Role of Aquaporins in *Brassica napus* Responses to Root Hypoxia and Re-aeration

by

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Abstract

Root oxygen deprivation (root hypoxia) in flooding-prone areas is detrimental to most terrestrial plants and results in growth reductions and mortality. Although plants widely vary in their waterlogging tolerance, our understanding of these variations is limited to the ability of some plants to improve root aeration through morphological and structural features. My thesis research focused on the importance of aquaporins in plant waterlogging tolerance. Aquaporins play multifunctional roles in plant stress responses including root hypoxia. However, their role in gas and water transport as well as their regulation mechanisms during and following hypoxic events have been rarely addressed. In this thesis, I studied the responses of canola (*Brassica napus* cv Westar) plants to root hypoxia and re-aeration to determine the role of aquaporins in these processes. To reveal the significance of oxygen transport through aquaporins, *NtPIP1*;*3* from tobacco (*Nicotiana tabacum*) was overexpressed in canola and plant responses to hypoxia were examined.

In Chapter 2, I examined the effects of root hypoxia (waterlogging) on canola (*Brassica napus*) plants at the seedling, flowering and podding growth stages. The plants were waterlogged for up to eight days and their growth, gas exchange, leaf water potentials, and root hydraulic conductance (K_r) were examined. In addition, relative contributions of the aquaporin-mediated and apoplastic root water transport, gene expression levels of the plasma membrane intrinsic proteins (*BnPIP*s), as well as the lignin and suberin deposition in roots were detected. Waterlogging decreased dry weights, gas exchange, leaf water potentials K_r and the effect was greater in plants at the seedling stage compared with other growth stages. Waterlogging

accelerated root suberization and lignification resulting in an increased contribution of aquaporin-mediated water transport. The transcript levels of *BnPIP*s in roots increased with the increasing waterlogging duration. The study demonstrated that maintaining functional aquaporins is critical to the survival of waterlogged plants.

In the second study (Chapter 3), hydroponically-grown canola plants were exposed to three days of root hypoxia (waterlogging) followed by re-aeration. Hypoxia impacted gas exchange and plant water relations. However, the most severe reduction of L_{pr} was on one day following re-aeration and it was accompanied by an increase in apoplastic transport. After one day of re-aeration, a sharp increase of root reactive oxygen species was measured together with a decrease in transcript abundance of most of the *BnPIP2*s in roots and leaves. A water permeability assay of these *PIP2*s overexpressed in yeast confirmed that they are fast water transporters. The yeast H_2O_2 survival assay demonstrated that *Bn*PIP1;2 and *Bn*PIP1;3 facilitate H_2O_2 transport and, therefore, the upregulations of these aquaporins likely contributed to plant waterlogging recovery. A gradual recovery of L_{pr} following re-aeration was accompanied by upregulations of *BnPIP*s in roots and leaves and the activation of antioxidant enzymes in roots. Net photosynthesis, transpiration rates, and shoot water contents remained depressed one day after re-aeration but recovered over time. Collectively, the results indicate that oxidative burst had a decisive impact on modulating the hydraulic recovery of plants upon re-aeration.

To determine the significance of oxygen-transporting aquaporins in waterlogging tolerance, in the third study (Chapter 4), I overexpressed *NtPIP1;3* aquaporin from tobacco (*Nicotiana tabacum*) in canola (*Brassica napus* cv Westar) plants and examined its effects on growth, physiological parameters, and energy metabolism in plants subjected to three and eight days of

root hypoxia. The overexpression of *NtPIP1*;*3* did not affect physiological parameters in well-aerated plants, but maintained greater dry biomass and higher gas exchange rates, root hydraulic conductivity, leaf water potentials, root respiration rates, as well as root ATP concentrations under root hypoxia stress. Moreover, metabolic profiling revealed that overexpression of *NtPIP1*;*3* enabled plants to respond to a decreased oxygen environment by altering the glycolysis, pyruvate metabolism, and TCA cycle in roots. The results collectively demonstrate that the overexpression of *NtPIP1*;*3* improved the waterlogging tolerance of canola plants by increasing root oxygen availability, which impacted the energy metabolism, improved the plant growth and physiological performance. The results provide direct evidence for the functional importance of aquaporin-mediated oxygen transport in plants.

Preface

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

Chapter 2 of this thesis has been published as "Liu M, Tan X, Sun X, Zwiazek JJ. (2020) Properties of root water transport in canola (*Brassica napus*) subjected to waterlogging at the seedling, flowering and podding growth stages. Plant Soil 454: 431-45. Xiangfeng Tan and I designed the experiments with the guidance of Dr. Janusz Zwiazek. I performed the experiments with the guidance of Dr. Zwiazek and with the help of Dr. Xiangfeng Tan and Xuehui Sun.

Chapter 3 and 4 are intended for publication. I designed all the experiments with the guidance of Dr. Janusz Zwiazek. The data collection and analysis were completed by myself with help of Qiang Li and Xuehui Sun. The metabolic analysis in Chapter 4 was done with the help from Dr. Shanjida Khan.

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Chapter 1. Introduction

1.1 General introduction

Root oxygen supply is one of the major determinants of plant survival and growth. Soil oxygen concentration is typically lower compared with the atmosphere (Bailey-Serres et al. 2012) and can be further reduced by factors such as excessive soil moisture and soil compaction. Periodic flooding events induce hypoxia stress to plants in natural ecosystems and agricultural areas causing substantial crop losses in many parts of the world (Bailey-Serres and Voesenek 2008). As the frequency of flooding and other environmental disasters have increased due to climatic changes, oxygen deprivation stress to plants has also become more common (Garssen et al. 2015). Rapid plant recovery following waterlogging is important for subsequent plant survival. When soil oxygen supply to plants increases, physiological processes are gradually restored and plant growth resumes. The recovery of plants from waterlogging and especially the processes of root water transport following waterlogging have received relatively little attention. Thus, it is imperative to develop better understanding of the mechanisms of plant responses to oxygen deprivation and their recovery following re-aeration of roots.

Since oxygen is a final electron acceptor in the respiratory electron transport chain, hypoxia impairs the mitochondrial respiration processes and lowers the plant energy status. Insufficient ATP has a negative impact on plant growth and yield, through the impairment of physiological processes such as photosynthesis, respiration, and water transport. However, some plants have developed various adaptations at different developmental stages to cope with low oxygen availability including modifications of cell walls and aerenchyma formation (Que et al. 2018). In

addition, plants accumulate excessive reactive oxygen species (ROS) during the re-aeration process, which may potentially affect the recovery of plants from hypoxia (Yeung et al. 2019).

Hypoxia may alter root water transport properties in sensitive plants (Tan et al. 2018). An inhibition of root hydraulic conductivity by hypoxia was attributed to the effects on root aquaporins (Tan and Zwiazek 2019). Oxygen deficiency results in cellular acidosis, which has an impact on the aquaporin protein conformation (Tournaire-Roux et al. 2003). During the re-aeration process, the overproduction of ROS has been reported to impose additional stress on plants and result in lipid peroxidation (Yeung et al. 2019), suggesting that the ROS can affect membrane function, including its water transport properties. Previous studies also demonstrated that aquaporin expression patterns were affected during the re-aeration process following hypoxia (Yeung et al. 2019).

Since the tobacco *Nt*PIP1;3 aquaporin was shown to be involved in the oxygen transport when expressed in yeast (Zwiazek et al. 2017), it can be hypothesized that some of the aquaporins may play a role in plants during oxygen deprivation and re-aeration. Therefore, overexpression of the *NtPIP1*;*3* gene in waterlogging-sensitive plants such as canola (*Brassica napus*), could facilitate oxygen transport to the root cells and improve plant hypoxia tolerance. Studies reported in this thesis were carried out to reveal the mechanisms of responses to oxygen deprivation and during hypoxia recovery in canola plants. The main objectives of this research were to: (1) investigate the responses of water relations to root oxygen deprivation and re-aeration; (2) examine the aquaporin expression patterns under oxygen deprivation and re-aeration process in canola; (3) generate overexpression lines of *NtPIP1*;*3 Brassica napus* and examine its hypoxia tolerance. Therefore, the root water transport properties and aquaporin expression patterns were

investigated at different stages of canola following waterlogging stress in Chapter 2. The responses of root water relations to the re-aeration following root hypoxia were studied in Chapter 3. In Chapter 4, the physiological traits were compared between the *NtPIP1*;*3* overexpression lines of canola and the wild-type plants.

1.2 Oxygen deficiency (hypoxia and anoxia) in plants

Oxygen deprivation is generally associated with hypoxia (low oxygen) or anoxia (no oxygen) conditions. For plants, this problem typically arises during flooding because of the 10^4-fold slower diffusion of gases dissolved in water compared with the air (Bailey-Serres and Voesenek 2008). Flooding is a serious concern in agriculture and forestry since global climate change is predicted to increase flooding events (Bailey-Serres et al. 2012). In contrast to animals, plants do not possess an active oxygen distribution system but depend on the diffusion of gas in their tissues to support respiration (Armstrong and Armstrong 2005). Most plants poorly tolerate flooding, and even flooding-tolerant plants cannot tolerate persistent flooding for a long term since the amount of oxygen dissolved in water is too low to support their energy demands (Bailey-Serres and Voesenek 2008). In addition, the timing of oxygen deficiency is important. Plants at different developmental stages have different levels of hypoxia tolerance and the juvenile seedlings are more oxygen-demanding than mature plants, which have higher carbohydrate reserves and lower growth respiration demands. Some plants have also evolved a wide variety of morphological (e.g. adventitious roots and aerenchyma), physiological, and metabolic adaptations at different developmental stages allowing them to survive root oxygen deprivation (Bailey-Serres and Voesenek 2008).

A sufficient supply of oxygen is essential for plant survival and growth. Mitochondrial electron transport that is linked to oxidative phosphorylation consumes the largest proportion of oxygen supplied to the tissues. This process provides ATP for cellular metabolism and regenerates NAD^+ from NADH. Oxygen is the final electron acceptor in the mitochondrial electron transport chain. When oxygen is depleted, ATP production via oxidative phosphorylation in the mitochondria is highly limited and plant cells can only rely on the small amount of ATP that is produced through glycolysis (Ricard et al. 1994). Aerobic respiration is an energy pump of the whole plant, especially in organs and tissues with a very high metabolic activity. Therefore, waterlogged roots are susceptible to a decrease of oxygen in the soil (Whipple 2012).

1.3 Physiological responses of plants under oxygen deficiency

Since plants depend on a steady supply of oxygen to all tissues and cells, any disruption of oxygen availability causes disturbance of plant metabolism (Bailey-Serres and Voesenek 2008). Depending on the level of plant tolerance, soil oxygen depletion can cause disruption of the cellular metabolism, eventually leading to reduced plant growth and mortality. The physiological responses of plants to oxygen deficiency vary between the plant species, developmental stage, type of oxygen deficiency (waterlogging, soil compaction, submergence), as well as the oxygen concentration and the and duration of plant exposure.

Different plant species possess different strategies to cope with oxygen deficiency stress. Terrestrial plants originated from the aquatic ancestors and flooding-tolerant plants regained their aquatic adaption (Voesenek and Pierik 2008; Jackson et al. 2009). Highly adapted plant species survive waterlogging up to several months without any injuries. Flooding-tolerant

species like rice (*Oryza sativa*) and lotus (*Lotus corniculatus*) have the ability to adjust their physiology through metabolic, morphological and anatomical modifications. There is some evidence that flooding-tolerant plants exhibit higher rates of alcoholic fermentation, stronger ability to maintain adequate carbohydrate supply, and higher root respiration over prolonged periods of waterlogging (Ferner et al. 2012; Le Provost et al. 2012) compared with flooding-sensitive plants (Parelle et al. 2006). However, such patterns are not always consistent (Ferner et al. 2012; Martínez-Alcántara et al. 2012).

Oxygen deficiency tolerance varies at different developmental stages (de Lima Tartaglia et al. 2018). It has been commonly observed that adult trees tolerate waterlogging better than seedlings of the same species (Siebel and Blom 1998). In herbaceous plants, the leaf formation stage was found to be the most sensitive to oxygen-deprivation stress in the soil, following with the appearance of the floral buds and silique formation stages (Zhou and Lin 1995). Moreover, it has been reported that although root oxygen deprivation during the vegetative phase reduces stomatal conductance and photosynthetic rate, the greatest effects are observed when the hypoxia stress is applied during the reproductive stage (Pociecha et al. 2008). Duration of hypoxia is also of great importance. Three days of oxygen-deprivation stress resulted in a reduction of grain yield in canola (*Brassica napus*) (Boem et al. 1996).

In addition to reduced oxygen availability to plants, waterlogging may alter the electrochemical properties of soil, since some soil microorganisms use oxidized chemicals as electron acceptors (Laanbroek 1990). The toxic compounds in soil, such as the reduced forms of Mn^{2+}, Fe^{2+}, H_2S and S^{2-}, might accumulate. These compounds can enter the roots and transfer to shoots, adversely affecting both organs (Armstrong and Armstrong 2005). Additionally, volatile

fatty acids, such as propionic and butyric acids, can increase in waterlogged soils and cause substantial root damage (Armstrong and Armstrong 1999). These organic acids and high CO_2 concentrations can cause 'acid loads' in waterlogged roots (Greenway et al. 2006).

Submergence occurs when floodwaters rise to high levels which cover the shoots preventing the gas exchange between the plant and the atmosphere. Submergence generally results in poor light penetration, which is aggravated by the high turbidity of the floodwater. Deficiency of light and CO_2 hampers the photosynthesis rate under water (Mommer and Visser 2005). During short-term flooding, the main stress is oxygen deficiency, resulting in an energy crisis in roots. Long-term oxygen deficiency stress will also alter plant morphology and structure.

Both short- and long-term oxygen deficiency can alter multiple processes in plants such as gene expression, energy consumption, cellular metabolism, thereby affecting plant growth and development (Bailey-Serres and Voesenek 2008; Yeung et al. 2019). Commonly described physiological changes in plants exposed to root hypoxia include reductions in root respiration, root water transport, shoot water potentials, and photosynthesis (Hossain and Fujita 2010; Tan and Zwiazek 2019).

1.3.1 Water relations under oxygen deficiency

Although soil is saturated with water during flooding, plants may experience water deficit stress. Roots, which are very efficient in taking up water from the soil to meet the water demands of the transpiring shoots, may not properly function and may be damaged by waterlogging. Root diameter, specific root length (SRL, root length per mass), and tissue density are important functional traits that may be affected by oxygen deficiency. As a result, the ability of root water

uptake is commonly affected. Numerous studies have reported that oxygen deficiency alters root hydraulics (Kamaluddin and Zwiazek 2002; Holbrook and Zwieniecki 2003), which is accompanied by the stomatal closure and xylem sap acidification (Tan et al. 2018). The reduction of root hydraulic conductivity (L_{pr}) or/and root hydraulic conductance (K_r) has been observed in many plant species and leads to a decrease in shoot water potential. However, in some plants, including tobacco (*Nicotiana tabacum*) and Norway spruce (*Picea abies*), waterlogging had no effect on L_{pr} (Wang et al. 2013; Tan and Zwiazek 2019). In trembling aspen (*Populus tremuloides*), the decline of root hydraulics was associated with the downregulation of aquaporin expression (Kamaluddin and Zwiazek 2002). It has also been reported that the oxygen deficit-induced acidification of root cell cytoplasm causes the closure of aquaporin channels (Tournaire-Roux et al. 2003).

Water flow follows the least resistant pathway in plant tissues. According to the composite transport model, radial water transport in plant roots can occur through the apoplastic, symplastic, and transcellular pathways (Steudle and Peterson 1998; Steudle 2001). Symplastic and transcellular pathways are often referred together as the cell-to-cell pathway. The apoplastic pathway is mainly regulated by cell wall modifications, whereas the cell-to-cell pathway is mainly regulated by aquaporins (Steudle 2001; Kerbler et al. 2019). Cell wall modifications in the exodermis and endodermis, such as a deposition of lignin and suberin, limit radial root water transport through the apoplastic pathway (Kreszies et al. 2018). Therefore, higher aquaporin abundance in the inner cortex and endodermis can facilitate water flow in the root tissues where apoplastic transport is restricted by cell wall modifications (Gambetta et al. 2013; Hachez et al. 2006). The presence of high densities of aquaporins in cells where water flow through the apoplast is restricted supports the notion of the primary role of aquaporins in regulating water

flow across the root. Under abiotic drought conditions, switches between the two pathways were reported (Siemens and Zwiazek 2004). However, the contributions of apoplastic and cell-to-cell pathways under root hypoxia conditions have not been thoroughly studied.

Root hypoxia usually has negative effects on leaf water relations. Decreases in leaf water potential under low oxygen conditions have been commonly reported in various plants (Else et al. 2001; Domingo et al. 2002; Nicolás et al. 2005). The decline of transpiration is a common phenomena in hypoxic plants (Mielke et al. 2003; Rodriguez-Gamir et al. 2011). Stomatal closure is responsible for inhibiting transpiration under low oxygen conditions (Islam et al. 2003; Blanke and Cooke 2004; Atkinson et al. 2008). Plants adjust their hydraulic conductivity and transpiration rates depending on water supply and the shoot-to-root communication may involve both hydraulic and chemical signals (Liu et al. 2014).

1.3.2 Anatomical and morphological changes under oxygen deficiency

In response to root hypoxia stress, anatomical changes including increased cell wall thickening, lignification and suberization of the exodermis, endodermis and sclerenchyma have been observed in rice (*Oryza sativa*), maize (*Zea mays*) and *Glyceria maxima* roots (Soukup et al. 2007; Mostajeran and Rahimi-Eichi 2008). Lignification and suberization of the endodermis help plants to maintain a certain level of oxygen concentration inside the roots by forming a barrier to radial oxygen loss (Katsuhiro Shiono et al. 2014). The enhanced apoplastic barriers are also responsible for preventing the entry of reduced phytotoxic compounds (e.g., Fe^{2+} with the hydrated ionic radius of approximately 300 pm) and H_2S (approximate radius of 193 pm) (Watanabe et al. 2013). The permeability of apoplastic barriers depends on growth conditions and the duration of oxygen deprivation. However, the deposition of lignin and suberin also

provides a significant barrier to root radial water transport by reducing the diameter of pores in the cell walls (Ranathunge et al. 2011).

Another feature often associated with oxygen deficiency response is the formation of adventitious roots or aerenchyma cells. Adventitious roots are produced under oxygen deficiency when the primary root system of the plant is impaired (Calvo-Polanco et al. 2012). Adventitious roots with a high proportion of intercellular spaces facilitate longitudinal oxygen transport and help maintain the efficiency of water transport (Tan and Zwiazek 2019). Waterlogging tolerant species such as rice (*Oryza sativa*) typically possess a large volume of aerenchyma. However, most flooding-sensitive species including *Brassica napus* do not form aerenchyma or adventitious roots in response to waterlogging (Voesenek et al. 1999).

1.3.3 Metabolism under oxygen deficiency

Another class of hypoxic responses includes the downregulation of a suite of energy and oxygen-consuming metabolic pathways (Geigenberger 2003). When oxygen availability decreases in roots, plant respiration is affected. The activity of enzymes in the tricarboxylic acid cycle including malate dehydrogenase and succinate dehydrogenase declines accordingly (Paul et al. 2016). In addition to ethanol and lactate, metabolites such as alanine, succinate, and γ-aminobutyric acid (GABA) can increase in hypoxic plant tissues (Kreuzwieser et al. 2009; Zabalza et al. 2009; Narsai et al. 2011). Under hypoxic conditions, glycolysis is the major source of energy. Fermentative metabolism produces either lactate through lactate dehydrogenase (LDH) pathway, or ethanol through two subsequent reactions catalyzed by pyruvate decarboxylase (PCD) and alcohol dehydrogenase (ADH) (Tadege et al. 1999). These enzymes are vital for the regeneration of NAD^+ to sustain glycolysis under hypoxic conditions (Yin et al.

2019). Additionally, it was found that the cortex of adventitious roots of tamarack (*Larix laricina*) under oxygen deficiency conditions accumulated abundant starch grains in response to oxygen deficiency (Calvo-Polanco et al. 2012). Starch accumulation in leaves has also been observed in soybean (*Glycine max*) response to flooding (Mutava et al. 2015).

1.4 Recovery from oxygen deficiency

Re-exposure of plants to aerated conditions during the post-hypoxic period causes more severe challenges than the hypoxia itself (Yuan et al. 2017b). The degree to which plants can recover after a period of oxygen deficiency depends on the duration of hypoxia and on the changes in metabolism resulting in acclimation of plant tissues to a low oxygen environment (Szal et al. 2003). When the root tissue is exposed to air after hypoxic treatment, the oxygen concentration in the tissue rapidly increases, resulting in higher respiration as well as excess ROS, which may cause lipid peroxidation and induce membrane leakiness leading to excessive water loss (Fukao et al. 2011).

1.4.1 Water uptake recovery under oxygen deficiency

Plants slowly recover from water deficit conditions after oxygen is re-introduced. Since roots are frequently damaged by waterlogging, uptake of mineral nutrients and water is reduced. Reductions of relative water content were observed in *Arabidopsis* and rice (*Oryza sativa*) during post-hypoxia (Fukao et al. 2011; Yeung et al. 2019). The re-opening of stomata following re-aeration increases plant water loss. However, the roots only slowly regain their water transport capacity. Additionally, the cycles of hypoxia and re-entry of oxygen can increase levels of oxidative stress (Goggin and Colmer 2005), which may also have an adverse effect on the water

uptake recovery process (Leon et al. 2021). During re-aeration, L_{pr} recovered over time above the pre-hypoxia levels in wheat (*Triticum aestivum*) and lupin (*Lupinus luteus*) (Bramley et al. 2010). A similar phenomenon was also reported for the recovery phase from low root temperature stress (Lee et al. 2005), which might occur due to the activation of aquaporins and recovery of root growth.

1.4.2 Post-hypoxia injury

As floodwaters recede, oxygen re-enters tissues adjusted to a hypoxic environment triggering a post-hypoxia injury. This injury is attributed primarily to the excessive generation of reactive oxygen species (ROS) including $O_2\bullet^-$, H_2O_2, OH•, and $^1O_2$ (Monk et al. 1987; Crawford et al. 1996). ROS act in the signaling processes, but high concentrations can damage lipid, protein and DNA molecules (Chen et al. 2018). H_2O_2 is one of the most prominent signaling molecules due to its relatively high stability and mobility (Miller et al. 2007). ROS are formed by stepwise electron reduction of molecular oxygen and are mainly produced in chloroplasts and mitochondria, which are characterized by highly oxidizing metabolic activities and high electron flow rates. Cellular ROS levels are balanced by antioxidant systems (Corpas and Barroso 2013). Environmental stresses can skew this balance, leading to oxidative stress. Re-aeration following hypoxia results in low energy-charge values, high reducing-equivalent levels, saturated electron-transport components, leading to bursts of ROS (Yeung et al. 2019).

Alleviation of post-hypoxic ROS burst is linked with dedicated antioxidant systems (Blokhina et al. 2003). Nonenzymic components (eg. ascorbate, glutathione, NADPH) and enzymatic antioxidants (eg. superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidases (POD)) contribute together to eliminate or reduce damaging effects of cellular ROS (Noctor et al. 2016).

Normally, each cellular compartment contains more than one enzymatic activity that detoxifies a particular ROS (Noctor et al. 2016).

1.5 Aquaporins

1.5.1 Aquaporin structure

Aquaporins (AQPs) are a group of transmembrane proteins belonging to the membrane intrinsic protein (MIP) family consisting of six tilted transmembranes of α-helices 1 to 6, packed together with the pore-forming domains (Heymann and Engel 2000; Verkman and Mitra 2000). Aquaporins usually exist as tetramers and each monomer forms an independent water channel. The central pore is predicted to be able to transport gas molecules (Hub and de Groot 2006; Wang and Tajkhorshid 2007). Molecular structure analysis of two plant aquaporins, kidney bean (*Phaseolus vulgaris*) *Pv*TIP3;1 and spinach (*Spinacia oleracea*) *So*PIP2;1, confirmed the typical tetrameric conformation found in plant aquaporins (Karlsson et al. 2003). It also indicated that aquaporin structure joined by five loops (A–E) with the N- and C-termini facing the cytosol joining the transmembrane helices. B- and D-loop are cytosolic, while the A-loop, C-loop, E-loop are extracytosolic which creating a pore with high specificity with two filter regions (Kirch et al. 2000; Ishibashi 2006). One is formed by the conserved asparagine–proline–alanine (NPA) motifs and another is formed by an aromatic/arginine (ar/R) region (Murata et al. 2000; de Groot and Grubmüller 2001). Besides being water channel proteins, aquaporins also permit other molecules to pass through the plasma membrane, i.e. urea, glycerol, H_2O_2, NH_3, O_2, CO_2, etc. (Zwiazek et al. 2017; Tan et al. 2018).

1.5.2 Diversity of plant aquaporins

Plant aquaporin homologs include seven subfamilies classified according to subcellular localization and structural characteristics (Anderberg et al. 2011), 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) (Liu et al. 2009; Venkatesh et al. 2015). The XIP subfamily is absent in Brassicaceae and monocots (Danielson and Johanson 2008; Lopez et al. 2012), whereas the GlpF-like Intrinsic Proteins (GIPs) and the Hybrid Intrinsic Proteins (HIPs) are present typically in mosses and not in vascular plants. PIPs, TIPs, NIPs and SIPs exist in all terrestrial plants and the PIPs are the only ones that are exist in both algae and higher plants.

Aquaporin genes have been described in plant species including maize (*Zea mays*), rice (*Oryza sativa*), soybean (*Glycine max*) , tomato (*Solanum lycopersicum*), grapevine (*Vitis vinifera*) etc. Regarding the number of aquaporin homologs in plants, 35 aquaporin genes have been identified in the *Arabidopsis* genome (Johanson and Gustavsson 2002) and 36 expressed aquaporin genes have been identified in maize (*Zea mays*) (Chaumont et al. 2001). The record number of 121 aquaporin genes may be held by the canola (*Brassica napus*) (Yuan et al. 2017a).

1.5.3 Structure and function of PIPs

PIPs localized in the plasma membrane and are subdivided into two isoform groups, PIP1s and PIP2s. PIP1 and PIP2 isoforms share about 80% amino acid identity. But PIP1s are characterized by a longer N-terminal extension but shorter C-terminal end than PIP2s (Chaumont et al. 2000). PIP2s mainly act as water channels while PIP1s are not directly involved in water transport, but

support water transport with PIP2s(Li et al. 2013). Some PIP2s can also transport H_2O_2, glycerol (Chaumont et al. 2000; Chaumont et al. 2005), CO_2 (Wang et al. 2016; Israel et al. 2021) and ions (Byrt et al. 2018; Kourghi et al. 2018). However, PIP1 members have been shown to be permeable to molecules such as glycerol (Moshelion et al. 2002), boric acid (Martinez-Ballesta et al. 2008), CO_2 (Uehlein et al. 2003) and O_2 (Zwiazek et al. 2017). Extensive studies demonstrated that some PIP1 require a PIP2 partner to reach the plasma membrane. When *ZmPIP1*;*2* was co-expressed with *ZmPIP2*;*1*, *ZmPIP2*;*4* or *ZmPIP2*;*5*, an increase in P_f was observed compared with the P_f of oocytes injected with *ZmPIP2*;*5* cRNA alone (Fetter et al. 2004).

Advanced genetic manipulation techniques including gene overexpression and suppression provide new perspectives regarding the function of aquaporins. Overexpression of rice (*Oryza sativa*) *OsPIP1*;*3* in tobacco (*Nicotiana tabacum*) promoted plant growth and water uptake (Liu et al. 2020). Similarly, in rice (*Oryza sativa*), seed germination and salt stress resistance were enhanced by overexpression of *OsPIP1*;*1* (Liu et al. 2013). Plants of the *AtPIP2*;*2* knockout lines had lower hydrostatic root water transport rates compared with the wild-type plants (Da Ines et al. 2010)

1.5.4 Regulation of aquaporins gene expression

Gene expression is a process that coverting gene information into proteins. Thus, the factors affecting aquaporin gene expression are critical for protein functioning. Transcript abundance of *PIP*s may increase or decrease depending on the environmental conditions, plant species, developmental stage and cell type (Qiu et al. 2020). *PIP* transcripts in hydroponically grown two-week-old *Arabidopsis* roots were *AtPIP1*;*1*, *AtPIP1*;*2*, *AtPIP2*;*7* and *AtPIP2*;*8* (Jang et al.

2004), and in the roots of hydroponically grown 30-day-old plants *AtPIP2*;*2* was highly expressed following with *AtPIP2*;*1*, *AtPIP1*;*2* and *AtPIP1*;*1* (Boursiac et al. 2005). Relatively high or exclusive expression of aquaporins in roots as compared to shoot tissue was reported for numerous plant species (Beaudette et al. 2007; Besse et al. 2011; Hachez et al. 2006; Javot et al. 2003). Also, aquaporin expression can vary along the main axis of roots but is commonly highest in the root tips (Knipfer and Fricke 2010; Gambetta et al. 2013). In barley (*Hordeum vulgare*). High aquaporins expression levels were detected in the endodermis and stele of roots, highlighting their potential role in the regulation of radial water transport (Knipfer and Fricke 2010). In grapevine (*Vitis vinifera*), upregulated root L_{pr} were associated with peak *VvPIP*s expression in the root tips (Gambetta et al. 2013). Upon environmental stress, aquaporin transcripts alter depending on the type of stress, duration, plant species and age (Chaumont et al. 2005). Drought stress triggered downregulation of most of the *PIP* genes in leaves of the four-week old *Arabidopsis* plants, whereas *AtPIP1*;*4* and *AtPIP2*;*5* was upregulated (Alexandersson et al. 2005).

The expression of *PIP*s is also regulated by cellular signals including pH, Ca^{2+}, and H_2O_2 which could influence PIP gating, localization, and/or protein interactions (Tournaire-Roux et al. 2003; Maurel et al. 2016). *AtPIP2*s appears to be highly responsive to numerous sets of factors, including oxidative stresses and hormonal treatments (Prak et al. 2008; Engelsberger and Schulze 2012). It was reported that the activity of aquaporins in *Chara corallina* can be substantially and reversibly inhibited by OH^- produced from H_2O_2 (Henzler et al. 2004). The treatment of *Arabidopsis* roots with 2 mM H_2O_2 for 15 min was also reported to induce alterations of PIPs and TIPs (Boursiac et al. 2005).

1.5.5 Responses of aquaporins to oxygen deprivation and re-aeration processes

Plant aquaporins have been shown to be involved in plant responses to abiotic stresses and stress signaling including oxygen deficiency and re-aeration. One of the earliest responses in plants to oxygen deprivation is a reduction of root water uptake, which is manifested by changes in aquaporin regulation including aquaporin transcript levels. Waterlogging studies in *Sorghum bicolor* indicated that transcript abundance of *PIP2*;*6*, *PIP2*;*7*, *TIP2*;*2*, *TIP4*;*4*, and *TIP5*;*1* exhibited contrasting patterns in tolerant and sensitive genotypes (Kadam et al. 2017). In *Arabidopsis*, *AtNIP2*;*1* was found to be sharply induced by waterlogging (Choi and Roberts 2007). On the post-translational level, a rapid decrease in cytosolic pH resulting from oxygen deprivation of plant roots leads to the closure of aquaporin channels (Gerbeauet al. 2002; Gibbs and Greenway 2003; Verdoucq et al. 2008). The structural model of *So*PIP2;1 suggests low pH resulted in a conformational change (Tornroth-Horsefield et al. 2006). Additionally, oxygen deprivation in plants triggers a rapid increase in cytosolic free Ca^{2+} (Shabala et al. 2014). Free Ca^{2+} in plants can modulate the opening and closing of the water channels (Jozefkowicz et al. 2016).

Studies of aquaporin regulation upon re-aeration following oxygen deficiency are scarce. A key component in the recovery of a species after waterlogging could be the reconfiguration of new aquaporins in root cell membranes, particularly if oxygen deficiency leads to the breakdown of aquaporins or root death. Moreover, given that the turnover rate of some aquaporins can occur within a few hours, the resumption of water uptake and growth could also be rapid. After a short-term (0.5 h) mild hypoxia treatment followed by 1 h re-aeration, L_{pr} of wheat roots was 50% greater than initial values of L_{pr}, which was associated with an increase in aquaporin activity

(Bramley et al. 2007). Moreover, in a long-term glasshouse experiment waterlogged wheat roots rapidly resumed growth when the pots were drained and the growth rate was greater than when the plants were waterlogged (Bramley et al. 2007). An increase in aquaporin activity could not only facilitate the rapid transport of water to the shoots, but also mediate changes in growth rate during the recovery period (Bramley et al. 2007).

1.6 Studied plant species

Brassica napus (2n = 38) belongs to the Brassicaceae family, which is a natural hybrid of *Brassica rapa* (2n = 20) and *Brassica oleracea* (2n = 18). The cultivation of Brassica oilseed crops dates back to 2000 B.C. in India and was brought into China and Japan around 30 B.C. (www.canolacouncil.org). It is the second-largest oilseed crop in the world, providing a large proportion of the world's plant oil supply (Chalhoub et al. 2014). It is also known as “canola”, a registered trademark of the Canadian Canola Association. Canola production is mostly distributed in the Canadian prairie provinces, including Alberta, Saskatchewan and Manitoba. Canola oil is economically essential to Canada and contributed about $26.7 billion to the Canadian economy in 2014 (www.canolacouncil.org). It holds a prominent position among the crops that have been used for intense breeding and genetic manipulation (Raza et al. 2021). As the most productive oilseed crop species, canola production has been threatened by various abiotic stresses including drought, salinity, extreme temperatures, flooding as well as various biotic stresses. The seed yield and seed oil content of canola are greatly dependent on the water availability at each growth stage (Tesfamariam et al. 2010). Additionally, *B. napus* evolved different strategies to cope with environmental stresses at different growth stages (Gan et al. 2004). Previous studies indicated that *B. napus* plants are relatively salt- and heat-tolerant (Maas

and Grattan et al. 1999), but are considered to be sensitive to drought and waterlogging (Zhou and Lin 1995; Xu et al. 2015). Despite the importance of canola cultivation, little work has been done to assess its water transport properties under oxygen deprivation conditions.

1.6.1 Aquaporins in *Brassica napus* and other members of Brassicaceae family

Aquaporin genes have been identified for several members of the Brassicaceae family, with 60 aquaporins in *B. rapa* (Tao et al. 2014), 67 in *B. oleracea* (Diehn et al. 2015) and 35 in *A. thaliana* (Johanson et al. 2001). *B. napus* is an allopolyploid formed by natural hybridization of *B. oleracea* and *B. rapa* which resulted in the abundance of aquaporin in *B. napus*. To date, 121 aquaporins family genes were identified in *B. napus*. Additionally, the NPA motifs and ar/R selectivity filters of *Bn*PIPs were also highly conserved when compared with PIPs in barley (*Hordeum vulgare*) (Tombuloglu et al. 2016), common bean (*Phaseolus vulgaris*) (Ariani and Gepts 2015) and rubber tree (*Hevea brasiliensi*) (Zou et al. 2015), indicating that the PIPs have been subject to strong selection in different plant taxonomic lineages.

All TIP sub-families of *B. napus*, *B. rapa*, *B. oleracea*, and *A. thaliana* share two identical NPA motifs in HB and HE, as the ar/R selectivity filters displayed minor differences. It has been shown that *At*NIP5;1, *At*NIP6;1, *At*TIP5;1, *Os*PIP2;4, *Os*PIP2;7, *Os*PIP1;3, *Os*PIP2;6, and *Hv*NIP2:1 were essential in boron homeostasis of plants (Mosa et al. 2015). PIPs also function as the water channels, which mediate efficient water transport in plant cells (Mahdieh et al. 2008). Gene expression levels of most *PIPs* in *B. napus* were relatively higher than other aquaporins in all tissues, especially in the roots under 0.25 μM and 25 μM boron conditions (Yuan et al. 2017a). The *BnPIP2*;*5* and *BnPIP2*;*7* transcripts were predominantly expressed in flowers, consistent with the expression of *AtPIP2*;*5* and *AtPIP2*;*7* in flowers of *Arabidopsis*

(Alexandersson et al. 2005). It was also found that *BnPIP1*;*1*, *BnPIP1*, *BnPIP2*, and *BnTIP2* were upregulated during seed germination (Ge et al. 2014).

1.6.2 *Brassica napus* under oxygen deficiency stress

Oxygen deficiency stress frequently affects canola since it is grown in many regions as a rotation crop following rice and in colder regions, it is sown during the snow-melting period in early spring. Oxygen deficiency causes significant yield reductions in canola, which may occur after 3 to 30 days following waterlogging, depending on the climate and the developmental stage of the plants (Boem et al. 1996). The physiological reactions to waterlogging at the seedling and floral bud appearance stages of canola were associated with decreases in the photosynthetic rates, antioxidant enzyme activities, as well as accumulation of leaf malondialdehyde (MDA) and ethylene (Zhou and Lin 1995). As a result, waterlogging stress caused a significant decrease in, plant height, stem width, number of branches and branching position of canola plants, thereby reducing the number of siliques and yield (Zhou and Lin 1995). Waterlogging at the reproductive stage, e.g., during anthesis, impairs reproductive organs. The floret fertility can be diminished, which leads to reductions in grain number per spike and lower yields (Marti, Savin, & Slafer, 2015). It can be concluded that *B. napus* may be susceptible to waterlogging in vegetative stages compared with the reproductive stages (Xu et al. 2015). However, studies concern the waterlogging response on canola are still scarce (Zhou and Lin 1995; Xu et al. 2015).

Studies have been carried out to find solutions for alleviating the harmful effects of oxygen deficiency stress on canola. A foliar application of tricyclazole significantly alleviated the growth-inhibiting effects of waterlogging stress, as evinced by the increased leaf concentrations

of photosynthetic pigments, plant morphological characteristics and enhanced grain yield (Habibzadeh et al. 2012).

1.7 Hypotheses and objectives

1.7.1 Hypotheses

Based on previous studies of the responses of plants to hypoxia and our understanding of the functions of aquaporins, it could be expected that aquaporins play an important role in both root hypoxia and re-aeration processes. Aquaporins are essential in facilitating root water transport that is essential for plants to survive the root hypoxia and re-aeration conditions. However, their water transporting properties are known to be affected by oxygen deprivation and oxidative burst. Also, there is increasing evidence that the aquaporins are involved in the transport of gases and other small molecules, which could have a significant impact on plants exposed to root hypoxia. In addition, considering the high value of canola in Canada, excessive losses of canola crop could have a severe impact on the Canadian economy. Canola research is vital in obtaining better understanding of stress resistance mechanisms. More research studies are needed to provide scientific basis that are required to develop guidelines for canola growers and to develop canola cultivars with enhanced stress tolerance traits.

The research study described in Chapter 2 was carried out to examine the hypothesis that PIPs are the main water transporters in plants and that hypoxia would alter the apoplastic pathway in roots through the deposition of suberin and lignin. I hypothesized that the contribution of aquaporin-mediated root water transport of waterlogged canola plants would vary depending on their developmental stage.

Considering that re-aeration is pivotal for plant recovery from hypoxia stress and the overproduction of ROS were reported upon re-aeration. I hypothesized that ROS would alter the regulation of aquaporin expression and, consequently, affect hydraulic recovery of plants.

Since NtPIP1;3 was previously found to be involved in oxygen transport when expressed in yeast and *B. napus* is sensitive to root hypoxia, I also hypothesized that overexpression of *NtPIP1*;*3* would increase the hypoxia tolerance of *B. napus* plants by facilitating oxygen delivery to roots.

1.7.2 Study objectives

The primary goal of the studies described in this thesis was to advance understanding of the multifunctional roles of aquaporins in *B. napus* plants exposed to root hypoxia and following re-aeration. The specific objectives were to (1) investigate changes in gene expression patterns of the main *PIP* aquaporins in *B. napus* plants subjected to root hypoxia at different developmental stages, (2) examine the effects of waterlogging on the physiological responses and root anatomy, (3) determine whether the oxidative burst of ROS occurs during root re-aeration, (4) examine the effect of oxidative burst on aquaporin expression, (5) investigate the water permeability properties of selected BnPIPs, and (6) reveal changes in respiratory metabolisms of the *B.napus* *NtPIP1*;*3* overexpression lines compare with the wild-type.

In this thesis, the research studies are described in Chapters 2, 3, and 4. In Chapter 2, transcript profiles of main *BnPIP*s in waterlogged *B. napus* at different developmental stages are presented. In addition, the changes in root anatomy including suberin and lignin deposition in hypoxic plants are presented. Chapter 3 presents the responses of *PIP*s in *B. napus* to re-aeration

and water permeability of BnPIPs expressed in yeast. Chapter 4 describes the responses of *NtPIP1*;*3* overexpression lines to root hypoxia that point to the importance of this aquaporin in oxygen delivery to roots. General discussion of the main findings is presented in Chapter 5.

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Chapter 2. Properties of root water transport in canola (*Brassica napus*) subjected to waterlogging at the seedling, flowering and podding growth stages

2.1 Introduction

Waterlogging is a major constraint to plant growth and survival across the globe, especially in the areas with high precipitation and poor soil drainage. It is also a common factor limiting the productivity of many agricultural crops including canola (*Brassica napus*) (Perboni et al. 2012). Canola is one of the most important oilseed crops in the world including Canada. The crop is seeded shortly after the snowmelt in Canada and as a rice rotation crop in paddy fields in China (Xu et al. 2015b), which leads to high risks of waterlogging. Distinct yield reductions in canola may occur after 3 to 30 days of flooding, depending on other environmental stress factors and the developmental stage of plants (Habibzadeh et al. 2012).

Waterlogging reduces oxygen availability to plants. Oxygen diffusivity in water is approximately 10,000 times lower than it is in air, and the flux of oxygen in soil is approximately 320,000 times lower when water fills the soil pores (Armstrong and Drew 2002). This leads to root hypoxia, which triggers declines in metabolic processes and induces rapid functional and structural alterations in waterlogged plants (Mielke and Schaffer 2010; Tan and Zwiazek 2019). Upset plant water balance has been often reported in waterlogged plants and explained as effects of hypoxia on root structure and on aquaporin-mediated root water transport (Jitsuyama 2017; Kreszies et al. 2018). Roots are the sites of greatest resistance to water flow in most plants

(Steudle and Peterson 1998) and, in addition to waterlogging, root hydraulic conductivity is also commonly altered by other environmental stresses including salinity, drought, and low soil temperature (Lee et al. 2012; Grondin et al. 2016; Meng and Fricke 2017; Vaziriyeganeh et al. 2018).

Radial water flow in roots follows the apoplastic and cell-to-cell (across cell membranes and through plasmodesmata) pathways (Knipfer and Fricke 2010). The apoplastic pathway predominates when hydrostatic gradients are created by transpiration while cell-to-cell pathway, which is largely mediated by aquaporins, plays a critical role under stress (Steudle and Peterson 1998, Maurel et al. 2008). However, the dynamic changes of these pathways have not been thoroughly investigated in waterlogged plants. Relative contributions of apoplastic and cell-to-cell pathways may substantially change depending on the type and duration of environmental stress in different plants (Steudle and Peterson 1998; Martínez-Ballesta et al. 2003; Henzler et al. 2004). Roots can adjust their transport properties in response to stress through structural modifications altering the apoplastic pathway (Bárzana et al. 2012) or through changes in the properties of aquaporin-mediated cell-to-cell water transport (Barberon et al. 2016). Since waterlogging induces an accumulation of lignin and suberin (Abiko et al. 2012), their deposition in roots could potentially decrease root hydraulic conductivity. Cell-to-cell pathway is regulated mainly by the abundance and activity of aquaporins, therefore, it may also be affected by the acidification of cytoplasm as well as by impeded membrane trafficking and phosphorylation processes that are likely to occur as a result of waterlogging (Kamaluddin and Zwiazek 2002; Maurel et al. 2015). It is also plausible that waterlogging could affect the aquaporin-mediated transport of other molecules than water including O_2, CO_2, NO, H_2O_2 and lactic acid (Uehlein et al. 2012; Tian et al. 2016; Zwiazek et al. 2017; Tan et al. 2018) and contribute to stress tolerance.

Plants vary in their sensitivity and may use different strategies to tolerate waterlogging during different stages of growth (Ren et al. 2017; Romina et al. 2014). Therefore, in the present controlled-environment study, we examined the effects of waterlogging on root water flow properties in relatively hypoxia-sensitive canola (*Brassica napus*) plants during the seedling, flowering, and podding stages of growth. We used the tracer dye, light green SF yellowish and silver nitrate to determine relative contributions of apoplastic and cell-to-cell pathways in aerated and waterlogged plants.

The objectives of the present study were to examine the effects of waterlogging on root water transport pathways at different growth stages in canola to clarify their role in plant responses to root hypoxia. We examined the hypothesis that waterlogging triggers root suberization and lignification, which reduces the effectiveness of apoplastic bypass and increases the dependence of roots on the aquaporin-mediated water transport. Therefore, maintaining the functionality of aquaporins is an important element for plant tolerance to waterlogging.

2.2 Materials and methods

2.2.1 Plant material and treatments

Canola (*Brassica. Napus* cv Westar) seeds were surface-sterilized with 70% ethanol for 2 min followed by 20% (v/v) commercial bleach (1% [v/v] sodium hypochlorite) for 30 min. The seeds were germinated in half-strength Murashige and Skoog (MS) medium at 20°C. Shortly after germination, the seedlings were moved into autoclaved peat moss/vermiculite (2:1) in sterilized 1 L (10 x 10 x 10 cm) square plastic pots. The seedlings were grown in a controlled environment growth room with 16 h photoperiod, 22/18°C (day/night) temperature, 400 μmol m^{-2} s^{-1}

photosynthetic photon flux density (PPFD), and 50-60% relative humidity. The seedlings were watered every other day and fertilized weekly with 50% modified Hoagland's solution (Epstein 1972). When 4-week (seedling stage), 8-week (flowering stage) and 11-week (podding stage) old, 16 plants from each stage were randomly divided into the control and waterlogging treatment groups. For the waterlogging treatment, for each growth stage, six pots with plants (one plant per pot) were placed in each of the five replicated 40 L plastic tubs (60×40×20 cm) filled with water up to 2 cm above the top of the pot. The control (also referred to as aerated) plants were grown in well-drained soil throughout the experiment.

2.2.2 Measurements of gas exchange, leaf water potentials and dry weights

After 2, 5, and 8 days of waterlogging, net photosynthesis rate (P_n) and transpiration rate (E) were measured approximately from 9:00 to 12:00 h using a Licor-6400 portable photosynthesis system with a 2×3 cm^2 leaf chamber (LI-COR, Lincoln, NB, USA). The reference CO_2 concentration was set to 400 μmol mol^{-1}; the flow rate was 200 μmol s^{-1}. The leaf chamber temperature was maintained at 20°C, and PPFD was 400 μmol m^{-2} s^{-1} of the red-blue light spectrum. Six plants from the control group and six plants from the waterlogged group were randomly picked and three fully expanded uppermost leaves from each plant were measured. The averages of the three leaves from each plant was taken to calculate the mean gas exchange values for six plants from the each control and waterlogged groups (n = 6). After the measurements, the leaves were excised and immediately placed in a Scholander pressure chamber (PMS instruments; Corvallis, OR, USA) for the measurements of leaf water potential (Ψ_{leaf}) (Scholander et al. 1965; Wan and Zwiazek 1999) (n = 6). Dry weights of total plants were

determined after oven drying at 80°C for 48 h (n = 6). Leaf areas were calculated using Sigmascan Pro 5.0 software (Systat Sofware, San Jose, CA, USA).

2.2.3 Measurements of root hydraulic conductance (K_r) and apoplastic and cell-to-cell water transport pathways

After 2, 5, and 8 days of waterlogging, root hydraulic conductance (K_r) was measured with a hydrostatic pressure method (Wan and Zwiazek 1999; Wan et al. 2001). A 0.25 L glass container containing half-strength Hoagland's solution was placed in a pressure chamber (PMS Instruments, Corvallis, OR, USA). Roots were excised above the root collar, immersed in Hoagland's solution in the container and sealed in the pressure chamber with the short part of the stem protruding above the pressure chamber lid. The measurements of root water flow rates were carried out by determining the volume of excluded sap after pressurizing the chamber to 0.3, 0.4, 0.5 MPa for 2 min (Wan and Zwiazek 1999). Linear regression of the applied pressure and flow rate was used to obtain a slope of the relationship which represented K_r.

The light green SF yellowish dye (light green SF yellowish; Sigma-Aldrich Chemical) was used to determine the relative contributions of apoplastic and cell-to-cell pathways. (López-Pérez et al. 2007). Light green SF yellowish moves apoplastically and is not transported across the plasma membrane (Martínez-Ballesta et al. 2003). Detopped root systems were immersed in 250 μmol L^{-1} light green SF yellowish solution inside a Scholander pressure chamber for 5 min before raising pressure to 0.4 MPa in the chamber. The xylem exudate was collected for two minutes and the dye concentration were immediately measured with a spectrophotometer (Genesys 10 S-UV-VIS, Thomas Scientific, NJ, USA) at 630 nm (Bárzana et al. 2012). The

percentage of apoplastic pathway contribution was calculated from the ratio between dye concentrations of the collected xylem sap and the applied solution.

We also used an aquaporin water transport inhibitor, silver nitrate (Tharanya et al. 2018), to determine changes in the aquaporin-mediated water transport of waterlogged plants compared with well-aerated control. Following K_r measurements, the roots were immersed in 400 μM $AgNO_3$ for 3 h (Tharanya et al. 2018), and K_r was determined again as described above. The contribution of aquaporin-mediated water transport to hydraulic conductance was calculated from the difference in K_r before and after the addition of $AgNO_3$.

2.2.4 RNA transcription profiling

After 2, 5, and 8 days of treatment, 3-5-cm-long distal root segments from 4 plants in each treatment group were excised, quickly frozen in liquid nitrogen and then put in the -80˚C freezer. The samples were ground with a mortar and pestle in liquid nitrogen and total RNA extracted with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA USA). The relative transcript abundance was analyzed for six *PIP* genes cloned from *B. napus* (Ge et al. 2014): *BnPIP1*;*1* (NCBI accession number NC_027760), *BnPIP1*;*2* (NC_027761), *BnPIP1*;*3* (NW_019168728), *BnPIP2*;*2* (NC_027769), *BnPIP2*;*5* (NC_027765), *BnPIP2*;*7* (NC_027757) (Primer sequences are shown in Appendix 1). First-strand complementary DNA (cDNA) synthesis and the real-time quantitative PCR (qRT-PCR) were conducted as earlier described (Xu et al. 2015a). For qRT-PCR, three technical replicates of each cDNA sample were analyzed for each of the four biological replicates (n = 4). Relative transcript abundance was calculated using the $2^{-\Delta\Delta Ct}$ method. The relative transcript fold change of *PIPs* was normalized against geometric mean of the CT value of two reference genes *BnACTIN7* (AF_111812) and *BnPPR* (XM_013831783).

2.2.5 Root anatomy

Distal root segments (n = 5) from each treatment were prepared for microscopy according to Roschzttardtz et al. (2009). Root segments around 3-5 cm from the end were fixed in formalin-acetic acid-alcohol (FAA) solution. After fixation, the root segments were dehydrated in an ethanol series, placed in toluene. Fixed root segments were embedded in paraffin and sectioned with a microtome (model RM2125 RTS, Leica; Solms, Germany). Lignin autofluorescence was visualized following UV excitation at 330 nm to 380 nm with a fluorescent microscope (Carl Zeiss; Jena, Germany) (Donaldson and Radotic 2013; Jia et al. 2015). The light intensity of lignification was determined with Image J software (https://imagej.nih.gov/ij/) (n = 5 images per group) and quantified as previously described (Yamaguchi et al. 2010). For suberin visualization, the protocol developed by Lux et al. (2005) was adopted. Briefly, thin paraffin sections were incubated in a freshly prepared solution of Sudan 7B (0.01% w/v, in lactic acid) at room temperature for 30 min and then rinsed in 90% ethanol (two baths of 1 min each). Samples were then mounted on slides in 50% glycerol and observed under ZEISS AXIO compound light microscope (Carl Zeiss; Jena, Germany). Suberin was quantified in microscopic sections as described by Krezies et al. 2019

2.2.6 Statistical analysis

All data were analyzed using the SPSS 18.0 statistical package (SPSS Inc., Chicago, IL, USA) to determine statistically significant differences ($P \leq 0.05$). Two-way ANOVA was applied to examine the effects of waterlogging (control or waterlogging) and waterlogging duration (2, 5, and 8 days). The data which did not meet the ANOVA assumptions of normality of distribution and

homogeneity of variance were transformed with a log10 function. Comparisons between different treatment means were conducted using Tukey's test.

2.3 Results

2.3.1 Growth parameters, gas exchange and leaf water potentials

Total plant dry weights were significantly reduced compared with control plants after 8 days of waterlogging at the seedling and flowering stages (Figure 2.1a, b). Compared with aerated controls, leaf areas were smaller in waterlogged plants at the seedling and flowering stages after 8 days of waterlogging and after 2 days of waterlogging at the podding stage (Table 2.1; Appendix 2). Defoliation was observed after 8 days of waterlogging at both seedling and flowering stages (Table 2.1). Plant heights were affected after 8 days of waterlogging at the seedling stage, whereas root lengths were reduced when measured after 8 days of waterlogging at the seedling stage compared with aerated control plants (Table 2.1).

Net photosynthesis (P_n) and transpiration (E) rates had the highest values at the seedling stage, following by flowering and podding stages (Figure 2.2). Compared with control plants, P_n was significantly reduced after 5 days waterlogging at the seedling and flowering stages and 8 days of waterlogging at all growth stages (Figure 2.2a, b, c). Waterlogging also significantly decreased E after 5 and 8 days at the seedling and flowering stages (Figure 2.2d, e). However, no marked reductions in E were observed at the podding stage (Figure 2.2f; Appendix 2).

Compared with well-aerated control, leaf water potentials (Ψ_{leaf}) were sharply reduced at the seedling stage after 2 and 8 days of waterlogging (Figure 2.3a). Small reductions in Ψ_{leaf} were

also measured after 2 and 8 days of waterlogging at the flowering stage and after 2 days of waterlogging at the podding stage (Figure 2.3b, c).

2.3.2 Root water transport

Waterlogging reduced root hydraulic conductance (K_r) compared with control plants at durations at seedling stage and 5, 8 days at flowering stage. Mild reductions were observed in the podding stage (Figure 2.3d, e, f; Appendix 2). The largest, several-fold decreases in K_r were measured at the seedling stage when waterlogged for 2 days (Figure 2.3d).

The light green SF yellowish dye concentration in the root xylem sap was lower in waterlogged compared with control plants at all growth stages, indicative of a greater contribution of transmembrane, relative to apoplastic, water transport pathway during waterlogging. The dye concentration declined in plants with waterlogging over time starting at day 2 at the seedling and podding stages and day 8 at the flowering stage (Figure 2.4a, b, c).

K_r was reduced in both aerated and waterlogged roots treated with $AgNO_3$, and the decreases varied between the growth stages and waterlogging treatment. In aerated control plants, the reductions were relatively higher at the seedling and flowering stages compared with the podding stage (Figure 2.4d, e, f; Appendix 2). The inhibition was greater in waterlogged compared with aerated control plants, especially at the seedling and flowering stages (Figure 2.4d, e). The K_r reductions in $AgNO_3$ treated plants deepened with the waterlogging duration at all stages (Figure 2.4d, e, f).

2.3.3 Relative transcript abundance of *PIP* aquaporins

The relative transcript abundance, which indicates the gene transcriptional levels relative reference genes, of 6 *BnPIP*s aquaporins was quantified using qRT-PCR in the distal root segments of waterlogged and control plants. After waterlogging for 2 days, plants at the seedling stage showed sharp increases in *BnPIP2*;*7*, while the expression level of *BnPIP2;2* was upregulated after 5 days of waterlogging and *BnPIP1*;*3* expression level was upregulated after 8 days of waterlogging (Figure 2.5c, d, f). However, *BnPIP1*;*1* was downregulated after 2 days of waterlogging at the seedling stage (Figure 2.5a). At the flowering stage, most of the *BnPIP*s expression levels were downregulated in waterlogged plants, including *BnPIP1*;*1*, *BnPIP1*;*3*, and *BnPIP2;5* (Figure 2.5a, c, e; Appendix 2). However, *BnPIP1*;*2* was upregulated over time and peaked after 8 days of waterlogging (~102-fold). *BnPIP2*;*7* expression level increased with the duration of waterlogging at the flowering stage (Figure 2.5f). At the podding stage, *BnPIP1*;*2*, *BnPIP1*;*3* and *BnPIP2*;*5* were upregulated after 8 days of waterlogging (Figure 2.5b, c, e).

2.3.4 Effect of waterlogging on suberization and lignification in roots

Suberization of endodermis increased with increasing plant age starting at the inner side of the endodermis while lignification was not evident (Figure 2.6a, b). Suberization also increased in the waterlogged plants. The suberization induced by waterlogging was mostly observed at the seedling stage starting at 2 days following waterlogging and after 8 days of waterlogging at the flowering and podding stages (Figure 2.6a). To quantify the suberization level, the numbers of suberized cells in the root endodermis were calculated. The number of suberized cells in waterlogged roots was about twice as high as compared with the aerated control plants after 8

days of waterlogging at the seedling stage (Figure 2.7a). When waterlogged for 8 days of flowering and podding stage, waterlogged plants had significantly more suberized cells than aerated plants (Figure 2.7b, c).

The greatest lignin deposition was observed after 5 and 8 days of waterlogging at the seedling stage and 5 days of waterlogging at the flowering stage (Figure 2.7d, e, f). Plant stage of growth did not influence the degree of lignification (Figure 2.6b). The lignin autofluorescence intensity at the seedling and flowering stage was slightly higher in waterlogged plants, compared with control plants after 2 days of waterlogging but not significant (Figure 2.7d, e). The light intensity difference between waterlogged and control plants were not significant during the podding stage (Figure 2.7f; Appendix 2).

2.4 Discussion

Our study demonstrated that waterlogging differently affected root water transport properties as well as growth, gas exchange and leaf water potentials depending on the growth stage of plants. Since efficient delivery of water to shoots is essential for maintaining gas exchange and growth processes, overcoming the challenges of waterlogging to root water transport may be key to understanding plant tolerance of this challenging condition.

There is abundant literature demonstrating the importance of root water transport in plant tolerance of a variety of abiotic stresses and the role of aquaporins in these responses (Chaumont and Tyerman 2014, Kapilan et al. 2018). Radial water transport in roots follows the route of least resistance, which is a combination of apoplastic and aquaporin-mediated cell-to-cell pathways (Steudle and Peterson 1998; Steudle 2001). It has been previously demonstrated

that root hypoxia induced by waterlogging inhibits the aquaporin-mediated root water transport due to the cellular acidosis and depletion of ATP that is required for protein phosphorylation (Kamaluddin and Zwiazek 2001; Kamaluddin and Zwiazek 2002; Tournaire-Roux et al. 2003; Bramley et al. 2007). Waterlogging can also alter root structure with the consequences to apoplastic pathway. Increased suberization (Tylová et al. 2017) and lignification (Nguyen et al. 2016) have been reported in the roots of some plants in response to waterlogging, could have major consequences to water flow resistance in the roots. It is also well established that waterlogging tolerance varies at a different plant age and stage of development (Xu et al. 2015b). Our study sheds more light on the role of root water transport in this process. Longer waterlogging durations altered the contributions of apoplastic and aquaporin-mediated water transport at each growth stage. Notably, plants at the seedling stage relied more on the aquaporin-mediated water transport for their extensive water requirement and rapid vegetative growth comparing with other growth stages. In addition, their roots contained relatively little suberin and lignin which would otherwise restrict apoplastic water transport. Furthermore, we found that canola plants at the seedling stage were less flooding tolerant compared with the flowering and podding stages.

Waterlogged plants at the seedling stage exhibited the strongest, while those at the podding stage displayed the weakest, declines in the total plant weights, P_n and K_r. This is consistent with the previous studies, which showed that changes of morphological traits under waterlogging at the seedling stage among 25 variaties of *B. napus* were greater compared with other growth stages (Xu et al. 2015; Zou et al. 2014; Habibzadeh et al. 2013). It is noteworthy, that despite the larger root systems in aerated plants at the flowering and podding stages, their K_r was lower compared with the plants at the seedling stage, likely due to a higher proportion of older roots.

Waterlogging resistance at different developmental stages appears to be strongly linked to the water demand as root water transport capacity is sharply reduced by the hypoxic conditions. In addition to slower growth rates, partial defoliation that commenced at the flowering stage and resulted in a decrease in plant dry weights, also contributed to the lower water demand, especially for plants at the podding stage.

As expected, both Ψ_{leaf} and K_r support the notion of high water demand linked to high growth rates characteristic of young plants. This is likely one of the key factors that makes seedlings more sensitive to waterlogging. Additionally, mature plants are likely to have greater stored carbon resources (Groeneveld and Voesenek, 2003) that can be exploited for fuelling fermentation and lowering osmotic potential (Huber et al. 2012). The Ψ_{leaf} declined in plants after 2 days of waterlogging and, similarly to other measured parameters, it was affected more at the seedling stage compared with the flowering and podding stages. Depending on the growth stage, a partial or full recovery of Ψ_{leaf} was observed after 5 days of waterlogging due to reductions in *E*. However, the *E* decreases were not sufficient to prevent subsequent declines of Ψ_{leaf} after 8 days of waterlogging in plants at the seedling and flowering stages. The decreases in K_r were profound and occurred rapidly at the seedling stage and were relatively mild in plants at the flowering and podding stages.

The adjustment of K_r has been often reported for plants exposed to root hypoxia (Kamaluddin and Zwiazek 2002; Rasheed-Depardieu et al. 2015a; Jitsuyama 2017) and attributed to the effect on aquaporin function and a switch between apoplastic and cell-to-cell pathways (Siemens et al. 2003). Symplastic and transmembrane pathways are together referred to as cell-to-cell pathway since they are experimentally difficult to separate (Kjellbom et al.

1999). Apoplastic water transport is commonly affected by alterations of root structure (Kreszies et al. 2019) while changes in cell-to-cell pathway is mainly linked to rapid fluctuations of aquaporin gating and expression (Maurel et al. 2015). Our results of apoplastic dye light green SF yellowish and aquaporin water transport inhibitor $AgNO_3$ demonstrated that apoplastic pathway was dominant in aerated plants at the flowering stage and decreased after waterlogging for 5 and 8 days. The aquaporin mediated transport measured with aquaporin inhibitor $AgNO_3$ indicated that K_r reductions after $AgNO_3$ treated plants deepened with the waterlogging duration at all stages. This suggest that the ratio of apoplastic to cell-to-cell decreased in waterlogged plants following increased waterlogging duration at each stage. Short duration of waterlogging was mild stress to canola and longer duration exerted severe stress. Longer waterlogging duration exerted a greater inhibition of apoplastic transport within each stage which is likely due to the gradual decreased E and cell wall modification (Ranathunge et al. 2016; Kreszies et al. 2019). If apoplastic pathway is blocked, water follows the less resistant cell-to-cell pathway regulated by aquaporins to reach the apoplast of the stele. The high expression of aquaporins with high water permeability can improve the radial hydraulic conductance and confer an efficient mechanism of regulating K_r (Bramley et al. 2007).

We determined the transcript abundance of *PIP*1s and *PIP*2s in distal root segments where aquaporins are the most abundant (Gambetta et al. 2013). Since protein abundance is primarily determined by the transcript levels (Pfannschmidt et al. 1999), PIP gene expression is an important mechanism that is used to control membrane water permeability (Maurel et al. 2008; Macho-Rivero et al. 2018). The results indicated that some of the *BnPIP*s were downregulated in response to 2 days of the waterlogging treatment which was consistent with previous studies (Rasheed-Depardieu et al. 2015b). However, several *BnPIP*s showed a pronounced upregulation

after two days of waterlogging. Rapid changes in the expression levels of various aquaporin genes in response to waterlogging stress has been reported for several plant species including *Arabidopsis*, tobacco, soybean, and sorghum (Matsuo et al. 2012; Kadam et al. 2017; Tan and Zwiazek 2019). As waterlogged roots encounter a general cell energy crisis, the expression of numerous genes is likely to be suppressed to conserve energy whereas hypoxia-inducible aquaporin genes are required for stress acclimation (Gibbs and Greenway 2003). The impact of aquaporins on K_r is mainly controlled by the gating mechanisms. Aquaporin gating, which controls the opening and closure of the water channels, is regulated by post posttranslational modification such as phosphorylation, protonation, cytosolic Ca^{2+} and Rop-dependent H_2O_2 production (Baxter-Burrell et al. 2002). Waterlogged roots are likely to suffer from energy deprivation, which may be required to maintain the opening of aquaporins through phosphorylation (Kamaluddin and Zwiazek 2002). Aquaporin gating is also affected by the cytosol pH (Tournaire-Roux et al. 2003). Reduced cytosolic pH under hypoxic conditions lead to closure of aquaporin (Verdoucq et al. 2008) with K_r inhibition as a likely consequence. In our studies, the most upregulated genes by waterlogging included *BnPIP1;2, BnPIP1;3, BnPIP2;2 and BnPIP2;7* and the function of these genes have not been revealed yet. In *Arabidopsis*, AtPIP1;2 was demonstrated to participate in CO_2 transport (Uehlein et al. 2012), while NtPIP1;3 in tobacco is an effective oxygen transporter (Zwiazek et al. 2017). While the effectiveness of gas transport through PIP1s aquaporins has never been tested in canola, efficient CO_2 and O_2 transport in waterlogged roots would constitute an important hypoxia tolerance mechanism in plants (Boudichevskaia et al. 2015; Zwiazek et al. 2017). Since PIP2s are essential for water transport, BnPIP2;2, BnPIP2;7 might be critical for regulating water relations in

waterlogged roots (Ding et al. 2019) and their water transport properties and regulation mechanisms in canola should be further examined in the future.

To examine the possible reasons for the decrease in apoplastic transport, we compared the deposition of suberin and lignin in aerated and waterlogged roots. Waterlogging for 2 days enhanced the accumulation of suberin at the seedling stage. However, it took 5 days of waterlogging to see marked lignification of roots at the seedling stage. The presence of suberin and lignin restricts the root radial water uptake by reducing the diameter of pores of apoplast (Hose et al. 2001). Increased suberin and lignin levels in roots significantly reduced water and solute permeabilities in rice roots (Zimmermann et al. 2000). However, in our study, lignification of waterlogged roots in plants at the podding stage did not significantly increase and there was a marked decline of apoplastic water transport at that time, suggesting that suberin might be mainly responsible for this reduction. Suberin and lignin deposition was also reported to be enhanced by various other environmental stresses including salinity, drought and heavy metal pollution (Byrt et al. 2018; Enstone et al. 2002). In waterlogged plants, suberin and lignin deposition in roots can help prevent pathogen infection, reduce radial oxygen loss, as well as excessive loss of water and solutes (Kotula et al. 2009; Ranathunge et al. 2011; Kotula et al. 2014).

2.5 Conclusions

We observed dynamic changes in apolastic and cell-to-cell contributions to root water transport following waterlogging at different growth stages in canola. The apoplastic transport declined with increased waterlogging duration and was accompanied by a deposition of suberin in waterlogged roots and by an increase in the proportion of the aquaporin-mediated transport.

Several aquaporin genes including *BnPIP1*;*2*, *BnPIP1*;*3*, *BnPIP2*;*2* and *BnPIP2*;*7* were upregulated. Furthermore, waterlogging triggered significant declines in K_r, P_n and E within 5 days at the seedling growth stage, which was the most sensitive growth stage to waterlogging. The evidence points to the importance of maintaining functional aquaporins in waterlogged roots as a factor contributing to plant survival of waterlogging.

2.6 References

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Table 2.1 Plant heights, numbers of leaves, root lengths and leaf areas in aerated control (C) and waterlogged (WL) canola and in plants waterlogged for 2, 5, and 8 days at the three growth stages. Means ± SE are shown (n = 6). Letters indicate a difference determined by Tukey's test ($P \leq 0.05$, Tukey's test)

Stage		Height (cm)		Leaf number		Root length (cm)		Average leaf area (cm^2)	
		C	WL	C	WL	C	WL	C	WL
Seedling	2d	17.98±1.02bc	16.46±0.81c	6.81±0.21ab	5.84±0.24b	22.00±1.89ab	26.34±2.10a	115.84±15.49bc	77.16±12.32c
	5d	19.00±1.11b	18.46±1.38ab	7.25±0.35ab	7.25±0.25ab	27.07±0.86a	27.61±2.89a	148.05±9.59cb	99.78±9.35bc
	8d	22.67±0.84a	16.42±1.19bc	8.50±0.26a	6.75±0.37b	27.98±1.02a	15.96±3.62b	176.92±11.71a	82.47±9.75c
Flowering	2d	91.83±6.64ab	71.60±5.92b	12.58±0.70a	12.00±0.73ab	26.60±1.32a	30.85±2.28a	121.27±13.13a	89.64±8.90ab
	5d	89.33±7.36ab	72.25±3.42b	10.08±0.40b	10.25±0.43b	31.10±1.31a	31.13±0.63a	93.13±6.39ab	65.941±7.13bc
	8d	97.89±6.08a	85.25±5.45ab	11.00±0.50ab	6.75±0.25c	26.68±1.39a	28.45±2.61a	101.65±9.02a	42.99±4.46c
Podding	2d	115.70±11.75a	108.6±5.05a	7.67±0.84a	6.09±0.37ab	28.88±1.77a	26.05±1.26a	60.70±6.55a	32.00±3.76b
	5d	120.44±10.27a	116.78±8.60a	4.86±0.91abc	3.33±0.78bc	29.38±1.65a	27.63±1.15a	46.81±7.10ab	28.45±4.75b
	8d	115.81±9.55a	110.20±8.31a	3.38±0.69bc	2.13±0.55c	26.00±1.61a	25.25±1.94a	44.57±11.41ab	29.52±6.23b

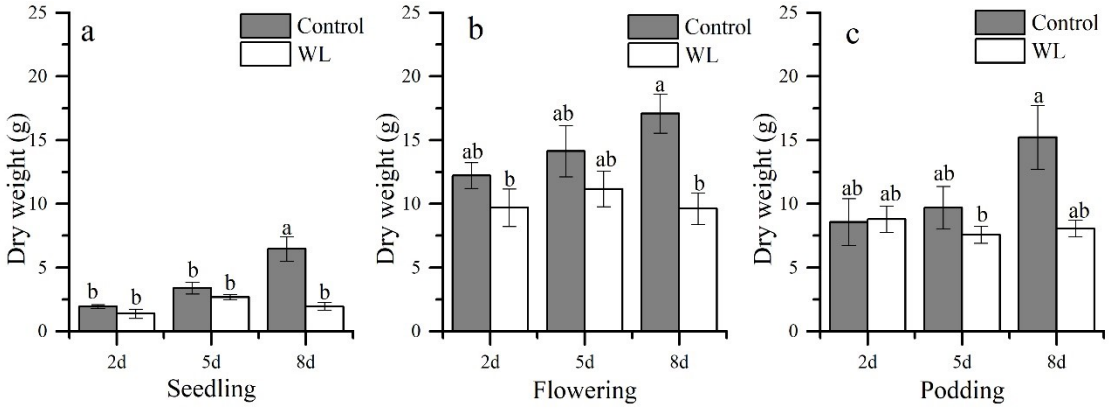

Figure 2.1 Plant dry weights of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage (a). F2, F5, F8 are 2, 5, and 8 days of waterlogging at the flowering stage (b). P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage (c). Means ± SE are shown (n = 6). Different letters above the bars indicate a difference determined by Tukey's test ($P \leq 0.05$).

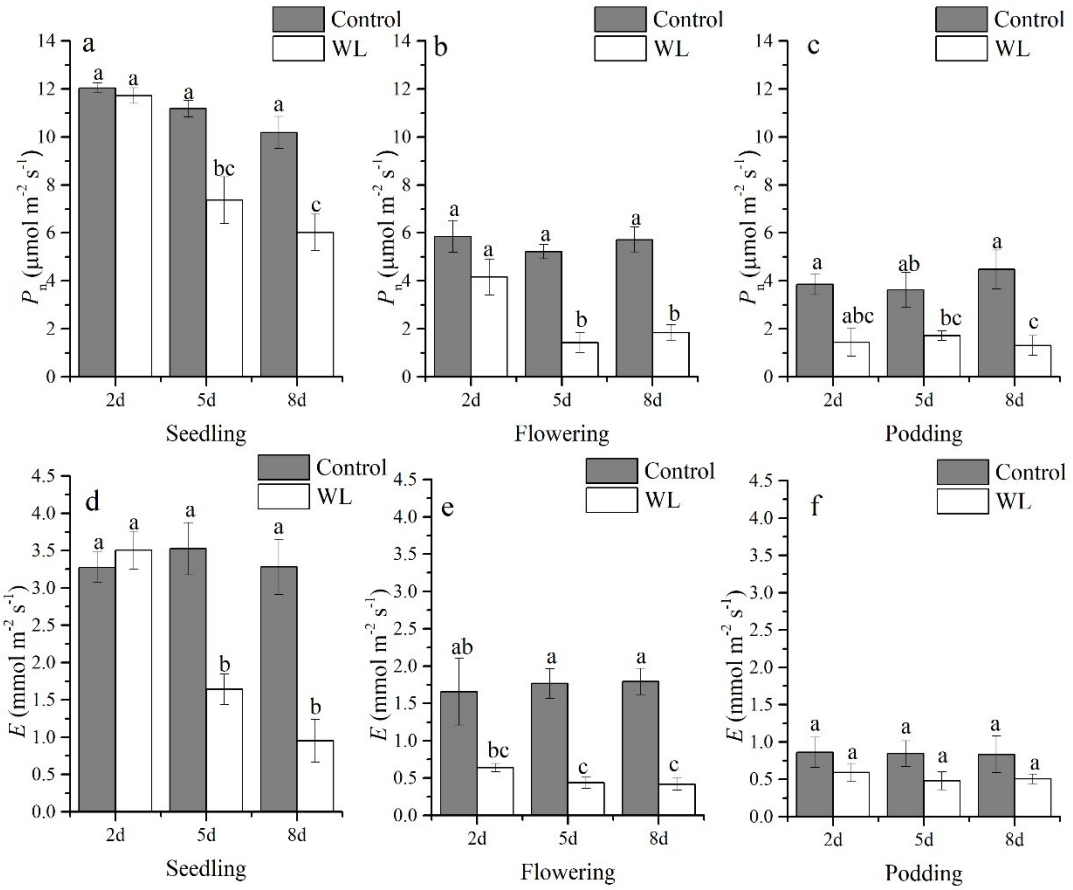

Figure 2.2 Plant net photosynthesis (P_n) and transpiration rate (E) of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage (a, d). F2, F5, F8 are 2, 5, and 8 days of waterlogging at the flowering stage (b, e). P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage (c, f). Means ± SE are shown (n = 6). Different letters above the bars determined by Tukey's test ($P \leq 0.05$).

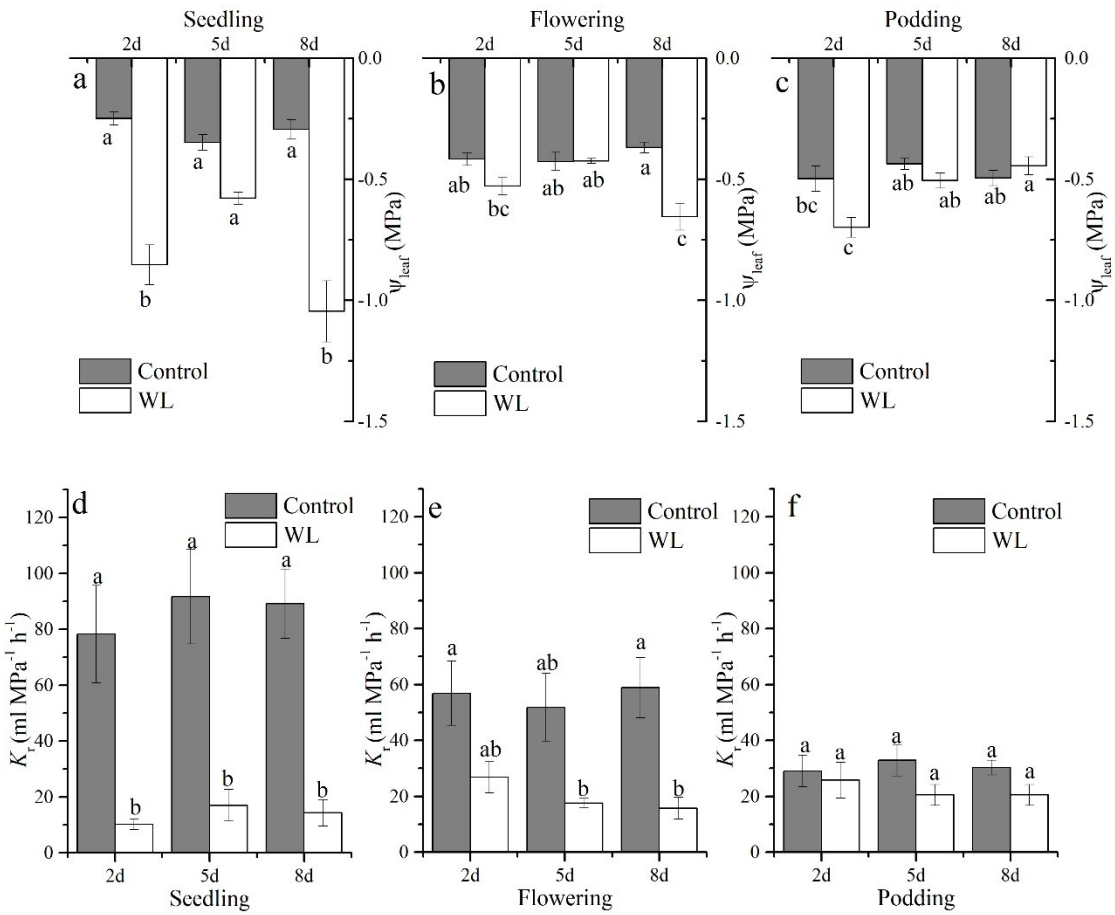

Figure 2.3 Leaf water potential (Ψ_{leaf}) and root hydraulic conductance (K_r) of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage (a, d). F2, F5, F8 are 2, 5, and 8 days of waterlogging at the flowering stage (b, e). P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage (c, f). Means ± SE are shown (n = 6). Different letters above the bars indicate a difference determined by Tukey's test ($P \leq 0.05$).

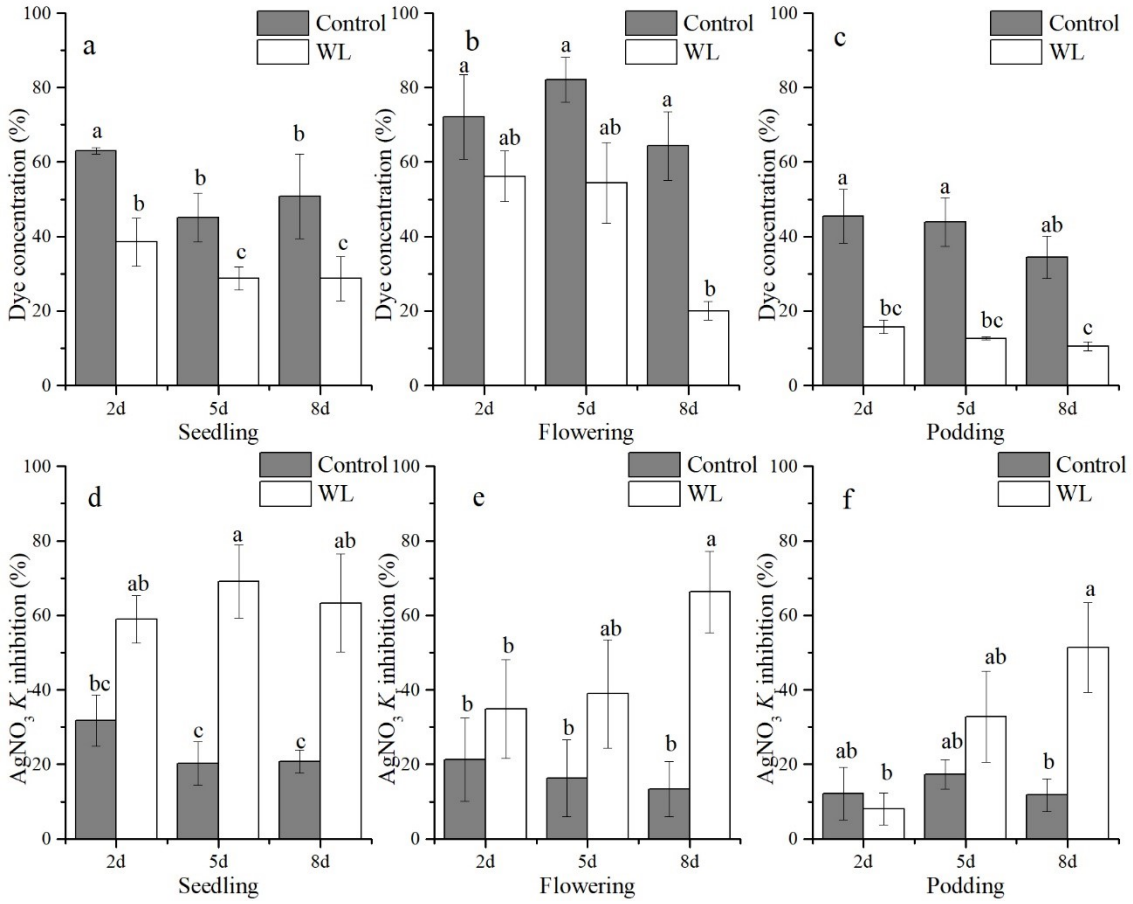

Figure 2.4 Dye concentration (Light green SF yellowish) and $AgNO_3$ K_r inhibition of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage (a, d). F2, F5, F8 are 2, 5, and 8 days of waterlogging at the flowering stage (b, e). P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage (c, f). Means ± SE are shown (n = 6). Different letters above the bars indicate a difference determined by Tukey's test ($P \leq 0.05$).

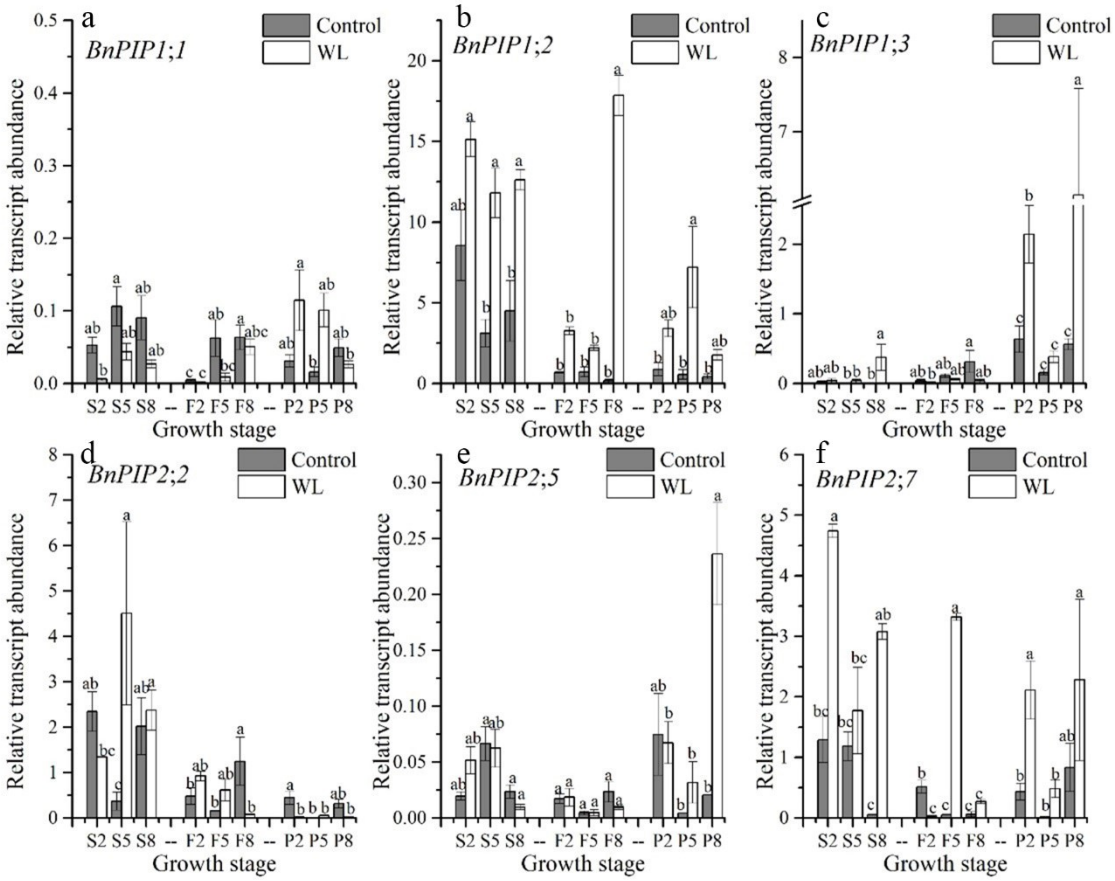

Figure 2.5 Aquaporin (*BnPIP*s) relative expression level of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage. F2, F5, F8 are 2, 5, and 8 days of waterlogging at the flowering stage. P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage. Means ± SE are shown (n = 4). Different letters above the bars indicate a difference determined by Tukey's test ($P \leq 0.05$).

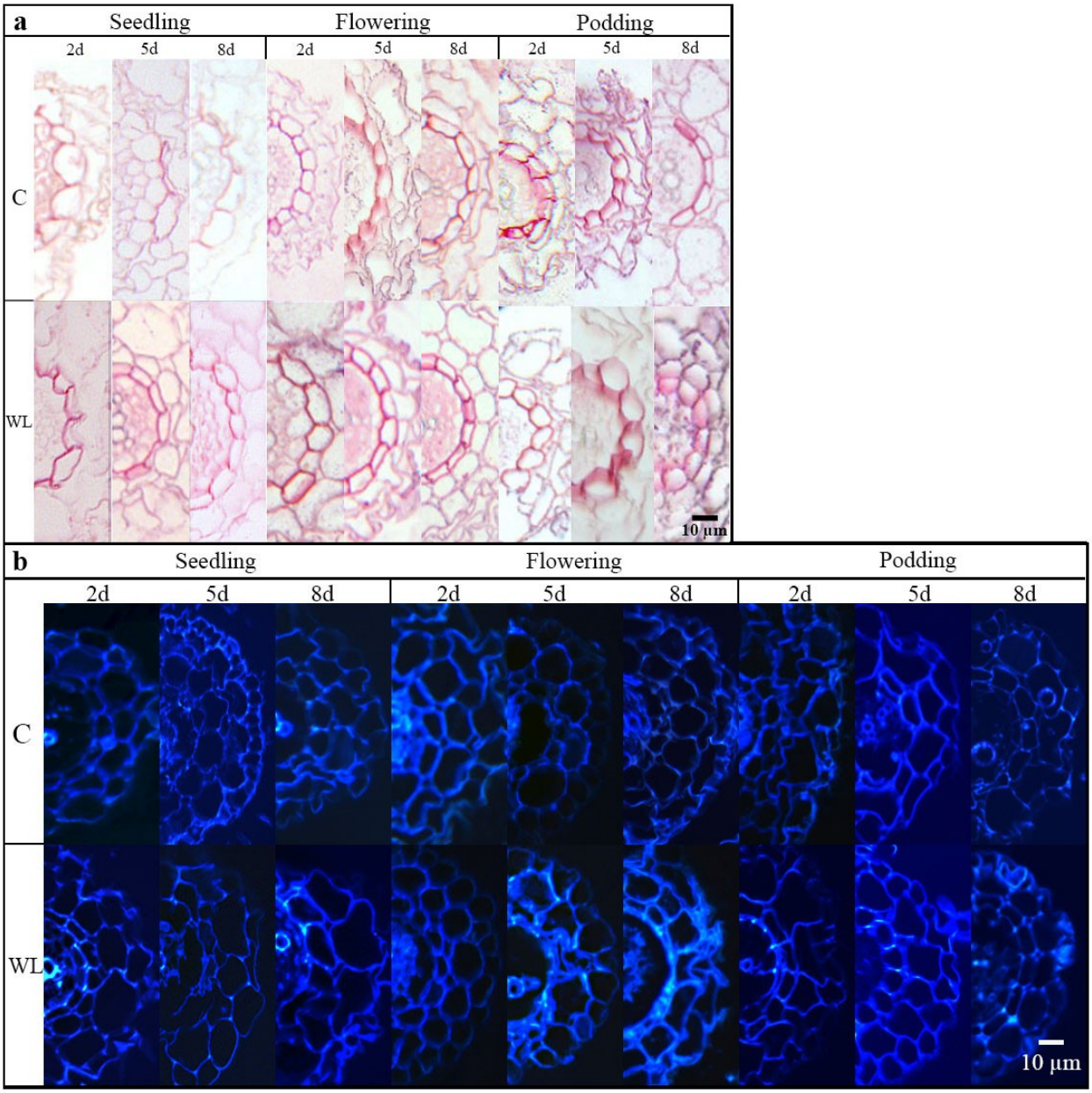

Figure 2.6 Root cross sections from 3-5cm from the end of root stained for suberin with Sudan 7B (a) and examined for lignin autofluorescence with blue light (b) in aerated control canola plants (C) and in plants subjected to waterlogging (WL) for 2, 5, and 8 days at the different growth stages. Bars =10 µm.

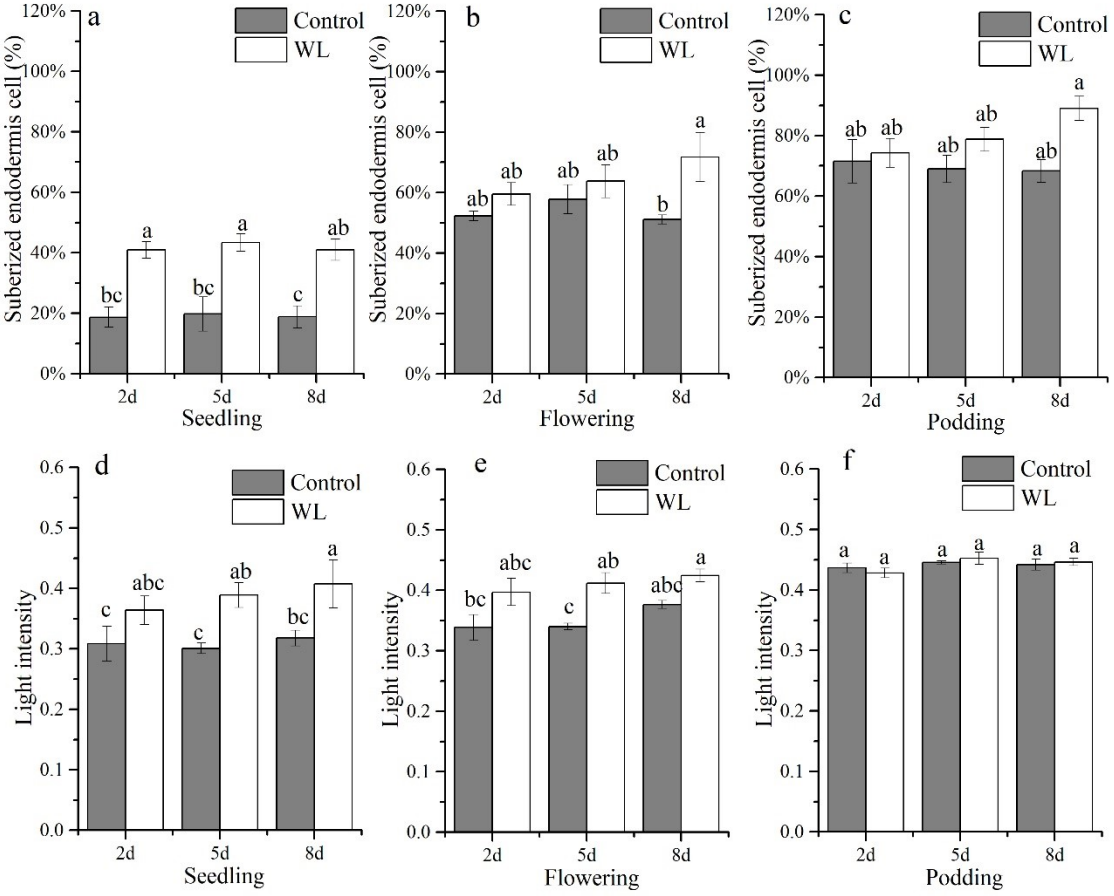

Figure 2.7 Percentage of suberized endodermis cell in distal root segments and light intensity of lignified structures in distal root segments of aerated (control) canola (*Brassica napus*) plants and plants subjected to the waterlogging treatment (WL) for 2, 5, and 8 days at different growth stages. S2, S5, S8 are after 2, 5, and 8 days of the waterlogging treatment at the seedling stage (a, d). F2, F5, F8 are 2, 5, and 8 days of waterlogging at the flowering stage (b, e). P2, P5, P8 are 2, 5, and 8 days of waterlogging at the podding stage (c, f). Means ± SE are shown (n = 5). Different letters above the bars indicate a difference determined by Tukey's test ($P \leq 0.05$).

Chapter 3. Recovery of canola (*Brassica napus*) plants from waterlogging is hindered by oxidative stress and inhibition of aquaporin-mediated root water transport

3.1 Introduction

Root oxygen deprivation (root hypoxia) caused by waterlogging affects energy-demanding processes in plants and leads to growth reductions and plant mortality (Tan et al. 2018; Pedersen et al. 2020). The occurrence of oxygen deprivation process is often transient and followed by a recovery of surviving plants with varying recovery rates depending on the ability of plants to tolerate hypoxic and post-hypoxic events (Considine et al. 2017; León et al. 2020). Plant re-aeration following hypoxia triggers a signaling pathway that regulates the recovery processes and may involve ROS defenses during a transient oxidative burst (Yuan et al. 2017; Yeung et al. 2018; Lee et al. 2019). Successful recovery of plants from root hypoxia requires a rapid resumption of efficient water delivery to leaves (Maurel et al. 2020) since root hydraulic conductivity is inhibited under low oxygen conditions (Kamaluddin and Zwiazek 2002) and the impairment of root hydraulic system is the primary factor leading to the decreases in leaf gas exchange and growth (Tournaire-Roux et al. 2003; Maurel et al. 2016).

The effect of re-aeration on plant water relations is frequently overlooked and commonly attributed to hypoxia (Shingaki-Wells et al. 2014). A potential aggravating effect of re-aeration on root hydraulic conductivity has been suggested (León et al. 2020), but there is little information concerning the hydraulic events in plants during hypoxia recovery. Since re-aeration

of plants may lead to oxidative burst that generates high ROS levels (Yuan et al. 2017; Yeung et al. 2018), and ROS have a strong inhibitory effect on aquaporin-mediated water transport (Ye et al. 2006), it could be logically expected that the recovery of root hydraulics from waterlogging may be affected by the effectiveness of the antioxidant defense system.

Reactive oxygen species (ROS), including superoxide anion ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH \cdot$), are generated in plants exposed to various environmental stresses (Foyer et al. 2016). On the one hand, ROS plays a vital role as signaling molecules in plant stress responses and recovery (Noctor and Foyer 2016a; Schmidt et al. 2018). On the other hand, their excessive accumulation is responsible for oxidative stress that leads to irreversible damage of plant cells (Mittler, 2017). Although ROS accumulation is commonly considered to be a negative consequence of stress (Choudhury et al. 2017), positive aspects of ROS in plants during aluminum toxicity recovery (Matsumoto et al. 2012), flooding (Sasidharan et al. 2018) and heat acclimation (Sun et al. 2018) have also been reported. Antioxidant enzymatic systems are activated together with the generation of ROS. It has been revealed that ROS produced by the cells are metabolized through dedicated antioxidant systems (Noctor and Foyer 2016a). Understanding of how main antioxidants interact with ROS to modulate oxidative stress under hypoxia conditions is fundamental to understanding plant recovery from hypoxia (Noctor and Foyer 2016a).

Early responses of root hypoxia include the adjustments of root hydraulic conductivity (L_{pr}), which is key to plant adaptation to climate change and a major target for crop improvement (Shahzad et al. 2016; León et al. 2020). Frequently reported reductions of L_{pr} have been explained as a consequence of cytosol acidification and energy deprivation leading to the

inhibition of the aquaporin-mediated water transport under low oxygen conditions (Tan et al. 2018). However, the processes of plant recovery following hypoxia and the role of root water transport in these processes have received little attention. Plant dehydration upon re-aeration was observed in the leaves of *Arabidopsis* (Tsai et al. 2014; Yeung et al. 2018) and rice (Fukao et al. 2011). It was proposed that the processes involved in plant dehydration upon recovery may be associated with the function of aquaporins (Yeung et al. 2019). Since ROS accumulation was detected during root re-aeration following hypoxia (Yuan et al. 2017; Yeung et al. 2018) and several examined ROS were demonstrated to affect the function of aquaporins (Henzler et al. 2004; An et al. 2016; Rodrigues et al. 2017), we expected that in flooding-sensitive canola plants (Habibzadeh et al. 2012, Liu et al. 2020), ROS burst would be a significant factor affecting plant recovery from root hypoxia through its effect on the water transport processes.

In the present study, we investigated the recovery of canola (*Brassica napus*) plants from three days of root hypoxia. We examined the root water transport properties, ROS generation and enzymatic scavenging systems, as well as the aquaporin gene expression as well as the water and H_2O_2 transport capacities of different canola aquaporins expressed in a heterologous yeast system. We hypothesized that oxidative burst following re-aeration of hypoxic plants would aggravate detrimental effects of hypoxia on root water transport properties, which may potentially be a critical factor impeding plant recovery from hypoxic stress.

3.2 Materials and methods

3.2.1 Experimental set-up and treatments

Seed sterilization of Canola (*Brassica napus* cv Westar) was done with 70% ethanol for 2 min followed by 20% commercial bleach (1% [v/v] sodium hypochlorite) for 30 min. Then seeds were germinated in Petri dishes with half-strength Murashige and Skoog (MS) medium (Huang et al. 2019) at pH 5.7 in a controlled-environment growth room (16 h photoperiod, 22/18°C (day/night) temperature, 400 μmol m^{-2} s^{-1} photosynthetic photon flux density, and 50-60% relative humidity). The seedlings were moved into the autoclaved peat moss/vermiculite (2:1) growth medium in sterilized plastic pots (10 cm in diameter, one seedling per pot) after germination and placed in the same growth room. When four weeks old, the plants were removed from the soil, their roots gently washed and moved into the hydroponic culture. The hydroponic set-up consisted of six 40 L plastic tubs filled with half-strength modified Hoagland's nutrient solution (Hoagland et al. 1950) and 15 seedlings in each tub. The solution was replaced every five days and was aerated with an air pump to maintain the dissolved oxygen level of approximately 8 mg L^{-1}. After one week, the plants in three tubs (45 plants) were subjected three days to hypoxia treatment by continuously flushing N_2 through the mineral solution to maintain a dissolved oxygen level of about 2±0.35 mg L^{-1}. After three days of hypoxia, re-aeration treatment started by increasing the oxygen level back to 8 mg L^{-1} for six days. The remaining 45 plants in the remaining three tubs were aerated throughout the experiment (8±0.71 mg L^{-1} O_2) and served as control. After three days of hypoxia (Hy3) and one, three, and six days of re-aeration (Re1, Re3, and Re6), six to eight plants from the control and treatment groups were randomly selected from the different tubs for the measurements.

3.2.2 Measurements of gas exchange and leaf water potentials

To assess gas exchange responses to hypoxia and re-aeration, net photosynthesis (P_n) and transpiration rates (E) were measured after three days of hypoxia treatment and one, three, and six days following re-aeration. The measurements were carried out between three and five hours after the start of photoperiod using a Li-Cor-6400 portable photosynthesis system equipped with the 2×3 cm^2 leaf chamber (Li-Cor, Lincoln, NB, USA). The leaf chamber temperature was maintained at 20°C, PPFD was 400 μmol m^{-2} s^{-1}, and the reference CO_2 concentration was set to 400 μmol mol^{-1}. Six plants from the control and six plants from the treatment tubs were randomly selected on each measurement day and three fully expanded uppermost leaves from each plant were used for the measurements. The mean values of the three leaves from each of the six plants per treatment were used for the P_n and E calculations (n = 6). After the measurements, three leaves were excised from each plant and the leaf blades were immediately placed in a Scholander pressure chamber (PMS instruments; Corvallis, OR, USA). The average values of three leaves from each of the six plants (n = 6) were calculated to determine mid-day leaf water potentials (Ψ_{leaf}) (Scholander et al. 1965).

3.2.3 Determination of plant fresh and dry weights and shoot water contents

Six plants from the control and treatment groups (subjected to hypoxia for three days and following re-aeration for one, three, and six days) were randomly taken from the containers and their shoots and roots and weighed to determine fresh weights (FW) separately. The shoots and roots were then placed in an oven at 70 °C for 72 h and weighed again to determine dry weights (DW). For plant dry weight determination, shoot and root dry weights were added. The shoot water contents (WC) were calculated as: WC (%) = [(FW−DW) / FW] × 100.

3.2.4 Measurements of root hydraulic conductivity and root water flow pathways

All measurements were carried out in roots of six plants from the control and treatment groups (subjected to hypoxia for three days and following re-aeration for one, three, and six days). Root hydraulic conductivity (L_{pr}) was measured to reveal the hydraulic recovery from hypoxia using the hydrostatic pressure method (Wan and Zwiazek, 1999). Roots were excised above the root collar, immersed in the half-strength Hoagland's solution in a 0.25 L glass cuvette placed in a pressure chamber (PMS Instruments, Corvallis, OR, USA), then it was sealed with the stem protruding through the lid. The measurements were carried out by determining the volume of exuded xylem sap after pressurizing the chamber to 0.3 to 0.6 MPa at intervals of 0.1 MPa for 2 min at each pressure to establish root water flow rates (Wan and Zwiazek, 1999). Linear regression between the applied pressure and flow rate was used to obtain a slope of the relationship, which represented hydraulic conductance (K_r). The L_{pr} was calculated according to the formula $L_{pr}=K_r$/root volume (Siemens and Zwiazek, 2004).

Light green SF yellowish dye (light green SF yellowish) were used for determination of the relative contributions of apoplastic transport pathways (López-Pérez et al. 2007). Light green SF yellowish moves apoplastically in roots and cannot transported across the cell membranes (Martínez-Ballesta et al. 2003). Detopped root samples were immersed in light green SF yellowish (250 µmol L^{-1}) for 5 min before applying pressure at 0.4 MPa. The xylem exudate was collected for two minutes, and the dye absorbance was detected via a spectrophotometer (Genesys 10 S-UV-VIS, Thermo Scientific, NJ, USA) at 630 nm (Bárzana et al. 2012). The ratio of apoplastic pathway was calculated as the percentage of the dye concentration in the collected xylem exudates of the original solution.

3.2.5 H_2O_2 determination

H_2O_2 concentration was measured in roots of four plants from the control and each treatment group (subjected to hypoxia for three days and following re-aeration for one, three, and six days). Fresh 3-cm distal root segments (0.5 g) were ground in 5 mL of 0.1% ice-cold trichloroacetic acid (TCA). Homogenates were centrifuged at 12,000 g at 4°C for 20 min, and 100 µL of the supernatant was added to the assay solution containing 250 µL of 10 mM potassium phosphate buffer (pH 7.0), 500 µL of 1 M KI and 150 µL of 0.1% (w/v) trichloroacetic acid (TCA). The samples were incubated in the dark on ice for 30 min before their absorbance was measured with a spectrophotometer at 390 nm. The H_2O_2 concentrations were calculated from a standard curve with known concentrations of H_2O_2 (Figure S3.1) (Wang et al. 2008).

3.2.6 Histochemical detection of ROS

DAB (3,3-diaminobenzidine) staining of H_2O_2 was carried out in distal 3-cm root segments from 6-8 plants from each treatment and control to detect H_2O_2 inside and outside of the dead cells (Zidenga et al. 2012). The root segments were immersed in 0.1 mg mL^{-1} solution containing DAB (Sigma-Aldrich) dissolved in 50 mM Tris-acetate buffer (pH 5.0) at 25°C in the dark for 5 h to facilitate DAB uptake and reaction with H_2O_2 (Barba-Espin et al. 2010).

For the detection of $O_2\cdot^-$, distal 3-cm root segments from 6-8 control and treated plants were incubated in 0.5 mM nitroblue tetrazolium (NBT) (Sigma-Aldrich) with 10 mM potassium phosphate buffer (pH 7.8) for 20 min (Liszkay et al. 2004). The samples were then rinsed in ethanol and mounted on microscope slides in water.

The DAB and NBT staining in root tip segments were observed with a ZEISS AXIO compound light microscope (Carl Zeiss, Jena, Germany) equipped with the MacroFire Digital Camera (Optronics, Goleta, CA, USA).

Intracellular ROS staining by CM-H_2DCFDA was carried out according to the method of Xiong et al. (2015) with modifications. Enzymatic deacetylation and subsequent oxidation by H_2O_2 generate 2,7-dichlorofluorescein (DCF), which can be detected as fluorescence (Cárdenas et al. 2008). After three days of hypoxia and one, three, and six days of re-aeration, 3-cm distal parts of root tip segments were excised (n = 6 - 8) and incubated for 30 min in the dark in 10 µM [5-(&-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester] (CM-H_2DCFDA) (Life Technologies, Carlsbad, CA, USA) dissolved in 0.00025% dimethyl sulfoxide. The root segments were then washed three times with distilled water in the dark. Fluorescence was visualized using a laser-scanning confocal microscope (Axio Imager M1, Carl Zeiss, Jena, Germany). The fluorescence signal was collected with excitation and emission wavelengths of 488 and 515 nm, respectively. Image J (https://imagej.nih.gov/) was used to assess the staining pixel intensity of the captured images as ROS level (n = 5 images per group).

3.2.7 Antioxidant enzyme assays

Distal 3 cm root segments from the control and treatment groups (subjected to hypoxia for three days and following re-aeration for one, three, and six days) were frozen at -80°C and grinded in a mortar (n = 6 - 8). For the enzyme assays, 0.15 g samples were homogenized with 1.5 mL of 0.1 M potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and 50 mg polyvinyl pyrrolidine in a chilled mortar kept in the ice bath. The homogenate was centrifuged at 15,000 r.p.m for 15 min at 4°C. The clear supernatant was taken for the enzyme assays (Noctor et al.

2016b). All spectrophotometric analyses were conducted using a UV/visible spectrophotometer (Genesys 10 S-UV-VIS, Thomas Scientific, NJ, USA) using appropriate controls.

Catalase (CAT)

The catalase activity (CAT) was measured by adding 40 µL H_2O_2 to 1760 µL of 0.1 M phosphate buffer, 1 mM EDTA (pH 7.5) gives a final H_2O_2 concentration of 40 mM (A240 = 1.6 for the initial control without extract). Reaction was started by adding 200 µL extract and the decrease in A240 was monitored for 1–2 min (Noctor et al. 2016b).

Glutathione reductase (GR)

Glutathione reductase (GR) activity was determined by adding 20 µL of 10 mM NADPH, 200 µL of extract and 1760 µL of 0.1 M phosphate buffer, 1 mM EDTA (pH 7.5) in cuvette at 25 °C. The reaction was started by adding 20 µL of 50 mM GSSG and the decrease in A340 was monitored for 2–3 min (Noctor et al. 2016b).

Ascorbate peroxidase (APX)

Ascorbate peroxidase (APX) activity was assayed by adding 100 µL of extract and 100 µL of 10 mM ascorbate to 1880 µL of 0.1 M phosphate buffer, 1mM EDTA (pH7.5). The reaction was started by adding 20 µL of 20 mM H_2O_2 and monitor decrease in A290 for 1–2 min. Control without the extract was used to correct for chemical oxidation by H_2O_2 (Noctor et al. 2016b).

Dehydroascorbate reductase (DHAR)

Dehydroascorbate reductase (DHAR) activity was assayed by adding 100 µL 4mM DHA and 50 µL 100 mM GSH to 1810 µL 0.1 M phosphate buffer, 1mM EDTA (pH7.0). The reaction was started by adding 40 µL of extract and monitor decrease in A265 for 2–3 min. Control measurements were performed to correct for chemical reduction of DHA by GSH by and subtracted from the rates obtained with the plant extracts. (Noctor et al. 2016b).

3.2.8 RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR)

To analyze the relative transcript abundance for six *PIP* genes in *B. napus* (Ge et al. 2014): *BnPIP1*;*1* (NCBI accession number KF277205), *BnPIP1*;*2* (KF277206), *BnPIP1*;*3* (KF277207), *BnPIP2*;*2* (KF277209), *BnPIP2*;*5* (KF277210), *BnPIP2*;*7* (KF277211) (primer sequences are shown in Appendix 1), leaves and 3 cm distal root segments from four plants in each group were harvested after plant exposure to hypoxia for three days and re-aeration for one, three, and six days. The samples were frozen immediately in liquid nitrogen and ground with a mortar and pestle. Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA USA) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) synthesis and qRT-PCR were conducted as described (Xu et al. 2015). Relative transcript abundance was calculated with the $2^{-\Delta\Delta Ct}$ method by normalization with the CT value of *BnPIP*s against two reference genes, *BnACTIN7* (AF_111812) and *BnPPR* (XM_013831783). To assess the impact of treatments on transcript abundance of these genes, the cDNA samples of leaves and roots in aerated plants were harvested at the same time points as treated plants.

3.2.9 Water permeability measurements in yeast

For water transport assays, the complete open reading frames of *BnPIP1;1*, *BnPIP1*;*2*, *BnPIP1*;*3*, *BnPIP2*;*2*, *BnPIP2*;*5* and *BnPIP2*;*7* were inserted into the yeast expression vector pAG426GAL-ccdB (http://www.addgene.org/yeast-gateway/), respectively, using the Gateway technology (Invitrogen, Carlsbad, CA, USA) (primer sequences are shown in Appendix 3). The attB-PCR fragment of above BnPIPs was transferred into pDONR221 with BP clonase and shuttled from the entry clones into the yeast expression plasmid pAG426Gal-ccdB-eGFP by Gateway LR cloning reaction to generate C-terminally tagged protein–enhanced green fluorescent protein (eGFP) fusions. The empty vector pAG426GAL - ccdB was used as the mock control. The S.c. EasyComp™ Transformation Kit (ThermoFisher Scientific, Waltham, MA, USA) was used to transform *S. cerevisiae* yeast strain (INVSc1 from ThermoFisher Scientific) with each plasmid DNA. The constructs were verified by sequencing and the localization of BnPIPs in yeast was examined by fluorescent microscopy (Carl Zeiss; Jena, Germany) through GFP filters (Chroma) photographed with the MacroFire Digital Camera (Optronics; Goleta, CA, USA) (Figure S3.2). Transformed yeasts were cultured in glucose containing synthetic complete medium without uracil for 20 h (1.2 g, 30°C). Cultures were adjusted to OD 0.6 at 600 nm, and heterologous protein expression was induced by growing in the medium with replacing the carbon source as galactose for 16 h (1.2 g, 30 °C). Cells were centrifuged (500 × g, 5 min), resuspended in 3 ml of equilibration buffer (50 mM potassium phosphate (pH 7.2) with 40 mM β-mercaptoethanol) and shaken at 30 °C, for 15 min. Six ml of digestion buffer (50 mM potassium phosphate (pH 7.2), 40 mM β-mercaptoethanol, 2.4 M sorbitol, 50 mg/ml bovine serum albumin, 2 mg Zymolyase 20T) was added, the mixture was vortexed and shaken for 60 min at 30 °C. Protoplasts were harvested by centrifugation (1700 g,

3 min), resuspended in buffer (1.8 M sorbitol, 50 mM NaCl, 5 mM $CaCl_2$, 10 mM Tris (pH 8) with HCl) and kept on ice before use.

Volume changes in yeast protoplasts, resulting from transmembrane water transport, were detected by a stopped-flow spectrophotometer at 436 nm with 90° light scattering (model SX18MV‐R; Applied Photophysics, Surrey, UK) as described (Bertl et al. 2007; Navarro‐RóDenas et al. 2015). Briefly, Yeast protoplasts were equilibrated in incubation buffer (1.8 M sorbitol, 50 mM NaCl, 5 mM $CaCl_2$ and 10 mM Tris/HCl, pH 8.0) and water transport was started by mixing the equilibrated protoplast suspension with an equal volume of test solution which had the same ionic composition but a lower osmolarity (1.2 M sorbitol). The signals were fitted by single-phase exponential decay functions using the Sigmaplot 14.0 (Systat Software Inc., Chicago, IL, USA). The osmotic water permeability coefficients (P_f) were calculated according to the equation: $P_f = (K \cdot V_o)/(S \cdot V_w \cdot C_{out})$ where K is the exponential rate constant, V_o is the initial mean vesicle volume determined by the image J software (https://imagej.nih.gov/ij/) (n = 5 cells per group), V_w is the molar volume of water, S is the mean vesicle surface area, and C_{out} is the external osmolarity (Fischer and Kaldenhoff, 2008). The measurements were obtained from 5 biological replicates with an average of 15 measurements for each sample.

3.2.10 Yeast H_2O_2 survival assay

Yeast survival assays were carried out in two independent experiments to examine H_2O_2 permeability of yeast cells overexpressing *BnPIP*s (Di Giorgio et al. 2016). Yeast cells were transformed with *BnPIP*s as described above for the water transport assay. The overexpression and mock strains (with an empty vector) were grown on the galactose-containing synthetic

complete medium without uracil in the presence of 0, 0.5 and 1 mM H_2O_2. Yeast cells which initially grown in liquid YNB medium supplemented with 2% (w/v) glucose were diluted in sterile water to an A600 of 1, 1/10, 1/100, 1/1000, and 5 μL were spotted on the solid medium. After four days of incubation under 30 °C, differences in growth and survival were recorded (Di Giorgio et al. 2016).

3.2.11 Statistical analysis

All data were analyzed using the SPSS 18.0 statistical package (SPSS Inc., Chicago, IL, USA) using one-way ANOVA and Tukey's test ($\alpha = 0.05$) to compare the differences between different treatments. The data that did not meet the ANOVA assumptions of normality of distribution and homogeneity of variance were transformed with a log10 function.

3.3 Results

3.3.1 Plant dry weights and gas exchange

Total dry weights of plants exposed to three days of root hypoxia were lower by approximately 40% compared with the aerated control group, but there was no difference between the dry weights of re-aerated and the control plants (Figure 3.1a). Net photosynthesis (P_n) and transpiration (E) rates followed similar patterns and were lower in plants exposed to hypoxia compared with aerated control (Figure 3.1b, c). Following re-aeration, both P_n and E remained lower after one day of re-aeration and went gradually back to the control level on days three and six (Figure 3.1b, c).

3.3.2 Water relations

Leaf water potentials (Ψ_{leaf}) were sharply reduced by hypoxia and slowly increased following re-aeration (Figure 3.2a). However, Ψ_{leaf} did not fully recover after six days of re-aeration (Figure 2a). Shoot water contents were markedly lower following re-aeration for one day compared with aerated control in plants (Figure 3.2b). There were no significant differences in shoot water contents between the control plants and the plants subjected to three days of hypoxia and following re-aeration for three and six days (Figure 3.2b). The root hydraulic conductivity (L_{pr}) was reduced by the hypoxia treatment and it further decreased by about five-fold compared with control plants following one day of re-aeration (Figure 3.2c). After three and six days of re-aeration, there were no significant differences in L_{pr} between the re-aerated and control plants (Figure 3.2c). The light green SF yellowish dye concentration in xylem exudates collected from plants subjected to hypoxia was about three-fold lower compared with aerated control (Figure 3.2d). However, following one day of re-aeration, the dye concentration was about two-fold higher than in control before it declined again below the control level after three and six days of re-aeration (Figure 3.2d).

3.3.3 ROS in roots

To assess the oxidative burst upon re-aeration following hypoxia conditions, the H_2O_2 distribution inside and outside of the dead cells of distal root segments was examined microscopically after staining with DAB. Brown staining, indicative of H_2O_2 accumulation, was observed mainly close to the root apical parts of the control and increased in intensity in plants exposed to hypoxia (Figure 3.3a). Following re-aeration, brown coloration extended through the whole distal segment of the root, with the highest intensity observed after one day of re-aeration

(Figure 3a). The pixel intensity of DAB staining showed the highest accumulation of H_2O_2 in the roots of hypoxic plants following one day of re-aeration (Figure 3.3d).

Accumulation of ROS in distal root segments was visualized by the CM-H_2DCFDA staining (Figure 3.3b). The fluorescence intensity was unaffected by the hypoxia treatment (Figure 3.3b). However, increased fluorescence intensity was noticeable in the re-aeration treatment, with maximum intensity observed in the roots of plants re-aerated for one day (Figure 3.3b), which was also confirmed by the pixel intensity of fluorescence (Figure 3.3e).

NBT staining intensity was examined in the distal root segments to detect superoxide accumulation. High purple staining intensity due to the presence of formazan was observed in the roots of plants treated with hypoxia and following re-aeration, with the highest intensity indicating superoxide accumulation observed in plants after one day of re-aeration (Figure 3.3c). This was also confirmed by the staining pixel intensity of the captured microscope images (Figure 3.3f).

The H_2O_2 content in the distal root segment was similar in control and hypoxia treatment and sharply increased after one day of re-aeration (Figure 3.3g). The H_2O_2 concentrations in roots returned to the control level after three and six days of re-aeration (Figure 3.3g).

3.3.4 Antioxidant enzyme activities in roots

The activities of enzymatic ROS scavengers, including CAT, GR APX and DHAR were measured in roots of aerated plants and in plants exposed to hypoxia and re-aeration treatments. Compared with the aerated plants, the activity of CAT increased in plants hypoxia for three days and re-aerated for one day following hypoxia (Figure 3.4a). GR activity

gradually increased upon hypoxia and peaked in three days of re-aerated plants (Figure 3.4b) while APX activity significantly increased in plants exposed to three days of re-aeration compared with control (Figure 3.4c). In contrast, the highest DHAR activities were measured following one day of re-aeration (Figure 3.4d).

3.3.5 Aquaporin profiling

To investigate the expression patterns of aquaporins under hypoxia and re-aeration conditions, the transcript abundance of six *BnPIP*s was examined in leaves and roots of aerated (control) plants, in plants subjected to three days of hypoxia and following one, three, and six days of re-aeration. In the leaves of plants exposed to hypoxia, the transcript abundance of *BnPIP2*;*5* decreased by over three-fold and *BnPIP2*;*7* decreased by 50% compared with the control group (Figure 3.5d, f). After one day of re-aeration, leaf *BnPIP1*;*3* sharply increased, whereas leaf *BnPIP2*s showed marked downregulation (Figure 3.5c-f). After three days of re-aeration, transcript abundance of *BnPIP1*;*1* significantly increased in the leaves and after six days of re-aeration, all *BnPIPs* significantly increased with the exception of *BnPIP1*;*3* (Figure 3.5a-f).

In roots, transcript abundance of *BnPIP1*;*2*, *BnPIP1*;*3*, *BnPIP2*;*2* and *BnPIP2*;*7* increased whereas transcript abundance of *BnPIP2*;*5* decreased in response to three days of hypoxia (Figure 3.6b, c, d, f). After one day of re-aeration, root *BnPIP2*s were all significantly downregulated compared with control plants and showed a similar pattern to the leaves (Figure 3.6d-f). After three days of re-aeration, the relative expression levels of all examined root *BnPIP1*s were similar or lower compared with aerated control and *BnPIP2*s were upregulated (Figure 3.6a-f). The transcript abundance of examined root *BnPIP1*s, *BnPIP2*;*2* and *BnPIP2*;*5*

were significantly elevated upon re-aeration for six days whereas *BnPIP*2;2 and *BnPIP2*;*7* were not significantly different from control (Figure 3.6a-f).

3.3.6 Water permeability of *BnPIP*s expressed in yeast

To examine the water permeability ability of *BnPIP*s, we expressed the six *BnPIP* genes into yeast cells. The rates of volume increase of yeast protoplasts were followed by the decrease of scattered light intensity in the stopped flow spectrophotometer (Figure S3.3). The yeast protoplasts expressing *BnPIP*s displayed a significantly higher osmotic water permeability coefficient (P_f) compared with the mock control. The P_f in yeast strains expressing *BnPIP1*s was significantly lower than that of *BnPIP2*s (Figure 3.7). The protoplasts expressing *BnPIP2*;*5* had the highest P_f, although no significant differences were detected between *BnPIP2*;*2*, *BnPIP2*;*5* and *BnPIP2*;*7* (Figure 3.7).

3.3.7 Yeast survival assay of *BnPIP* overexpression strains in H_2O_2 media

To determine the sensitivity of yeast strains overexpressing different *BnPIP*s to H_2O_2, the survival assays were performed. The number of colonies reflects the number of surviving cells after the treatment. The results demonstrated that the yeast cells transformed with *BnPIP1;2* and *BnPIP1;3* displayed the lowest survival in the 1 mM H_2O_2 medium of all of the examined strains (Figure 3.8), indicating increased H_2O_2 uptake from the external medium. Little or no effects of H_2O_2 on the growth and viability of yeast cells were observed in the *BnPIP2* overexpressing strains compared with the mock strain (Figure 3.8).

3.4 Discussion

Oxidative stress is frequently observed upon re-aeration following waterlogging (Yuan et al. 2017; Yeung et al. 2018; Lee et al. 2019). Plant recovery from waterlogging requires a rapid resumption of efficient root water transport that is depressed due to effects of hypoxia on root aquaporins (Tournaire-Roux et al. 2003; Liu et al. 2020). Our study demonstrated that in waterlogging sensitive *B. napus* plants, hydraulic recovery from waterlogging was affected by oxidative burst. The re-aeration of plants after one day was even more detrimental to L_{pr} than the three days of waterlogging to which the plants had been earlier subjected.

Similarly to the findings reported for canola and other waterlogging sensitive plants (Shahzad et al. 2016; Tan et al. 2019; Liu et al. 2020), in our study, root hypoxia triggered reductions in plant growth, gas exchange, leaf water potentials, and shoot water contents due to low oxygen availability, Following re-aeration, P_n and E rapidly recovered, whereas Ψ_{leaf} and L_{pr} showed delayed recovery. Additionally, the re-aeration process resulted in transient oxidative stress due to a sharp increase in ROS in roots, especially one day following re-aeration. It can be concluded that oxidative stress was an important determinant in plant hydraulic recovery.

To assess the extent of ROS accumulation in plant roots subjected to three days of hypoxia and following one, three, and six days of re-aeration, H_2O_2 content measurements and ROS staining were carried out in distal root segments. ROS accumulation was slightly enhanced after three days of hypoxia and sharply increased one day after re-aeration, followed by a progressive decline. A similar transient oxidative burst was previously reported for *Arabidopsis* (Yeung et al. 2018) and rice (Fukao et al. 2011). When dissolved oxygen concentration suddenly increases after hypoxia, the photosynthetic and mitochondrial electron transport systems are reactivated

and this may render excessive electron and proton leakage leading to ROS overproduction posing additional challenges to plants (Voesenek et al. 2015). Plants develop various scavenging mechanisms that counteract the effects of excessive generation of ROS (Foyer et al. 2005). In our study, hypoxia and re-aeration treatments resulted in the upregulation of the antioxidant enzymes. The CAT, GR, APX, and DHAR enzyme activities increased in the distal root segments, especially after one and three days of re-aeration, to maintain the ROS homeostasis, and their activities followed a similar pattern as ROS accumulation.

Notably, a significant decline of L_{pr} was detected during hydraulic recovery that was accompanied by an increase in the concentration of light green SF yellowish in the collected sap and which indicated an increased contribution of the apoplastic pathway to water flow (Steudle, 2001). According to the composite transport model, radial water transport in roots follows a combination of apoplastic and aquaporin-mediated cell-to-cell pathways (Steudle and Peterson 1998; Steudle 2001). It was demonstrated that root hypoxia inhibited apoplastic transport in canola under prolonged waterlogging (Liu et al. 2020). Apoplastic tracer dyes have been successfully used to detect changes in root water flow pathways (Voicu et al. 2004). However, due to their relatively larger size, the results can only be interpreted as relative changes in the transport pathways rather than a direct measurement of apoplastic flow (Voicu et al. 2009). The increase in apoplastic contribution to water flow and the decrease of L_{pr} point to the inhibition of cell-to-cell water transport, which is mainly regulated by aquaporins (Liu et al. 2020; Maurel et al. 2020). Since ROS are strong inhibitors of root water transport (Ye et al. 2006) and the re-aeration treatment resulted in a significant accumulation of H_2O_2 and other ROS in roots, we interpreted the inhibition of L_{pr} to be due to the effects of ROS on root hydraulic recovery. As reported in the leaves of rice and *Arabidopsis* (Alpuerto et al. 2016; Yeung et al. 2018), we also

observed a transient decline in shoot water contents and leaf water potential on one day of re-aeration, as a consequence of the reduced capacity of roots to transport water.

To explain the observed inhibition of L_{pr} and changes in the relative contribution to the water flow by hypoxia and re-aeration, I further examined the effects of these two factors on the expression of the several principal *Bn*PIP aquaporins in leaves and roots. Leaves are considered the a significant source of ROS in plants (Foyer et al. 2005) and are formed in the reaction centers of PSI and PSII in the thylakoids. The results demonstrated that the inhibition of L_{pr} and the increased contribution of apoplastic water transport were accompanied by changes in *BnPIP*s expression. All examined *BnPIP2*s were downregulated in both roots and leaves on day one of re-aeration. The downregulation of these aquaporin genes was accompanied by a decline in shoot water contents, likely triggered by the ROS burst. Exogenous applications of H_2O_2 caused a significant inhibition of L_{pr} in sugarcane (Silva et al. 2015) and *Arabidopsis* (Boursiac et al. 2008) and was also accompanied by changes in the aquaporin expression. It has been previously proposed that OH·, rather than H_2O_2, may cause the oxidation of aquaporins (Ye et al. 2006). It is plausible that OH· directly oxidizes amino acid residues such as the cysteine or (and) that C=C double bonds of the plasma membrane are targeted by OH·, resulting in a formation of aggressive radicals that, in turn, target aquaporins. In both cases, conformational changes of the aquaporins could result in channel closure and a subsequent decrease of L_{pr} (Henzler et al. 2004; Ye et al. 2006). In our study, after the declines on one day of re-aeration, transcript levels of most examined *BnPIP*s started increasing on three days following re-aeration, at the time when ROS levels decreased and L_{pr} recovered to the values measured in control plants. These events were accompanied by a recovery of growth processes and, likely, by an increased uptake of nutrients, including phosphorus that is required for PIP phosphorylation (Bramley et al. 2009).

However, the effects of ROS and mineral nutrition on aquaporin gating during plant hydraulic recovery from hypoxia still remain to be addressed in future studies.

Our results demonstrated that all three examined *Bn*PIP2s similarly increased water transport in yeast protoplasts, while *Bn*PIP1s had only a moderate effect when expressed in yeast. PIP2s are the main water transporters on the plasma membranes (Chaumont et al. 2000; Li et al. 2013), but the water transport properties of different PIP2s within a plant species usually show large differences (Almeida Rodriguez et al. 2010; Groszmann et al. 2017). Interestingly, different *BnPIP*s responded differently to re-aeration. Although one day of re-aeration suppressed the expression levels of most of the *BnPIP*s, in our study, *BnPIP1*;*3* in leaves and *BnPIP1*;*2* in roots were upregulated. Since some of the plant aquaporins transport H_2O_2 (Bienert et al. 2007; Tian et al. 2016; Rodrigues et al. 2017), we carried out the growth assay of yeast strains overexpressing different canola aquaporins. The H_2O_2 growth assay showed that the growth of *BnPIP1*;*2* and *BnPIP1*;*3* overexpression yeast strain was markedly reduced in the medium with H_2O_2, indicating that these two aquaporins are likely involved in H_2O_2 transport. Since the expression levels of *BnPIP1*;*2* and *BnPIP1*;*3* increased in canola during the re-aeration stage, we interpreted this increase as an important mechanism of ROS detoxification contributing to a successful plant recovery from waterlogging.

3.5 Conclusions

In conclusion, our study provides a detailed mechanistic understanding of plant hydraulic recovery following hypoxia. The results demonstrated that root re-aeration of *B. napus* following three days of hypoxia had an inhibitory effect on L_{pr} and increased the proportion of apoplastic water flow. This transient effect was accompanied by an oxidative burst and an increase in the

antioxidant enzymatic activity. The transcript abundance of the examined *BnPIP2*s that are involved in fast water transport decreased, suggesting that the reduced gene transcription of these aquaporins contributed to the decrease in cell-to-cell water transport shortly after the re-aeration. The yeast survival assay demonstrated that *Bn*PIP1;2 and *Bn*PIP1;3 likely facilitate H_2O_2 transport, and the increase in gene expression of these aquaporins during re-aeration was likely crucial to plant waterlogging recovery. Plant growth, gas exchange, leaf water potentials, and shoot water contents were reduced by the hypoxia treatment. Upon re-aeration, P_n and E recovered rapidly whereas Ψ_{leaf} and L_{pr} recovered relatively more slowly. These results confirm our hypothesis that rapid ROS accumulation following hypoxia is a major factor hindering the recovery of plants from hypoxia stress through its effects on root hydraulics.

3.6 References

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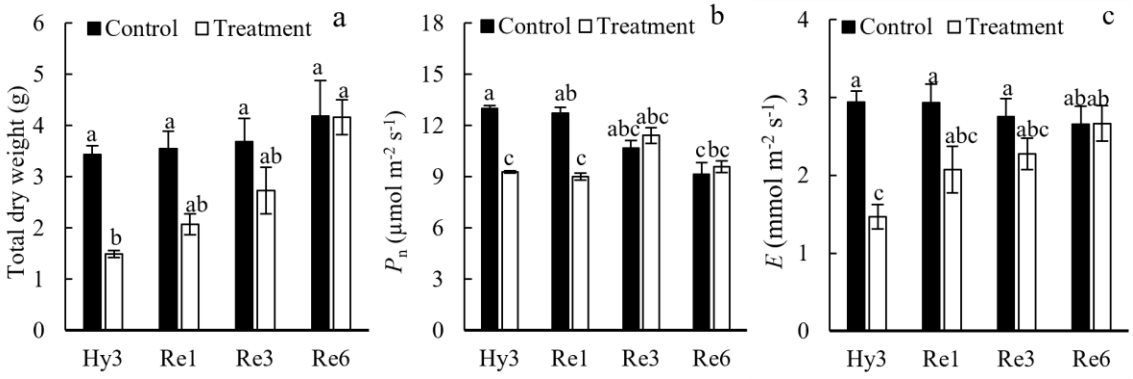

Figure 3.1 Dry weights (a), net photosynthesis (P_n, b), and transpiration (E, c) rates of aerated plants (Control), after three days of hypoxia (Hy3), and following re-aeration for one (Re1),

three (Re3), and six (Re6) days. Means ± SE are shown (n = 6 - 8). Values with different letters are significantly different at $P \leq 0.05$, as determined by ANOVA, Tukey's test.

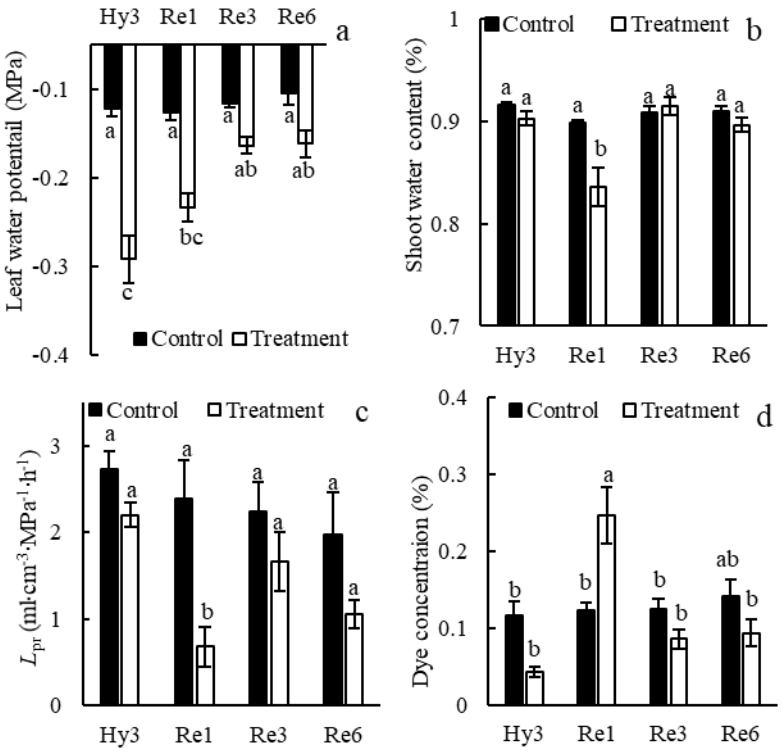

Figure 3.2 Leaf water potentials (a), shoot water contents (b), root hydraulic conductivity (L_{pr}) (c) and the light green SF yellowish dye concentration (d) in aerated plants (Control), after three days of hypoxia (Hy3) ,and following re-aeration for one (Re1), three (Re3), and six (Re6) days. Means ± SE are shown (n = 6 - 8). Values with different letters are significantly different at $P \leq 0.05$, as determined by ANOVA, Tukey's test.

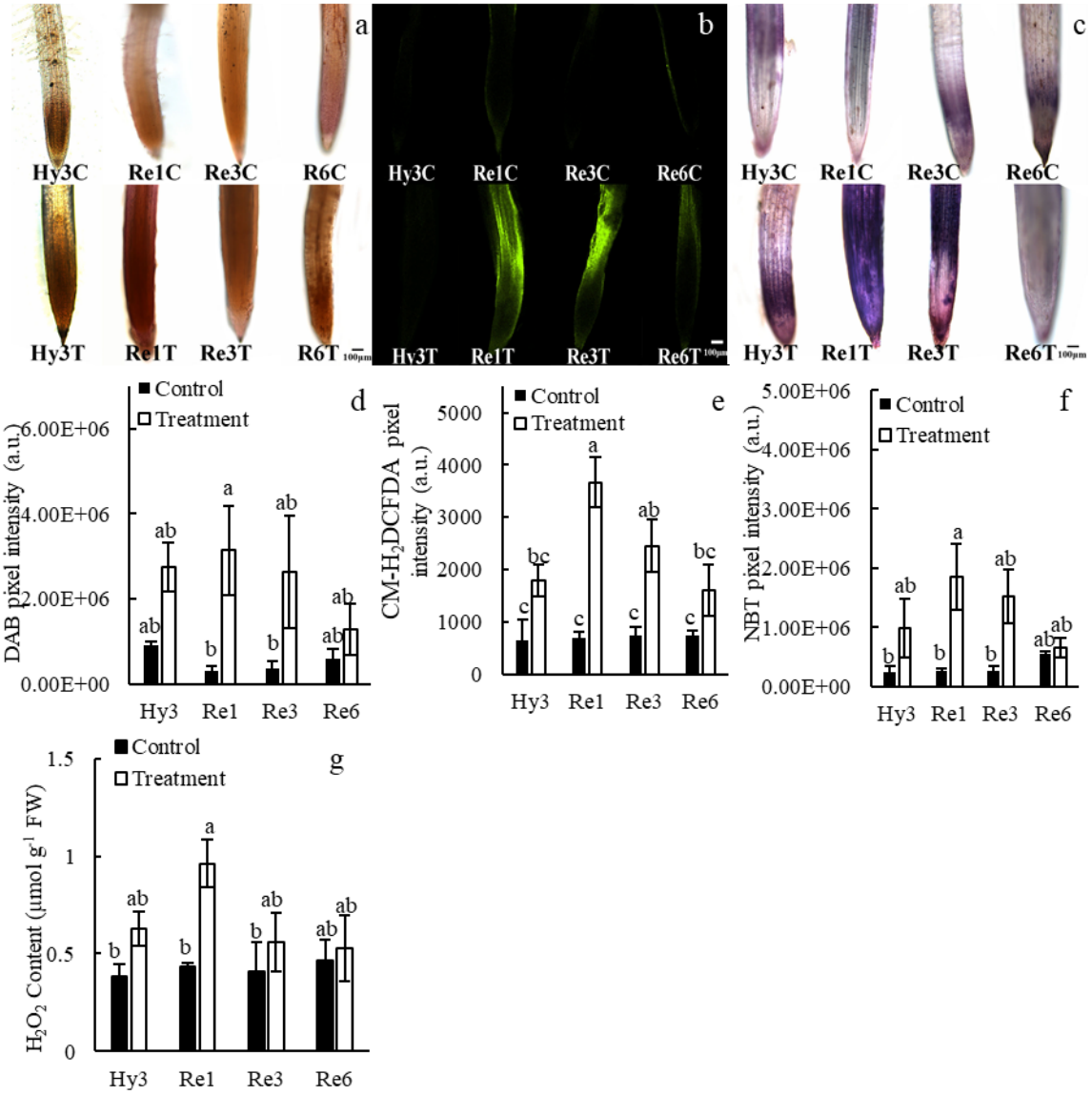

Figure 3.3 Distal root segments, which were exposed to three days of hypoxia (Hy3T) followed by re-aeration for one, three, and six days (Re1T, Re3T, Re6T), and aerated controls (Hy3C, Re1C, Re3C, Re6C). The roots were stained with DAB (A), CM-H_2DCFDA (B) and NBT (C), and examined microscopically. Bar = 100 µm. The staining pixel intensity of DAB (D), CM-H_2DCFDA (E), NBT (F) and root H_2O_2 content (G) in in aerated plants (Control), and in plants subjected to hypoxia for three days (Hy3) and re-aerated for one (Re1), three (Re3), and six

(Re6) days following hypoxia (Treatment). Means ± SE are shown (n = 6 - 8). Values with different letters are significantly different at $P \leq 0.05$, as determined by ANOVA, Tukey's test.

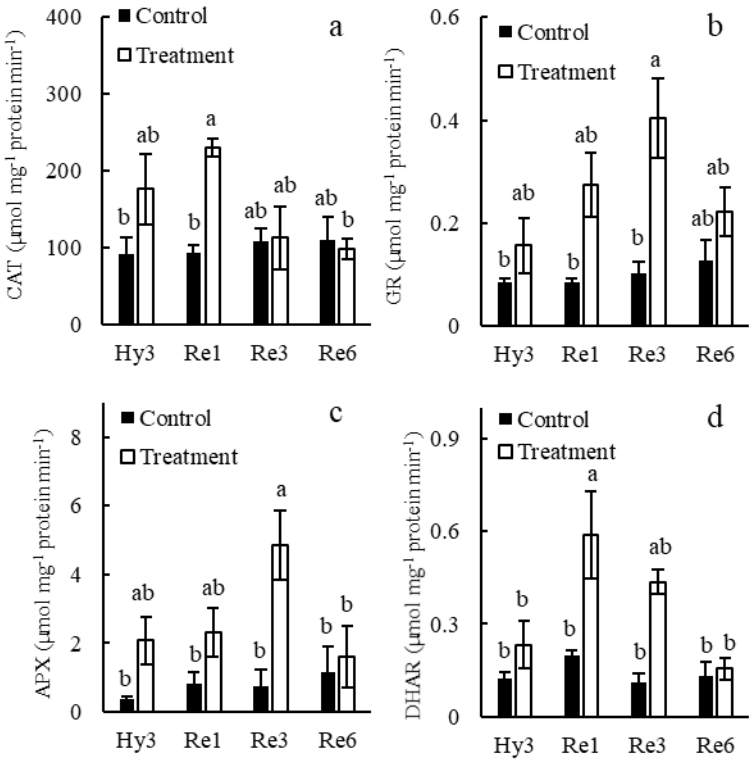

Figure 3.4 The antioxidant enzyme activities in distal root segments of aerated plants (Control), after three days of hypoxia (Hy3), and following re-aeration for one (Re1), three (Re3), and six (Re6) days. Means ± SE are shown (n = 6 - 8). Values with different letters are significantly different at $P \leq 0.05$, as determined by ANOVA, Tukey's test.

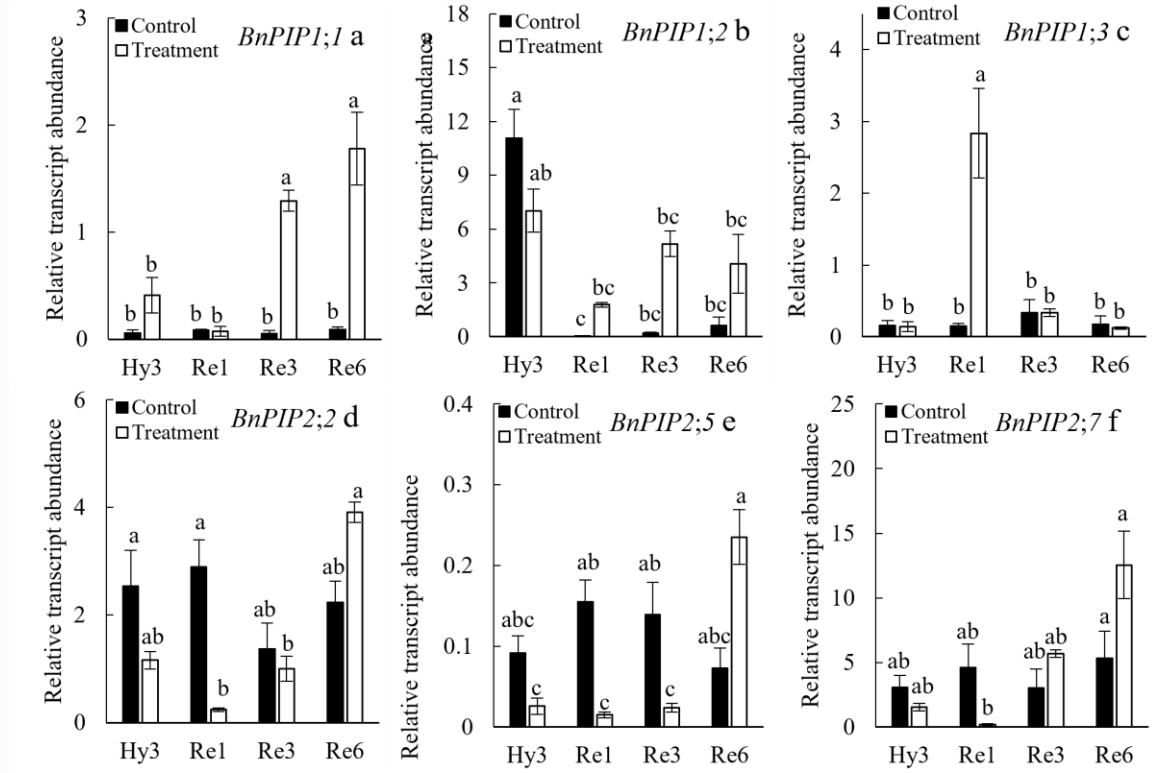

Figure 3.5 The *BnPIP*s relative transcript abundance in leaves of aerated plants (Control), after three days of hypoxia (Hy3) and following re-aeration for one (Re1), three (Re3), and six (Re6) days. Means ± SE are shown (n = 4). Values with different letters are significantly different at $P \leq 0.05$, as determined by ANOVA, Tukey's test.

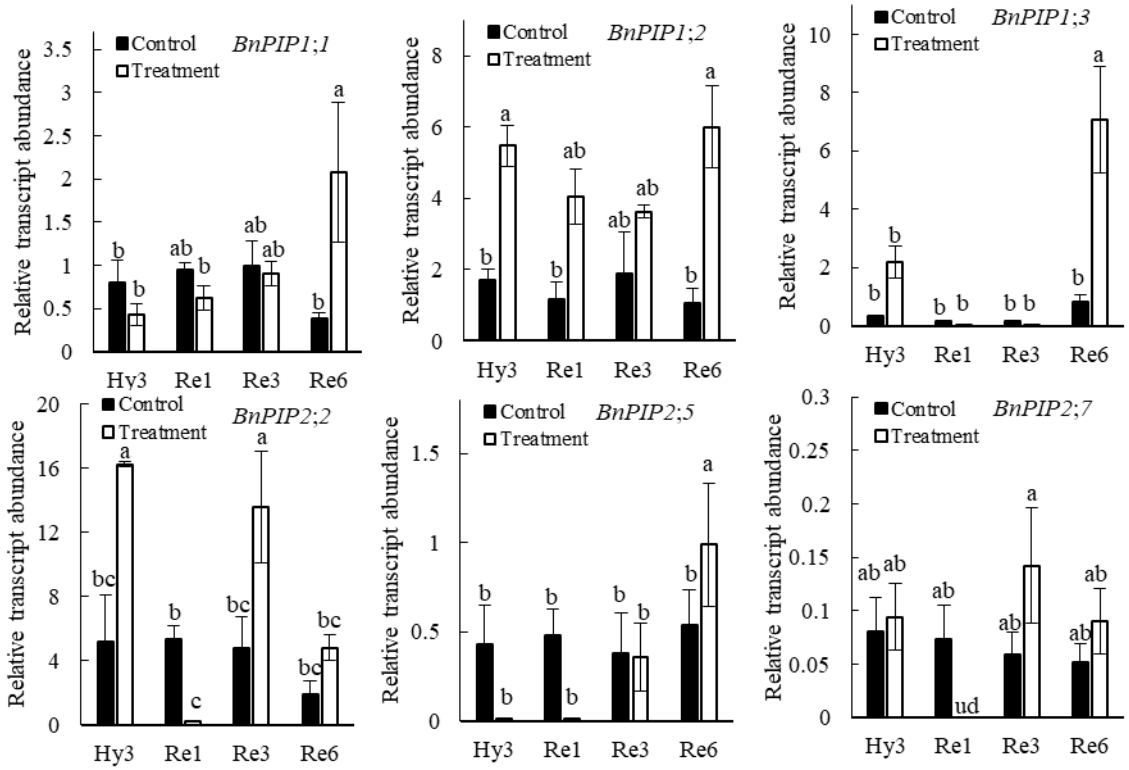

Figure 3.6 The *BnPIP*s relative transcript abundance in roots of aerated plants (Control), after three days of hypoxia (Hy3) and following re-aeration for one (Re1), three (Re3), and six (Re6) days. Means ± SE are shown (n = 4); ud, undetected. Values with different letters are significantly different at $P \leq 0.05$, as determined by ANOVA, Tukey's test.

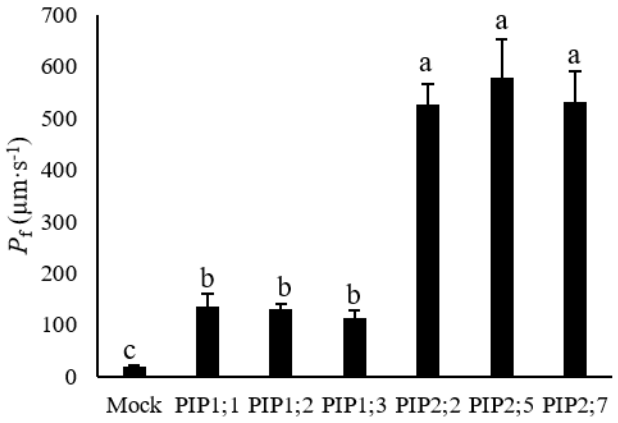

Figure 3.7 Osmotic water permeability coefficients (P_f) of *S. cerevisiae* cells expressing *BnPIP*s and mock control. Means ± SE are shown (n = 5). Values with different letters are significantly different at $P \leq 0.05$, as determined by ANOVA, Tukey's test.

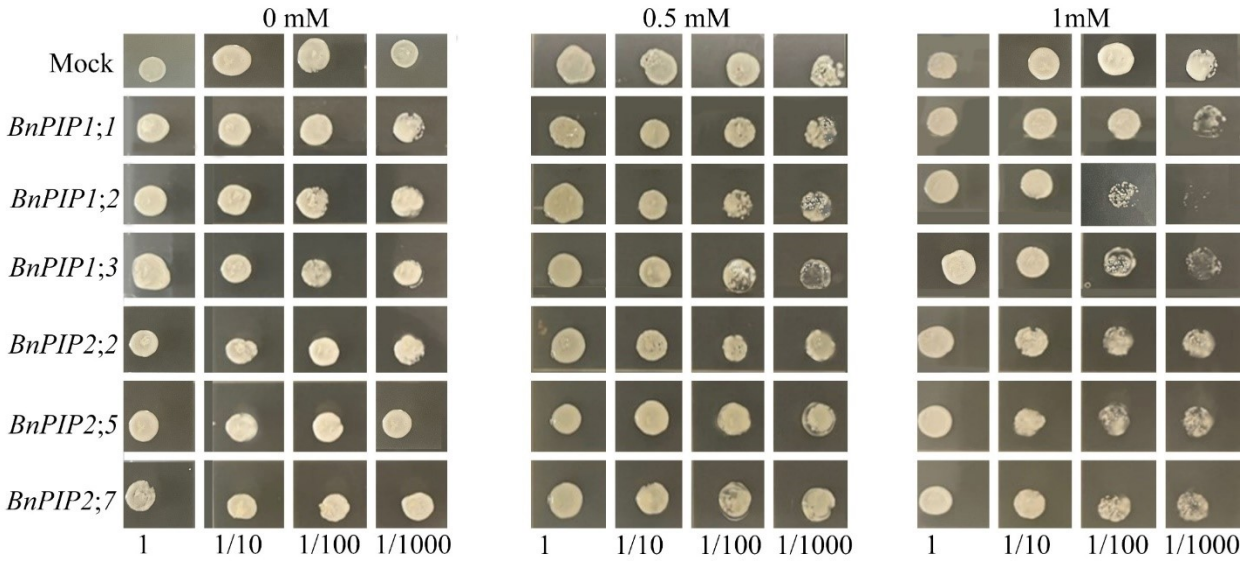

Figure 3.8 Hydrogen peroxide survival assays of *S. cerevisiae* cells expressing different *BnPIP*s and mock control. Serial dilutions (1, 1/10, 1/100 and 1/1000) of *S. cerevisiae* strains expressing *BnPIP*s were spotted onto medium containing the indicated concentration of H_2O_2. Cell survival was recorded after four days. Photos shown are representative of three independent experiments with consistent results.

Chapter 4. Overexpression of *NtPIP1;3* aquaporin in canola provides evidence for functional importance of aquaporins in oxygen transport

4.1 Introduction

Efficient delivery of oxygen is essential to all aerobic organisms for their survival. Plants have no metabolically activated oxygen dispersal mechanisms other than cytoplasmic streaming, which is ineffective for long-distance oxygen transport (Denison 1992). Low oxygen concentration in the root zone (root hypoxia) is the main factor affecting the survival and growth of plants in waterlogged soils due to the low oxygen dissolving capacity of water (Bailey-Serres and Voesenek 2008). While some wetland plants have developed anatomical and morphological adaptations to improve root aeration (Colmer and Greenway 2011), most of the terrestrial plant species cannot supply a sufficient amount of oxygen to waterlogged roots to support their aerobic respiration (Bailey-Serres et al. 2012). For these plants, oxygen diffusion across the tissues from shoots to roots or directly from the water to roots is too slow to meet their respiratory demands (Armstrong and Armstrong 2014).

Facilitated transport of oxygen has been demonstrated for several human (Ivanov et al. 2004; Nakhoul et al. 1998), mice (Al-Samir et al. 2016) and plant (Zwiazek et al. 2017) aquaporins. However, similarly to the views concerning water transport that had prevailed prior to the discovery of aquaporins, the notion of functional significance of facilitated gas diffusion across the cell membranes has not received overwhelming support since the existing models show

relatively high gas permeability of the lipid bilayer (Ivanov et al. 2004; Missner et al. 2008). Nevertheless, increasing recent evidence points to possible importance of aquaporins in the transport of gas molecules including CO_2 and O_2 (Ivanov et al. 2004; Kaldenhoff et al. 2014; Zwiazek et al. 2017), in addition to water (Agre and Kozono 2003), small neutral molecules (Beitz et al. 2006) and some ions (Tyerman et al. 2021).

Hypoxia stress affects the oxygen-dependent process in cells, including respiration. Respiration is an important process that releases energy from organic molecules to sustain cellular activity. The respiration rate is mostly dependent on cellular oxygen availability (Wagner et al. 2018). Additionally, compelling evidence has demonstrated that metabolites involved in the tricarboxylic (TCA) acid cycle are the most limiting factors for the ATP synthesis. Under hypoxia stress, maintaining the glycolytic pathway and pyruvate metabolism is essential to plant survival since under these conditions, a major energy source is fermentative metabolism (Rocha et al. 2010).

In our earlier study (Zwiazek et al. 2017), we demonstrated that the tobacco aquaporin *Nt*PIP1;3 increased oxygen uptake when expressed in yeast and was likely a contributing factor to waterlogging tolerance in tobacco plants. To provide unequivocal evidence for the functional significance of *Nt*PIP1;3 in waterlogging tolerance through its oxygen transport properties, in the present study, we constitutively expressed this aquaporin in canola (*Brassica napus*) plants, which are relatively sensitive to waterlogging (Liu et al. 2020a). Canola plants do not produce aerenchyma and exhibit a high rate of radial oxygen loss from the root base upon root hypoxia stress (Voesenek et al. 1999; Xu et al. 2015b). In the present study, we compared the responses of two *NtPIP1*;*3* overexpression (OE) canola lines and the wild-type (WT) plants to well-aerated and hypoxic (waterlogging) conditions in hydroponic culture. We examined the hypothesis that

the overexpression of *NtPIP1*;*3* in canola would increase oxygen delivery to roots and improve waterlogging tolerance of plants as evidenced by higher levels of aerobic respiration, improved physiological performance and higher plant growth rates compared with the WT plants.

4.2 Materials and Methods

4.2.1 Plasmid construction

The sequence information of *NtPIP1*;*3* (GenBank accession: NM_001325293) was obtained from the NCBI (National Center for Biotechnology Information). The open reading frame encoding *NtPIP1*;*3* was amplified from cDNA of tobacco leaves. The PCR products were then cloned into the Gateway® pCR8/GW/TOPO vector (Invitrogen, Carlsbad, CA) with the addition of the Gateway recombination site attB. The PCR product was then introduced in the destination vector pBRACT114 (John Innes Centre) through the LR reaction. The expression of the open reading frame was driven by a CAMV 35S promoter. Sequences were confirmed by the Sanger sequencing method. Constructs were confirmed by PCR and DNA sequencing analysis.

4.2.2 Generation of transgenic plants

All constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 (Dai et al. 2020) by heat shock which were then used for the transformation. Roots of five-days-old canola (*Brassica napus* cv Westar) seedlings were removed by a razor and the aerial part was transferred on the co-cultivation medium (Murashige and Skoog (MS) medium with 0.1 mg L^{-1} NAA, 2 mg L^{-1} BAP, 0.5 mg L^{-1} $AgNO_3$) for 24 h and then move into the callus induction medium (MS with 0.1 mg L^{-1} NAA, 2 mg L^{-1} BAP, 0.5 mg L^{-1} $AgNO_3$, 500 mg L^{-1} carbenicillin). After eight to ten weeks, the survivals the viable calli were transferred to the root

regeneration medium (MS with 2 mg L^{-1} BAP, 0.5 mg L^{-1} $AgNO_3$, 500 mg L^{-1} carbenicillin, 50 mg L^{-1} kanamycin) by inserting the hypocotyl tips into the medium. The culture continued in a plant tissue culture room at 24°C and 400 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) with 16 h photoperiod for additional three to four weeks until the new roots were formed. The transformed plantlets were then transferred into the soil in a growth chamber (16 h photoperiod, 22/18°C (day/night) temperature, 400 μmol m^{-2} s^{-1} PPFD, and 50-60% relative humidity), and the first pairs of true green leaves were harvested for the PCR detection of the transgene using the NpII specific primers.

Relative transcript abundance of *NtPIP1*;*3* in leaves of transgenic canola was quantified using the standard curve method of quantification, as previously described (Xu et al. 2015a). Relative transcript abundance was calculated. The $2^{-\Delta Ct}$ method was used to analyze the relative expression using *BnPPR* (XM_013831783) and *BnACTIN7* (AF_111812) as the internal control. Primer sequences are in Appendix 4. Four independent biological replicates and three technical replicates were used in this experiment.

Seeds were harvested and plated on the kanamycin (50 mg L^{-1}) selection medium to identify transgenic plants. Two independent homozygous OE lines (OE3 and OE11) with high *NtPIP1*;*3* expression levels were selected in the T2 generation for our current study.

4.2.3 Plant material and growth conditions

Seeds of wild-type, OE3 and OE11 T2 generation canola (*Brassica napus* cv Westar) were surface-sterilized (70% ethanol for 2 min followed by 20% (v/v) commercial bleach (1% [v/v] sodium hypochlorite, 30 min) and germinated in Petri dishes with half-strength MS medium at

24°C. After germination, the seedlings were moved into autoclaved peat moss/vermiculite (2:1) in sterilized 1 L (10 × 10 × 10 cm) plastic pots. Forty seedlings of each line (WT, OE3 and OE11) were grown in the growth chamber with the same conditions as mentioned above. The seedlings were watered every other day and fertilized with modified Hoagland's mineral solution every week (Epstein 1972).

When four-week-old, the plants were gently moved to the aerated hydroponic culture with 50% Hoagland's solution in eight 40 L plastic tubs (60×40×20 cm) and aerated to maintain a dissolved oxygen level of about 8 mg L^{-1}. After one week, half of the plants in the four tubs were randomly selected and subjected to the root hypoxia treatment by flushing nitrogen gas through the hydroponic solution to obtain oxygen concentration of about 2 mg L^{-1}. The second half of the plants remained well-aerated and served as control. For each group, six to eight plants were randomly picked from different tubs for measurements and harvested. Wild-type and transformed plants were randomly distributed in each tub.

4.2.4 Determination of gas exchange, leaf water potential, and plant dry weights

After three and eight days of root hypoxia treatment, net photosynthetic (P_n) and transpiration (E) rates were measured in three leaves of each of the 6 - 8 plants per treatment by a portable infrared gas analyzers with red/blue light source LI-6400XT (LiCor, Lincoln, NE, USA). The reference CO_2 concentration (400 μmol mol^{-1}), the flow rate (200 μmol s^{-1}), the leaf chamber temperature (20 °C) and PPFD (400 μmol m^{-2} s^{-1}) were set. The mean of each treatment were calculated from three leaves from each six to eight plants (n = 6 - 8). The measurements were conducted from 11:00 am to 13:00 pm. Leaves were subsequently excised and placed in a Scholander pressure chamber (PMS instruments; Corvallis, OR, USA) for mid-day leaf water

potential (Ψ_{leaf}) determination (Wan and Zwiazek 1999) (n = 6 - 8). Plant samples were dried at 80°C in an oven for 48 h. Dry weights were obtained from an average of six to eight plants (n = 6 - 8).

4.2.5 Root hydraulic conductivity

To compare the root hydraulic responses in wild-type and OE plants upon hypoxia, the root hydraulic conductivity (L_{pr}) was detected with the hydrostatic method (Wan and Zwiazek 1999) in WT and OE plants subjected to root hypoxia treatment for three and eight days and in control plants (n = 6 - 8). The plants were detopped and fixed in a Scholander pressure chamber (PMS Instrument, St. Albany, OR, USA). Pressure was applied from 0.3 MPa to 0.6 MPa at an interval of 0.1 MPa and the exuded xylem sap was collected with a micropipette. K_r was calculated from the slope of the linear regression between applied pressure and flow rate. Root hydraulic conductivity (L_{pr}) was obtained by dividing the K_r by root volume.

4.2.6 Root oxygen uptake

Roots from 5 - 8 plants per treatment (n = 5 - 8) were excised from wild-type and transgenic plants that subjected to root hypoxia for three and eight days and their distal 50 mm segments immediately placed in a cylinder containing aerated (O_2 level of ~ 8 mg L^{-1}) or stagnant (O_2 level of ~ 2 mg L^{-1}) hydroponic solution. Oxygen uptake by the root segments was determined by hmeasuring the dissolved oxygen concentrations in solution at 22ºC with the oxygen microsensor with a tip diameter of 50 μm (OX50, Unisense, Aarhus, Denmark). The signals were collected by a computer software (Sensortrace.PRO V3.1.3, Unisense, Denmark). Calibration was carried out before the measurements using zero O_2 (2 g ascorbate in 100 ml deionized (DI) H_2O in 0.1 N

NaOH) and O_2-saturated solution (257.9 µmol O_2 l^{-1} at 25°C, 20.6 kPa O_2). The oxygen concentration in the solution was continuously measured and the signal from the OXY meter (A/D converter, Unisense, Aarhus, Denmark) was recorded at 0, 5, 10, 20, 30, 40 min after placing the root segment in the medium. Root respiration rates were calculated based on the oxygen concentrations changes in the aerated and stagnant solutions over the initial 20 minutes. Fresh weights of root segments were determined by weighing following the measurements.

4.2.7 ATP determination

Root ATP concentrations were determined in WT and OE plants after three and eight days of hypoxia and in aerated plants (n=6 - 8). Distal root segments were ground on ice. Then samples (50 mg) were immediately placed with trichloroacetic acid (TCA, 600 µL, ice-cold, 5%) solution in 2 mL centrifuge tubes with vortex. Samples were put on ice for 10 min before centrifugation (10,000×g, 4°C, 10 min). Each 400 µL of supernatant was collected and added tris-acetate buffer (400 µL, pH 7.75, 1M). Then the mixture (4 µL) was added into 96 µL of ATP-free water into a well of the 96-well plate (Costar, Sigma-Aldrich, Oakville, ON, Canada). To quantify ATP concentration, luciferase/luciferin solution (50 µL) from ENLITEN ATP Assay Kit (Promega, Madison, WI, USA) was pipetted into each well, and the standard curve was developed following the manufacturer's instructions. A Microplate reader was used to detect bioluminescence signal (Fluostar Optima, BMG Labtech, Ortenberg, Germany) (Zwiazek et al. 2017).

4.2.8 Metabolic analyses

To evaluate the changes of metabolites in wild-type and OE plants under hypoxic conditions, metabolic levels were determined by a combination of direct injection mass spectrometry with a reverse-phase LC-MS/MS custom assay system (TMIC Prime) (Foroutan et al. 2020). Aerated and hypoxic roots of WT and OE11 (n = 4) were sampled after three days of the root hypoxia treatment and immediately frozen in liquid nitrogen. For all metabolites except organic acids, samples were thawed on ice, vortexed and centrifuged (13,000 X g). Metabolites were extracted by adding a mixture of ammonium acetate/methanol (5 mM ammonium acetate in methanol) to the upper 96-well filter plate, shaking at 330 rpm for 30 min, and then centrifuging the plates, loading extracts into the lower 96-deep well plate.

To analyze the organic acid, the mixture of ice-cold methanol (150 µL) and of the isotope-labeled internal standard (10 µL) was added with sample (50 µL) for overnight protein precipitation. After centrifugation (13,000 x g, 20 min), sample of supernatant (50 µL) was loaded into each well of a 96-deep well plate with adding 3-nitrophenylhydrazine (NPH) reagent. After incubation for 2 h, the BHT stabilizer and water were added before LC-MS injection.

The mass spectrometric determination was performed using an ABSciex 4000 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) with an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA). Delivery of sample to the mass spectrometer was done by an LC method following with a direct injection (DI) method. Data analysis was done by Analyst 1.6.2 software (Concord, ON, Canada).

4.2.9 Statistical analyses

To determine statistically significant differences, the data were analyzed by one-way ANOVA using SPSS 18.0 (SPSS Inc., Chicago, IL, USA) followed by Tukey's test ($P \leq 0.05$) to determine the differences between treatments. The data were transformed with a square root function if they did not meet the ANOVA assumptions of normality of distribution and homogeneity of variance.

4.3 Results

4.3.1 Confirmation of overexpression lines

Five OE lines of canola plants transformed with *NtPIP1;3* were generated under the control of the CaMV 35S promoter using *Agrobacterium* as a vector. The qRT-PCR analysis of the transcript abundance of *NtPIP1;3* in leaves showed the highest levels of *NtPIP1;3* in the OE3 and OE11 lines, which were subsequently used for the study (Figure S4.1). No amplification was detected with the *NtPIP1*;*3* primers in WT.

4.3.2 Overexpression of *NtPIP1*;*3* enhanced root respiration rates and ATP concentrations under hypoxia

To examine the responses of OE and WT plants to root hypoxia, the oxygen level in the hydroponic culture was reduced from ~ 8 (well-aerated plants) to ~ 2 mg L^{-1} for three and eight days. The respiration rates were measured in root segments over the 20 min period after their placement in the aerated (~ 8 mg L^{-1} O_2) (Figure 4.1a, b) and stagnant (~ 2 mg L^{-1} O_2) (Figure 4.1c, d) solutions. When measured in the aerated solution, respiration rates of the WT plants

subjected to three (Figure 4.1a) and eight days (Figure 4.1b) of hypoxia were reduced by two- and several-fold, respectively, compared with aerated plants. However, the root respiration rates in both OE lines subjected to three (Figure 4.1a) and eight days (Figure 4.1b) of hypoxia were similar to those measured in aerated plants. Similarly to the measurements in the aerated solution, when root respiration rates were measured in the low oxygen stagnant solution, the respiration rates in WT plants exposed to three (Figure 4.1c) and eight days (Figure 4.1d) of hypoxia were reduced by about three-fold compared with aerated plants. However, the root respiration rates measured in the stagnant solution for the OE plants subjected to three and eight days of hypoxia were similar to those of the aerated OE plants (Figure 4.1c, d). Root ATP concentrations were reduced significantly after three (Figure 4.1e) and eight days (Figure 4.1f) of hypoxia in the WT plants. There were no effects of both three and eight days of hypoxia on the root ATP concentrations in plants of the two OE lines (Figure 1e, f). The percentage of oxygen loss from the aerated and hypoxic hydroponic solutions was measured over 40 min after immersing the excised WT and OE roots in their original growth medium (Figure S4.2a-d). In both hypoxic (three and eight days) solutions, the rates of oxygen loss from the solution with the root segments of both OE lines were higher compared to the solution with the WT roots (Figure S4.2b, d).

4.3.3 Root hypoxia had little or no effect on growth and gas exchange in OE plants

Three days of exposure to root hypoxia resulted in significantly lower plant total dry weights in WT canola compared with well-aerated plants, whereas the hypoxic plants of OE3 and OE11 lines had similar dry weights to well-aerated plants (Figure 4.2a). After eight days of hypoxia, small decreases in dry weights were observed in the OE hypoxic plants compared with the well-

aerated plants (Figure 4.2b). However, the OE hypoxic plants still had higher dry weights compared with the WT hypoxic plants (Figure 4.2b).

Similarly to the plant dry weights, net photosynthetic (P_n) and transpiration (E) rates in the OE plants were not significantly affected by three days of root hypoxia, while they significantly decreased in the WT plants under three days of hypoxic conditions (Figure 4.2c, e). After eight days of hypoxia, P_n and E further declined in the WT plants (Figure 4.2d, f) and only relatively small decreases in P_n and E compared with the aerated plants were observed in the OE lines (Figure 4.2d, f).

4.3.4 Overexpression of *NtPIP1*;*3* improved plant water relations under hypoxia

To evaluate the effects of hypoxia on water relations in wild-type and OE plants, midday leaf water potential (Ψ_{leaf}) and root hydraulic conductivity (L_{pr}) were determined. After three days of root hypoxia, midday Ψ_{leaf} decreased in the WT plants but was not significantly different from aerated plants in both OE lines (Figure 4.3a). The Ψ_{leaf} declined further to about -2.5 MPa in the WT plants after eight days of root hypoxia compared with about -1 MPa measured in well-aerated plants (Figure 4.3b). The Ψ_{leaf} in both OE lines declined after eight days of root hypoxia to about -1.5 MPa compared with approximately -1 MPa measured in aerated controls (Figure 4.3b).

Root hydraulic conductivity (L_{pr}) was reduced in the WT plants after three and eight days of root hypoxia to about 50% of the values measured in aerated plants (Figure 4.3c, d). Only relatively minor and not statistically significant decreases in L_{pr} compared with aerated plants were observed after three and eight days of root hypoxia in both OE lines (Figure 4.3c, d).

4.3.5 Overexpression of *NtPIP1;3* affected energy metabolism under hypoxia

To investigate the role of NtPIP1;3 in orchestrating metabolic changes under the early stages of root hypoxia stress, metabolic profiling was compared between aerated WT and OE11 plants and the plants subjected to three days of root hypoxia. The metabolic pathway analysis (MetPA) of aerated and hypoxia roots in WT and OE plants was performed. Interestingly, the top 3 altered metabolic pathways in WT roots were (i) Histidine metabolism; (ii) Sulfur metabolism; (iii) Aminoacyl-tRNA biosynthesis (Figure 4a) whereas (i) Glycolysis / Gluconeogenesis; (ii) Pyruvate metabolism; (iii) TCA cycle compromised the top 3 altered metabolic pathways (Figure 4.4b) in the OE11 roots.

Schematic representation displays the hypoxia response mechanisms of OE root cells. The glycolysis, pyruvate metabolism and TCA cycle were the major energy metabolism pathways altered in OE roots under hypoxia. Specifically, the perturbation of pyruvate, citrate, aconitate, succinate, and fumarate contributed to glycolysis and TCA pathways (Figure 4.5).

4.4 Discussion

The importance of aquaporins in controlling water transport across cell membranes has been well acknowledged and supported by solid experimental evidence. However, there is also growing evidence for the multifunctional role of aquaporins in transporting gases, small neutral molecules, and ions (Tyerman et al. 2021). Several mammalian aquaporins (Al-Samir et al. 2016) and the plant *Nt*PIP1;3 aquaporin from tobacco (Zwiazek et al. 2017) were demonstrated to facilitate oxygen transport and *Nt*PIP1;3 was also suggested to contribute to waterlogging tolerance in tobacco plants (Tan and Zwiazek 2019). Compared with mammalian aquaporins,

plant plasma membrane intrinsic proteins (PIPs) appear to show a greater degree of specialization in their transporting properties since their homotetramers have little or no water transporting capacity (Chaumont et al. 2000). Therefore, the responses of organisms overexpressing PIP1s are less likely to be due to water transport.

The importance of gas transport across aquaporins remains a contentious topic. Aquaporins form homo- and heterotetramers with each subunit containing a functional water pore (Tornroth-Horsefield et al. 2006). At the center of the four monomers lies the fifth pore, which is composed mainly of hydrophobic amino acids, and which may provide an additional path for the passage of non-polar molecules including CO_2 and O_2 (Herrera and Garvin 2011). However, it has been argued that aquaporins may not significantly decrease membrane resistance to gases since the lipid bilayers are considered to be gas-permeable (Wang et al. 2007). We earlier proposed that the aquaporin-mediated oxygen transport through *Nt*PIP1;3 is functionally essential to plants (Zwiazek et al. 2017). This hypothesis was tested in the present study by overexpressing this aquaporin in canola and comparing the responses of OE and WT plants to root hypoxia. Canola plants are relatively sensitive to root hypoxia and do not develop morphological or anatomical features such as adventitious roots or aerenchyma in response to waterlogging (Liu et al. 2020a). Our results clearly demonstrate that the overexpression of *NtPIP1*;*3* improved plant waterlogging tolerance through increased oxygen delivery, as evidenced by higher root respiration rates, elevated root ATP levels, as well as enhanced growth and physiological performance compared with the WT plants.

Waterlogging leads to the energy crisis since aerobic respiration is depressed due to low oxygen concentrations in stagnant water (Munir et al. 2019). In our study, respiration rates of the

root segments excised from the WT plants, that had been subjected to three days of root hypoxia and subsequently placed in aerated (~ 8 mg L^{-1} O_2) and stagnant (~ 2 mg L^{-1} O_2) hydroponic solutions, were sharply reduced compared to those measured in the root segments from aerated plants. However, after three days of root hypoxia, there was no significant decrease of root respiration rates in both OE lines. The respiration rates were still several-fold higher in both OE lines compared with the WT plants that had been subjected to eight days of hypoxia, especially when measured in the aerated hydroponic solution. The higher root respiration rates of the OE plants compared with the WT plants were also reflected by the root ATP concentrations, which, contrary to the WT plants, were not significantly reduced after three and eight days of hypoxia. These results demonstrate that the roots of OE plants subjected to root hypoxia were supplied with more oxygen compared with the WT plants.

The enhanced oxygen delivery to the root cells of intact hypoxic plants could be accomplished by the improved root oxygen uptake directly from the hydroponic solution or (and) by the enhanced transport of oxygen from the shoots. Since the root respiration measurements were carried out in the excised root segments, our results demonstrate that the increased oxygen diffusion directly from the aerated and stagnant media was among the factors contributing to higher respiration rates in the OE plants. The improved root oxygenation helped the OE plants maintain relatively higher root hydraulic conductivity compared with the WT plants, resulting in higher leaf water potentials, transpiration rates, net photosynthetic rates, and, consequently, higher plant dry biomass. *Os*PIP1s are not the main water transporters in *Oryza sativa* (Liu et al. 2020b) and show negligible water channel activity; hence the overexpression of *NtPIP1*;*3* in canola was not likely to have a significant direct effect on root water transport. Root hydraulic conductivity is highly dependent on the energy supply and maintenance of cytosolic pH to

maintain the function of aquaporins involved in root water transport (Kamaluddin and Zwiazek 2001; Kamaluddin and Zwiazek 2002; Tournaire-Roux et al. 2003). The inhibition of aquaporin-mediated water transport in hypoxic roots is the principal factor leading to the decreased water supply to leaves affecting plant water balance and leading to gas exchange and growth reductions (Liu et al. 2020b; Maurel and Nacry 2020). These parameters were greatly improved in the present study in the OE canola plants exposed to three and eight days of root hypoxia.

We also carried out metabolic analyses to compare the effects of three days of root hypoxia on the metabolite levels in the WT and OE11 plants. The results demonstrated that the overexpression of *NtPIP1*;*3* in canola plants altered major energy metabolism pathways, the glycolysis pyruvate metabolism and TCA cycle pathways (Millar et al. 2011), which are responsible for the production of ATP, reducing power and generating molecules needed for biosynthesis and N-assimilation in plants (O'Leary and Plaxton 2020). These findings provide additional evidence that *NtPIP1*;*3* overexpression increased the supply of oxygen for oxidative phosphorylation, which helps sustain sufficient ATP production to maintain growth and physiological functions under root hypoxia conditions.

The study had some important limitations. Since the cellular oxygen concentration is difficult to determine directly and precisely, the oxygen concentrations in different cells and tissue types in OE lines could not been compared with the wild-type plants upon hypoxia stress. Appropriate experimental techniques to measure oxygen fluxes in cells and tissues still need to be developed by future studies. In addition, as my results indicated that OE plants have higher root hydraulic conductivity under hypoxia, the expression patterns of *BnPIP*s in OE plants under both aerated

and hypoxic conditions should be compared with the wild-type plants. These data may provide more information on possible interactions between *NtPIP1*;*3* and other local *BnPIP* genes.

4.5 Conclusion

My results demonstrated that the overexpression of *NtPIP1*;*3* in canola substantially improved plant tolerance of the root hypoxia (waterlogging) treatments that were applied to plants for three and eight days. This was clearly evidenced by the measured physiological and growth parameters. Compared with the WT plants, the OE plants exposed to root hypoxia exhibited higher root respiration rates, ATP concentrations, and levels of metabolites that are associated with energy metabolism, indicating improved oxygen supply to the roots. The study results confirmed our hypothesis that the oxygen-transporting properties of *Nt*PIP1;3 are of functional importance to plants. The study points to the need for additional research on the aquaporin-mediated oxygen transport involving other members of the aquaporin family in plants and in other organisms.

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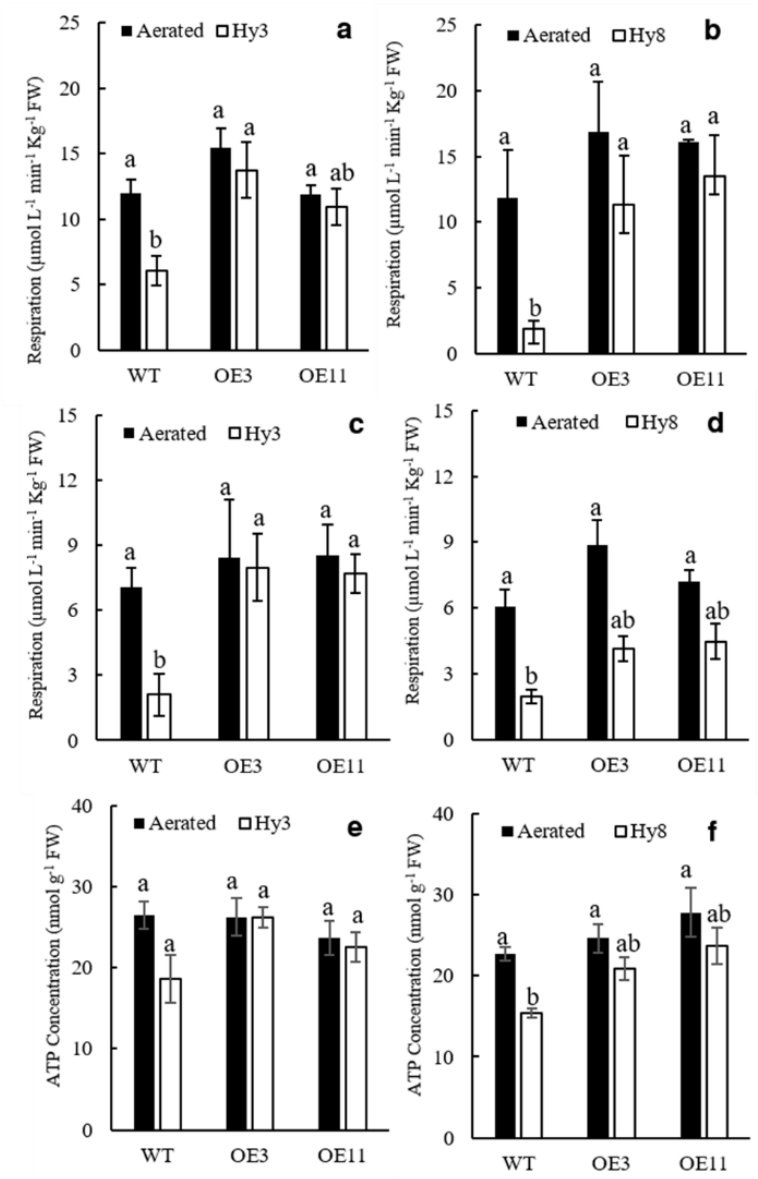

Figure 4.1 Overexpression of *NtPIP1*;*3* improved root respiration and maintained stable ATP concentrations under hypoxia. The respiration rates in the distal root segments of aerated wild-type (WT) and transgenic (OE3, OE11) plants and in plants subjected to root hypoxia treatments for three (Hy3) and eight (Hy8) days. The respiration measurements were carried out in the aerated (~ 8 mg L-1 O_2) (a, b) and stagnant (~ 2 mg L-1 O_2) (c, d) hydroponic media; ATP

concentrations in the distal root segments of aerated wild-type (WT) and transgenic (OE3, OE11) plants and in plants subjected to root hypoxia treatments for three (Hy3) and eight (Hy8) days (e, f). Means ± SE are shown (n = 6 - 8). Values with different letters are significantly different at P ≤ 0.05 (ANOVA, Tukey's test).

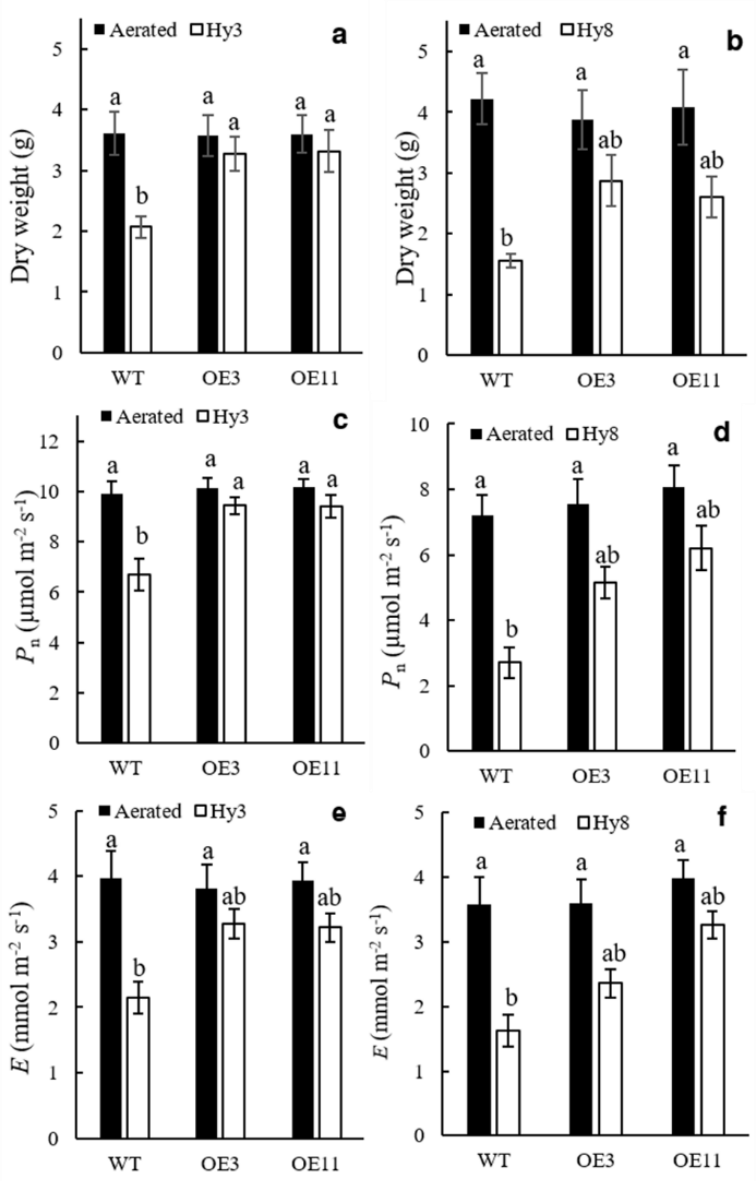

Figure 4.2 Overexpression of *NtPIP1*;*3* improved growth and gas exchange under hypoxia. The total plant dry weights (a, b), net photosynthesis (P_n) (c, d) and transpiration (E) (e, f) rates in the wild-type (WT) and transgenic (OE3, OE11) aerated plants and in plants exposed to root hypoxia for three (Hy3) and eight (Hy8) days. Means ± SE are shown (n = 6 - 8). Values with different letters are significantly different at P ≤ 0.05 (ANOVA, Tukey's test).

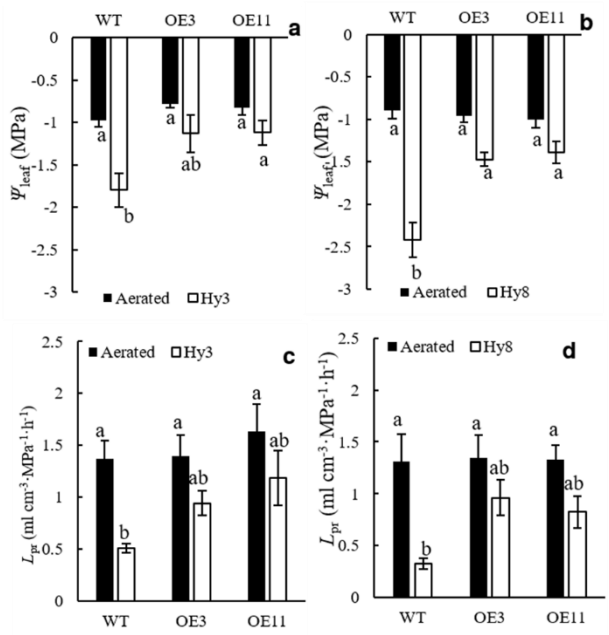

Figure 4.3 Overexpression of *NtPIP1*;*3* improved plant water relations under hypoxia. The leaf water potential (Ψ_{leaf}) (a, b) and root hydraulic conductivity (L_{pr}) (c, d) in the wild-type (WT) and transgenic (OE3, OE11) aerated plants and in plants exposed to root hypoxia for three (Hy3) and eight (Hy8) days. Means ± SE are shown (n = 6 - 8). Values with different letters are significantly different at P ≤ 0.05 (ANOVA, Tukey's test).

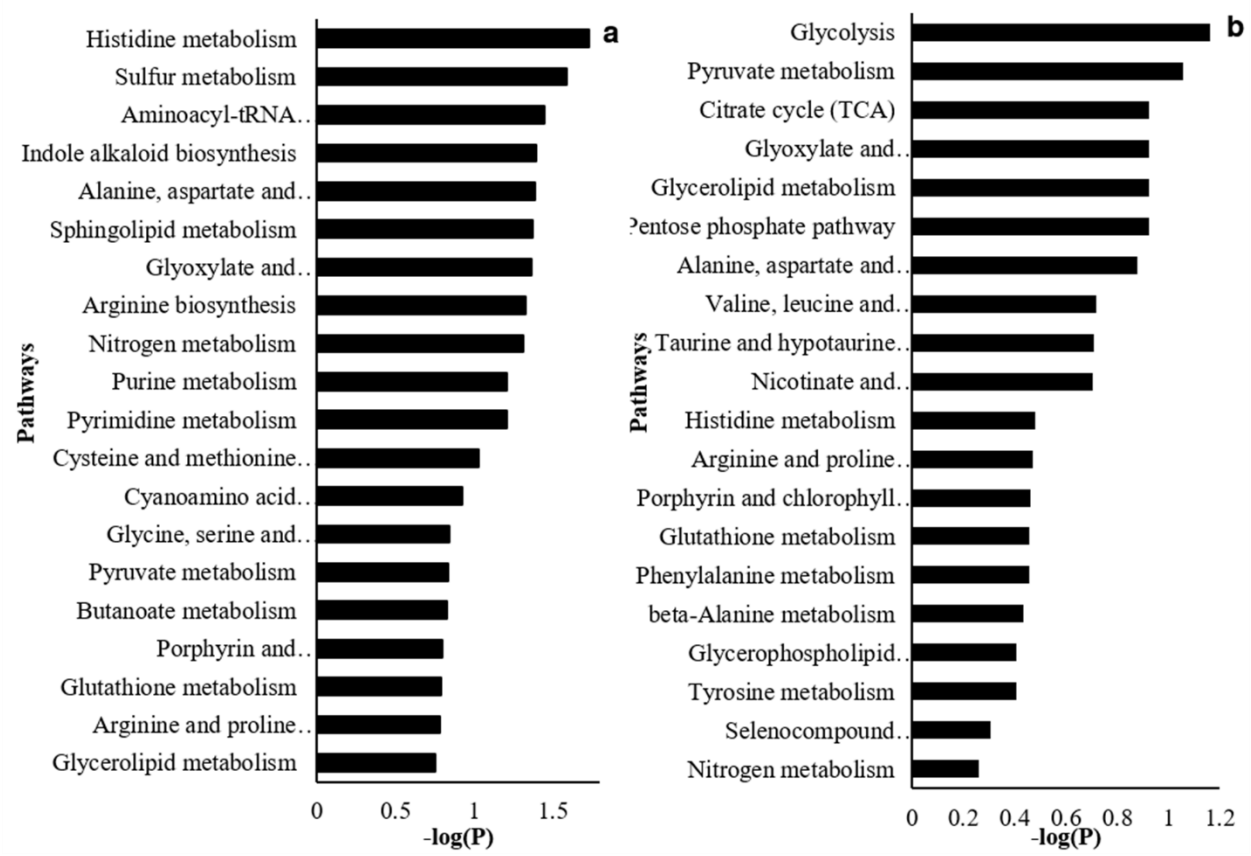

Figure 4.4 Overexpression of *NtPIP1*;*3* altered energy metabolism under hypoxia. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for differentially expressed metabolites induced by hypoxia in wild-type (WT, a) and transgenic (OE11, b) plants. The top 20 pathways of differential metabolites between aerated and hypoxia plants are shown in the histogram. The pathway and enrichment score for −Log10 (p-value) of the corresponding pathway are presented in the vertical and horizontal axes, respectively.

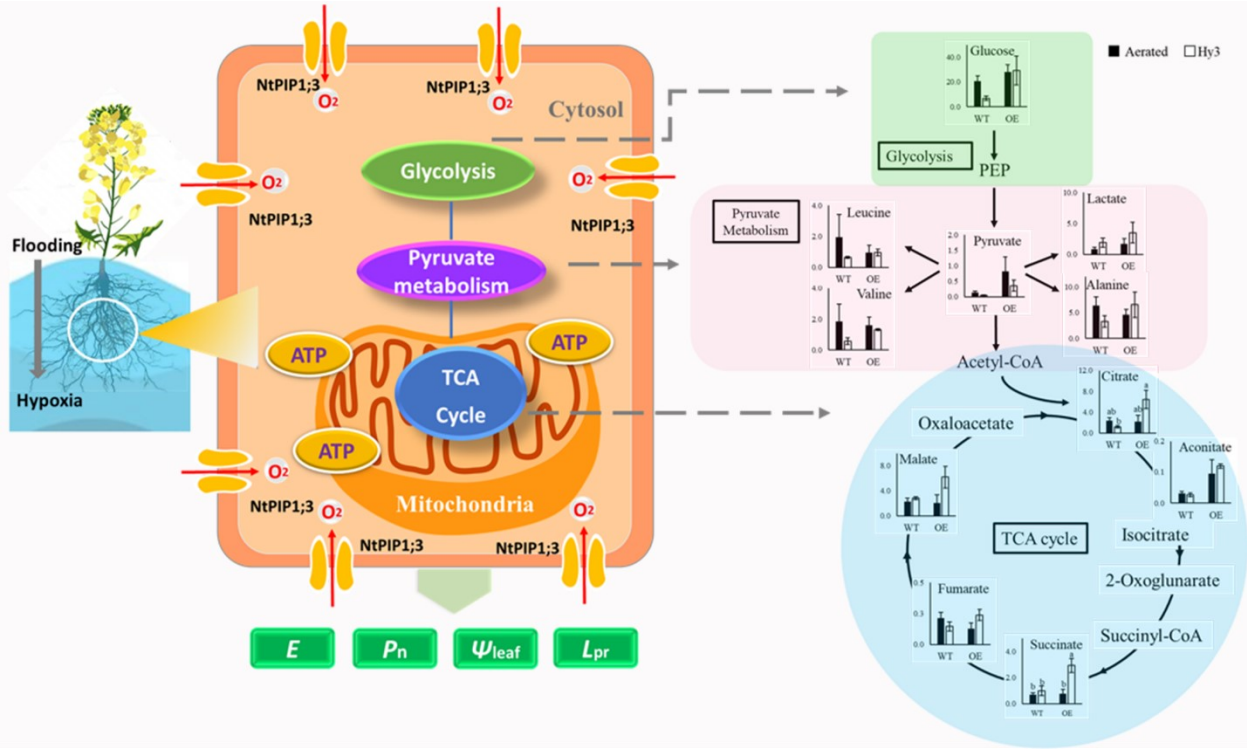

Figure 4.5 Schematic representation of the effects of *NtPIP1;3* overexpression on the energy metabolism. Overexpression of *NtPIP1;3* improved canola hypoxia tolerance by the effects on glycolysis, pyruvate metabolism and TCA pathways. Metabolites involved in the main pathways of the wild-type (WT) and transgenic (OE11) aerated plants and in plants exposed to root hypoxia for three days (Hy3). Values with different letters are significantly different at $P \leq 0.05$ (ANOVA, Tukey's test).

Chapter 5. General Discussion and Conclusions

5.1 General discussion

Oxygen deficiency frequently occurs as a result of flooding or soil compaction and may affect plants at different growth stages resulting in their mortality and decreased growth (Bailey-Serres and Colmer 2014). It has been frequently observed that plants exposed to root hypoxia suffer from the upset water balance (Tan et al. 2018). This response in flooded plants had puzzled researchers for a long time prior to the discovery of aquaporins and their importance in controlling root water transport. Aquaporins are known to play multifunctional roles in response to environmental stresses in plants (Chaumont and Tyerman 2014; Tyerman et al. 2021) and, in addition to water transport, they may be involved in the transport of other molecules affecting plant responses to root hypoxia including CO_2, O_2, and ions (Uehlein et al. 2012; Zwaizek et al. 2017; Tyerman et al. 2021). To address the functions of plant aquaporins in response to oxygen deficiency and re-aeration, three studies (Chapters 2, 3, 4) were conducted under controlled environment conditions in *B. napus*. The hypotheses presented in Chapter 1 were verified and stated objectives were fulfilled. The studies described in this thesis enhanced the understanding of water transport properties in canola and revealed multiple roles of aquaporins under hypoxia and re-aeration conditions.

Plants may vary in their responses to root hypoxia depending on developmental stages. In Chapter 2, the study was carried out to examine the responses to waterlogging of *Brassica napus* plants during the seedling, flowering, and podding developmental stages. This study was aimed at generating detailed information concerning the water relations of plants at these different

stages when subjected to root hypoxia. The study demonstrated that the seedling stage was the most susceptible to root hypoxia and, therefore, all subsequent studies were carried out with seedlings. The results of the study described in Chapter 2 showed that the apoplastic root water transport significantly decreased in waterlogged plants (Figure 5.1). Aquaporin-mediated cell-to-cell transport increased with increasing exposure to waterlogging at the three growth stages. The *BnPIP1*;*2* and *BnPIP2*;*7* aquaporins were markedly upregulated in waterlogged plant roots indicating their potential role in water transport under hypoxic conditions. Some of the plant aquaporins were downregulated in response to oxygen deprivation stress. These results are in agreement with previous studies that were carried out with other plants species. The expression levels of *GmPIP2*;*2* in soybean (*Glycine max*) and its three homologs were downregulated in roots after a three-hour exposure to hypoxic conditions (Matsuo et al. 2012). Aquaporins were also found to be downregulated in tamarack (*Larix laricina*) (Calvo-Polanco et al. 2012) and avocado (*Persea americana*) (Reeksting et al. 2016) upon oxygen deprivation. The aquaporin channel closure might be due to cytoplasm acidification triggered by hypoxia (Tournaire-Roux et al. 2003). However, it is still unclear if all aquaporins are equally sensitive to the cytoplasm acidification. The upregulation of aquaporins induced by root hypoxia could also suggest that they might have other functions than water transport under hypoxic conditions.

In the study described in Chapter 3, *B. napus* seedlings were subjected to three days of hypoxia followed by re-aeration. Within six days of re-aeration, the plants recovered from the hypoxia treatment. Interestingly, an oxidative burst was detected one day following re-aeration and resulted in a sharp decrease of L_{pr} and downregulation of most of the *BnPIP2*s (Figure 5.1). Oxidative bursts upon re-aeration were also reported in other plants including *Arabidopsis* (Yeung et al. 2018). In my study, the functional assays of *Bn*PIP2s in yeast indicated that,

contrary to *Bn*PIP1s, *Bn*PIP2s are fast water transporters (Figure 5.1), which is consistent with previous findings (Chaumont et al. 2005). The upregulation of *BnPIP1*;*2* in this study could be potentially associated with gas transport and (or) with the transport of H_2O_2 and other signaling molecules (Figure 5.1). Various PIP1s were shown to transport gases and other molecules in previous studies (Uehlein et al. 2012; Liu et al. 2020). However, the functionality of *Bn*PIP1;2 as an H_2O_2 transporter remains to be determined.

The notion that aquaporin-mediated oxygen transport may alleviate flooding stress was examined in canola plants overexpressing the tobacco *NtPIP1*;*3* aquaporin gene (Chapter. 4). This study demonstrated that this aquaporin was a key in conferring root hypoxia tolerance to canola seedlings. Higher respiration rates and ATP levels in plants overexpressing *NtPIP1*;*3* indicated higher oxygen supply that was likely responsible for enhanced physiological performance including P_n, E and L_{pr}. Since *Os*PIP1;3 are not mainly responsible for transporting water (Liu et al. 2020), the overexpression of *NtPIP1*;*3* was not likely a significant direct factor responsible for the increased root water transport in hypoxic OE plants.

In conclusion, my thesis research has confirmed the stated hypothesis of the key importance of aquaporins in plant tolerance of root hypoxia. A conceptual model summarizing the responses of canola plants to root hypoxia and the potential tolerance mechanisms is presented in Figure 5.1. Root hypoxia (waterlogging) induced the deposition of suberin and lignin, which reduced the water transport of apoplastic pathway. Re-aeration following hypoxia triggered accumulation of ROS and decreased *BnPIP2*s transcript levels (Figure 5.1). An increase in transcript levels of several *BnPIP*s could have potentially played a role in transporting H_2O_2 out of the cytosol as part of the ROS detoxification mechanism (Tian et al. 2016) and this notion deserves further

attention. Since *Nt*PIP1;3 was confirmed to transport oxygen when expressed in yeast (Zwiazek et al. 2017), this aquaporin was overexpressed in *B. napus* in my study. A significant enhancement of root hypoxia tolerance in the overexpression plants was accompanied by increased root respiration and ATP levels confirming the significance of *Nt*PIP1;3 in O_2 transport (Figure 5.1). It is also plausible that some of the *Bn*PIPs may be involved in CO_2 transport as part of the hypoxia tolerance strategy by plants.

5.2 Perspectives for future studies

The survival and growth of plants in flood-prone areas are important, especially in agriculture and forestry. My studies addressed only root hypoxia and the re-aeration process in canola plants. However, the submergence of smaller plants also frequently occurs in flood-prone areas (Fukao et al. 2019). In addition to roots, submergence affects physiological traits of plant shoots, including light capture and gas exchange. Future studies will be needed to address the effects of submergence on plant water relations and aquaporin function.

The task of elucidating roles of aquaporins in the transport of water and other molecules is highly challenging. As the gas transport is difficult to determine experimentally, transport models based on the 3D structure of the protein could be developed to simulate the transport process and estimate the oxygen transport ability of *Nt*PIP1;3. In addition, my study involved only PIPs in *Brassica napus* and the *Nt*PIP1;3 from *Nicotiana tabacum*. However, with the diversity of aquaporins in various plant species and within the same plant, other studies demonstrated that different isoforms of aquaporins including NIPs and TIPs, could also potentially play a pivotal role under the hypoxia and re-aeration conditions. For example, *Prunus*

NIP1;1 was reported as a putative lactic acid transporter (Mateluna et al. 2018) and proposed to play a key role in the survival of root hypoxia.

The *NtPIP1*;*3* overexpression lines of canola showed increased waterlogging tolerance by maintaining higher root respiration rate and the plants were characterized by enhanced growth and physiological performance under hypoxic conditions There is a limitation in understanding the underlying regulatory mechanisms of *Nt*PIP1;3 at both the transcript and translational levels in my study. For instance, transcriptome analysis of overexpression lines could be done and post-translational modification mechanism of NtPIP1;3 remain to be investigated. In addition, as a variety of reverse genetic approaches have been developed in plants including CRISPR-Cas9 and RNA interference technique (Ma et al. 2020), the *NtPIP1*;*3* knock-out lines should also be generated with the gene-editing methods to confirm that knock-out lines of tobacco are more sensitive to oxygen deprivation stress. Additionally, the hypoxia tolerance of overexpression lines was only tested under controlled-environment conditions. Future experiments should be conducted under different field conditions to confirm these results and evaluate the potential use of the *NtPIP1*;*3* overexpression lines in agriculture.

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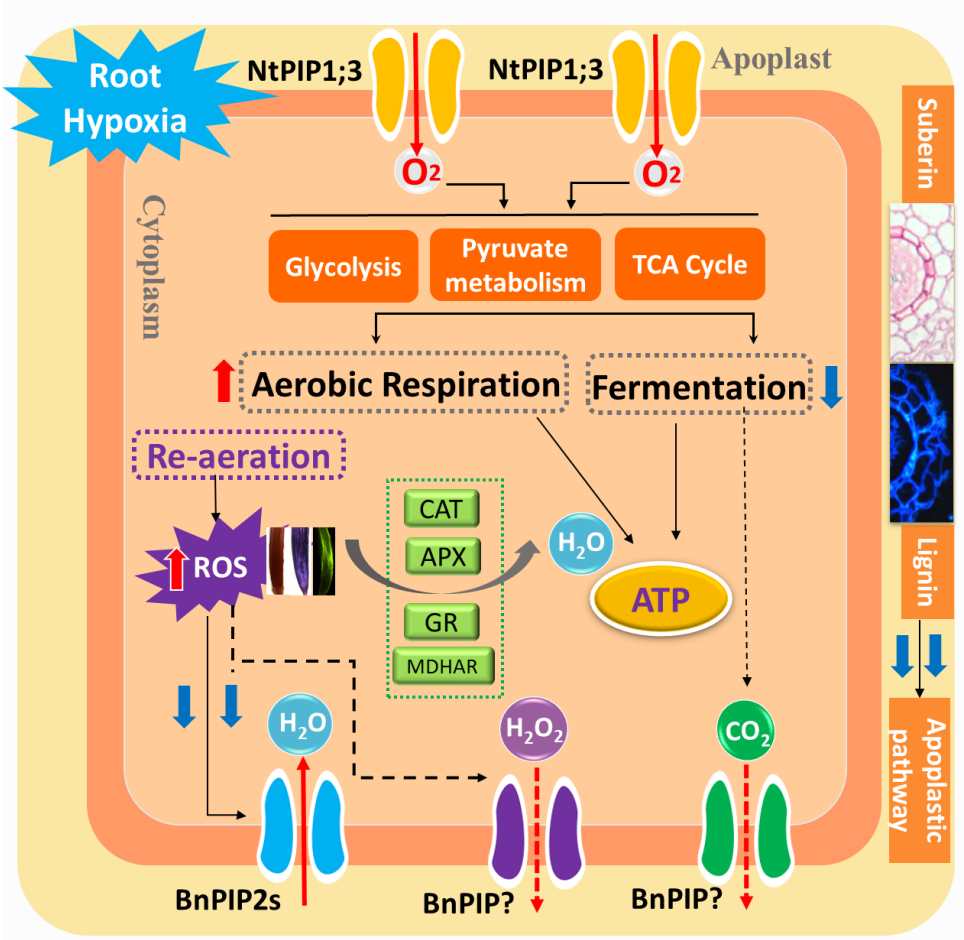

Figure 5.1 Schematic representation of the processes in root cells of canola plants contributing to the tolerance of root hypoxia and re-aeration. *Nt*PIP1;3 is shown for the canola overexpression lines. Root hypoxia triggered the accumulation of suberin and lignin which limited the apoplastic pathway of root water transport. Aquaporin-mediated pathway dominated root water transport with upregulated *BnPIP*s (Chapter 1). Re-aeration following hypoxia caused an accumulation of ROS, which had an inhibitory effect on *BnPIP* expression and was accompanied by upregulation of the antioxidant enzymes. *Bn*PIP2s were confirmed as the main water transporters in heterologous yeast systems (Chapter 2). Overexpression of *NtPIP1;3* in *B. napus* promoted oxygen uptake and increased aerobic respiration upon root hypoxia compared with the wild-type *B. napus* (Chapter 3). Some *Bn*PIPs may be responsible for the efflux of H_2O_2, and CO_2, which

needs to be further investigated. Dashed lines with arrows indicate hypothesized processes, solid lines with arrows are processes confirmed by this study, red arrows indicate increases, and blue arrows indicate decreases.

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Appendices

Appendix 1. Primers for qRT-PCR in chapter 2

Genes		Sequence (5'-3')
BnPIP1;1	Forward	GCTAAGAACAGACCAAAAGTG
	Reverse	GGCATTCGGTGGTATGATA
BnPIP1;2	Forward	GAATGGTCCAACCCAAAAG
	Reverse	TGTTCCCATTCTTGCACCT
BnPIP1;3	Forward	CTGGAAGCCTTTGACAACG
	Reverse	CATAAACCCAGCGGTGACA
BnPIP2;2	Forward	GGAGTCTCGGGCATTTCTT
	Reverse	CGATGGATACAGCACAGGG
BnPIP2;5	Forward	GTGTAGTAAGAAGACTGGAAGGA
	Reverse	GAAAGTGACGTTGGTGAGG
BnPIP2;	Forward	TGGGATGGTAGCCAAATGC
	Reverse	TTCTCCGCGACTGACCCT
BnaPPR	Forward	TGGTGTGCGATAAGTGTGAGA

	Reverse	GGTGTCCATCTGTTCTTCTTGG
Bnactin7	Forward	CCCTGG AATTGCTGACCGTA
	Reverse	TGGAAAGTGCTGAGGGATGC

Appendix 2. Two-way ANOVA tests of effects with waterlogging treatment (treatment) and time durations (time) treatments on measured parameters

Variable	Stage	Treatment	Time	Treatment × Time
Height	Seedling	0.003	0.145	0.034
	Flowering	0.001	0.17	0.819
	Podding	0.468	0.758	0.983
Leaf number	Seedling	<0.001	<0.001	0.015
	Flowering	<0.001	<0.001	<0.001
	Podding	0.014	<0.001	0.969
Root length	Seedling	0.074	0.06	0.002
	Flowering	0.13	0.106	0.452
	Podding	0.29	0.161	0.806
Average leaf area	Seedling	<0.001	0.013	0.04
	Flowering	<0.001	<0.001	0.161
	Podding	<0.001	0.329	0.564
Total dry weight	Seedling	<0.001	<0.001	<0.001
	Flowering	<0.001	0.294	0.213
	Podding	0.065	0.015	0.432
Net photosynthesis (P_n)	Seedling	0.002	<0.001	0.094
	Flowering	<0.001	0.003	0.042
	Podding	<0.001	0.923	0.348
Transpiration rate (E)	Seedling	<0.001	0.06	0.024
	Flowering	<0.001	0.662	0.262
	Podding	0.035	0.934	0.961
Dye concentration	Seedling	<0.001	<0.001	0.326
	Flowering	<0.001	0.006	0.442
	Podding	<0.001	0.29	0.775

Leaf water potential (Ψ_{leaf})	Seedling	<0.001	<0.001	<0.001
	Flowering	<0.001	0.13	0.003
	Podding	0.442	<0.001	0.31
Root hydraulic conductance (K_r)	Seedling	<0.001	0.816	0.857
	Flowering	0.002	0.876	0.739
	Podding	0.015	0.795	0.795
$AgNO_3$ K_r inhibition	Seedling	<0.001	0.023	0.114
	Flowering	0.002	0.938	0.808
	Podding	0.015	0.623	0.572
BnPIP1;*1*	Seedling	<0.001	0.061	0.893
	Flowering	0.008	<0.001	0.107
	Podding	0.209	0.106	0.004
BnPIP1;*2*	Seedling	<0.001	0.026	0.665
	Flowering	<0.001	<0.001	<0.001
	Podding	<0.001	0.058	0.087
BnPIP1;3	Seedling	0.015	0.079	0.026
	Flowering	0.05	0.132	0.241
	Podding	<0.001	<0.001	<0.001
BnPIP2;*2*	Seedling	<0.001	0.168	0.072
	Flowering	0.786	0.627	0.019
	Podding	<0.001	<0.001	<0.001
BnPIP2;5	Seedling	0.191	0.002	0.482
	Flowering	0.636	0.034	0.279
	Podding	0.761	0.868	0.309
BnPIP2;7	Seedling	0.002	0.171	0.063
	Flowering	<0.001	<0.001	<0.001
	Podding	0.001	<0.001	0.106
Suberized endodermis cell	Seedling	<0.001	0.365	0.713
	Flowering	0.01	0.534	0.428
	Podding	0.056	0.813	0.487
Light intensity	Seedling	0.108	0.133	0.568
	Flowering	<0.001	0.014	0.18
	Podding	0.987	0.199	0.611

Appendix 3. Primers for yeast transformation in Chapter 3

Genes		Sequence (5'-3')
BnPIP1;*1*	Forward	ATGGAAGGCAAGGAAGAAGA
	Reverse	TTAGTTTCTGGACTTGAAGGGGATT
BnPIP1;*2*	Forward	ATGGAAGGCAAGGAAGAAGAT
	Reverse	TCAGCTTCTGGACTTGAATGG
BnPIP1;*3*	Forward	ATGGAAGGGAAAGAAGAGGA
	Reverse	TCAGGATCTGGTCTTGAAAGG
BnPIP2;*2*	Forward	ATGGCGAAAGAGGTGGAAG
	Reverse	TTAAACGTT GGCCGCACT
BnPIP2;*5*	Forward	ATGACGAAAGATGTGGCTGGA
	Reverse	TTAAACGTGAGACTGGCTCCT
BnPIP2;*7*	Forward	ATGTCGAAAGAAGTGAGCGAAG
	Reverse	TCAGTTTGTTGCGCTGCTT
BnPIP1;*1* (Gateway)	Forward	GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAAGGCAAGGAAGAAGA
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTTTCTGGAC

		TTGAAGGGGATT
BnPIP1;*2* (Gateway)	Forward	GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAAGGCAAGGAAGAAGAT
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCTTCTGGACTTGAATGG
BnPIP1;*3* (Gateway)	Forward	GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAAGGGAAAGAAGAGGA
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGATCTGGTCTTGAAAGG
BnPIP2;*2* (Gateway)	Forward	GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGAAAGAGGTGGAAG
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCAACGTTGGCCGCACT
BnPIP2;*5* (Gateway)	Forward	GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACGAAAGATGTGGCTGGA
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCAACGTGAGACTGGCTCCT
BnPIP2;*7* (Gateway)	Forward	GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCGAAAGAAGTGAGCGAAG
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTTTGTTGCGCTGCTT

Appendix 4. Primers used in Chapter 4

Genes		Sequence (5'-3')
Clone *NtPIP1*;*3* cDNA	Forward	ATGGCAGAGAACAAGGAAGAGG
	Reverse	TTAGCTCTTGAATGCAATGGC
Gateway primers	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCAGAG AACAAGGAAGA
	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGCTCTTGA ATGCAATGGCTCT
CAMV 35S primer for sanger sequencing	Forward	GAGGAGCATCGTGGAAAAAG
NpII specific primers	Forward	GAGGCTATTCGGCTATGACTGG
	Reverse	ATCGGGAGCGGCGATACCGTA
NtPIP1;*3* qRT-PCR	Forward	TGGTATGATCTTTGCCCTTGTCT
	Reverse	CAAATGTCACTGCTGGGTTAATG
Bnactin7	Forward	TGGTGTGCGATAAGTGTGAGA
	Reverse	GGTGTCCATCTGTTCTTCTTGG
BnPPR	Forward	CCCTGG AATTGCTGACCGTA

Reverse	TGGAAAGTGCTGAGGGATGC

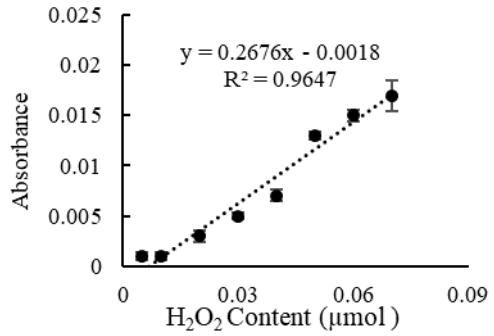

Figure S3.1 The standard curve for H_2O_2 determination (n = 3).

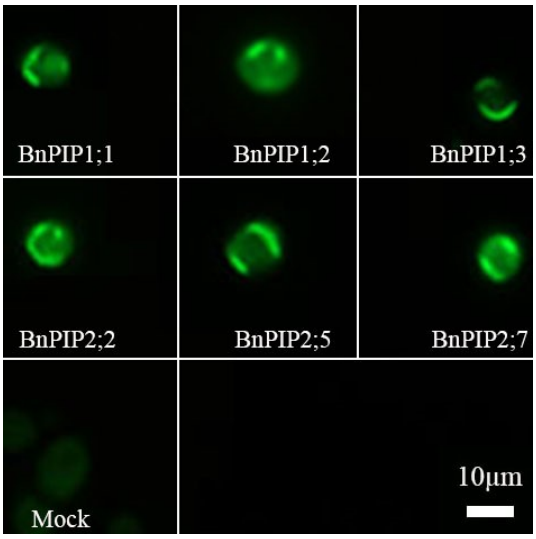

Figure S3.2 Images of yeast cells showing the localization of GFP-tagged *Bn*PIP proteins. Scale bars = 10 μm.

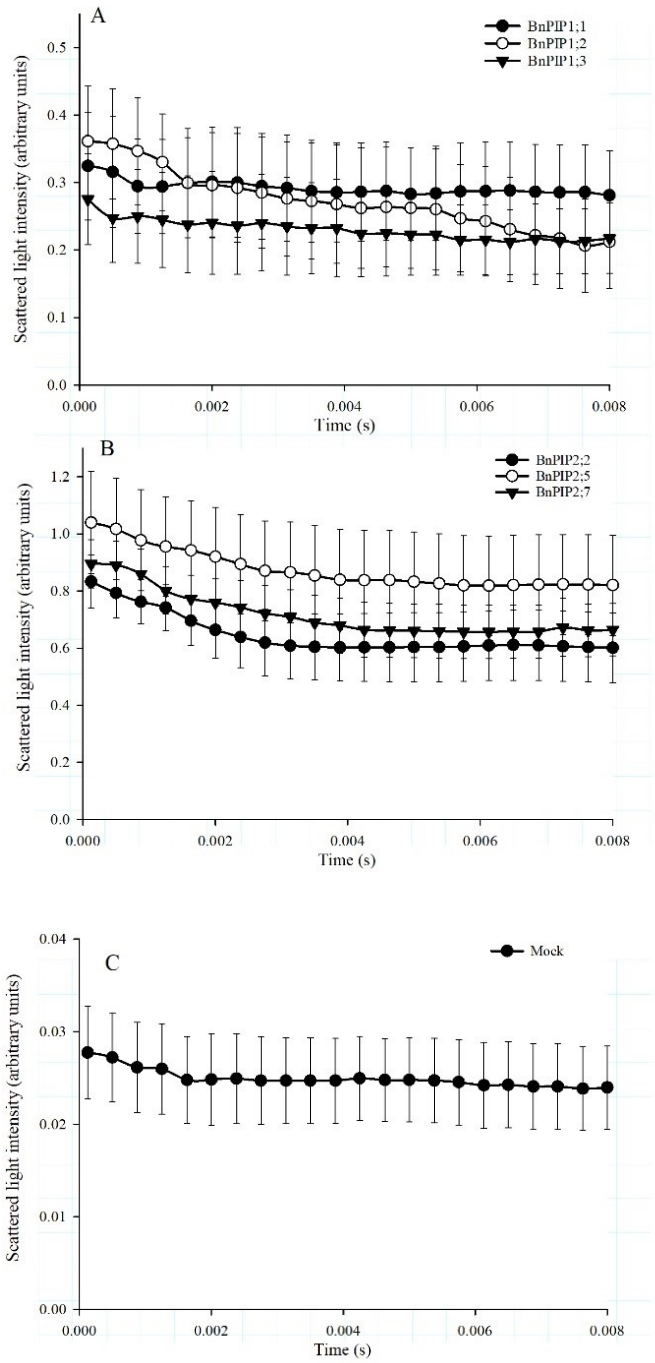

Figure S3.3 Kinetics of *S. cerevisiae* cells expressing *BnPIP*s and Mock control (A. BnPIP1;1, BnPIP1;2, BnPIP1;3 ; B. BnPIP2;2, BnPIP2;5, BnPIP2;7; C. Mock). Means ± SE are shown (n = 10).

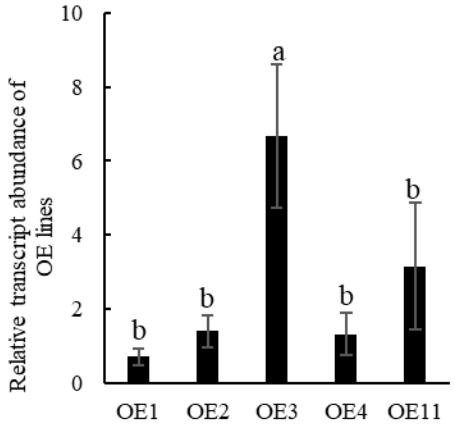

Figure S4.1 The *NtPIP1*;*3* expression levels in leaves of wild-type (WT) and OE lines (OE1, OE2, OE3, OE4, OE11) of T1 generation. Means ± SE are shown (n = 4). Values with different letters are significantly different at $P \leqslant 0.05$ (ANOVA, Tukey's test).

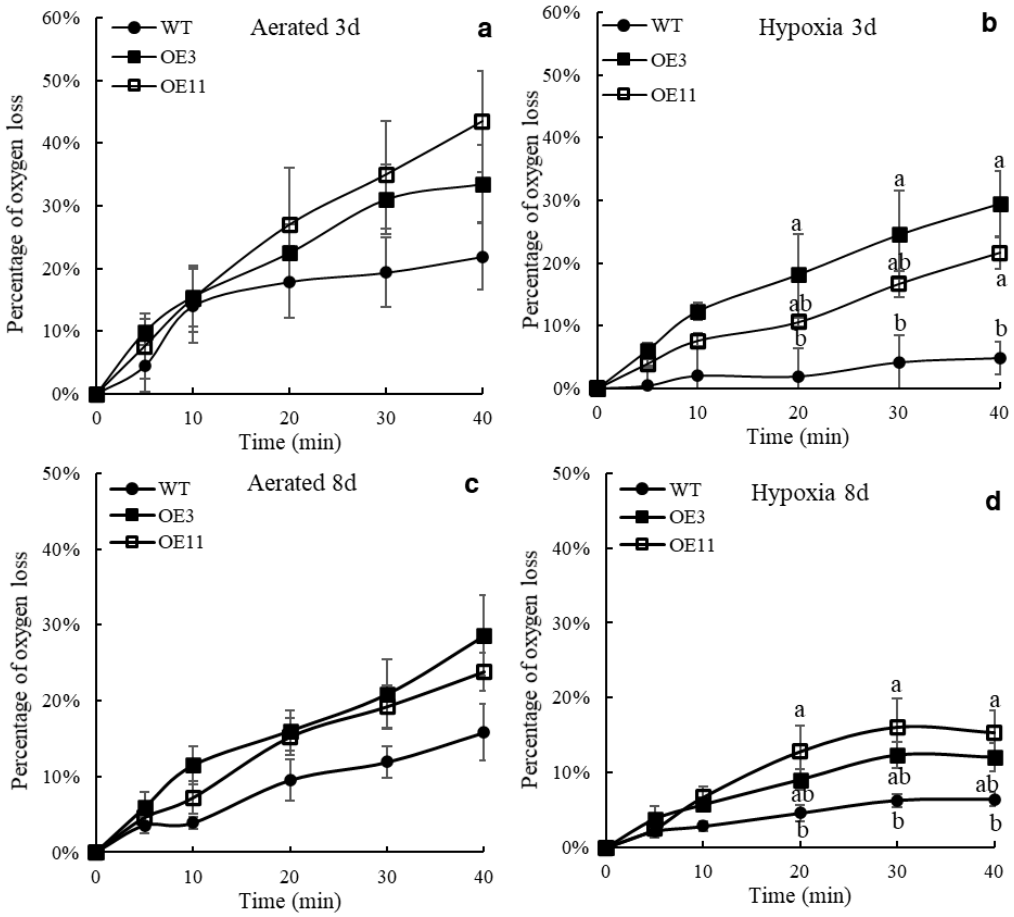

Figure S4.2 The percentage of oxygen loss from the respective hydroponic media in which distal root segments of the wild-type (WT) and transgenic plants (OE3, OE11) were placed for the measurements. The plants were grown under aerated (a, c) or hypoxic conditions for three (b) and eight days (d), and the oxygen loss was measured over time from the aerated (~ 8 mg L^{-1} O_2) (a, c) and hypoxic (~ 2 mg L^{-1} O_2) (b, d) media in which the root segments were placed. Means ± SE are shown (n = 6 - 8). Values with different letters are significantly different at $P \leq 0.05$ (ANOVA, Tukey's test).