereas a slightly higher fluorescence intensity suggesting increased H_2O_2 permeability in *At*PIP1;2 strain was not statistically significant (Fig 5.11).

5.3.3 Yeast O_2 consumption capacity

Yeast cells heterologously expressing *Nt*PIP1;3 and *Hs*AQP1 showed 2.3-fold and 1.8-fold higher O_2 uptake rates, respectively, compared with mock control (Fig 5.12a) and depleted O_2 from the solution significantly faster ($P \leq 0.05$) (Fig 5.12b and 13). The O_2 uptake rates of yeast cells expressing *At*PIP1;2 and the time for O_2 depletion from the solution were not significantly ($P \leq 0.05$) different from the mock controls (Fig 5.12). Yeast cell diameter was not significantly affected by the heterologous expression of aquaporins and measured 2.87 ± 0.05, 2.74 ± 0.06, 2.98 ± 0.06, and 2.87 ± 0.08 µm (mean, n = 50 ± SE) in mock, *Hs*AQP1, *Nt*PIP1;3, and *At*PIP1;2 strains.

5.3.4 PIP transcript abundance and ATP level in tobacco roots under hypoxia

We examined the relative transcript abundance of tobacco plants subjected to flooding-induced hypoxia in mineral solution culture for two and seven days. After two days of hypoxia (≈ 125 µmol L^{-1} O_2), leaf and root relative transcript abundance of *NtPIP1;3* (the aquaporin showing the high rate of O_2 transport) increased by about four-fold compared with well-aerated (≈ 500 µmol L^{-1} O_2) plants (Fig 5.14a and b). In well-aerated plants, aquaporin relative transcript abundance remained similar on days two and seven in leaves and roots (Fig 5.14 a-d). Relatively minor increases were also measured for relative transcript abundance of *NtPIP1;4* in leaves and *NtPIP1;1* and *NtPIP1;2* in roots (Fig 5.14a and b). After seven days, a sharp increase of *NtPIP1;3* was measured in hypoxic leaves (about 12-fold higher than aerated control) and roots (about 22-fold higher than aerated control) (Fig 5.14c and d). There was also about three-fold increase in *NtPIP2;1* in the leaves (Fig 5.14c). The increase in *NtPIP1;3* relative transcript abundance in leaves and roots between day two and day seven of hypoxia treatment were accompanied by a sharp increase in root ATP levels, which

were measured in the root segments at different distances from the root tips (Fig 5.15a and b). After seven days of treatment, hypoxic and well-aerated roots had similar concentrations of ATP in all root segments regardless of the distance from the root tip (Fig 5.15b). In our study, hypoxic plants had healthy and green appearance and did not show chlorosis or other visible signs of O_2 deficiency.

5.4 Discussion

In this study, I investigated the potential contribution of aquaporins to transmembrane O_2 transport in yeast whole cells and yeast protoplasts by measuring absorbance near the peak wavelengths of myoglobin over time. In the whole-cell assay, A_{541} increased over the first 60 s, which enabled us to screen strains that expressed putative O_2-transporting aquaporins (Fig 5.5b). Changes in A_{541} likely represent a combination of several processes including O_2 diffusion, oxygenation of deoxymyoglobin, conversion between oxymyoglobin and metmyoglobin, and O_2 consumption. The presence of cell walls might hinder the changes in absorbance in myoglobin and lead to an underestimation of O_2 diffusion in the preliminary screening. In addition, possible artifacts on absorbance reading might be caused during the mixing of the yeast suspension and aerated buffer. The yeast protoplast assay aimed to eliminate these potential pitfalls with numerous precautions and more replications and to maximize the signal of myoglobin oxygenation. The results suggested that $\Delta A_{319}/\Delta A_{341}$ in yeast protoplast assay may also be a highly sensitive parameter in measuring O_2 transport through aquaporins.

Human aquaporin *Hs*AQP1, which I found to enhance myoglobin oxygenation by facilitating O_2 passage, was also reported to facilitate CO_2 transport when heterologously expressed in *Xenopus laevis* oocytes (Nakhoul et al. 1998). However, other major CO_2-transporting aquaporins including *At*PIP1;2 (Uehlein et al., 2012), *Nt*PIP1;2 (Uehlein et al., 2003) and *Lb*AQP1 (Navarro-Ródenas et al., 2015) did not facilitate O_2 transport when expressed in yeast (Fig 5.5b). This suggests that aquaporin orthologues have developed certain degree of specificity for O_2 transport. The alignment of all the 20 analyzed aquaporins does not show consensus residues that are exclusive to O_2-transporting aquaporins (Appendix 4). It appears that the conserved residues are species-dependent rather than being relevant to transport capacity. However, it is noteworthy that all six O_2-transporting aquaporins have

well-conserved 29 amino acid residues across species, including most of the Asn-Pro-Ala (NPA) signature motifs and the selective filters of Ar/R residues (Appendix 5). In *Nt*PIP1;3, the asparagine residue commonly in the second NPA motif is substituted by threonine (Thr-235), which may be potentially relevant to its highly enhanced O_2-transporting capacity.

Calculations of permeation of hydrophobic gases (O_2, CO_2, and NO) have consistently shown similar values of an energy barrier of 5-6 kcal mol^{-1} through water pores (Wang et al., 2007; Hub and de Groot, 2008). Membrane protein simulation systems of the human *Hs*AQP1 tetramer have demonstrated the presence of a pore located in the center between the four monomers that is lined by largely hydrophobic residues and may be involved in the transport of gases rather than water (Wang et al., 2007; Hub and de Groot, 2006). It could be speculated that the presence and the exact structure of this pore imparts gas transport specificity to different aquaporins. In proven correct, the gating properties of this pore could be targeted to alter rates of the transmembrane passage of gases.

The results of yeast O_2 uptake rate corroborate those of the O_2 transport assays, pointing to the significance of pore-mediated transport for respiration. Increased relative transcript abundance of *HsAQP1*, also sometimes accompanied by other aquaporins, have been commonly reported for cancerous cells (Hoque et al., 2006; Wang et al., 2015), with the level of *HsAQP1* expression often correlated with cell growth, grade of tumor (Wang et al., 2015; Wei and Dong, 2015), and metastasis (Xie et al., 2011; Esteva-Font et al., 2014). It has been also reported that the deletion of *HsAQP1* was effective in reducing breast tumor size and lung metastasis (Esteva-Font et al., 2014) and *HsAQP1* silencing inhibited the proliferation and invasiveness of osteosarcoma cells (Wu et al., 2015). Although the proposed explanations for the links between *HsAQP1* expression and cancerous growth have largely focused on water transport, the association between high O_2 demand of rapidly growing cancerous cells and facilitation of O_2 transport by *Hs*AQP1 should also be considered.

Since the reports of hypoxia-induced expression of *HsAQP1* (Leggett et al., 2012; Zhang et al., 2013) suggest that aquaporin-mediated transport processes may be especially important under low O_2 conditions, I examined relative transcript abundance of *N. tabacum* plants subjected to flooding-induced hypoxia. Although the ATP concentrations showed some decline in well-aerated plants after 7 days compared with 2 days (Fig 5.15a and b), the reverse trend was observed in plants

subjected to root hypoxia resulting in similar ATP levels in leaves and all root segments of hypoxic and well-aerated plants after 7 days of hypoxia (Fig 5.15a and b). The results suggest that after the initial hypoxic stress, plants likely received sufficient O_2 to support aerobic respiration, as hypoxic plants had healthy and green appearance and did not show chlorosis or other visible signs of O_2 deficiency. While the resistance to root hypoxia can be explained in some plants by an increased supply of O_2 to the root cells through the development of specialized aerating structures such as aerenchyma, the processes of plant resistance to hypoxia in the absence of obvious structural changes remain obscure. In our study, there were no structural features present in the roots and stems of plants exposed to root hypoxia that could be indicative of improved O_2 delivery. Therefore, the increase in *NtPIP1;3* relative transcript abundance (Fig 5.14) could be among important factors contributing to improved root aeration, similarly to the increased relative transcript abundance of *HsAQP1* in hypoxic human tissues (Leggett et al., 2012; Zhang et al., 2013). Clearly, the link between pore-mediated O_2 transport and hypoxia deserves further attention.

In conclusion, our results indicate that some of the studied plant and human aquaporins are likely to be involved in O_2 transport. Yeast cells heterologously expressing these aquaporins maintained higher O_2 uptake rates in liquid culture and tobacco plants exhibited sharp increases in the putative O_2-transporting aquaporin after their roots were subjected to hypoxic conditions. These increases in O_2 transporting aquaporins were accompanied by increases in leaf and root ATP concentrations in hypoxic plants. The results of the study support the notion that functional significance of pore-mediated O_2 transport should receive more attention.

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Table 5.1 Subcellular localization of expressed aquaporin proteins predicted by TargetP 1.1 server

Aquaporins	Length	mTP	SP	Other	Loc	RC
HsAQP1 [*DQ895575*]	269	0.016	0.927	0.171	S	2
NtPIP1;3 [*U62280*]	284	0.077	0.298	0.828	-	3
AtPIP1;2 [*AT2G45960*]	301	0.137	0.283	0.744	-	3

Note: Subcellular localization of the aquaporins was predicted using TargetP1.1. The highest score is the most likely location according to TargetP. Loc stands for the prediction of localization: M for mitochondrion, i.e. the sequence contains mTP, a mitochondrial targeting peptide; S for secretory pathway, i.e. the sequence contains SP, a signal peptide; - for any other location. RC stands for reliability class, from 1 to 5, where 1 indicates the strongest prediction.

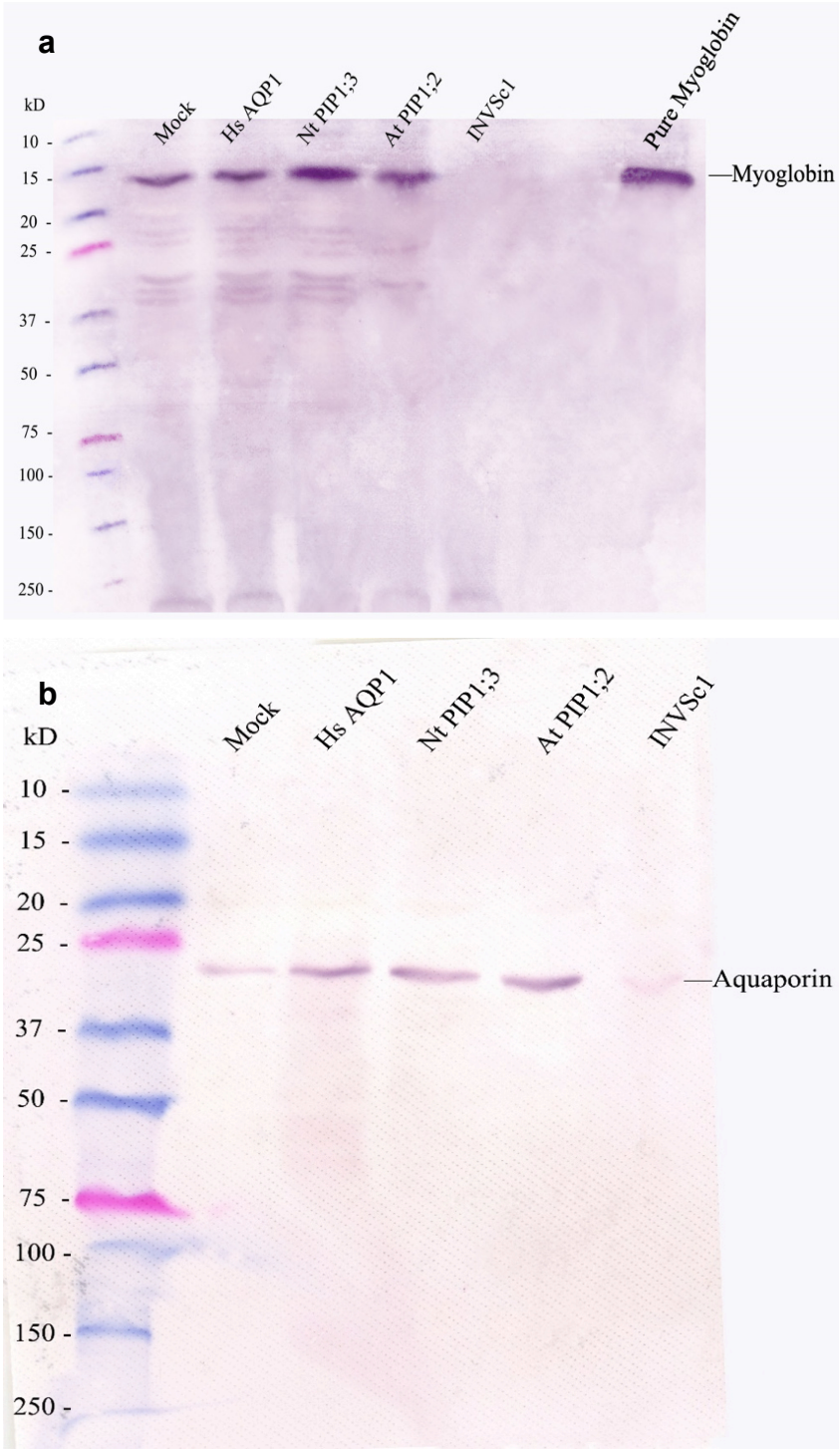


Figure 5.1 Immunoblot probed with the anti-myoglobin antibody and the anti-human aquaporin 1 antibody. (a) The yeast total proteins were immunoblotted with the primary anti-myoglobin antibody. (b) The yeast total proteins were immunoblotted with the primary anti-human aquaporin 1 antibody.

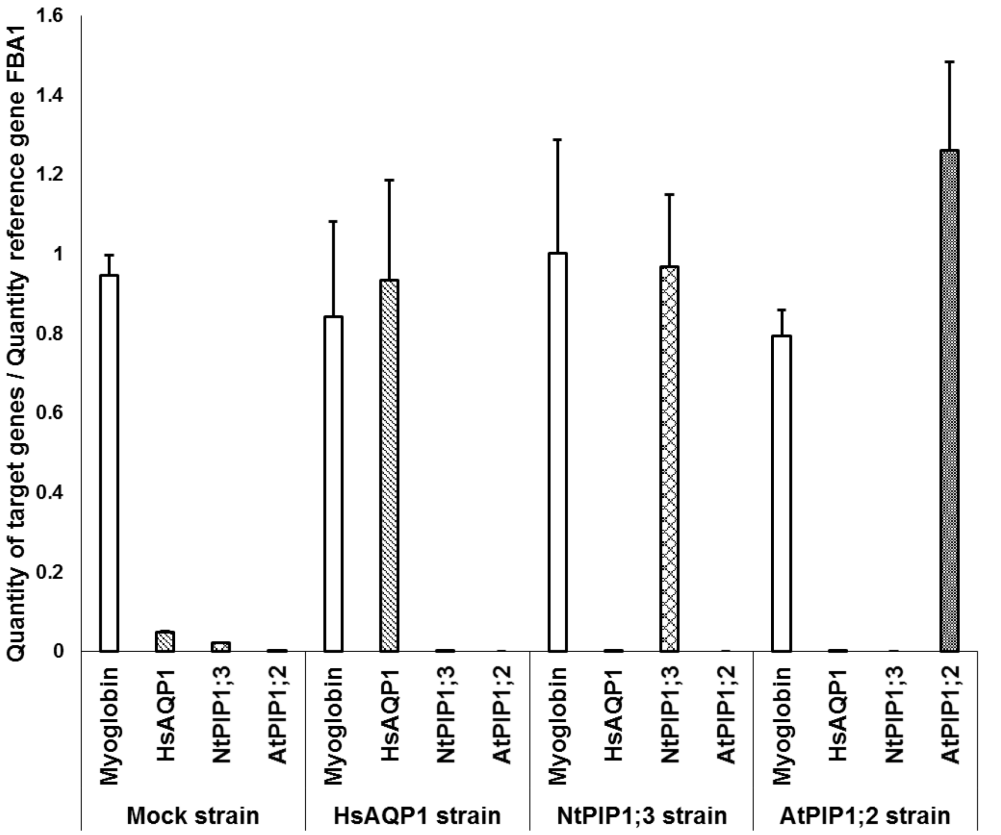


Figure 5.2 Transcript level of myoglobin and aquaporins in the transformed yeast strains. The transcript abundance of myoglobin, *HsAQP1*, *NtPIP1;3* and *AtPIP1;2* in each strain was normalized to the yeast reference gene *ScFBA1*, by relative quantification of standard curve method with correction for amplification efficiency (n = 3).

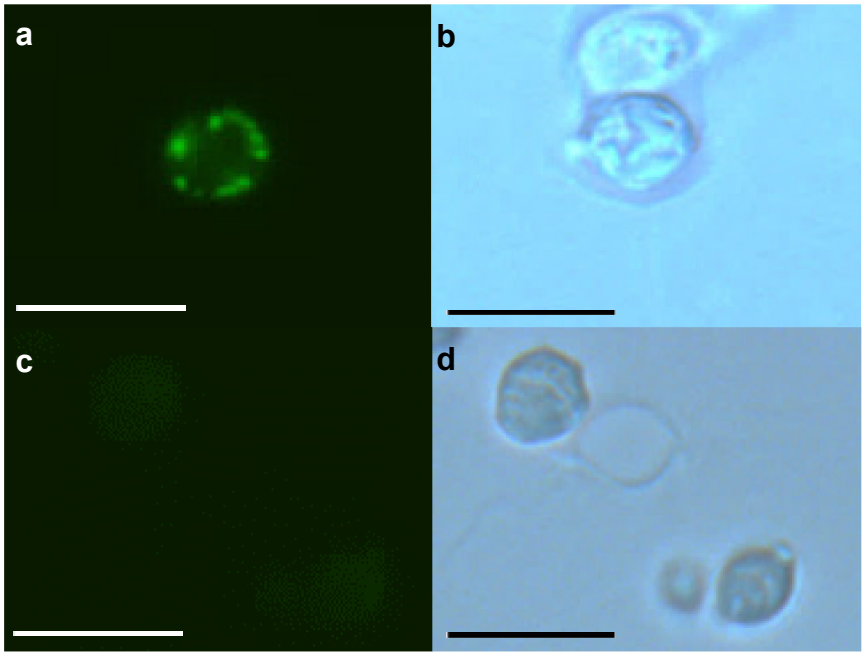


Figure 5.3 Indirect immunofluorescence of paraffin-embedded yeast cells of *Hs*AQP1 strain after the incubation with the primary anti-aquaporin 1 monoclonal antibody and the fluorescein-conjugated secondary antibody. (a) *Hs*AQP1 strain under blue light excitation. (b) *Hs*AQP1 strain in bright field. (c) Mock strain under blue light excitation. (d) Mock strain in bright field. The length of bars is 10 μm.

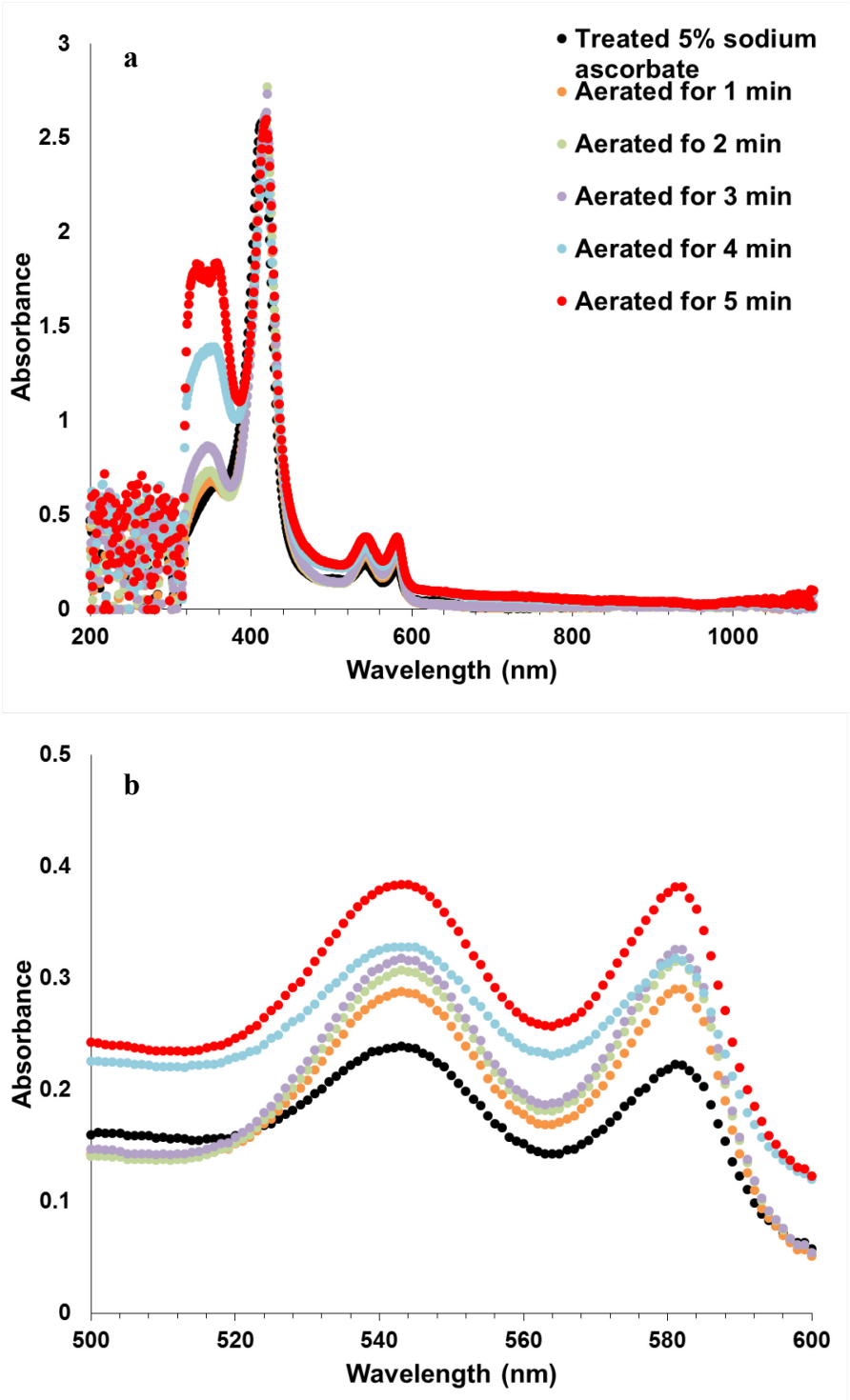


Figure 5.4 Effect of aeration on absorbance spectra of purified myoglobin protein. (a) Absorbance spectrum from 190 nm to 1100 nm. (b) Absorbance spectrum from 500 nm to 600 nm.

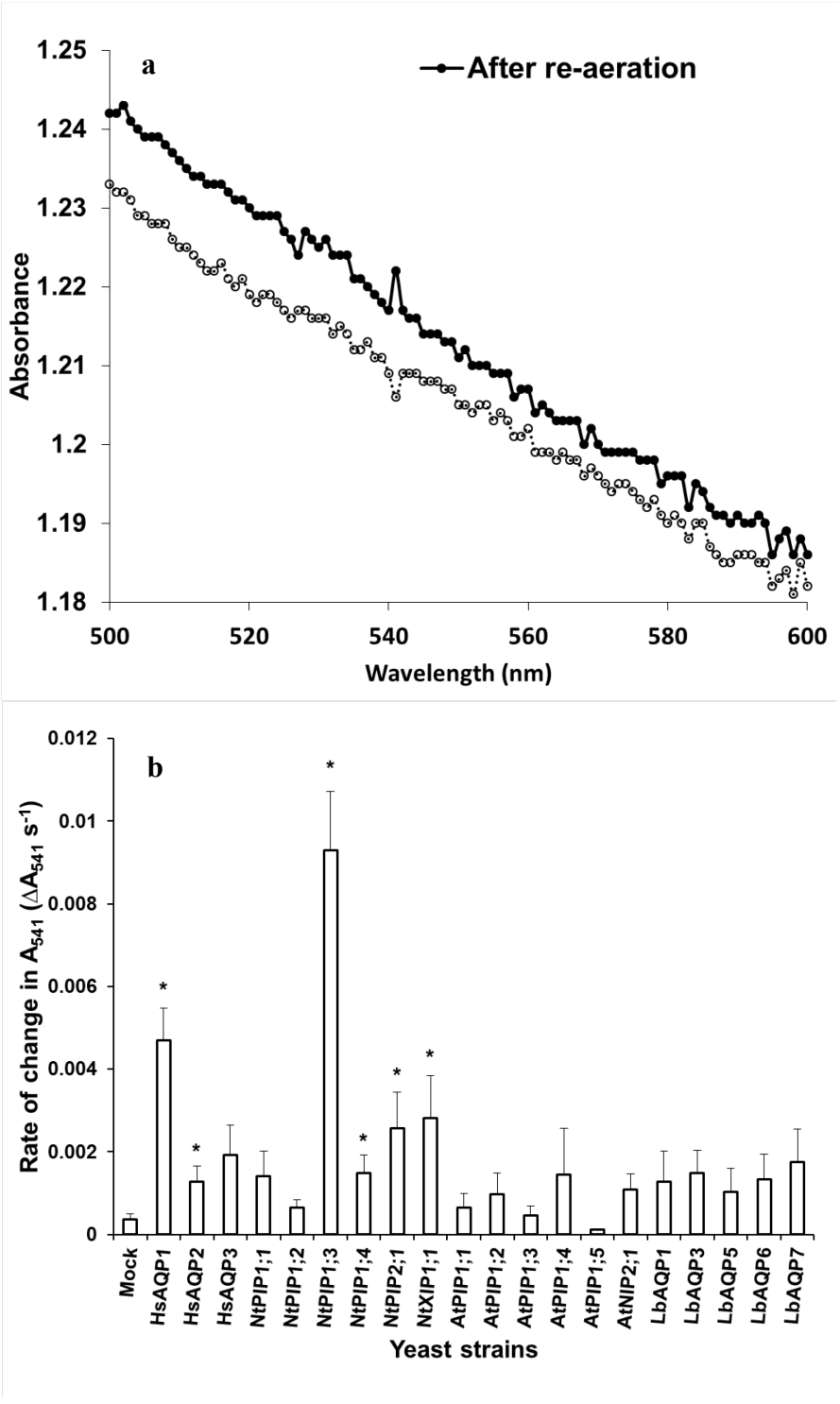


Figure 5.5 The expression of O_2-transporting aquaporins in yeasts led to higher rate of increase in A541 of cell suspension due to oxygenation of the co-expressed myoglobin. (a) Absorbance spectrum between 500 nm and 600 nm for *Hs*AQP1 yeast strain after oxygen depletion or re-aeration. (b) Rate of increase in A_{541} in 20 strains of yeasts co-expressing myoglobin and different human (*Hs*), *Nicotiana tabacum* (*Nt*), *Arabidopsis thaliana* (*At*) and *Laccaria bicolor* (*Lb*) aquaporins and in a mock strain

(control)**.** The sperm whale myoglobin and one of the 20 different aquaporins were co-expressed in yeast strain INVSc1, and the rate of O_2 uptake was reflected by the rate of increase in A_{541} (ΔA_{541} s^{-1}) due to myoglobin oxygenation. A_{541} of 1 mL yeast suspension of each strain was recorded for 2 min at 1 s interval immediately after the addition of 1 mL of air-saturated KH_2PO_4 buffer or N_2-saturated KH_2PO_4 as negative control, respectively, to calculate ΔA_{541} s^{-1}. The means and standard errors were calculated from six biological replications ($n = 6 \pm$ SE). Asterisks indicate statistically significant difference (ANOVA, Tukey's test, $P \leq 0.05$).

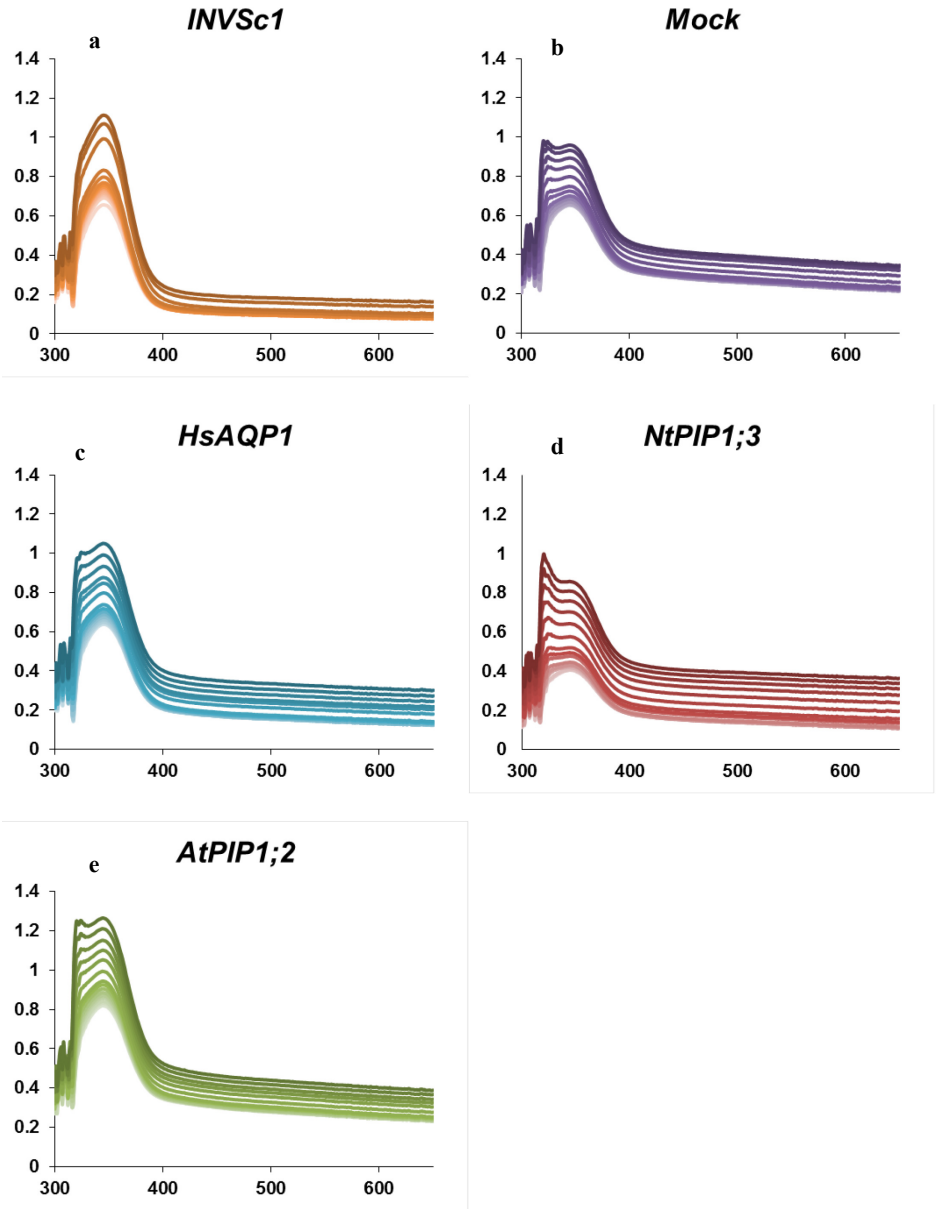


Figure 5.6 Scanning of absorbance spectra of yeast protoplasts from 300 nm to 650 nm during 10 min with multiple times of aeration. (a) INVSc1, (b) Mock, (c) *Hs*AQP1, (d) *Nt*PIP1;3, (e) *At*PIP1;2.

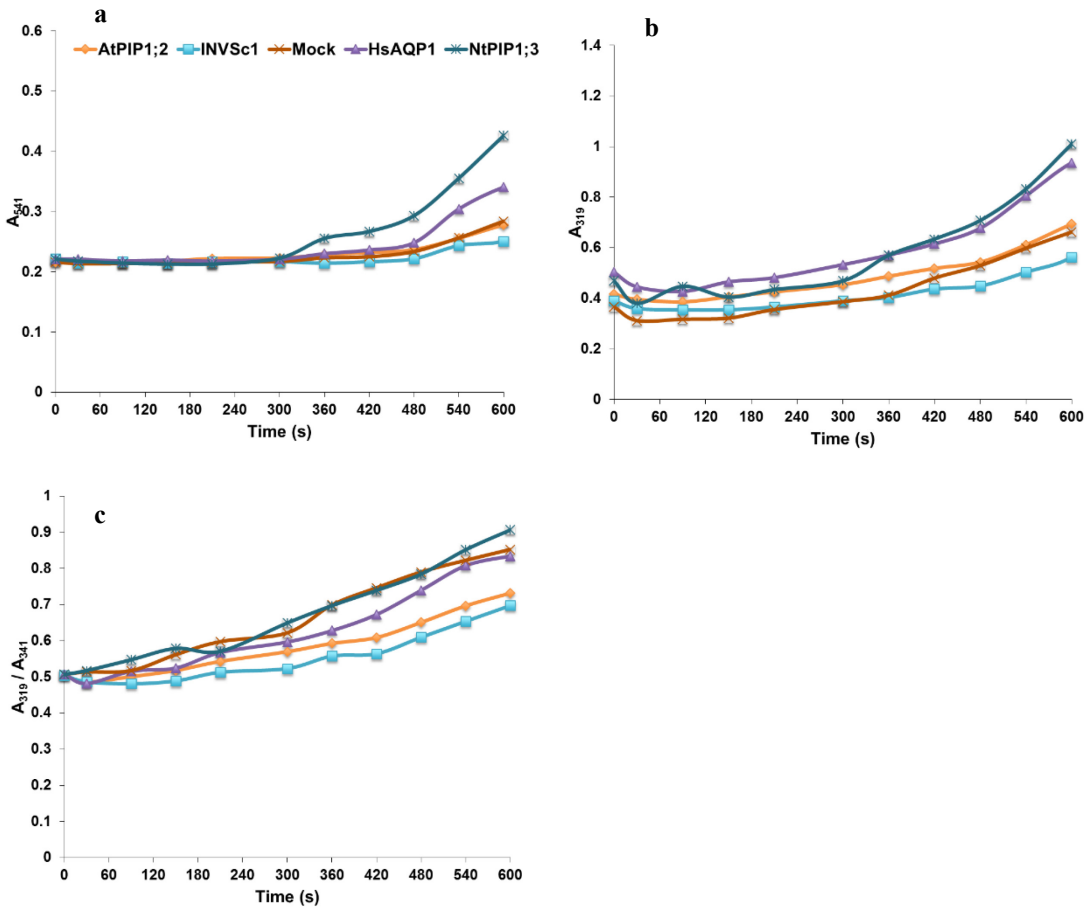
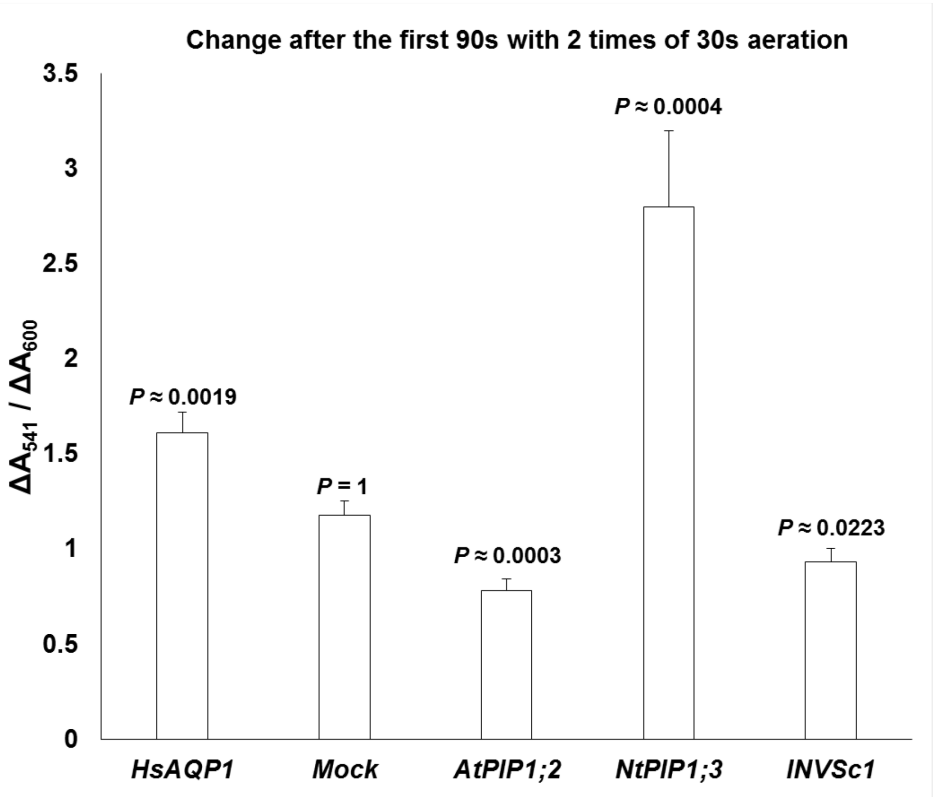
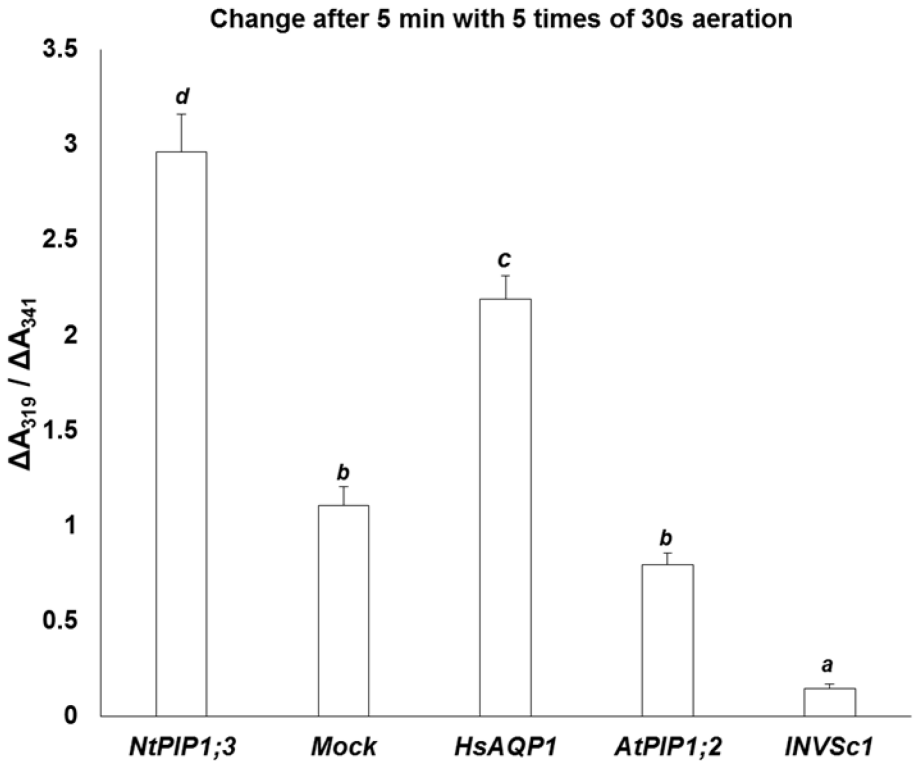


Figure 5.7 Change in A_{541}, A_{319} and A_{319}/A_{341} of yeast protoplasts during 10 min with multiple times of aeration. (a) A_{541}, (b) A_{319}, (c) A_{319}/A_{341}.



Pairwise Comparisons		*P*
Factor 1	***Factor 2***	
*Hs*AQP1	Mock	0.502801684
*Hs*AQP1	*At*PIP1;2	0.023656001
*Hs*AQP1	*Nt*PIP1;3	0.000393702
*Hs*AQP1	INVSc1	0.100915775
Mock	*At*PIP1;2	0.587724108
Mock	*Nt*PIP1;3	9.05412E-05
Mock	INVSc1	0.89713671
*At*PIP1;2	*Nt*PIP1;3	9.03061E-05
*At*PIP1;2	INVSc1	0.979490423
*Nt*PIP1;3	INVSc1	9.03076E-05

Figure 5.8 $\Delta A_{541}/\Delta A_{600}$ of yeast protoplasts after the first 90s with 2 times of 30s aeration. *P* values for statistical significance between the aquaporin-expressing strains and the mock strain are shown above the bars for standard errors. *P* values for statistical significance across all the strains are shown in the table below. (ANOVA, Tukey's test, $P \leq 0.05$, $n = 19$).



Pairwise Comparisons		*P*
Factor 1	***Factor 2***	
*Nt*PIP1;3	Mock	0.000101938
*Nt*PIP1;3	*Hs*AQP1	0.000779584
*Nt*PIP1;3	*At*PIP1;2	0.000101938
*Nt*PIP1;3	INVSc1	0.000101938
Mock	*Hs*AQP1	0.000112793
Mock	*At*PIP1;2	0.343082001
Mock	INVSc1	0.000140061
*Hs*AQP1	*At*PIP1;2	0.000101946
*Hs*AQP1	INVSc1	0.000101938
*At*PIP1;2	INVSc1	0.004806013

Figure 5.9 $\Delta A_{319}/\Delta A_{341}$ of yeast protoplasts after 5 min with 5 times of 30s aeration. Different letters indicate statistically significant difference across all the strains (*P* values shown in the table below) (ANOVA, Tukey's test, $P \leq 0.05$, $n = 6$).

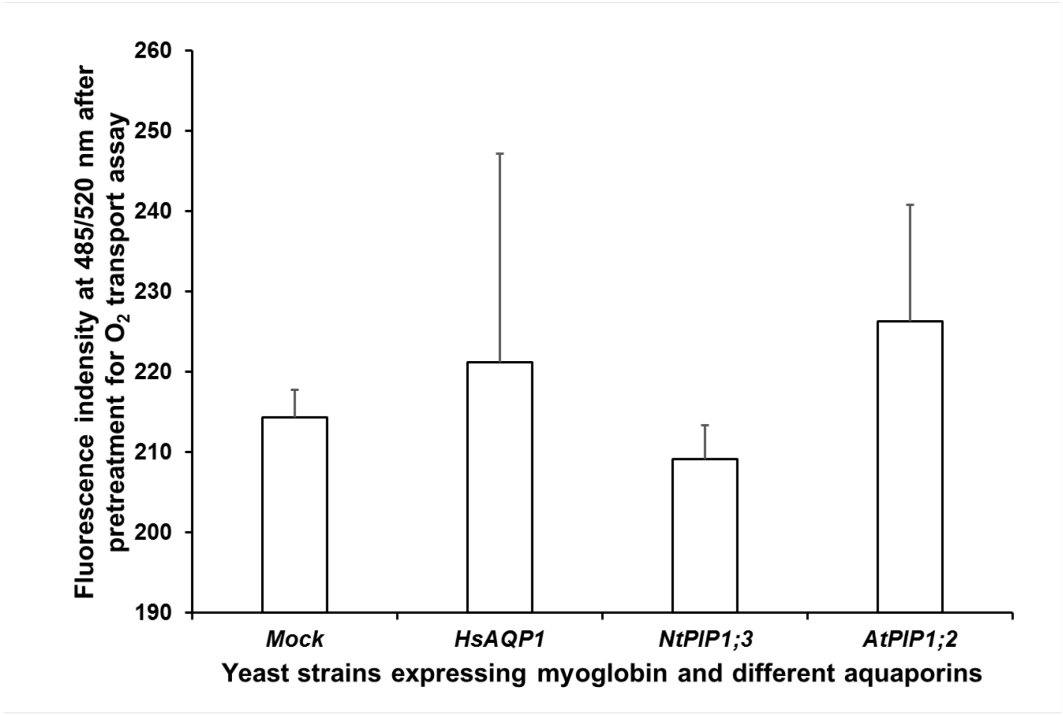


Figure 5.10 Cellular oxidative stress in yeast strains after pre-treatment for O_2 transport assay using CM-H_2DCFDA as the ROS indicator. The bars show mean fluorescence intensity over 3 min (n = 6 ± SE).

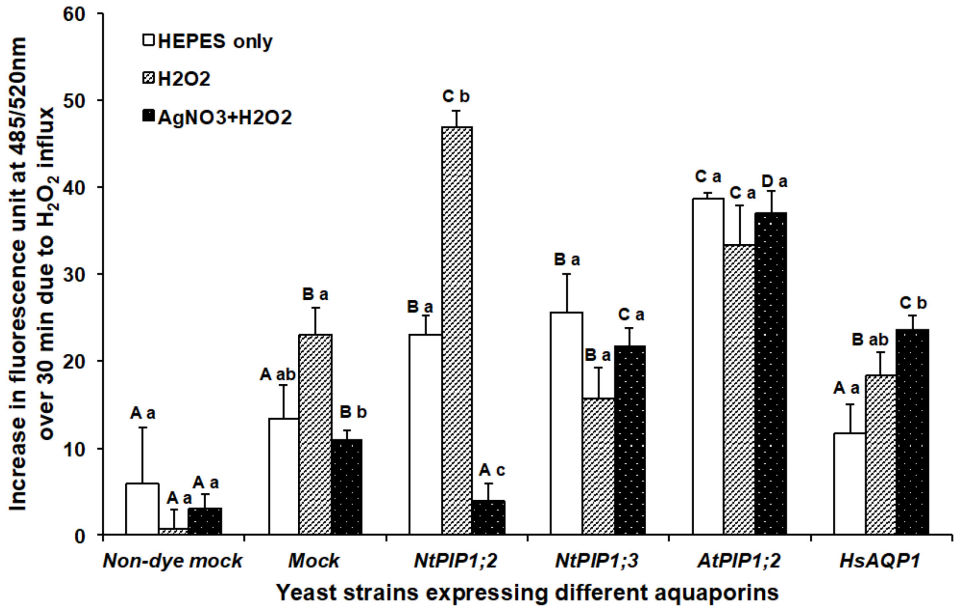


Figure 5.11 Effect of the expression of *Nt*PIP1;2 (*Nt*AQP1), *Nt*PIP1;3, *Hs*AQP1, *At*PIP1;2, and the mock control of empty expression vector on H_2O_2 permeability of yeast plasma membrane. The bars show mean increases in fluorescence generated by CM-H_2DCFDA caused by the influx of H_2O_2 (n = 6 ± SE). Different uppercase letters above bars indicate statistically significant differences between yeast strains under the same treatment at $P \leq 0.05$, whereas different lowercase letters indicate statistically significant differences between treatments within each strain at $P \leq 0.05$, as determined by ANOVA, Tukey's test.

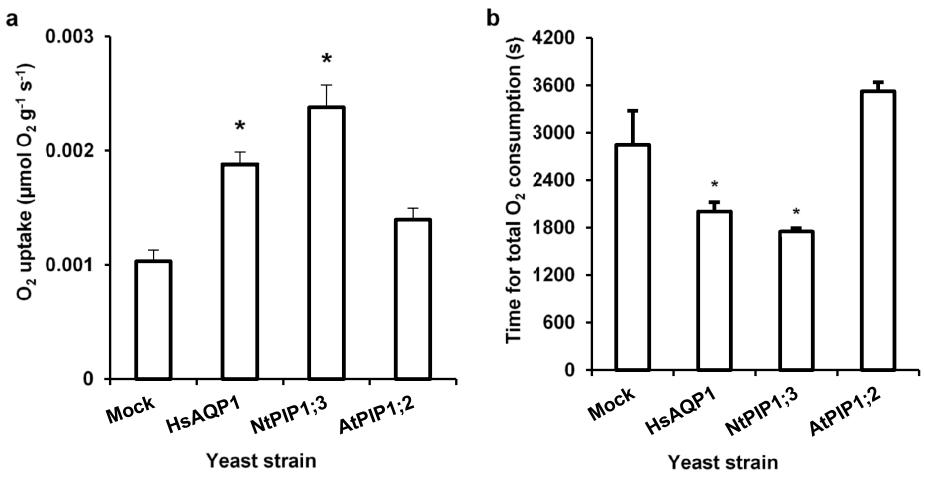


Figure 5.12 O_2 consumption of yeast strains over 1000 s. (a) Respiration rates in *Hs*AQP1, *Nt*PIP1;3, *At*PIP1;2, and mock strain (control). (b) Time for total O_2 consumption in *Hs*AQP1, *Nt*PIP1;3, *At*PIP1;2, and mock strain (control). Immediately after air was supplied to the yeast suspension in N_2-bubbled SD-L-U + glucose medium to reach the saturation concentration of soluble O_2 of 235 µmol L^{-1}, the decrease of O_2 concentration in yeast suspension was monitored and logged per second using an O_2 microsensor (n = 6 ± SE). Different letters indicate statistically significant difference (ANOVA, Tukey's test, $P \leq 0.05$).

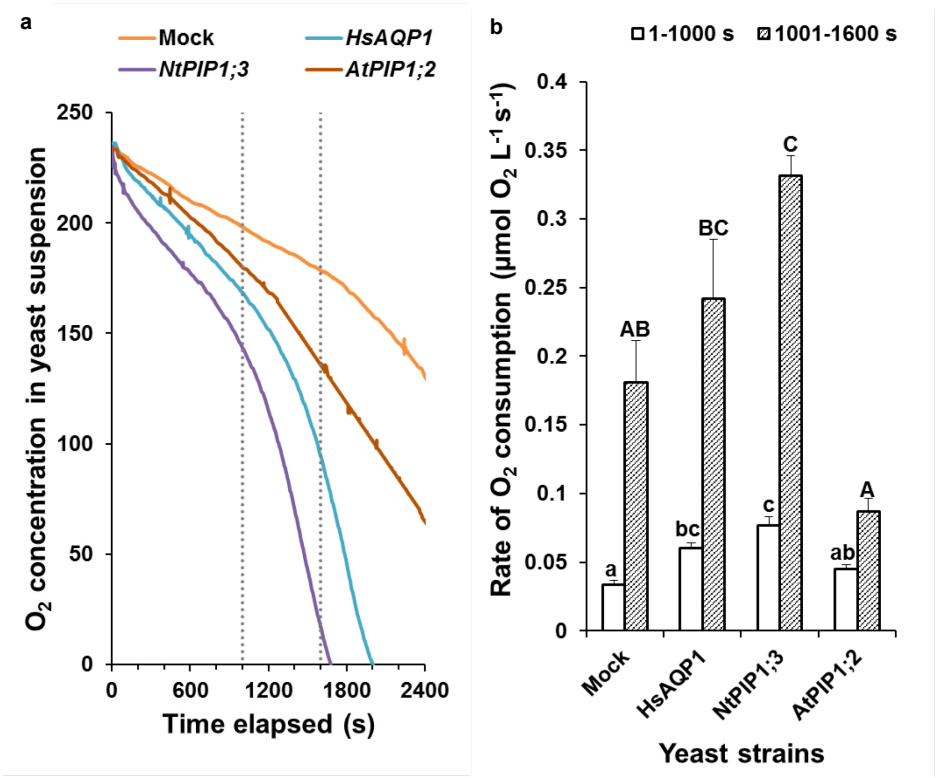


Figure 5.13 Characteristics of O_2 consumption of yeast strains. (a) Changes in concentration of soluble O_2 over 40 min in cell suspension of yeast mock strain and the strains that expressed *Hs*AQP1, *Nt*PIP1;3 and *At*PIP1;2 aquaporins, and (b) their rates of O_2 consumption over 1600 s.

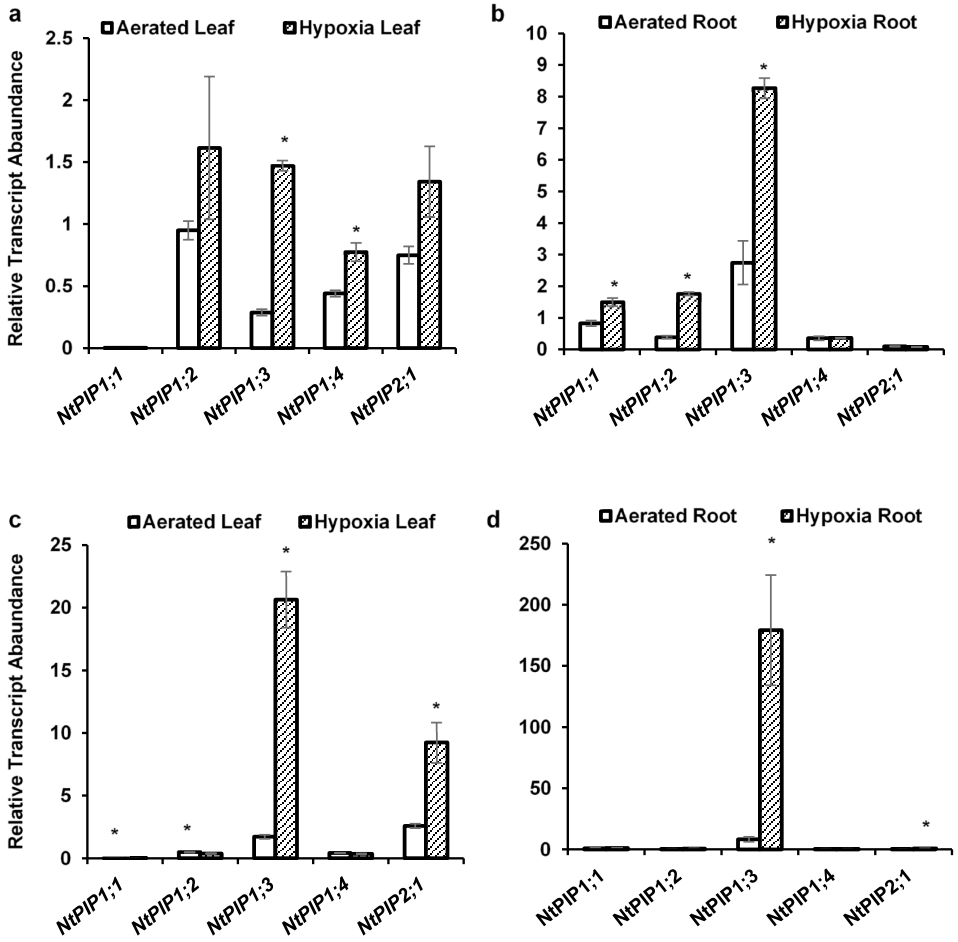


Figure 5.14 Transcript abundance of tobacco plasma membrane intrinsic proteins (PIPs) after exposure to well-aerated and hypoxic conditions. Relative transcript abundance of selected *PIP*s in (a) leaves and (b) roots after 2 days of exposure, and in (c) leaves and (d) roots after 7 days of exposure**.** Transcript abundance of *PIP*s was measured by the standard curve method in qRT-PCR assay, with normalization against geometric mean of that of the two reference genes, *EF1-α* and *L25*. Means ($n = 6$) ± SE are shown. Asterisks indicate significance difference in gene expression between well-aerated and hypoxic treatment (ANOVA, Tukey's test, $P \leq 0.05$).

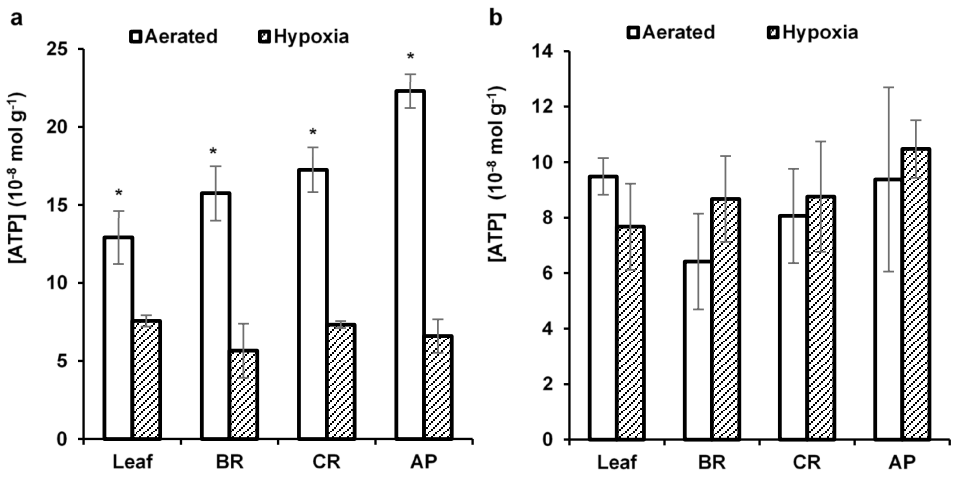


Figure 5.15 ATP levels in leaves and basal (BR), central (CR) and apical (AP) root segments of tobacco plants subjected to hypoxic and well-aerated conditions. (a) ATP levels after 2 days of the treatments. (b) ATP levels after 7 days of the treatments. ATP concentration was determined by detecting bioluminescence in Luciferase/Luciferin reaction. Means (n = 6) ± SE are shown. Asterisks indicate significant differences between well-aerated and hypoxic treatments for the same tissue (ANOVA, Tukey's test, $P \leq 0.05$).

6 General Discussion and Conclusions

6.1 Outcomes of this thesis

To address the functions of plant aquaporins under O_2 deficiency, four studies (Chapters 2, 3, 4 and 5) were conducted in this thesis. These studies deepen our understanding of the functions and responses of plant aquaporins under O_2-deficient conditions, as well as the effects of exogenous ethylene and formation of adventitious roots on aquaporin relative transcript abundance under hypoxia. Transport analyses in yeast expression system verified the hypothesis of the transporting role of specific aquaporins in O_2 transmembrane movement.

i. O_2 transport through aquaporins: Twenty aquaporin genes from human, plant, and fungus were heterologously expressed in yeast. O_2 permeability was examined by co-expressing heterologous aquaporin and myoglobin in yeast. *Hs*AQP1 and *Nt*PIP1;3 were identified as two promising O_2 transporters. Overexpression of *Nt*PIP1;3 increased the O_2 uptake rates of yeast in suspension culture. Hypoxic tobacco roots showed increased relative transcript abundance of *NtPIP1;3* accompanied by the increased apical root ATP levels.

ii. The involvement of aquaporins in ethylene-induced hypoxia resistance of trembling aspen: Physiology and transcripts of several aquaporins were examined in hypoxic aspen seedlings. Exogenous ethylene enhanced the root aeration, root hydraulic conductance, leaf photosynthesis and transpiration of hypoxic hydroponic-grown aspen. Transcripts of *P. tremuloides PIP2;4* was induced by exogenous ethylene under hypoxia, which possibly, at least partially, contribute to the enhanced root hydraulic conductance under hypoxia.

iii. Relative transcript abundance of aquaporins and hypoxia-responsive genes in roots of hypoxic tobacco: Tobacco seedlings were subjected to hypoxia treatment for one week. Formation of adventitious roots were induced by low O_2 conditions. Different aquaporins expression patterns were detected in induced adventitious roots and existing lateral roots. Relative transcript abundance of most aquaporins were reduced by hypoxia with the exception of *NtPIP1;3*. In Chapter 5, however, the relative transcript abundance of *NtPIP1;3* was upregulated under hypoxic conditions. It is possible that gene expression patterns in response to hypoxia may

vary between different sampling tissues or between different developmental stages at which plants were treated with hypoxia. Adventitious roots received more O_2 than existing lateral roots and helped maintain the functions and stable expression of aquaporins. These differences between adventitious roots and lateral roots may play a role in maintaining root conductance and gas exchange.

iv. Transcripts of aquaporins in waterlogged *Arabidopsis*: *Arabidopsis* seedlings were subjected to well-aerated and waterlogging treatment. Gas exchange of waterlogged *Arabidopsis* plants was inhibited. *AtPIP*s expression decreased after 24 h of waterlogging, but *AtPIP1;1* and *AtPIP1;4* exhibited increased expression after 48 h of waterlogging. These results have demonstrated that aquaporin expression may change along with the waterlogging treatment time. It needs to be determined in the future whether these changes in aquaporin expression are translated into physiological changes.

6.2 General discussion

Plant aquaporins exhibit high diversity in higher plants. The roles of aquaporins in plant water relations have been well acknowledged, but the other functions of aquaporins remain unclear, especially in pore-mediated gas transport. The transcriptional and post-transcriptional regulation of aquaporins is affected by various abiotic factors, including O_2 deprivation.

In this study, physiological and molecular responses of tobacco (Chapter 2, Chapter 5), trembling aspen (Chapter 3) and *Arabidopsis* (Chapter 4) in O_2-deficient conditions were studied. Different species showed different strategies to cope with oxyen deficiency. Hydroponically-grown tobacco produced more ARs when exposed to root hypoxia, and no adventitious roots were observed in aspen and *Arabidopsis*. With more air space than in existing LRs, ARs can conduct more O_2 into roots from shoots under O_2-deficient conditions. Better aeration helped ARs maintain stable relative transcript abundance of PIPs and hypoxia-responsive genes, which also partially contributed to maintaining K_r of tobacco plants exposed to root hypoxia. The delivery of O_2 from shoots is the only alternative to increase O_2 supply for plants subjected to root hypoxia (Tan et al., 2018). A few hypoxia-tolerant plants can develop adaptations to low O_2 conditions that reduce tissue resistance to O_2 transport (aerenchyma and ARs) or increase barriers to O_2 loss from roots (Bailey-Serres and

Voesenek, 2008). These adaptations can help plants mininmize the consequences of O_2 deficiency but not eliminate the O_2 conditions in roots. The formation of ARs is effective in helping plants escape from low O_2 conditions but is also energy-consuming (Bailey-Serres and Voesenek, 2008). Thus, plants need to integrate diffetent strategies coping with challenging low O_2 conditions. In aspen and *Arabidopsis* plants, hypoxia did not induce the formations of ARs, but the adjustments in gene expression, gas exchange and root hydraulics were observed. The different strategies of different plant species in response to O_2 deprivation are the results of the diverse evolutionary choices (Bailey-Serres and Voesenek, 2008). Transcription of different aquaporin isoforms exhibited diverse responses to O_2 deprivation. The up-regulation or down-regulation of transcirpt levels in O_2-deficient conditions have been widely reported in *Arabidopsis* (Liu et al., 20015), soybean (Matsuo et al., 2012), *Sorghum bicolor* (Kadam et al., 2017) and *Persea americana* (Reeksting et al., 2016). The down-regulation of PIP genes is generally thought to be associated with the decrease in Lp_r. The up-regulation of *Arabidopsis NIP2;1* was linked to the transmembrane transport of lactic acid (Choi and Roberts, 2007), which is the end-product of lactic fermentation. However, the physiological significance of the up-regulation of other aquaporin isoforms is still unclear. The transcription of genes under O_2 deprivation is regulated differently compared to well-aerated conditions, and may be affected by various biotic and abiotic factors (Branco-Price et al., 2008). In my studies, the changes of aquaporin transcripts under O_2 deprivation conditions were analysed together with the difference between different root tissues (Chapter 2), ethylene (Chapter 3) and duration of hypoxia treatment (Chapter 4). These changes may be associated with hydraulic adjustments, as well as other potential functional roles of aquaporins, in roots under O_2 deprivation conditions and contribute to the resistance of plants to hypoxia.

Plant aquaporins show high diversity and the functions and regulation of aquaporins are still little understood. Facillitated transport of water and other small molecules, such as H_2O_2 (Dynowski et al., 2008) and lactic acid (Choi and Roberts, 2007), has been well acknowledged to be associated with the physiological processes in response to O_2 deprivation (Tan et al., 2018) (Fig 6.1). O_2 is thought to diffuse freely across biological membranes, which however, has been challenged by the concept of gas channels in biological membranes (Uehlein et al. 2003). One of the

central hypotheses in this thesis is that some aquaporin isoforms are involved in the pore-mediated transmembrane transport of O_2. A new method of detecting cellular oxygen by expressing a whale myoglobin in yeast was developed. In this thesis, aquaporin-mediated O_2 transport was discussed, which should be considered in the future studies. Compared to detecting transmembrane movement of other gases, for example, using pH difference representing CO_2 transport (Uehlein et al., 2003), the lack of sound techniques detecting intracellular oxygen concentration limits the research of subcellular O_2 dynamics (van Dongen and Licausi, 2014). Expressing O_2-binding proteins is a promising method in detecting cellular O_2 levels since the process of binding O_2 is reversible and can be monitored by detecting changes in absorbance. However, improvements of this method are still needed.

6.3 Perspectives for future studies

Pore-mediated gas transport across biological membranes is arelatively little studied area, which may have a profound significance and change our understanding of some of the fundamental biological processes. In this thesis study, advances have been made in detecting cellular O_2 by expressing whale myoglobin in yeast. However, in order to detect the transient process of O_2 movement across membrane, further improvements are still needed. Possible directions include the amplification of signals. In the results of Chapter 6, it was shown that the background signals in spectrophotometry are quite strong, which may weaken signals of O_2 transport. Efforts has been made in this thesis by using yeast protoplasts instead of intact yeast cells, while other solutions should also be explored, including increasing the concentrations of subcellular myoglobin. The possibility of controlling the rate of transmembrane O_2 transport should also be further explored. In water transmembrane transport, aquaporin blockers, for example, Hg^{2+} (Agre et al., 1993), Ag^{+} and Au^{2+} (Niemietz and Tyerman, 2002) have been proven to be effective in manipulating aquaporin-mediated water transport. It would be interesting to explore pore-mediated O_2 transport blockers and their possible physiological significance.

Regulation of aquaporins under abiotic stresses and in different plant developmental stages, has attracted a lot of research interests. In addition to the vital role in plant hydraulics, the roles of plant aquaporins have been integrated into wide ranges of biological processes, including root water uptake (Martre et al., 2002), gas

exchange (Laur and Hacke, 2013), ripening (Bots et al., 2005), and plant-microbe interactions (Navarro-Ródenas et al., 2015; Xu et al., 2015). In response to hypoxia, changes in plant hydraulics caused by the inhibition of aquaporin functions can be seen either as injury or acclimation. This needs to be addressed in the future studies manipulating aquaporin activities in order to promote plant performance under low O_2 conditions. Interactions between aquaporin activities and plant hormones, especially ethylene, need to be further addressed. The regulation of aquaporin activities by ethylene may be through protein phosphorylation (Qing et al., 2016), while more research is still needed to demonstrate the interactions between ethylene and other hormones.

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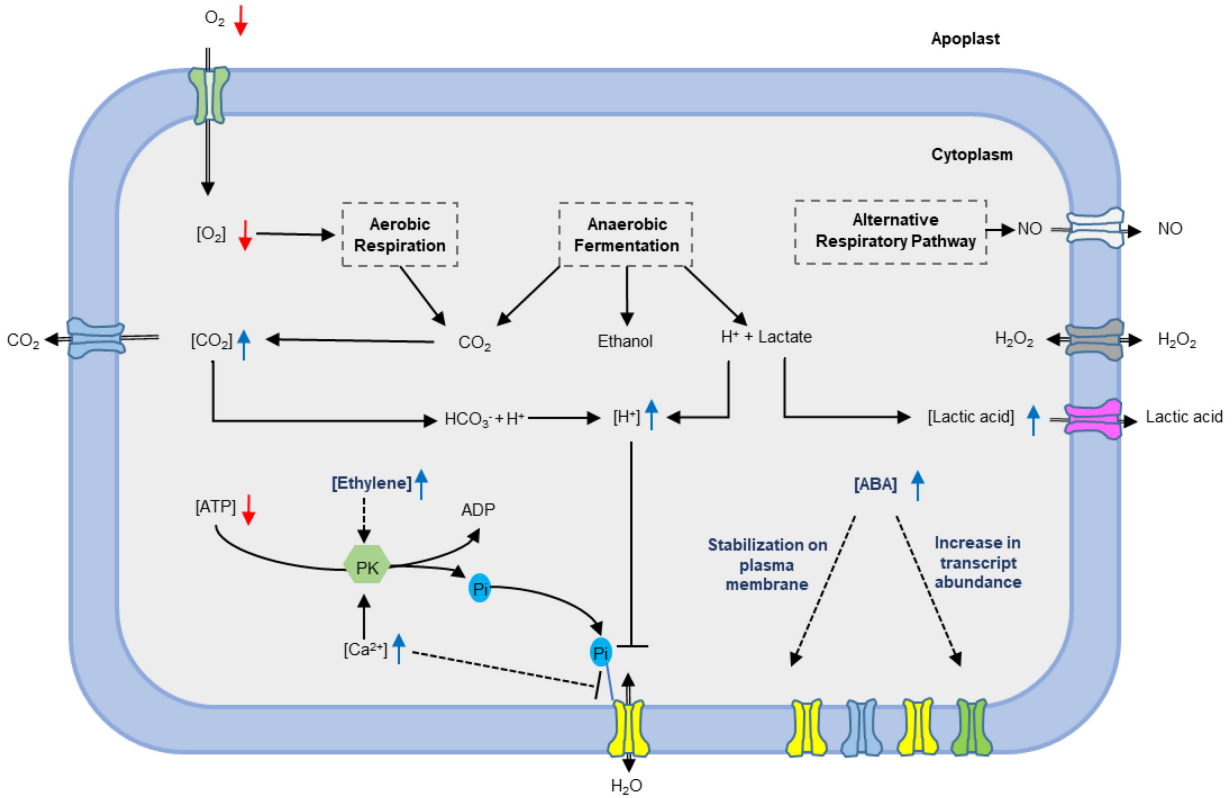

Figure 6.1 A conceptual model of the regulating mechanisms and transporting activities of plant aquaporins in oxygen deprivation conditions (Tan et al., 2018). The diagram was re-produced with the permission of authors. Under oxygen deprivation conditions, aerobic respiration and its ATP production are restricted. Anearobic fermentation and alternative respiratory pathway are triggered to meet the energy demand in cells, which

however, may lead to the accumulation of CO_2 and lactic acid, and increase proton concentration in cell. Cytosolic acidosis and decreasing cellular ATP content hamper the abundance and open conformation of aquaporins. However, some aquaporins can facilitate the diffusion of O_2, CO_2, lactic acid, NO and H_2O_2, which may contribute to the alleviation of damages to cells by low oxygen conditions. Phytohormornes, including ethylene and abscisic acid (ABA) can regulate aquaporin activities on both transcriptional and posttranscriptional levels. Phosphorylation of aquaporins by protein kinases (PKs) may also be regulated by Ca^{2+} and ethylene.

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Appendices

Appendix 1 The qPCR primers for gene transcript abundance assays

	Genes	Primers (5'-3')	
Saccharomyces cerevisiae	*Myoglobin*	Forward	TCATCCATGTTCTGCATTCT
		Reverse	GCTCGAGAGCTTTGTTCATA
	HsAQP1	Forward	CTACACTGGCTGTGGGATTAACC
		Reverse	GGTTGCTGAAGTTGTGTGTGATC
	NtPIP1;3	Forward	TGGTATGATCTTTGCCCTTGTCT
		Reverse	CAAATGTCACTGCTGGGTTAATG
	AtPIP1;2	Forward	GTCCTCTTCTTTGCCTAATGGAGAC
		Reverse	GTTGCCTGCTTGAGATAAACCCAG
	ScFBA1	Forward	GCACCCAATCTCTCCAAACTTC
		Reverse	TCTGGTCTCAAAGCGATGTCA
Nicotiana tabacum	*NtPIP1;1*	Forward	CCAATTACTGGAACTGGCATCA
		Reverse	CCATGCCTGTTTTTTGTTGAAG
	NtPIP1;2	Forward	TGCTGGTGTTGTGAAGGGATT
		Reverse	AACATTAGCACCACCACCAAGTC
	NtPIP1;3	Forward	TGGTATGATCTTTGCCCTTGTCT
		Reverse	CAAATGTCACTGCTGGGTTAATG
	NtPIP1;4	Forward	GCTGGTGTTGTGAAGGGATTC
		Reverse	AACATTAGCACCACCACCAAGTC
	NtPIP2;1	Forward	TTGGGTGCTGAGATTATTGGAA
		Reverse	ATGGGAGTCACGGGCACTT
	NtEF1-α	Forward	TGAGATGCACCACGAAGCTC
		Reverse	CCAACATTGTCACCAGGAAGTG
	NtL25	Forward	CCCCTCACCACAGAGTCTGC
		Reverse	AAGGGTGTTGTTGTCCTCAATCTT
	NtADH1	Forward	AAGCTGGAGGAATTGTGGAGAG
		Reverse	ACCAGTGAACACAGGAAGAACA
	NtPDC1	Forward	CCCGAACACCATTCACATTTC
		Reverse	CATCAGCAGATTCCACGATTTC
	NtACS	Forward	AAACGAGCCATTGCAACAAAGA
		Reverse	TGAATCCTGGTAAGCCCATGTC
Populus Tremuloides	*PtPIP1;1*	Forward	CTGCAATCATCTTCAACAAGG
		Reverse	GCTCTGATCACAACCTGTGG
	PtPIP1;2	Forward	CGAGAATCACATCTCTCAACAAC

		Reverse	ACCACAATACCAAATTCACATG
	PtPIP2;1	Forward	GTAAGAGAGATCGGATTACGATGG
		Reverse	CACGAGAAAACACAAAACTACCAC
	PtPIP2;2	Forward	ACAGTTACCAAGCCGAGAGAGAC
		Reverse	GATCCTTTCTCTGTAGTCGGTAC
	PtPIP2;3	Forward	CAACGTTATGTCATGGTTGTGTG
		Reverse	AAGACTAACACAGAGCACTTGC
	PtPIP2;4	Forward	AGAGGGTCTGGGAATCATGG
		Reverse	TGAAGTGAATTCTAAGGCAAGC
	PtPIP2;5	Forward	ATGCAAAGATTTAAGCAGTCC
		Reverse	ATACACAGGGAAACCTTATGCAG
	PtACO1	Forward	GCGATCAGATTGAGGTCCTTAG
		Reverse	GGTGGCCTTAAGTGATGGATTA
	PtACS1	Forward	GGTGGCCTTAAGTGATGGATTA
		Reverse	TCTCCGCTACGCTAATGAAATC
	PtACS2	Forward	CCCTGGACAGAGAGACACTAAA
		Reverse	CCTCTTCGACAACCTCAGAAATG
	PtERF17	Forward	CGAATACCAACATCAGGAAACAAA
		Reverse	CCAGCAGCTCCTCCAACAA
	PtERF18	Forward	CAGGATCCAAGCTCTGCCA
		Reverse	CCAGCAGCTCCTCCAACAA
	PtERF34	Forward	GCTCAACTTCCCCCCTCA
		Reverse	GTTTCAGGACCCAAGATGCAA
	PtERF35	Forward	ACCTGACCAGATGGCTGCT
		Reverse	TGACGTCGATACTGATGATGTGT
	PtERF71	Forward	AACCTCTTTAATCAGCAACCTTTTG
		Reverse	CCTTCCCATGTTCCTGTTGA
	PtERF76	Forward	CTTCTCCCGCTTCTCCATCT
		Reverse	CGAATCGCCCAGTGAACTT
	PtJip1	Forward	CTGGGAATGATCATGTGTTAATG
		Reverse	ATACACAGGGAAACCTTATGCAG
Arabidopsis thaliana	*AtPIP1;1*	Forward	CTG GCC TTG TCC TTA GTT GCT TC
		Reverse	ATT CTC CTT TGG AAC TTC TTC CTT G
	AtPIP1;2	Forward	GTC CTC TTC TTT GCC TAA TGG AGA C
		Reverse	GTT GCC TGC TTG AGA TAA ACC CAG
	AtPIP13	Forward	TCT CAG CCA CTC TCA TTA CCC ATT G
		Reverse	GGA ACT TGT TAG CTC CCA CTC GAA C
	AtPIP1;4	Forward	TCTGGGTTGGACCATTCATTG
		Reverse	GAAAGGAATCGCTCTGATGACA

AtPIP15	Forward	GTT GGT GGT GAT AGA GTG AGA GAG
	Reverse	CCG GTG GTG GTT CCT TAT AGT CC
AtPIP21	Forward	ACA AAT TAA CAA GAA GGA GAG CTC
	Reverse	ACT TCT TCT TCT TTC AAA CAA TAG C
AtPIP22	Forward	CAG AAG TGC AGC CAA CGT TTG AG
	Reverse	CAT TGG CAT TGG CAC ATA TCA AAG
AtPIP23	Forward	CAA GCC CTA CAA CAA CCA CAA AGC
	Reverse	GCG GAG GAT CTT CGT AAT CTC TCG
AtPIP24	Forward	GGA TGA TTA CAA ATG AAT TAA GTG G
	Reverse	CCA CAT TTA CAA TTA CAC GAA TGG
AtPIP25	Forward	GAT ATG CTC TTC CCT GAG TAC ATC
	Reverse	AAT ATC TCT CCT CAC CAA AGC TAG
AtPIP26	Forward	TTT CGA ACT AGC GAA GAG GTG AAG
	Reverse	AGA CAC AGT AAA TGT CAC TCA CCG
AtPIP27	Forward	GCT CCT CTT CTC GAC ATG GGT GAG
	Reverse	CTT GTG GCC GAT GAC AGT AGC GAC
AtActin28	Forward	GGT AAC ATT GTG CTC AGT GGT GG
	Reverse	AAC GAC CTT AAT CTT CAT GCT GC
AtADH1	Forward	GAATCGCTGGTGCTTCTAGG
	Reverse	CTCAGCGATCACCTGTTGAA
AtPDC1	Forward	GGTGGTCCTAAGTTGCGTGT
	Reverse	CTGCTCCCCAATAAGTTCCA

Appendix 2 Accession numbers and full-length open reading frames of the 20 aquaporin genes transformed into yeast

>HsAQP1[DQ895575][Homo sapiens]

ATGGCCAGCGAGTTCAAGAAGAAGCTCTTCTGGAGGGCAGTGGTGGCCGAGTTCCTGGCCACGACCCTC
TTTGTCTTCATCAGCATCGGTTCTGCCCTGGGCTTCAAATACCCGGTGGGGAACAACCAGACGACGGTC
CAGGACAACGTGAAGGTGTCGCTGGCCTTCGGGCTGAGCATCGCCACGCTGGCGCAGAGTGTGGGCCAC
ATCAGCGGCGCCCACCTCAACCCGGCTGTCACACTGGGGCTGCTGCTCAGCTGCCAGATCAGCATCTTC
CGTGCCCTCATGTACATCATCGCCCAGTGCGTGGGGGCCATCGTCGCCACCGCCATCCTCTCAGGCATC
ACCTCCTCCCTGACTGGGAACTCGCTTGGCCGCAATGACCTGGCTGATGGTGTGAACTCGGGCCAGGGC
CTGGGCATCGAGATCATCGGGACCCTCCAGCTGGTGCTATGCGTGCTGGCTACTACCGACCGGAGGCGC
CGTGACCTTGGTGGCTCAGCCCCCCTTGCCATCGGCCTCTCTGTAGCCCTTGGACACCTCCTGGCTATT
GACTACACTGGCTGTGGGATTAACCCTGCTCGGTCCTTTGGCTCCGCGGTGATCACACACAACTTCAGC
AACCACTGGATTTTCTGGGTGGGGCCATTCATCGGGGGAGCCCTGGCTGTACTCATCTACGACTTCATC
CTGGCCCCACGCAGCAGTGACCTCACAGACCGCGTGAAGGTGTGGACCAGCGGCCAGGTGGAGGAGTAT
GACCTGGATGCCGACGACATCAACTCCAGGGTGGAGATGAAGCCCAAATAG

>HsAQP2 [CR542024][Homo sapiens]

ATGTGGGAGCTCCGCTCCATAGCCTTCTCCAGGGCTGTGTTCGCAGAGTTCCTGGCCACACTCCTCTTC
GTCTTCTTTGGCCTCGGCTCTGCCCTCAACTGGCCACAGGCCCTGCCCTCTGTGCTACAGATTGCCATG
GCGTTTGGCTTGGGTATTGGCACCCTGGTACAGGCTCTGGGCCACATAAGCGGGGCCCACATCAACCCT
GCCGTGACTGTGGCCTGCCTGGTGGGCTGCCACGTCTCCGTTCTCCGAGCCGCCTTCTACGTGGCTGCC
CAGCTGCTGGGGGCTGTGGCCGGAGCCGCTCTGCTCCATGAGATCACGCCAGCAGACATCCGCGGGGAC
CTGGCTGTCAATGCTCTCAGCAACAGCACGACGGCTGGCCAGGCGGTGACTGTGGAGCTCTTCCTGACA
CTGCAGCTGGTGCTCTGCATCTTCGCCTCCACCGATGAGCGCCGCGGAGAGAACCCGGGCACCCCTGCT
CTCTCCATAGGCTTCTCTGTGGCCCTGGGCCACCTCCTTGGGATCCATTACACCGGCTGCTCTATGAAT
CCTGCCCGCTCCCTGGCTCCAGCTGTCGTCACTGGCAAATTTGATGACCACTGGGTCTTCTGGATCGGA
CCCCTGGTGGGCGCCATCCTGGGCTCCCTCCTCTACAACTACGTGCTGTTTCCGCCAGCCAAGAGCCTG
TCGGAGCGCCTGGCAGTGCTGAAGGGCCTGGAGCCGGACACCGATTGGGAGGAGCGCGAGGTGCGACGG
CGGCAGTCGGTGGAGCTGCACTCGCCGCAGAGCCTGCCACGGGGTACCAAGGCCTGA

>HsAQP3[CR541991][Homo sapiens]

ATGGGTCGACAGAAGGAGCTGGTGTCCCGCTGCGGGGAGATGCTCCACATCCGCTACCGGCTGCTCCGA
CAGGCGCTGGCCGAGTGCCTGGGGACCCTCATCCTGGTGATGTTTGGCTGTGGCTCCGTGGCCCAGGTT
GTGCTCAGCCGGGGCACCCACGGTGGTTTCCTCACCATCAACCTGGCCTTTGGCTTTGCTGTCACTCTG
GGCATCCTCATCGCTGGCCAGGTCTCTGGGGCCCACCTGAACCCTGCCGTGACCTTTGCCATGTGCTTC
CTGGCTCGTGAGCCCTGGATCAAGCTGCCCATCTACACCCTGGCACAGACGCTGGGAGCCTTCTTGGGT
GCTGGAATAGTTTTTGGGCTGTATTATGATGCAATCTGGCACTTCGCCGACAACCAGCTTTTTGTTTCG

GGCCCCAATGGCACAGCCGGCATCTTTGCTACCTACCCCTCTGGACACTTGGATATGATCAATGGCTTC
TTTGACCAGTTCATAGGCACAGCCTCCCTTATCGTGTGTGTGCTGGCCATTGTTGACCCCTACAACAAC
CCCGTCCCCCGAGGCCTGGAGGCCTTCACCGTGGGCCTGGTGGTCCTGGTCATTGGCACCTCCATGGGC
TTCAACTCCGGCTATGCCGTCAACCCTGCCCGGGACTTTGGCCCCCGCCTTTTTACAGCCCTTGCGGGC
TGGGGCTCTGCAGTCTTCACGACCGGCCAGCATTGGTGGTGGGTGCCCATCGTGTCCCCACTCCTGGGC
TCCATTGCGGGTGTCTTCGTGTACCAGCTGATGATCGGCTGCCACCTGGAGCAGCCCCCACCCTCCAAC
GAGGAAGAGAATGTGAAGCTGGCCCATGTGAAGCACAAGGAGCAGATCTGA

>NtPIP1;1[AF440271][Nicotiana tabacum]

ATGGCAGAAAACAAAGAAGAAGATGTGAATCTTGGAGCAAACAAATACAGAGAAACACAACCCTTAGGA
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TTATCATCTTGGTCATTTTACAGAGCTGGGATTGCAGAATTTATGGCCACTTTCTTGTTCTTGTACATT
ACAATCTTGACTGTAATGGGACTTAAAAGGTCAGATAGTTTGTGTTCTTCTGTTGGTATTCAAGGTGTT
GCTTGGGCTTTTGGTGGTATGATCTTTGCTCTTGTTTACTGTACTGCTGGTATCTCAGGAGGCCACATT
AATCCAGCTGTGACCTTTGGTCTATTCTTAGCAAGGAAACTTTCCTTAACCAGGGCAGTATTCTACATG
GTAATGCAATGCCTTGGTGCTATTTGTGGTGCTGGTGTTGTTAAAGGTTTCATGAAAGGTCCATACCAA
AGACTTGGTGGTGGTGCCAATGTGGTTAACCCTGGCTATACTAAAGGTGATGGACTTGGTGCTGAAATT
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CATGTTCCTATTTTGGCACCTCTTCCTATTGGATTTGCTGTGTTCTTGGTTCATTTGGCCACCATCCCA
ATTACTGGAACTGGCATCAACCCTGCTAGGAGTCTTGGAGCTGCTATTATCTTCAACAAAAAACAGGCA
TGGGATGACCATTGGATCTTCTGGGTTGGACCATTCATTGGAGCTGCTCTTGCTGCAGTTTACCACCAG
ATTATTATCAGAGCCATTCCATTCAAGAGCTAA

>NtPIP1;2[AF024511][Nicotiana tabacum]

ATGGCAGAAAACAAAGAAGAAGATGTTAAGCTTGGAGCTAACAAATTCAGAGAAACACAGCCATTAGGA
ACAGCTGCTCAAACAGACAAAGATTACAAAGAACCACCACCAGCTCCTTTGTTTGAACCAGGGGAATTA
TCATCATGGTCATTTTACAGAGCTGGAATTGCAGAATTTATGGCTACTTTCTTGTTTTTGTACATCACT
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TGGGCTTTTGGTGGTATGATCTTTGCTTTGGTTTACTGTACTGCTGGTATCTCAGGAGGACACATCAAC
CCAGCTGTGACCTTTGGATTGTTCTTGGCAAGGAAACTGTCCTTAACCAGGGCTATTTTCTACATAGTG
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CTTGGTGGTGGTGCTAATGTTGTTAACCATGGTTACACCAAAGGTGATGGCCTTGGTGCTGAAATTATT
GGCACTTTTGTCCTTGTTTACACTGTTTTCTCTGCTACTGATGCTAAGAGAAATGCCAGAGACTCATAT
GTTCCTATTTTGGCACCACTTCCCATCGGATTCGCGGTTTTCTTGGTTCATTTGGCCACCATTCCCATC
ACCGGAACTGGCATCAACCCCGCTAGGAGTCTTGGAGCTGCGATCATCTACAACACAGACCAGGCATGG
GACGACCACTGGATCTTTTGGGTTGGACCATTCATTGGAGCTGCACTTGCTGCAGTTTACCATCAAATA
ATCATCAGAGCCATTCCATTCCACAAGTCGTCTTAA

>NtPIP1;3[U62280][Nicotiana tabacum]

ATGGCAGAGAACAAGGAAGAGGATGTTAAGCTAGGAGCAAACAAGTACAGAGAAACACAACCTTTGGGT
ACAGCAGCTCAAACAGACAAGGATTATAAGGAGCCACCACCAGCTCCTTTGGTTTGGAGGCAGAAGAGT
TGTCGTCATGGTCTTTTTACAGAGCTGGAATTCAGCATGGCCACTTTCTTGTTCTTGTACATCACTATC
TTGACTGTTATGGGTCTTAAAAGATCTGATAGTTTGTGTTCTTCTGTTGGTATTCAAGGAGTTGCTTGG
GCTTTTGGTGGTATGATCTTTGCCCTTGTCTACTGCACTGCTGGTATCTCAGGAGGACACATTAACCCA
GCAGTGACATTTGGTCTGTTCTTGGCAAGAAAGTTGTCTTTAACAAGGGCTGTGTTCTACATGGTGATG
CAGTGCCTAGGTGCAATCTGTGGTGCTGGTGTTGTTAAAGGTTTTATGGTGGGTCCATACCAGAGACTT
GGTGGTGGGGCCAACGTGGTTAACCCTGGCTACACTAAAGGTGATGGACTTGGTGCTGAGATTATTGGC
ACCTTTGTCCTTGTTTACACTGTTTTCTCTGCCACTGATGCCAAGAGAAATGCTAGAGATTCACATGTT
CCTATTTTGGCACCTCTTCCTATTGGATTCGCGGTGTTCTTGGTTCATTTGGCCACCATCCCAATCACC
GGAACCGGTATCACCCCCGCCCGGAGCCTTGGAGCTGCTATCATCTTCAACCAAGACCGGGCATGGGAT
GATCACTGGATCTTCTGGGTTGGACCATTCATTGGAGCTGCACTTGCTGCAGTTTACCACCAGATAATC
ATCAGAGCCATTGCATTCAAGAGCTAA

>NtPIP1;4[DQ914525][Nicotiana tabacum]

ATGGCAGAAAACAAAGAAGAAGATGTTAAGCTTGGAGCTAACAAATTCAGAGAAACACAGCCATTAGGA
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TCATCATGGTCATTTTACAGAGCTGGAATTGCAGAATTTATGGCTACTTTCTTGTTTTTGTACATCACT
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TGGGCTTTTGGTGGTATGATCTTTGCTTTGGTTTACTGTACTGCTGGTATCTCAGGAGGACACATCAAC
CCAGCTGTGACCTTTGGATTGTTCTTGGCAAGGAAACTGTCCTTAACCAGGGCTATTTTCTACATAGTG
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CTTGGTGGTGGTGCTAATGTTGTTAACCATGGTTACACCAAAGGTGATGGCCTTGGTGCTGAAATTATT
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GTTCCTATTTTGGCACCACTTCCCATCGGATTCGCGGTTTTCTTGGTTCATTTGGCCACCATTCCCATC
ACCGGAACTGGCATCAACCCCGCTAGGAGTCTTGGAGCTGCGATCATCTACAACACAGACCAGGCATGG
GACGACCACTGGATCTTTTGGGTTGGACCATTCATTGGAGCTGCACTTGCTGCAGTTTACCATCAAATA
ATCATCAGAGCCATTCCATTCCACAAGTCTTCTTAA

>NtPIP2;1[AF440272][Nicotiana tabacum]

ATGTCAAAGGACGTGATTGAAGAAGGACAAGTTCATCAACAGCACGGGAAGGATTACGTGGACCCACCA
CCAGCTCCTTTGCTTGATTTTGCAGAACTCAAGCTCTGGTCTTTTCACAGAGCTCTTATTGCTGAGTTC
ATTGCTACTCTTCTTTTCCTTTACGTCACTGTTGCAACTGTAATTGGTCACAAGAAGTTGAATGGTGCT
GATAAATGTGATGGGGTTGGTATTCTTGGTATTTCTTGGGCTTTTGGTGGCATGATTTTTGTTCTTGTT
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AAAGTGTCATTGTTAAGAGCAGTGGGATATATTATTGCACAGTCATTAGGTGCAATTTGTGGTGTTGGT
TTAGTGAAAGGTTTCATGAAACATTACTACAACACATTAGGTGGTGGTGCTAATTTTGTGCAACCTGGT

TATAACAAGGGCACAGCTTTGGGTGCTGAGATTATTGGAACTTTTGTTCTTGTTTACACTGTTTTCTCT
GCTACTGACCCTAAAAGAAGTGCCCGTGACTCCCATGTCCCTGTTTTGGCCCCTCTGCCAATTGGTTTT
GCTGTTTTCATGGTTCATTTGGCTACTATTCCTATTACTGGAACTGGTATTAACCCTGCTAGGACCTTT
GGAGCTGCTGTCATTTACAACACTGAAAAAATCTGGGATGATCAATGGATTTTCTGGGTTGGACCATTT
GTGGGAGCATTGGTAGCAGCAGTATATCATCAGTATATCTTGAGAGGTTCAGCAATTAAGGCATTGGGT
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>NtXIP1;1[HM475294][Nicotiana tabacum]

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>AtPIP1;1[AT3G61430][Arabidopsis thaliana]

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>AtPIP1;2[AT2G45960][Arabidopsis thaliana]

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>AtPIP1;3[AT1G01620][Arabidopsis thaliana]

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>AtPIP1;4[AT4G00430][Arabidopsis thaliana]

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>AtPIP1;5[AT4G23400][Arabidopsis thaliana]

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>AtNIP2;1[AT2G34390][Arabidopsis thaliana]

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>LbAQP1[JQ585592][Laccaria bicolor]

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>LbAQP3[JQ585593][Laccaria bicolor]

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>LbAQP5[JQ585594][Laccaria bicolor]

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>LbAQP6[JQ585595][Laccaria bicolor]

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>LbAQP7[JQ585596][Laccaria bicolor]

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Appendix 3 Amino acid sequences of aquaporins in this study deduced by NCBI ORF Finder

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>AtPIP1;2

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>AtPIP1;3

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>AtPIP1;5

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>HsAQP1

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>HsAQP2

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>HsAQP3

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>NtPIP1;2

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>NtPIP2;1

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MASNASHVLGDEESQLSGGSNRVQPFSSTPKNRNIDDEGKKHTSLTVAQRLGISDFFSLDVWRASVGEL
LGSAVLVFMLDTIVISTFESDVKMPNLIMSILIAIVITILLLAVVPVSGGHINPVISFSAALVGIISMS
RAIIYMVAQCVGAILGALALKAVVSSTIAQTFSLGGCTITVIAPGPNGPITVGLEMAQALWLEIFCTFV
FLFASIWMAYDHRQAKALGLVTVLSIVGIVLGLLVFISTTVTMKKGYAGAGMNPARCFGAAVVRGGHLW
DGHWIFWVGPTIACVAFYVYTKIIPPKHFHADGYKYDFIGVVKASFGLHE*

>LbAQP1

MHPQVASLFDNVYEDLAAATLEFIGTAFFLLFGLGGIQASTAEDTASSQPPASGIEHVLYISTCMGFSL
VVSAWLFFRVTGGLFNPNISFALLLVGGLKPLRFVLFCIAQLTGAIAGAAIVRGLTSAPLSVNNVLQQG
TSAAQGVFIEMFITAALVLSVLMLAAEKHEATPFAPVGIGLTLFACHLFAVYYTGAAMNSARAFGPAVI
SGFPEPQHWVYWVGPFLGSLLGAGFYATLKHYKYWRLNPDQATSDYRKSPSDPVALLKSTAETFINVGD
EETRNGCASNEEGVRATGDEKSSNATSSRTNFSPV*

>LbAQP3

MSATPIIHLRDVKKRTGVLNAWERVRNKPQVHWAMECFAEALGVFFYVYFGLGSTAAWVIGNILKQSGL
SSVFQIGFAYAFGILFAIGVCAATSGGHFNPCVTIAFTIFRGFPPLKAVRYIVAQILGAYIASALVYNQ
WKVLIVESELLLKQAGVYETTMFTPNGPAGIFALYLLPGAQTLPRAFLNEFVNCFVLALVIWAALDPTS
FMIPPVMAPFIIAAAYAGSIWGYAVPAISLNSARDIGCRLFALTIWGKSAAGGSYSAIAALVNIPATLL
AAVVYELFLVDSDRVVAGSHLEFMNVAANHRRHRQQAEDDNLVEADDSSQEKPV*

>LbAQP5

MAEFVGVALLVIFGAGAACQVILSTNPGVSPSERGSFLSINFGWAIGIATGAWVSTGMSEGHINPAITI
GMATYRGFPWREVPGYIFGQVLGGFVGAALVYANYFHAIDIFEGGHRTQATASLFATFALPYMTQASCF
FSEFLATAVLFIVFLALNDKHNGALTNGLLPFALFILFIGLGASLGMQTGYAVNPARDFGPRLFLAMAG
YGKAVFNYRRQYWIWAPIIAPILGAQAGGLLYDTFIYNGDDSPIKWR*

>LbAQP6

MDDKFDDDALPNSKTTAKDYEDKLPEYDYTTTFPNTWMRLREPFREYFAEFVGVAVLIIFGVGADCQVV
LSANTGVASSPKGSYLSLNCGWAIGTAMGVWISGGISGGHINPAVTLAMATWRGFPWWKVPGFIFAQLL
GGIVGAGLVYVNYIHAIDIVEGGRHIRTLDTAGLFATYAADYMTNLSCFFSEFLATAVLIIVIHAMNDK
RNTPPPAGIVPFVLFFLILGIGASLGMETGYAINPARDLGPRMLTAMVGYGRQVFAFRNQYWIWCPVLA
PFLGAQVGTIFYDLFFYKGQDNVFGRLGSHIHISPA*

>LbAQP7

MSGQHQITEQPSGNPLSRTSTLIQEKPLTPTSSHAETQKHLEAPRQSSFLIQLQDIRHAIRMPMAEFFG
VALLIIFGAGSACQVVLSTNPNVASSDRGSFLSINLGWAIGIAMGAWVSGGISGGHINPAITIAMATYR
GFPWRRVPSYIFAQVLGGVVGAALVYANYIHAIDIFEGGRHVRTQATASLFATYALPYMTQVSCFFSEF
LATAVLSMMVLALTDNRNGAPTNGLLPFALFVLFIGLGASLGMETAYALNPARDFGPRLFLAMSGYGKA
LFNYRSQYWLWAPIIAPVLGAQAGGLLYDTFLYDGDNSPIKWRRASSQECQLAEVV*

Appendix 4 Alignment of amino acid sequences of the aquaporins in Chapter 5 by CLUSTAL O (V.1.2.1)

```
LbAQP3          ------------------------------MSATPIIH---------LRDVKKRTGVLNAW
HsAQP3          ----------------------------------------MGRQKELVSRCGEM----L
LbAQP6          -------MD------DKFDDDAL----------PNSKTTAKDYEDKLPEYDYTTTFPNTW
LbAQP5          ------------------------------------------------------------
LbAQP7          -------MSGQHQITEQPSGNPLSRTSTLIQEKPLTPTSSHAETQKHLEAPRQSSFLIQL
AtNIP2;1        --------MDDISVSKSNHGNVVVLNI---KASSL-ADTSLPSNKHE----SSS------
NtXIP1;1        MASNASHVLGDEE-SQLSGGSNRVQPF---SST-P-KNRNIDDEGKK----HTSLTVAQR
LbAQP1          ------------------------------------------------MHPQVASL
NtPIP2;1        ----------------------MSKDV-----I-E-EGQVHQQHGKD----YVDPPPAPL
AtPIP1;5        --------MEGKE-EDVNVGANKFPER-----Q-P-IGTAAQTESKD----YKEPPPAPF
AtPIP1;4        --------MEGKE-EDVRVGANKFPER-----Q-P-IGTSAQSTDKD----YKEPPPAPL
AtPIP1;3        --------MEGKE-EDVRVGANKFPER-----Q-P-IGTSAQ-TDKD----YKEPPPAPF
AtPIP1;1        --------MEGKE-EDVRVGANKFPER-----Q-P-IGTSAQ-SDKD----YKEPPPAPF
AtPIP1;2        --------MEGKE-EDVRVGANKFPER-----Q-P-IGTSAQ-SDKD----YKEPPPAPL
NtPIP1;3        -------MAENKE-EDVKLGANKYRET-----Q-P-LGTAAQT-DKD----YKEPPPAPL
NtPIP1;1        -------MAENKE-EDVNLGANKYRET-----Q-P-LGTAAQTENKD----YIEPPPAPL
NtPIP1;2        -------MAENKE-EDVKLGANKFRET-----Q-P-LGTAAQT-DKD----YKEPPPAPL
NtPIP1;4        -------MAENKE-EDVKLGANKFRET-----Q-P-LGTAAQT-DKD----YKEPPPAPL
HsAQP1          ------------------------------------------------------------
HsAQP2          ------------------------------------------------------------

LbAQP3          ERVRNKPQVHWAMECFAEALGVFFYVYFGLGSTAAWVIGNI--LKQ--SGLSSVFQIGFA
HsAQP3          HIRYR-----LLRQALAECLGTLILVMFGCGSVAQVVLSRG--------THGGFLTINLA
LbAQP6          MRLRE-----PFREYFAEFVGVAVLIIFGVGADCQVVLSANTGVAS--SPKGSYLSLNCG
LbAQP5          ---------------MAEFVGVALLVIFGAGAACQVILSTNPGVSP--SERGSFLSINFG
LbAQP7          QDIRH-----AIRMPMAEFFGVALLIIFGAGSACQVVLSTNPNVAS--SDRGSFLSINLG
AtNIP2;1        ---PPLLSVHFLQKLLAELVGTYYLIFAGCAAIAVNAQHNH---------VVTLVGIAVV
NtXIP1;1        LGISDFFSLDVWRASVGELLGSAVLVFMLDTIVISTFESDV---------KMPNLIMSIL
LbAQP1          FDN----VYEDLAAATLEFIGTAFFLLFGLGGIQASTAEDTASSQPPASGIEHVLYISTC
NtPIP2;1        LDFAELKLWSFHRALIAEFIATLLFLYVTVATVIGHKKLNG-ADKCD---GVGILGISWA
AtPIP1;5        FEPGELKSWSFYRAGIAEFIATFLFLYVTVLTVMGVKRA---PNMCA---SVGIQGIAWA
AtPIP1;4        FEPGELSSWSFYRAGIAEFIATFLFLYITVLTVMGVKRA---PNMCA---SVGIQGIAWA
AtPIP1;3        FEPGELSSWSFYRAGIAEFIATFLFLYITVLTVMGVKRA---PNMCA---SVGIQGIAWA
AtPIP1;1        FEPGELSSWSFWRAGIAEFIATFLFLYITVLTVMGVKRS---PNMCA---SVGIQGIAWA
AtPIP1;2        FEPGELASWSFWRAGIAEFIATFLFLYITVLTVMGVKRS---PNMCA---SVGIQGIAWA
NtPIP1;3        VWRQKSCRHGLF-TELEFSMATFLFLYITILTVMGLKRS---DSLCS---SVGIQGVAWA
NtPIP1;1        FEPGELSSWSFYRAGIAEFMATFLFLYITILTVMGLKRS---DSLCS---SVGIQGVAWA
```

```
NtPIP1;2        FEPGELSSWSFYRAGIAEFMATFLFLYITILTVMGLKRS---DSLCS---SVGIQGVAWA
NtPIP1;4        FEPGELSSWSFYRAGIAEFMATFLFLYITILTVMGLKRS---DSLCS---SVGIQGVAWA
HsAQP1          -MASEFKKKLFWRAVVAEFLATTLFVFISIGSALGFKYPVG-NNQTT---VQDNVKVSLA
HsAQP2        --MWELRSIAFSRAVFAEFLATLLFVFFGLGSALNWPQA-----------LPSVLQIAMA
                                ..    :                              :

LbAQP3          YAFGILFAIGVCAATSGGHFNPCVTIAFTIFRGFPPLKAVRYIVAQILGAYIASALVYNQ
HsAQP3          FGFAVTLGILIAGQVSGAHLNPAVTFAMCFLAREPWIKLPIYTLAQTLGAFLGAGIVFGL
LbAQP6          WAIGTAMGVWISGGISGGHINPAVTLAMATWRGFPWWKVPGFIFAQLLGGIVGAGLVYVN
LbAQP5          WAIGIATGAWVSTGMSEGHINPAITIGMATYRGFPWREVPGYIFGQVLGGFVGAALVYAN
LbAQP7          WAIGIAMGAWVSGGISGGHINPAITIAMATYRGFPWRRVPSYIFAQVLGGVVGAALVYAN
AtNIP2;1        WGIVIMVLVYCLGHLS-AHFNPAVTLALASSQRFPLNQVPAYITVQVIGSTLASATLRLL
NtXIP1;1        IAIVITILLLAVVPVSGGHINPVISFSAALVGIISMSRAIIYMVAQCVGAILGALALKAV
LbAQP1          MGFSLVVSAWLFFRVTGGLFNPNISFALLLVGGLKPLRFVLFCIAQLTGAIAGAAIVRGL
NtPIP2;1        FGGMIFVLVYCTAGISGGHINPAVTFGLFLARKVSLLRAVGYIIAQSLGAICGVGLVKGF
AtPIP1;5        FGGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRALFYIVMQCLGAICGAGVVKGF
AtPIP1;4        FGGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRAVFYMIMQCLGAICGAGVVKGF
AtPIP1;3        FGGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRAVFYIVMQCLGAICGAGVVKGF
AtPIP1;1        FGGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRALYYIVMQCLGAICGAGVVKGF
AtPIP1;2        FGGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRAVYYIVMQCLGAICGAGVVKGF
NtPIP1;3        FGGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRAVFYMVMQCLGAICGAGVVKGF
NtPIP1;1        FGGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRAVFYMVMQCLGAICGAGVVKGF
NtPIP1;2        FGGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRAIFYIVMQCLGAICGAGVVKGF
NtPIP1;4        FGGMIFALVYCTAGISGGHINPAVTFGLFLARKLSLTRAIFYIVMQCLGAICGAGVVKGF
HsAQP1          FGLSIATLAQSVGHISGAHLNPAVTLGLLLSCQISIFRALMYIIAQCVGAIVATAILSGI
HsAQP2        FGLGIGTLVQALGHISGAHINPAVTVACLVGCHVSVLRAAFYVAAQLLGAVAGAALLHEI
               .             : . :** ::..          .   :   *  *.  .   :

LbAQP3          WKVLIVESELLLKQAGVYETTMFTPNGPAGIFALYLLPGAQTLPRAFLNEFVNCFVLALV
HsAQP3          YYDAIWHFAD-------NQLFVSGPNGTAGIFATYPSGHLD-MINGFFDQFIGTASLIVC
LbAQP6          YIHAIDIVEG-------GRHI--RTLDTAGLFATYAADYMT-NLSCFFSEFLATAVLIIV
LbAQP5          YFHAIDIFEG-------GH----RTQATASLFATFALPYMT-QASCFFSEFLATAVLFIV
LbAQP7          YIHAIDIFEG-------GRHV--RTQATASLFATYALPYMT-QVSCFFSEFLATAVLSMM
AtNIP2;1        FDLNND---------VCS--------KKHDVFLG-SSPSG-SDLQAFVMEFIITGFLMLV
NtXIP1;1        VSSTIAQTFSL---GGCTITV--IAPGPNGP----ITVGL-EMAQALWLEIFCTFVFLFA
LbAQP1          TSAPLS----V-----------------NNV----LQQGT-SAAQGVFIEMFITAALVLS
NtPIP2;1        MKHYYN----T---LGGG----------ANF----VQPGY-NKGTALGAEIIGTFVLVYT
AtPIP1;5        QPGLYQ----T---NGGG----------ANV----VAHGY-TKGSGLGAEIVGTFVLVYT
AtPIP1;4        QPTPYQ----T---LGGG----------ANT----VAHGY-TKGSGLGAEIIGTFVLVYT
AtPIP1;3        QPNPYQ----T---LGGG----------ANT----VAHGY-TKGSGLGAEIIGTFVLVYT
AtPIP1;1        QPKQYQ----A---LGGG----------ANT----VAHGY-TKGSGLGAEIIGTFVLVYT
```

```
AtPIP1;2        QPKQYQ----A---LGGG----------ANT----IAHGY-TKGSGLGAEIIGTFVLVYT
NtPIP1;3        MVGPYQ----R---LGGG----------ANV----VNPGY-TKGDGLGAEIIGTFVLVYT
NtPIP1;1        MKGPYQ----R---LGGG----------ANV----VNPGY-TKGDGLGAEIIGTFVLVYT
NtPIP1;2        MVGPYQ----R---LGGG----------ANV----VNHGY-TKGDGLGAEIIGTFVLVYT
NtPIP1;4        MVGPYQ----R---LGGG----------ANV----VNHGY-TKGDGLGAEIIGTFVLVYT
HsAQP1          TSSLTG--------NSLG----------RND----LADGV-NSGQGLGIEIIGTLQLVLC
HsAQP2        TPADIR--------GDLA----------VNA----LSNST-TAGQAVTVELFLTLQLVLC
                                                          .  ::.    :

LbAQP3          IWAALDPTSFMIPP----------VMAPFIIAAAY-AGS--IWGYAVPAISLNSARDIG
HsAQP3          VLAIVDPYNNPVPR----------GLEAFTVGLVV-LVIGTSMGF-NSGYAVNPARDFG
LbAQP6          IHAMNDKRNTPPPA----------GIVPFVLFFLI-LGIGASLGM-ETGYAINPARDLG
LbAQP5          FLALNDKHNGALTN----------GLLPFALFILF-IGLGASLGM-QTGYAVNPARDFG
LbAQP7          VLALTDNRNGAPTN----------GLLPFALFVLF-IGLGASLGM-ETAYALNPARDFG
AtNIP2;1        VCAVTTTKRTTE------------ELEGLIIGATVTLNVI--FAGEVSGASMNPARSIG
NtXIP1;1        SIWMAYDHRQAKALGLV-----TVLSIVGIVLGLLVFISTTVTMKKGYAGAGMNPARCFG
LbAQP1          VLMLAAEKHEAT------------PFAPVGIGLTLFACHL--FAVYYTGAAMNSARAFG
NtPIP2;1        VFSATDPKRSARDSHVP--------VLAPLPIGFAVFMVHL--ATIPITGTGINPARTFG
AtPIP1;5        VFSATDAKRSARDSHVP--------ILAPLPIGFAVFLVHL--ATIPITGTGINPARSLG
AtPIP1;4        VFSATDAKRSARDSHVPVWTPLLVPILAPLPIGFAVFLVHL--ATIPITGTGINPARSLG
AtPIP1;3        VFSATDAKRSARDSHVP--------ILAPLPIGFAVFLVHL--ATIPITGTGINPARSLG
AtPIP1;1        VFSATDAKRNARDSHVP--------ILAPLPIGFAVFLVHL--ATIPITGTGINPARSLG
AtPIP1;2        VFSATDAKRNARDSHVP--------ILAPLPIGFAVFLVHL--ATIPITGTGINPARSLG
NtPIP1;3        VFSATDAKRNARDSHVP--------ILAPLPIGFAVFLVHL--ATIPITGTGITPARSLG
NtPIP1;1        VFSATDAKRNARDSHVP--------ILAPLPIGFAVFLVHL--ATIPITGTGINPARSLG
NtPIP1;2        VFSATDAKRNARDSYVP--------ILAPLPIGFAVFLVHL--ATIPITGTGINPARSLG
NtPIP1;4        VFSATDAKRNARDSHVP--------ILAPLPIGFAVFLVHL--ATIPITGTGINPARSLG
HsAQP1          VLATTDRRRRDLG------------GSAPLAIGLSVALGHL--LAIDYTGCGINPARSFG
HsAQP2        IFASTDERRGENP------------GTPALSIGFSVALGHL--LGIHYTGCSMNPARSLA
                                          . :                . .:. ** :.

LbAQP3          CRLFA-LTIWGKSAAGGS--YSA-IAALVNIPATLLAAVVYELFLVDSDRVVAGSHL-EF
HsAQP3          PRLFTALAGWGSAVFTTG-QHWWWVPIVSPLLGSIAGVFVYQLMIGCHLEQPPPSNE-EE
LbAQP6          PRMLTAMVGYGRQVFAFRNQYWIWCPVLAPFLGAQVGTIFYDLFFYKGQDNVFGRLG-SH
LbAQP5          PRLFLAMAGYGKAVFNYRRQYWIWAPIIAPILGAQAGGLLYDTFIYNGDDSPIKWR*---
LbAQP7          PRLFLAMSGYGKALFNYRSQYWLWAPIIAPVLGAQAGGLLYDTFLYDGDNSPIKWRR-AS
AtNIP2;1        PALVW-------GCY-----KGIWIYLLAPTLGAVSGALIHKMLPSIQNAE--PEFS--K
NtXIP1;1        AAVVRGG-----HLW-----DGHWIFWVGPTIACVAFYVYTK-IIPPKHFH-ADGYKYDF
LbAQP1          PAVISGF------PE-----PQHWVYWVGPFLGSLLGAGFYATLKH---------YKYWR
NtPIP2;1        AAVIYNTE----KIW-----DDQWIFWVGPFVGALVAAVYHQYILRGSAIKALGSFR---
AtPIP1;5        AAIIYNKD----HAW-----DDHWIFWVGPFIGAALAALYHQIVIRAIPFKSKT------
```

```
AtPIP1;4        AAIIYNKD----HSW-----DDHWIFWVGPFIGAALAALYHQIVIRAIPFKSKS------
AtPIP1;3        AAIIYNKD----HAW-----DDHWIFWVGPFIGAALAALYHQLVIRAIPFKSRS------
AtPIP1;1        AAIIYNKD----HSW-----DDHWVFWVGPFIGAALAALYHVVVIRAIPFKSRS------
AtPIP1;2        AAIIFNKD----NAW-----DDHWVFWVGPFIGAALAALYHVIVIRAIPFKSRS------
NtPIP1;3        AAIIFNQD----RAW-----DDHWIFWVGPFIGAALAAVYHQIIIRAIAFKS*-------
NtPIP1;1        AAIIFNKK----QAW-----DDHWIFWVGPFIGAALAAVYHQIIIRAIPFKS*-------
NtPIP1;2        AAIIYNTD----QAW-----DDHWIFWVGPFIGAALAAVYHQIIIRAIPFHKSS*-----
NtPIP1;4        AAIIYNTD----QAW-----DDHWIFWVGPFIGAALAAVYHQIIIRAIPFHKSS*-----
HsAQP1          SAVIT-------HNF-----SNHWIFWVGPFIGGALAVLIYDFILAPRSSDLTDRVKVWT
HsAQP2        PAVVT-------GKF-----DDHWVFWIGPLVGAILGSLLYNYVLFPPAKSLSERLAVLK
                :.                      :    .          .


LbAQP3          -MNVAANHRRHRQQ---------AEDDNLVEADDSSQE----------------------
HsAQP3          --NVKLAHVKHKEQ---------I*-----------------------------------
LbAQP6          IHISPA*------------------------------------------------------
LbAQP5          ------------------------------------------------------------
LbAQP7          SQECQLAEVV*--------------------------------------------------
AtNIP2;1        TGS------SHKRVTDLPL------------------------------------------
NtXIP1;1        IGVVKASFGLHE*------------------------------------------------
LbAQP1          LNPDQATSDYRKSP-SDPVALLKSTAETFINVGDEETRNGCASNEEGVRATGDEKSSNAT
NtPIP2;1        SNP---TN-*---------------------------------------------------
AtPIP1;5        ------------------------------------------------------------
AtPIP1;4        ------------------------------------------------------------
AtPIP1;3        ------------------------------------------------------------
AtPIP1;1        ------------------------------------------------------------
AtPIP1;2        ------------------------------------------------------------
NtPIP1;3        ------------------------------------------------------------
NtPIP1;1        ------------------------------------------------------------
NtPIP1;2        ------------------------------------------------------------
NtPIP1;4        ------------------------------------------------------------
HsAQP1          SGQVEEYD-L--------------------DADDINSRVEMK--PK*-------------
HsAQP2        GLE-PDTD-W--------------------EEREVRRRQSVELHSPQSLPRGTK------

LbAQP3        ------KPV*
HsAQP3        ----------
LbAQP6        ----------
LbAQP5        ----------
LbAQP7        ----------
AtNIP2;1      ----------
NtXIP1;1      ----------
```

```
LbAQP1          SSRTNFSPV*
NtPIP2;1        ----------
AtPIP1;5        ----------
AtPIP1;4        ----------
AtPIP1;3        ----------
AtPIP1;1        ----------
AtPIP1;2        ----------
NtPIP1;3        ----------
NtPIP1;1        ----------
NtPIP1;2        ----------
NtPIP1;4        ----------
HsAQP1          ----------
HsAQP2          ---A*
```

Appendix 5 Alignment of amino acid sequences of six O_2-transporting aquaporins in Chapter 5 by CLUSTAL O (V.1.2.1)

NPA motifs and Ar/R residues are highlighted in orange and blue, respectively.

```
NtXIP1;1        MASNASHVLGDEESQLSGGSNRVQPFSSTPKNRNIDDEGKKHTSLTVAQRLGISDFFSLD
NtPIP2;1        -----------------MSKDVI------EEGQVHQQHGKDYVDPPPAPLLDFAELKLWS
NtPIP1;3        MAE-------NKEEDVKLGANKYRE---TQPLGTAAQTDKDYKEPPPAPLVWRQKSCRHG
NtPIP1;4        MAE-------NKEEDVKLGANKFRE---TQPLGTAAQTDKDYKEPPPAPLFEPGELSSWS
HsAQP1          ------------------------------------------------MASEFKKKL
HsAQP2       ----------------------------------------------------MWELRSIA
                                    .

NtXIP1;1        VWRASVGELLGSAVLVFMLDTIVISTFES--D--V-KMPNLIMSILIAIVITILLLAVVP
NtPIP2;1        FHRALIAEFIATLLFLYVTVATVIGHKKLNGADKCDGVGILGISWAFGGMIFVLVYCTAG
NtPIP1;3        LF-TELEFSMATFLFLYITILTVMGLKRS--DSLCSSVGIQGVAWAFGGMIFALVYCTAG
NtPIP1;4        FYRAGIAEFMATFLFLYITILTVMGLKRS--DSLCSSVGIQGVAWAFGGMIFALVYCTAG
HsAQP1          FWRAVVAEFLATTLFVFISIGSALGFKYPVGNNQTTVQDNVKVSLAFGLSIATLAQSVGH
HsAQP2       FSRAVFAEFLATLLFVFFGLGSALNWPQA-------LPSVLQIAMAFGLGIGTLVQALGH
                       .  : .   :.: ::::.    .:              ::  :.  *  *  .

NtXIP1;1        VSGGHINPVISFSAALVGIISMSRAIIYMVAQCVGAILGALALKAVVSSTIAQTFSLGGC
NtPIP2;1        ISGGHINPAVTFGLFLARKVSLLRAVGYIIAQSLGAICGVGLVKGFMKHYYN----TLGG
NtPIP1;3        ISGGHINPAVTFGLFLARKLSLTRAVFYMVMQCLGAICGAGVVKGFMVGPYQ----RLGG
NtPIP1;4        ISGGHINPAVTFGLFLARKLSLTRAIFYIVMQCLGAICGAGVVKGFMVGPYQ----RLGG
HsAQP1          ISGAHLNPAVTLGLLLSCQISIFRALMYIIAQCVGAIVATAILSGITSSLT-----GNSL
HsAQP2       ISGAHINPAVTVACLVGCHVSVLRAAFYVAAQLLGAVAGAALLHEITPADI-----RGDL
                      :**.*:**.::..  :   :*: **  *:  * :**: ..  :  .

NtXIP1;1        TITVIAPGPNGPITVGLEMAQALWLEIFCTFVFLFASIWMAYDHRQAKALGLVTVLSIVG
NtPIP2;1        GANFVQP--------GYNKGTALGAEIIGTFVLVYTVFS-ATDPKRSARDSHVPVL--AP
NtPIP1;3        GANVVNP--------GYTKGDGLGAEIIGTFVLVYTVFS-ATDAKRNARDSHVPIL--AP
NtPIP1;4        GANVVNH--------GYTKGDGLGAEIIGTFVLVYTVFS-ATDAKRNARDSHVPIL--AP
HsAQP1          GRNDLAD--------GVNSGQGLGIEIIGTLQLVLCVLA-TTDRRRRD----LGGS--AP
HsAQP2       AVNALSN--------STTAGQAVTVELFLTLQLVLCIFA-STDERRGE----NPGT--PA
                      . :         .   . .:  *:: *: ::   :  : * ::

NtXIP1;1        IVLGLLVFISTTVTMKKGYAGAGMNPARCFGAAVVR-GGHLWDGHWIFWVGPTIACVAFY
NtPIP2;1        LPIGFAVFMVHL--ATIPITGTGINPARTFGAAVIYNTEKIWDDQWIFWVGPFVGALVAA
NtPIP1;3        LPIGFAVFLVHL--ATIPITGTGITPARSLGAAIIFNQDRAWDDHWIFWVGPFIGAALAA
```

```
NtPIP1;4        LPIGFAVFLVHL--ATIPITGTGINPARSLGAAIIYNTDQAWDDHWIFWVGPFIGAALAA
HsAQP1          LAIGLSVALGHL--LAIDYTGCGINPARSFGSAVIT---HNFSNHWIFWVGPFIGGALAV
HsAQP2       LSIGFSVALGHL--LGIHYTGCSMNPARSLAPAVVT---GKFDDHWVFWIGPLVGAILGS
           : :*: * :         :* .:.*** :. *::     :. :*:**:** :.

NtXIP1;1           VYTK-IIPPKHFHADGYKYDFIGVVKASFGLHE*-----------------------
NtPIP2;1           VYHQYILRGSAIKALGSFRSN---P---------------------TN*--------
NtPIP1;3           VYHQIIIRAIAFKS*-----------------------------------------
NtPIP1;4     VYHQIIIRAIPFHKSS*----------------------------------------
HsAQP1       LIYDFILAPRSSDLTDRVKVWTSGQVEEYDLDADDINSRVEMK--PK*---------
HsAQP2       LLYNYVLFPPAKSLSERLAVLKGLE-PDTDWEEREVRRRQSVELHSPQSLPRGTKA*
           :  . ::
```