Expression of Class 1 Phytoglobin (*HvPgb1*) promotes waterlogging tolerance responses in barley (*Hordeum vulgare* L.)

by

James De Castro

A Thesis submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Plant Science

University of Manitoba

Winnipeg

ACKNOWLEDGEMENTS

The completion of my thesis was made possible *only* because of the support of my advisory committee, lab members, friends, and family. My progression over the past 2.5 years was not the smoothest, given the time of the Covid 19 pandemic as well as other personal developments over this time period. I want to first thank CAP Ag Action MB, Manitoba Crop Alliance for the financial support. My co-supervisor, Dr. Badea, has been the most pleasant surprise. Her commendable dedication to research is beyond admirable and I was not expecting the amount of empathy she had provided me. Dr. Hill's encyclopedic knowledge in the field was very intimidating, but he continues to be an equally great resource for morale. Dr. Renault has been a great mentor in my project as well as my coursework, and I have her to thank for my improvements in writing. To Doug D., Dr. Mohammed M., Dr. Mohamed Y. and Shimaa: to say that all of your combined work ethic is what scaffolds the lab, is an understatement. I, as well as the rest of the lab, have all of you to thank for maintaining its rigorous practice, as well as nourishing my now expanded skillset. To my lab mates Beth, Sam, Sonia, Ehsanul, Lakmini and Samadhi: I am so proud of what we have all achieved and will achieve, and I aim to be a long-term colleague and friend. To Dr. Claudio, who has been the most patient P.I., I do not think there will come a time when I would accept that I deserved your patience, and for that I will always be grateful. To my parents: it is not hyperbolic to say I have come this far because of the sacrifices you have made for me and my brothers. The completion of my higher education was done not out of obligation to you, but because through this, I can show my deepest appreciation for the hard work you both had to do for us. And finally, to my dearest Leclaire. You have been the one true witness of this stage not just in my life, but in yours as well. Its conclusion merely symbolizes the start of the rest, and I cannot wait.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABA- Abscisic acid

ADH – alcohol dehydrogenase

$AgNO_3$ – silver nitrate

AlaAT – alanine aminotransferase

ctrl – control

ERVIIs – Group VII Ethylene response factors

Eth – Ethephon

HvPgb1 – Class 1 phytoglobin

HvPgb1(OE) – HvPgb1-overexpressing transgenic line

NO – Nitric oxide

NR – Nitrate reductase

PDC – pyruvate decarboxylase

PSR – Photosynthetic rate

ROS – Reactive oxygen species

SC – Stomatal conductance

WT – Wild type

wtlg – waterlogged

ABSTRACT

De Castro, James. M.Sc., The University of Manitoba, July, 2022. **Expression of Class 1 Phytoglobin (HvPgb1) promotes waterlogging tolerance responses in barley (*Hordeum vulgare* L.).** Co-supervisors: Claudio Stasolla and Ana Badea

Barley (*Hordeum vulgare* L.) is the most susceptible cereal species to excess moisture stress. Waterlogging-induced hypoxia causes major physiological changes that disrupt the photosynthetic machinery of seedlings. The signaling molecule nitric oxide (NO) regulates numerous metabolic processes but increases to toxic levels during hypoxia. Phytoglobin1 (Pgb1) functions as a NO-scavenging hemeprotein that influences hypoxic stress responses such as ethylene-mediated adaptations and nitrate use. This study demonstrated that overexpression of *HvPgb1* maintains photosynthetic rate in barley during waterlogging, but also confirms previously reported decreases in physiological performance during normoxic (control) conditions. Foliar ethylene was found to decrease in waterlogged wild type (WT) but remain unchanged in the *HvPgb1*-overexpressing line [HvPgb1(OE)]. It is proposed that ACC from the hypoxic root mobilizes to the shoot, where it induces HvPgb1 activity and nitrate generation. Foliar nitrate was measured as well and showed a significant increase in leaves of waterlogged HvPgb1(OE) plants, while remaining unchanged in waterlogged WT leaves. These changes were associated to a putative increased in the levels of polyamines. Finally, *HvPgb1* expression was measured from a group of selected waterlogging-tolerant and susceptible genotypes. A sharp increase in gene expression was seen in the leaves of waterlogged tolerant genotypes, thus positioning *HvPgb1* expression as a potential molecular marker for waterlogging tolerance in barley.

FOREWORD

This thesis follows the paper style outlined by the Department of Plant Science and Faculty of Graduate Studies at the University of Manitoba. The manuscript follows the style of recommended by the Journal of Plant Physiology. This thesis is presented as a single manuscript, containing an abstract, introduction, materials and methods, results, discussion, and conclusion section. A literature review precedes the manuscript, while supplementary figures are positioned after the body of the manuscript.

1. LITERATURE REVIEW

1.1 Introduction

In global cereal production, barley is the fourth most important following wheat, rice, and maize. In 2019, barley production totaled 159 million tonnes (M t) over 50 million hectares (M ha), giving a yield of over 3 tonnes per hectares (t/ha) led by the Russian Federation, France, Germany, with Canada ranking fourth (FAOSTAT, 2021). In Canada, it is the third largest crop after wheat and canola. Barley is a versatile cereal grain with varieties that have high protein and low starch grown for animal feed, as well as varieties with high starch and low protein used for malting (Giraldo et al., 2019). Strict requirements on malting quality make it difficult for farmers worldwide to grow malting varieties that meet all those parameters. In 2019, the top worldwide exporters of malt were France ($474M), Belgium ($426M), Germany ($376M), Australia ($343M), and Canada ($339M) (Malt OEC, 2019). It is assumed that barley was first used as human food but evolved into a feed, malting and brewing grain, in part due to the rise in prominence of wheat and rice. However, recent research into barley's low glycemic index, low cholesterol and high antioxidants and vitamins (Ames and Storsley, 2015; Chillo et al., 2011) is slowly increasing the demand for the crop to be grown for human consumption as a health-forward product.

Barley production is often limited by unsuitable landscapes and presence of diverse conditions of abiotic stress, including excess moisture. From the years 2006 to 2016, 65% of global crop loss due to abiotic stresses was caused by excess moisture (FAOSTAT, 2021). An

estimated 10% of total arable land is hindered by waterlogging (Yaduvanshi et al., 2012). In Canada, the prairie regions – where barley is primarily produced – are the most affected. The combination of factors such as the flat topography, the impact of land management practices, the large accumulation of snowfall and early spring rainfall, among others, participate in making the prairies susceptible to flooding and other excess moisture problems (Buttle et al., 2016). In Saskatchewan, heavy precipitation caused 49% of crop losses from 2006 – 2015 [Saskatchewan Crop Insurance Corporation (SCIC), 2019]. In Manitoba, excess moisture was the leading cause of crop loss at 38% from 1966 – 2015. Data from 2016 – 2017 showed that it remained to be the leading cause, yet at a much higher value of 71% [Manitoba Agricultural Services Corporation (MASC), 2019]. In 2019, wet harvest conditions caused pre-harvest sprouting, a higher seed moisture content, and higher susceptibility to excess moisture stress (Canadian Grain Commission, 2019). The year's harvest had lower protein content and thus a higher malt extract, but the impact of excess moisture still lowered malting quality. Because of this, the need for different solutions in enhancing barley production and imparting excess moisture tolerance persist, especially in maintaining Canada's economic position in high quality malt production.

With the advent of climate change, precipitation will also continue to be drastic in the coming years (Westra et al., 2014). An increase in both intensity and frequency is expected, along with unpredictable storm patterns. Simulation studies that use climate models predict large increases in flooding in the areas of Southeast Asia, India, and East Africa (Hirabayashi et al., 2013). In Canada, Eastern Manitoba, Western and Southern Ontario and the Atlantic provinces are expected to be affected the most (Province of Manitoba, 2019). To prepare for the worsening probabilities of excess moisture stress in the future, extra attention must be directed in

understanding the physiological and molecular mechanisms contributing to hypoxia tolerance in barley, as its demand continues to grow.

1.2 Hypoxic Stress: Waterlogging and oxygen deprivation

Plants exposed to excess moisture can experience diverse forms of oxygen deprivation conditions which are referred to hypoxia or anoxia. Hypoxia occurs when the partial pressure of O_2 limits ATP production in mitochondria (Pradet and Bomsel, 1978). Anoxia on the other hand, occurs when ATP production via oxidative phosphorylation is negligible compared to the output of glycolysis and fermentation (Pradet and Bomsel, 1978). Hypoxia tends to occur during waterlogging, which is when plant roots are fully submerged in water while the shoot is above the water level. In contrast, anoxia's definition is more specific: true anoxia is only achievable if there is no capacity to perform photosynthesis and respiration inside plant tissues, therefore the environment would need to be totally devoid of oxygen and sunlight which rarely occurs in nature (Sasidharan et al., 2017). For instance, research work that impose anoxic conditions would also substitute atmospheric air with an inert gas in addition to darkness, to come as close to an anoxic environment as possible (Loreti et al., 2005). Waterlogging-induced hypoxia occurs when the pores or air pockets in poor-draining soil become saturated with water, leading to the slow movement of dissolved O_2 into the roots (Drew, 1997), since O_2 diffuses through water 10^4-fold slower than in air (Armstrong and Drew, 2002). Hypoxic conditions can further develop if oxygen level is further lowered by the aerobic activity of the soil microflora competing with the root for oxygen (Barrett-Lennard, 2003). Waterlogging is often dependent on soil characteristics, with poor drainage enhancing the stress, but it can also occur because of cultivation practices, such as a rice-wheat rotation that leads to periodic soil compaction (Samad et al., 2001).

1.2.1 Effects of waterlogging on plants

Development and function of both shoot and root tissue are compromised during hypoxia, since the limited availability of O_2 interferes with metabolic pathways (Visser and Voesenek, 2005). A switch from aerobic respiration to fermentation contributes to the production of ATP in the roots albeit at much lower levels than under normoxic conditions (Barrett-Lennard, 2003). Accumulation of the products of fermentation such lactate and ethanol cause cytoplasmic acidosis that cause cell death and root injury (Drew, 1997), although the disruption of metabolic processes because of ATP limitation are the major causes of cell and tissue damage. For example, the lack of ATP disrupts phosphorylation of aquaporins – proteins that facilitate water transport – in root cells, thus lowering the total soil-to-leaf hydraulic conductance that results in wilting, which is observed in waterlogged plants including *Arabidopsis,* wheat, and maize (Tan et al., 2018). In addition to having lowered access to O_2, hypoxic roots exhibit symptoms of nutrient deficiency and senescence (Dordas, 2009). Root's access to sugars from the shoot is also impeded as ATP is often required for active transport of sugars from the phloem to sink tissues (Armstrong and Drew, 2002). Hypoxia also damages the root apical meristem (RAM) as reported in maize seedlings subjected to the stress, resulting in growth arrest and cell death (Mira et al., 2016).

In shoot tissue, chloroplasts are damaged through the deterioration of the membranes and grana lamellae (Zhang et al., 2015), causing chlorosis and eventually tissue senescence (Luan et al., 2018b). Hypoxia also increases the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) which can cause extensive damage to the photosynthetic machinery leading to decreased photosynthetic rate and stomatal conductance

(Bai et al., 2013; Ploschuk et al., 2018). These effects contribute to chlorosis-reduced growth and ultimately decreased seed production (Huang et al., 1994). It is therefore apparent that waterlogging-induced hypoxic stress not only compromises the function of the root, the initial organ perceiving the stress, but also many other tissues and organs which are above the water level.

1.2.2 Plant response to waterlogging

To cope with waterlogging stress, plants have evolved effective mechanisms that allow survival in O_2-deprived environments. Plant cells undergo metabolic changes to accommodate the energy deficit following the stress, as well as anatomical changes to grant access to more O_2. Some of these mechanisms are avoidance-based, wherein tissues undergo morphological changes like selective cell death during aerenchyma formation or shoot elongation away from flooded soil, to gain more access to O_2 (Justin and Armstrong, 1987). Other mechanisms are tolerance-based which ensure longer-term survival in prolonged waterlogged conditions by involving alternative pathways to return to an energy status fitting of the new hypoxic environment (Armstrong and Drew, 2002)

1.2.2.1 Structural changes

As a crop that requires a flooded environment for its cultivation, rice has been one of the preferred systems to study hypoxia. During submergence, an increase in the gaseous hormone ethylene stimulates the elongation of rice coleoptiles and internodes ~~t~~o maintain the above-water access to O_2 (Ishizawa and Esashi, 1984); this avoidance strategy was also documented in the

petioles of the wetland species *Rumex palustris* (Bailey-Serres and Colmer, 2014). Other aquatic species have cell walls reinforced with suberin and lignin around the root tip to further conserve and distribute O_2 near the meristematic zone and prevent radial loss out into the soil pores (Clark and Harris, 1981). Without this adaptation, the low redox potential of the anaerobic soil environment would have otherwise been a strong sink for O_2, redirecting the minute amount of O_2 available away from the meristematic zone of the root (Drew, 1997). Other cereal species not adapted to flooded conditions such as wheat and barley do not tend to elongate during waterlogging, but rather undergo structural modifications in the root system to favor O_2 acquisition. This includes one of the most documented and most studied avoidance mechanisms against hypoxia, which is the morphological adaptation of forming aerenchyma. These large intracellular spaces within the root (and most of the time, shoot tissue as well) allow low-resistance pathways for gas exchange from shoot to root (Armstrong, 1980). Aerenchyma can form through cell separation (schizogenous formation) or the controlled death of cells to produce these air spaces (lysigenous formation) (Evans, 2004). Like the adaptations mentioned previously, this mechanism is not present in all species. For instance, aerenchyma were found to occupy an area of 20-22 % in wheat roots and 13 -19 % in barley roots, but other waterlogging-susceptible crop species such as rapeseed and field pea are devoid of aerenchyma in addition to a decrease in photosynthetic capabilities (Ploschuk et al., 2018).

Another commonly employed strategy to cope with hypoxia involves the formation of adventitious roots (ARs) that re-route water and nutrient uptake away from the site of the stress (Luan et al., 2018b). They demonstrate negative geotropism and develop horizontally from the stem tissue, granting them more access to O_2 and eventually replacing the damaged primary root

system (Jackson and Drew, 1984). A positive correlation exists between formation of ARs and aerenchyma and tolerance to waterlogging, as demonstrated in maize (Yu et al., 2015), cucumber (Qi et al., 2017), and barley (Luan et al., 2018). In barley, aerenchyma formation also occurs in ARs to further facilitate the movement of oxygen and it has thus been proposed that because of the positive correlation between tolerance and AR formation, the presence of ARs represents a phenotypic indicator for hypoxic tolerance (Luan et al., 2018b).

1.2.2.2 Physiological changes

The anatomical adaptations occurring during hypoxia-induced waterlogging are the results of changes in cell physiology triggered by O_2 deprivation. During anaerobic conditions plant cells rely on fermentation pathways, producing ethanol and lactic acid, to generate ATP, since regular mitochondrial respiration is impaired in O_2 limiting environments (Gibbs and Greenway, 2003). An increase in starch and other soluble sugars was also observed in waterlogged plants and in the root tissue; this increase in starch is quickly utilized to maintain proper root functioning (Sauter, 2013). The starch fuels the transition to anaerobic fermentation, causing the increase in activity of fermentation enzymes such as alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC), often used as biochemical markers in hypoxic studies (Bailey-Serres and Colmer, 2014; Luan et al., 2018b). Other markers used in hypoxic stress tolerance studies include lactate dehydrogenase (LDH), and ethylene-response transcription factors (ERFs) (Voesenek and Bailey-Serres, 2015).

Under hypoxic stress, nitric oxide (NO) is generated through the reduction of nitrite (Cochrane et al., 2017). While acting as an important signal molecule in many developmental and stress-related processes (Groß et al., 2013), as described in the next section, NO can also cause severe cellular damage by favoring the production of reactive oxygen species (ROS). Reactive oxygen species induce oxidative stress when present in excess (Youssef et al., 2016), contributing to cell and tissue damage, especially in photosynthetic tissues (Yordanova et al., 2004).

Alterations in NO levels in hypoxic tissues also contribute to changes in hormone synthesis and response. For example, the levels of jasmonic acid (JA), indole-3-acetic acid (IAA) and ethylene are found to increase in hypoxic tissue (reviewed in Hill, 2012). In rice, besides inducing the expression of *SNORKEL* and *ERF-VII* which are involved in internode elongation responses (Bailey-Serres and Colmer, 2014), hypoxia induced by waterlogging or submergence increases the expression of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and ACC synthase, which participate in ethylene biosynthesis (reviewed in Sasidharan and Voesenek, 2015). Jasmonic acid can also alter NO homeostasis, which modulates the activity of several stress-related enzymes including glutathione S-transferase (GST), an attenuator of oxidative damage (Kamal and Komatsu, 2016), as well as the expression of the pathogenesis-related protein *TaBWPR-1.2* in root tissue (Haque et al., 2014).

An increase in gibberellic acid is also observed in hypoxic tissues; in waterlogged rice this increase promotes internode elongation (Voesenek and Bailey-Serres, 2015). A similar response is observed in *R. palustris* where it induces petiole elongation (Voesenek and Bailey-

Serres, 2015). When it comes to physiological changes in the shoot, abscisic acid (ABA) regulates systemic responses as demonstrated with the use of ABA-insensitive mutants (Hsu et al., 2011). A rise in ABA following waterlogging contributes to the closing of the stomata to reduce transpiratory processes minimizing and oxygen escape (Bai et al., 2013).

The generation and development of ARs are mainly mediated by the interaction between ethylene and auxin (Sauter, 2013) where ethylene first triggers auxin activity in order to promote the development of root primordia. This has been observed in maize, soybean, cucumber, tomato, and barley exposed to hypoxic stress (reviewed in Luan et al., 2018). Adventitious root formation allows the tolerant plants to obtain O_2 from the atmosphere as, in most cases, they grow out of shoot tissue above the flooded level.

1.3 Nitric oxide

1.3.1 Roles in plant development

Nitric oxide is a gaseous, water and lipid-soluble metabolite that has signaling functions in plants (Neill et al., 2003). It is involved in pathways that pertain to development, including those that regulate seed germination and dormancy, flowering, and root development (Gupta et al., 2011), but also in homeostatic pathways such as transpiration management and tissue senescence (Dordas, 2009). Higher NO concentrations (40-80 ppm) have been reported to impede growth of tomato, lettuce, and pea, while lower concentrations stimulated their growth (Hufton et al., 1996; Leshem and Haramaty, 1996). Nitric oxide is a signal molecule involved in

the ABA-driven stomatal closure by regulating K^+ and Cl^- channels (Guo et al., 2003). As a radical, its reactivity makes it unstable around O_2 and ROS, and readily available to form reactive NO species (RNOS) that cause damage via oxidative reactions (Kim et al., 2001). Because of these properties, extensive information on NO physiology relates to cell death.

1.3.2 NO synthesis

The main enzymatic pathway proposed in the synthesis of NO during hypoxia has nitrate reductase (NR) as its main catalyst. During root hypoxia, NR itself is upregulated and it uses NAD(P)H to reduce NO_2^- to NO (Igamberdiev and Hill, 2004). Other hemeproteins that synthesize NO during hypoxia include cytochrome *c* oxidase and the bc_1 complex, both of which also reduce NO_2^- and both belonging to the mitochondrial electron transport chain (Stoimenova et al., 2007). However, some hemeproteins retain an oxygenated state for extended periods of time and have a very high affinity for O_2, such as phytoglobins – previously termed nonsymbiotic hemoglobins in plants (Hill et al., 2016) – and therefore cannot reduce NO_2^- (Igamberdiev et al., 2010). Instead, they operate the reverse reaction of scavenging NO to produce NO_2 or NO_2^- (Igamberdiev et al. 2010).

1.3.3 Roles of NO in biotic and abiotic stress

Nitric oxide plays signaling roles during diverse conditions of stress. In biotic stresses, NO involvement in the hypersensitive response (HR) has been well documented, with NO being a messenger during the necrotic response around the site of infection following pathogen attack

(Neill et al., 2003). Soybean inoculated with *Pseudomonas syringae* accumulated NO, as well as exhibited high expression of defense-related genes such as phenylalanine ammonia-lyase (*PAL*) (Delledonne et al., 1998). In potatoes treated with NO donors, there was an accumulation of the phytoalexin rishitin, an antimicrobial terpenoid (Neill et al., 2003). The increased NO production soon after infection and the results of its exogenous application suggest NO acts upstream in defense signaling against biotic stress.

Abiotic stresses such as hypoxia, drought, ultraviolet radiation and ozone exposure induce plant responses that are also found to be moderated by NO and, one such example is the signaling role it has in stomatal closure (Neill et al., 2003). In *Tradescantia* spp. and *Vicia faba*, NO has been confirmed to be downstream of ABA in the signal transduction for stomatal closure (Mata and Lamattina, 2001). Relative to normoxic conditions, NO is produced at the onset of hypoxia, as observed in maize and alfalfa cells (Dordas et al., 2003a). The same authors suggested the rise in NO to be sufficient to contribute to the death program of the cortical cells and formation of aerenchyma (Dordas et al., 2003b).

Despite its roles in signal transductions during stress responses, the accumulation of NO could also lead to the formation of toxic reactive NO species (RNOS) such as peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3), which would lead to nitrosative stress (Neill et al., 2003). Damages resulting from nitrosative stress include single strand breaks in DNA, inhibition of repair mechanisms (Kim et al., 2001), and interference with mitochondrial respiration (Neill et al., 2003).

1.4 Reactive oxygen species (ROS)

1.4.1 Deleterious effects of ROS

Reactive oxygen species (ROS) such as H_2O_2 and O_2^- are produced due to homeostatic processes but also in response to stresses (Miller et al., 2010). In peroxisomes and mitochondria, they are synthesized in various electron transport reactions and membrane-bound NADPH oxidase, with O_2^- reacting with superoxide dismutase (SOD) to synthesize the more stable H_2O_2 (Mhamdi and van Breusegem, 2018). In chloroplasts, the non-radical singlet oxygen ($^1O_2$) is a product of photodynamic reactions and produced by photosystem II (Triantaphylidès and Havaux, 2009). Overproduction of ROS has been reported from plant exposure to pathogen infection (Yang et al., 2017), heavy metals (Vanhoudt et al., 2010), drought (Lee et al., 2012), salinity (Miller et al., 2010), pollutants (Ahammed et al., 2017) and waterlogging (Yamauchi et al., 2017) stresses. As strong oxidants, they damage plant cells by causing oxidative damage on lipids, proteins and nucleic acids (Apel and Hirt, 2004). A well-studied effect is the PCD-mediated aerenchyma formation induced by ROS as a mechanism to cope with hypoxic stress (Yamauchi et al., 2014). Depending on the response, plants can limit the amount of oxidative damage through activation of antioxidant enzymatic reactions and production of antioxidants such as glutathione, ascorbic acid, and polyphenols (Yordanova et al., 2004).

In hypoxic maize roots, the rise in ROS is governed by the rapid increase in ethylene driven by an overproduction of NO (Mira et al., 2016). The cell death caused by the rise in ROS was observed primarily in the quiescent center (QC) of the root apical meristem (RAM),

preventing root growth (Mira et al., 2016). Therefore, a system that scavenges NO as one of the earlier players in the stress response would be an important factor in avoiding further ROS-oxidative damage and excess PCD.

1.4.2 Reactive oxygen species in signaling and stress response

Reactive oxygen species (ROS) have long been hypothesized to have a signaling function during homeostatic physiological processes including plant growth (Foreman et al., 2003), PCD (Levine et al., 2003), and especially in responding to environmental stimuli (Miller et al., 2009). In response to stress and other stimuli, a burst of ROS is produced as well as that of antioxidant enzymes that scavenge and detoxify them (Apel and Hirt, 2004). It is the interplay between production and scavenging of ROS that determines the final effects of these species (Mittler et al., 2011). In particular, H_2O_2 has been linked to waterlogging stress signaling; it promotes aerenchyma formation in rice (Steffens et al., 2011) and ARs formation in cucumber (Qi et al., 2017). Knocking out respiratory burst oxidase homologs (*RBOH*) in rice roots decreases the ROS accumulation required to trigger aerenchyma formation during waterlogging stress (Yamauchi et al., 2017).

As previously mentioned, ROS are synthesized typically as by-products by enzymes active in homeostatic processes including photorespiration (Mittler, 2002). The enzymes responsible for ROS synthesis are a family of NADPH oxidases called respiratory burst oxidase homologs (RBOHs) (Torres and Dangl, 2005). Environmental stimuli such as drought, salinity, cold temperatures, wounding, and excess moisture (Qi et al. 2019) increase ROS through

induced RBOH (Baxter et al., 2014). In Arabidopsis, two of these oxidases, AtrbohD and AtrbohF work together to generate ROS responsible for the induction of hypoxia markers alcohol dehydrogenase (*ADH*), lactate dehydrogenase (*LDH*) and pyruvate decarboxylase (*PDC*) (Liu et al., 2017). Other RBOHs are reported to mediate the establishment of crucial symbiotic nodules in *Medicago*, as well as having functions in the hypersensitive response against pathogens (Suzuki et al., 2011). Because of their involvement in signal transduction, RBOHs are also commonly utilized as markers for ROS production (Youssef et al., 2016).

ROS and ethylene control of aerenchyma formation is also a heavily studied response following excess moisture induced hypoxia: H_2O_2 and ethylene together induce the development of aerenchyma in deepwater rice, as well as the hypoxic response of Arabidopsis (Pucciariello and Perata, 2017). In maize roots, O_2^- and H_2O_2 levels increased due to sulfate starvation in cells that underwent PCD to form aerenchyma (Evans, 2004). Given that NO is required to destabilize the ERFs that sense fluctuations in O_2, it is the crosstalk between ROS and NO that is then believed to be the basis for anaerobic metabolism and stress acclimation (Pucciariello and Perata, 2017).

1.5 Phytoglobins (Pgb)

Plant hemoglobins are hemeproteins that were first discovered in soybean's nitrogen-fixing root nodules (Kubo, 1939). Like their counterparts in animals, they bind O_2 as well as other ligands including NO, carbon monoxide, and hydrogen sulfide (Jackson, M B; Drew, 1984). The symbiotic leghemoglobins (LegHgbs), as they were later referred to, are involved in

the regulation and provision of O_2 for N_2-fixing bacteria (Dordas, 2009). Eventually, two other types of Hgbs were discovered: nonsymbiotic Hgbs – recently and appropriately renamed as phytoglobins (Pgbs) (Hill et al., 2016) – which are subdivided into class 1, found in both monocots and dicots, and class 2 which is exclusive to dicots (Landsmann et al., 1986) as well as the truncated Pgbs (class 3), termed as such due to the different protein structure (Wittenberg et al., 2002). Class 1 Pgbs have the strongest affinity for O_2 (Smagghe et al., 2009), with class 3 having the least (Watts et al., 2001).

1.5.1 Classes of Pgb

LegHgbs are like animal myoglobins in their association with oxygen. They are expressed in root nodules of leguminous plants that established symbiotic relationships with N_2-fixing soil bacteria such as *Rhizobium* (Duff et al., 1997) and function to buffer oxygen concentrations in the nodules (Appleby, 1984). Their pentacoordinate structure permits ligand binding with O_2 (Gupta et al., 2011). The mobility of its distal histidine sidechain allows O_2 to bind and be released (Appleby, 1992).

Given the high affinity of Pgbs for O_2, it has been speculated that Pgbs are not involved in the transport of O_2 (reviewed by Hill, 2012). Rice Pgb1's O_2 affinity, for instance, was calculated to be 78-times stronger than soybean LegHgb *a* (Arredondo-Peter et al., 1997), showing that once O_2 binds with Pgb, it is stabilized and unlikely to be released. Instead, class 1 Pgbs scavenge NO and participate in the redox balance during the waterlogging-induced hypoxic stress (reviewed in Gupta et al., 2011) and other abiotic stresses (Mira et al., 2021a, 2017;

Sørensen et al., 2019). Class 1 Pgbs also have roles in different plant organs at varying stages of development. Rice Pgbs were found expressed in the cytoplasm of differentiating tissues including the scutellum, root cap, and tracheary elements (Garrocho-Villegas et al., 2007). However, increased levels of transcripts and proteins occur when plants are stressed (Andrzejczak et al., 2020; Chen et al., 2014; Choudhury et al., 2013; Torres and Dangl, 2005). Focus on class 1 Pgb and its involvement in hypoxic responses will be discussed in later sections.

Class 2 Pgbs, studied extensively from *A. thaliana* and *L. esculentum*, are interestingly closer to symbiotic LegHgbs in structure and O_2 binding traits, despite not having a symbiotic function, as well as having a lower O_2- affinity relative to class 1 Pgbs (Smagghe et al., 2009). Transgenic *Arabidopsis* overexpressing class 2 Pgb showed an increase in fatty acid content in seed tissue, leading to the assumption that it has roles in delivering O_2 to developing tissues (Vigeolas et al., 2011). Although it shares a NO-scavenging function with class 1 Pgbs, a greater expression of class 2 Pgbs was observed following cytokinin and low temperature treatments (Hunt et al., 2002), making it unlikely to have primary roles in hypoxic stress tolerance.

Class 3 or the truncated Pgbs (TruncPgbs) are the least studied with still unclear functions in plant development. There has been more attention paid to their roles on bacterial and unicellular eukaryotes (Garrocho-Villegas et al., 2007). In the algae *Chlamydomonas eugamentos*, TruncPgbs are induced during active photosynthesis in the thylakoid membranes and in the cyanobacterium *Nostoc commune*, at the cytoplasmic side of the membrane during anaerobic conditions (Wittenberg et al., 2002).

1.5.2 Class 1 Phytoglobin: Structure and role in NO scavenging

The discovery of Pgbs in non-leguminous plant species that do not form mutualistic relationships with N_2-fixing bacteria (Landsmann et al., 1986) including barley and other monocot species (Taylor et al., 1994), broadened the physiological functions of Pgbs. The hexacoordinate structure of class 1 Pgb is what allows it to have a very strong affinity for O_2 compared to the pentacoordinate LegHgbs and class 2 Pgbs, therefore allowing it to function even during low O_2 availability (Smagghe et al., 2009). In hypoxia-tolerant genotypes, a notable decrease in NO concentrations was reported when the gene *Pgb1* is upregulated (reviewed in Hill, 2012). The strong affinity for O_2 and its antagonistic relationship with NO is what contributes to Pgb's NO-scavenging function, thus permitting the PCD-derived adaptations without the uncontrolled damage from excess NO and ROS.

Class 1 Pgbs in cereals are expressed in aleurone and embryo tissue during germination (Taylor et al., 1994). In roots, they are expressed during abiotic stresses such as nutrient deprivation (Garrocho-Villegas et al., 2007), drought (Mira et al., 2017), and hypoxia (Taylor et al., 1994), but almost undetectable in normal conditions. Dordas et al. (2003a) have hypothesized that, since it is unlikely for them to function as O_2 transporters, class 1 Pgbs would instead have regulatory functions on NO levels that increase during hypoxic stress. This was confirmed when hypoxic alfalfa roots down-regulating *Pgb1* had NO levels 2.5-fold higher than that of Pgb-over-expressing roots (Dordas et al., 2003a).

The scavenging properties of Pgbs have been documented in the Pgb/NO cycle elaborated in transgenic maize and alfalfa root culture (Igamberdiev et al., 2005) (Fig 1). Class 1 Pgb's strong affinity for O_2 allows it to retain its oxygenated form (OxyPgb) which is the form responsible for scavenging NO (Dordas et al., 2003b). In the cytoplasm where OxyPgb and NAD(P)H levels are high, OxyPgb oxidizes NO to produce nitrate (NO_3^-) and ferric Pgb (MetPgb). MetPgb is then reduced by MetPgb reductase with NAD(P)H. The high affinity of Pgb for O_2 causes an almost immediate oxygenation back to its OxyPgb form, and the cycle repeats (Igamberdiev et al., 2005).

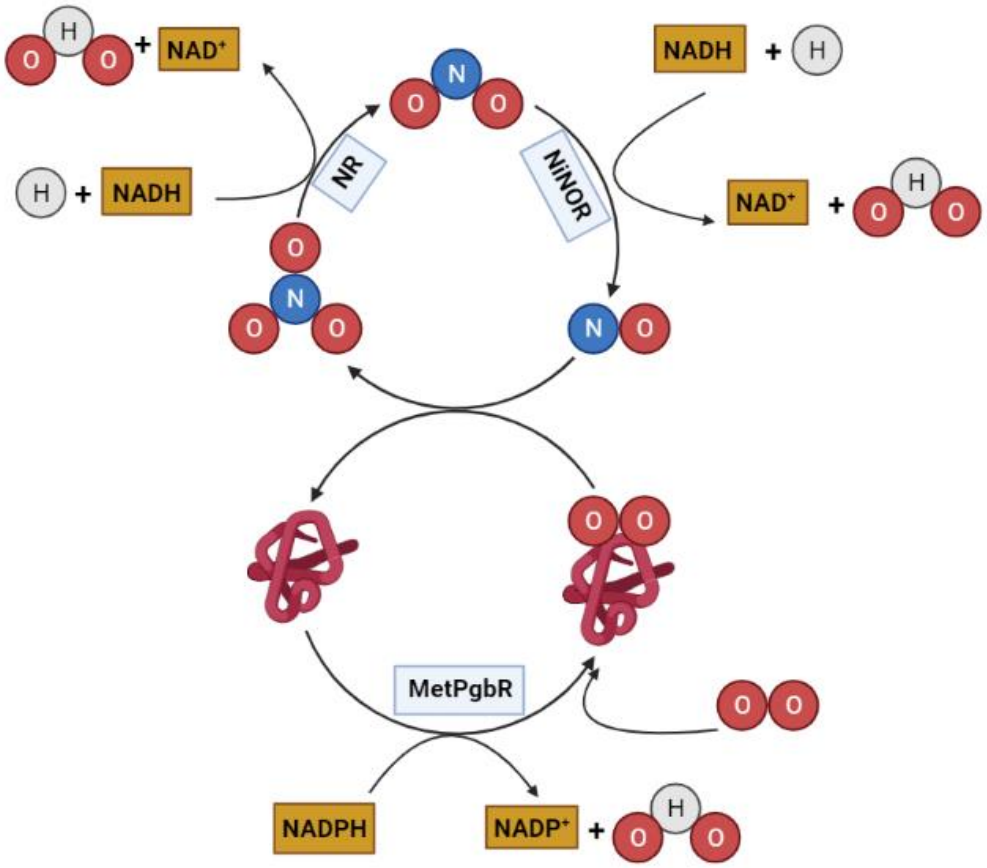


Figure 1. Phytoglobin1-Nitric oxide cycle (adapted from Gupta et al., 2011). Nitrate is reduced to nitrate by nitrite reductase (NR), nitrite is oxidized to nitric oxide (NO) by nitrate-NO reductase (NiNOR), and NO is reduced to nitrate by oxygenated Phytoglobin1. The metphytoglobin form can then be reduced back to its oxygenated form by metphytoglobin reductase (MetPgbR) to scavenge more NO.

1.5.3. Class 1 Phytoglobin and stress response

Instances of class 1 Pgb (Pgb1) induction following biotic stresses have been reported in some species, such as *Lotus japonicus* and cotton during fungal infection (Dordas, 2009).

Alteration of Pgb expression even enhanced defense mechanisms, as in the case of Pgb1-over-expression in *Arabidopsis* conferring tolerance to *Verticillium dahliae* and *Pseudomonas syringae* (Qu et al., 2006). The role of Pgb1 has been associated more with abiotic stress responses, and especially hypoxia where Pgb1 exercises a protective role (Hill, 2012; Silva-Cardenas et al., 2003).

In hypoxic maize cultures overexpression of the barley Pgb, maintained a high energy status needed to cope with the stress (Sowa et al., 1998). When the same Pgb gene was overexpressed in alfalfa root cultures subjected to oxygen deprivation, root growth persisted; this was in contrast to roots suppressing Pgb1 which exhibited a 30-70% decline in growth (Dordas et al., 2003a). The protective role of Pgbs was also demonstrated in whole plant systems: a strong positive correlation was observed between the expression of the maize Pgb1, *ZmPgb1,* and the ability to tolerate waterlogging stress (Youssef et al., 2016). In the same study, transgenic hypoxic lines over-expressing *ZmPgb1* retained a high photosynthetic rate, and this effect was linked to a reduced accumulation of O_2^- and H_2O_2 in leaves (Youssef et al., 2016). In contrast, transgenic lines downregulating *ZmPgb1* had higher leaf injury, decreased photosynthetic rate, and increased ROS production. The reduction in ROS and thus the reduction in oxidative damage in leaves, can therefore be attributed to the NO scavenging properties of Pgb upstream of ROS production (Youssef et al., 2016).

The deleterious effect of ROS during conditions of stress is most apparent at the root tip harboring the meristematic cells (Dordas et al., 2003a). In water stressed Arabidopsis roots, ROS accumulates within the quiescent center (QC) – the undifferentiated organizing center of the

RAM that maintains root growth, triggering the death program. These effects were mitigated by an elevation in Pgb1 and exacerbated by its suppression (Mira et al., 2017). A similar effect was also documented in hypoxic maize root tips (Mira et al., 2016).

Other types of abiotic stress modulated by Pgb through ROS are cadmium toxicity (Bahmani et al., 2019), and iron deficiency (Mira et al., 2021b). Transgenic Arabidopsis plants over-expressing the tobacco Pgb1 (*NtHb1*) exhibited diminished ROS levels and better growth under cadmium toxicity (Bahmani et al., 2019).

1.5.4 Phytoglobin roles in development of plant meristematic tissues and organs

The function of Pgbs has also been investigated, albeit to a lesser extent, during plant development. Phytoglobins are expressed in germinating barley embryos (Guy et al., 2002) and in Arabidopsis meristematic tissue (Hebelstrup et al., 2006), and their roles in organ development were demonstrated in a study that altered the expression of both class 1 and 2 Pgbs (*AHb1* and *Ahb2*). Silencing *Ahb1* caused abnormal floral bud and leaf hydathode development, yet still yielding a viable plant. While normal growth was observed in plants where *Ahb2* was mutated, silencing of both AHB1 and 2 proved to be lethal (Hebelstrup et al., 2006). The function of Ahb2 was further explored by Elhiti et al. (2013), who showed that suppression of Ahb2 increased somatic embryo formation through an elevation in auxin levels. Using maize somatic embryogenesis as a model system, Huang et al. (2014) linked the function of Pgb in the prevention of PCD, with embryo development; and further demonstrated that the specific localization of Pgbs can evoke different responses. Suppression of *ZmPgb1* expressed

ubiquitously in the embryogenic tissue triggered the abortion of the embryos. This was in contrast to suppression of *ZmPgb1* present only in the "anchor cells" connecting the developing embryos to the subtending embryogenic tissue, which caused their degradation of the "anchor cells" thus favoring the growth of the embryos (Huang et al., 2014).

1.6 Ethylene

Ethylene is a gaseous plant hormone with functions in plant development, germination, senescence, and ripening (reviewed in Ju and Chang, 2012). It has major roles in alleviating hypoxic stress from excess moisture, including soil waterlogging and total submergence (Voesenek and Sasidharan, 2013). Ethylene biosynthesis begins when 1-aminocyclopropane-1-carboxylic acid (ACC) synthase is induced by low O_2 levels, converting *S*-adenosylmethionine to ACC, which is then oxidized to ethylene by ACC oxidase (Vriezen et al., 1999).

1.6.1 Roles in aerenchyma formation and stomatal closure

The induction of ethylene following hypoxia induces numerous downstream effects. With regards to waterlogging stress, ethylene has been classically correlated with leaf epinasty as a symptom (Jackson and Campbell, 1975) but also with stress avoidance strategies including AR formation ~~via~~ and aerenchyma formation in the root tissue (Drew et al., 1979). Ethylene is required for the formation of lysigenous aerenchyma in barley (Shiono et al., 2019), as well as other monocots including wheat (Mc Donald et al., 2001), and maize (Jackson et al., 1985). The

ethylene-induced formation of aerenchyma is mediated by ROS signaling, culminating in programmed cell death in specific root domains (Evans, 2004), most commonly in the root cortex (Yamauchi et al., 2011).

Abscisic acid (ABA) is one of many hormones that interact with ethylene during plant development, with ethylene having an antagonistic effect in ABA-mediated germination and root growth (Ghassemian et al., 2000). More importantly, ABA is known for its major role in regulating stomatal closure through its effects on ion channels within the guard cells (reviewed in Armstrong et al., 1995). In WT Arabidopsis, the co-application of ABA with ethylene precursor ACC increased the size exclusion limits of the stomata, relative to plants only treated with ABA (Tanaka et al., 2005). A similar observation was found when the same treatments were performed on a mutant line overproducing ethylene, showing that both exogenous and endogenous ethylene inhibit ABA-induced stomatal closure (Tanaka et al., 2005). Since stomatal conductance is also being decreased by waterlogging (Ploschuk et al., 2018; Youssef et al., 2016), it can be assumed that the ABA-induced stomatal closing is also mediated by crosstalk with ethylene.

1.6.2 Ethylene Response Factors and the N-end rule pathway

One of the more recent developments in ethylene-mediated hypoxia responses is the relevance of Ethylene Response Factors (ERFVII) in triggering the expression of hypoxia-tolerance genes (Voesenek and Bailey-Serres, 2015). Under normoxic conditions, O_2 and NO partake in the N-end rule pathway, where PROTEOLYSIS6 (PRT6) degrades ERVIIs, thus

preventing the expression of anaerobic metabolism genes including *ALCOHOL DEHYDROGENASE* (ADH), *PYRUVATE DECARBOXYLASE* (PDC) (Gibbs et al., 2015) and *ALANINE AMINOTRANSFERASE* (AlaAT) (Hartman et al., 2021). In barley, *HvPRT6* RNAi lines were able to maintain total chlorophyll relative to the wild type in waterlogging stress, thus showing that the reduction in *HvPRT6* expression increases waterlogging tolerance (Mendiondo et al., 2016). During hypoxia, the presence of ethylene promotes Pgb1 production which decreases NO, thus halting the degradation of ERFVIIs and allowing its induction of hypoxia-tolerance genes (Hartman et al., 2019).

1.7 Barley

1.7.1 Susceptibility to waterlogging

From an agronomic perspective, tolerance to waterlogging is perceived as the capacity of the plant to maintain grain yield despite the stress (de San Celedonio et al., 2014) and compared to other cereal crops, barley is the most susceptible to waterlogging stress (Borrego-Benjumea et al., 2020; Setter and Waters, 2003). A comparative study between wheat and barley under natural waterlogging resulted in 19-82% yield reduction in wheat, and 51-84% in barley (Setter et al., 1999). The yield trait that waterlogging affected in barley was also different from wheat: a greater reduction in the number of spikes per plant was more prominent in barley, while wheat tended to show a greater reduction in the number of fertile florets per spike (de San Celedonio et al., 2018). The growth stage of the crop is also crucial in determining susceptibility to

waterlogging for barley, the range between leaf emergence and booting has been identified as the most vulnerable (Borrego-Benjumea et al., 2019). In barley excess moisture also causes a significant reduction (up to 50%) in chlorophyll a + b content and this decline was alleviated when Pgb was up regulated (Andrzejczak et al., 2020). Another common symptom observed in waterlogged barley is a reduction in stomatal conductance. After a week of early-waterlogging, stomatal conductance was reduced to 38% of its control and even more so to 27% of control after prolonged waterlogging (Ploschuk et al., 2018). Despite being very susceptible to excess moisture, when compared to other cereals, barley has demonstrated capacity to recover, with most of its gas exchange parameters recovering after a week of removing them from waterlogging stress (Ploschuk et al., 2018; Setter et al., 1999). Improving this characteristic is the focus of several current breeding programs.

Total crop losses due to waterlogging has historically averaged around $74 billion USD (Voesenek and Sasidharan, 2013). In Australia, $300 million AUD make up total crop losses due to waterlogging, with wheat and barley as the two most affected (Manik et al., 2019). In two of Canada's provinces, Manitoba and Saskatchewan, excess moisture insurance claims were on par with those of drought and heat from 1966-2005 (MASC, 2020). But from 2005-2015, excess moisture claims have surpassed drought claims by almost 30% (MASC, 2020). With excess moisture as the new lead cause of yield loss in barley and the projected 30% increase in precipitation by 2030 (Rosenzweig et al., 2002), the need for new tolerance strategies is clearly becoming more urgent. Currently, most studies that plan to utilize waterlogging tolerance mechanisms rely on work related to quantitative trait loci (QTLs). Those identified in barley so far, have involvements in root aerenchyma formation (Zhang et al., 2016), root membrane

potential (Gill et al., 2017), and ROS formation (Gill et al., 2019); yet these traits are not necessarily the primary genes responsible for the response to waterlogging (Zhang et al., 2017). Proteomic analyses done by Luan et al. (2018) have also identified pyruvate decarboxylase (PDC), ACC oxidase (ACO) and glutathione S-transferase (GST) as candidate genes after observing upregulation in a waterlogging-tolerant genotype.

1.7.2 Class 1 Phytoglobin in barley

Taylor et al. (1994) were the first to characterize the barley Pgb1, demonstrating its expression within the aleurone layers, and its induction at the onset of hypoxia. Barley class 1 Pgb (Pgb1) has an O_2 affinity two magnitude of orders higher (2-3 nM) than that of cytochrome c oxidase (COX), which is an enzyme that converts NO_2^- to NO (Stoimenova et al., 2007). It is also mobile and soluble, unlike COX which is membrane-bound (Stoimenova et al., 2007), thus making Pgb a more efficient factor in the prevention of NO toxicity.

One of the earliest studies demonstrating the effect of the barley Pgb during excess moisture employed transgenic alfalfa root cultures (Dordas et al., 2003a). The hypoxic inhibition of root growth was attenuated by the over-expression of Pgb1, while augmented in roots suppressing the same gene. Suppression of the barley Pgb1 also resulted in the degradation of cortical cells, an observation that the authors interpreted as the formation of aerenchyma (Dordas et al. 2003a). These effects were mediated by the ability of Pgb1 to scavenge NO.

Class 1 phytoglobin is also expressed in the seed's aleurone layer and was hypothesized to have major influences in germination (Taylor et al., 1994). Germination requires a high

amount of energy and, especially upon imbibition, O_2 is consumed thus creating a hypoxic environment. Zafari et al. (2020) observed decreased germination rates in *Pgb*-knockdown barley lines compared to control, while enhanced germination rate and longer roots were observed in *Pgb*-overexpressing lines (Zafari et al., 2020). These effects were ascribed to the ability of the barley Pgb1 to scavenge NO, which suppresses the activity of key enzymes essential for the maintenance of the redox balance.

1.7.3 Yield penalty in Phytoglobin-overexpressing barley

A study by Cochrane et al. (2017) demonstrated that there is a considerable trade-off to *Pgb*-overexpressing plants in normal "unstressed" conditions. Transgenic Pgb-overexpressing barley had lower biomass, an almost 50% reduction in seed yield, and an overall delayed development compared to *Pgb*-suppressed and WT lines (Cochrane et al., 2017). Since NO is a component of flower induction in cereals and the triggering of the HR against pathogens, Hebelstrup et al. (2014) also noted *Pgb*-overexpression's deleterious effect on flowering (Hebelstrup et al., 2014) as well as the partial breakdown in resistance to the fungus *Blumeria graminis* (Sorensen et al. 2019). A later proteomic study found a decrease in the hypersensitive response protein Downy Mildew Resistance 6 (BAJ89811.1) in a *Pgb*-overexpressing line (Andrzejczak et al. 2020), further supporting the idea that a strong reduction in NO by increasing Pgb could weaken defenses against biotic stresses. These results show that, without further research, the prospect of utilizing transgenic lines with enhanced *Pgb* expression would not be optimal for breeding purposes (Hebelstrup et al. 2014).

Based on these premises, the purpose of this thesis was to further refine the effects of barley *Pgb1* over-expression under waterlogged conditions, by measuring several morpho-physiological parameters known to be affected by oxygen deficiency. For this purpose, we used a previously characterized barley line over-expressing *HvPgb1* (Hebelstrup et al., 2014) and compared its responses to root waterlogging to those of wild type (WT) plants. The objectives of the research project were: to confirm if *HvPgb1* alteration induces waterlogging tolerance in barley, to investigate how HvPgb1 influences ethylene and nitrate activity during waterlogging stress, and to examine the potential of *HvPgb1* expression as a selection tool for waterlogging-tolerant barley cultivars.

2. MATERIALS AND METHODS

2.1 Plant Material

22 barley (*Hordeum vulgare* L.) genotypes were provided by Dr. Ana Badea (Agriculture and Agri-Food Canada, Brandon). Seeds were soaked in reverse osmosis water in the dark overnight. The following day, they were potted in 8.5 cm^3 pots (one plant per pot) layered with half an inch of silica sand covered with soilless potting mix (*Sunshine* mix #4: sphagnum peat moss and perlite). Plants were grown in a growth chamber [16-hour day/8-hour night at 24°C (day) and 20°C (night)] at 3500 μE $m^{-2}s^{-1}$ until the two-leaf stage of development. To prevent potting mix from floating during the waterlogging treatment a layer of silica send was applied on top of the potting mix. Pots were then placed in rectangular 94 x 38.3 x 16.3 cm plastic bins in a randomized complete block design and waterlogging stress was imposed then maintained a water level of 2.0 cm above the soil. Control plants were placed in the same bins without the stress and watered every other day. For all experiments described, each treatment had three biological replicates with each replicate consisting of al least three plants. The same protocol was followed for experiments comparing *HvPgb1*-overexpressing transgenic line and its wild type, cv. Golden Promise.

2.2 Measurement of Gas exchange parameters

The photosynthetic rate, stomatal conductivity, internal CO_2 level, and transpiration rate were measured using an infrared gas analyser (IRGA; LI-6400, LI-COR, Inc., Lincoln, NE,

USA). Measurements were carried out on the second oldest leaves under photosynthetically active radiation of 400 µmol/m^2s^{-1}, at 28°C, and with a CO_2 flow of 400 µmol/mol. All measurements were conducted between 9am and 4pm after 7 days of waterlogging.

2.3 Chlorophyll quantification

Chlorophyll measurements were conducted on second leaves after 7 days of waterlogging treatment using the procedure described in Metzner et al. (1965). Leaf discs (0.4 cm wide) were macerated in 80% acetone in a tissuelyser (*Precellys24*, Bertin technologies) for two minutes. After centrifugation at 12,000 g for five minutes at 20°C, the supernatant was collected for spectrophotometric analyses (Ultrospec 2100 Pro, Buckinghamshire, UK). Total chlorophyll content was quantified by measuring the absorbance at 645 nm and 663 nm and the concentration was calculated using the following formula:

$$\text{Total chlorophyll (µg/ml)} = 20.2\ (A_{645}) + 8.02\ (A_{663})$$

2.4 RNA extraction and Gene expression

The third youngest leaf was sampled for RNA extraction on 0-, 1-, 2- and 8-days post-waterlogging from 10 am – 1 pm. Extraction was performed following the protocol for *Qiagen*'s RNeasy Plant mini kit. RNA concentration and optical density were checked using a NanoDrop spectrophotometer (ThermoScientific). cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Measurements of relative gene expression of *HvHb1* was taken via quantitative real time PCR (qRT-PCR) and analyzed using Livak's $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). *HvGADPH* was selected as the reference gene.

2.5 Pharmacological treatments

Silver nitrate ($AgNO_3$), an ethylene receptor blocker (Drew et al. 1981) and ethephon, an ethylene donor (Roblin and Perault, 1985), were used as foliar sprays to alter shoot ethylene activity. 1.0 mM of $AgNO_3$ solution was made with RO water, 0.01% Tween 20, with crystalline $AgNO_3$ (Mallinckrodt, UK). 1.0 mM of ethephon (2-chloroethylphosphonic acid, ACROS Organics, NJ, USA) solution was prepared similarly using RO water and Tween 20. Sprays were applied two times prior to imposition of waterlogging to ensure droplets were allowed to dry. Total application rates were approximately 16.67 mL per plant. Leaves and roots were collected for ethylene measurements 24 hrs post-WL. Chemical concentrations were selected based on Labraña and Araus (1991).

2.6 Nitrate and nitrogen quantification

Entire shoot tissue from control and stressed plants after 2 days of waterlogging were harvested and oven-dried at 90°C for 5 days, with 500 – 1000 mg of dried tissue per sample used

for further analysis. Measurements of nitrate and nitrogen were conducted with *Agvise* (Agvise Laboratories Inc., Northwood, ND, USA; https://www.agvise.com/). Extraction of both elements were performed using 1M ammonium acetate, and determination was carried out by means of inductively coupled plasma atomic emission spectroscopy (ICP-AES).

2.7 Ethylene gas quantification

At 1-day post-waterlogging, root (about 0.15 g) and shoot (about 0.60 g) samples were collected and sealed in test tubes and incubated in the dark for 2 h at 22°C. A 10.0-mL syringe was used to transfer 3.0-mL of gas from the tube and into the headspace of a Bruker 450-GC gas chromatograph. Ethylene gas was quantified and analyzed using the Bruker Compass Data analysis 3.0 software, as reported in Mira et al. (2017).

2.8 Cytological analysis

Root samples from plants 3-days-post-waterlogging were free-hand sectioned 2 cm from the root tip and stained with toluidine blue O (modified from Trump et al., 1961). Sections were visualized and captured using Leica (Model DMRE).

2.9 Statistical analysis

Data analysis was conducted by one-way t-test and ANOVA using SPSS (IBM Corp. Released 2013. 466 IBM SPSS Statistics for Windows, Version 22.0 Armonk, NY: IBM Corp.).

Treatment means were compared by Tukey-HSD test ($P < 0.05$) to find the significant differences between parameters. Experiments were conducted using at least three biological replicates.

3. RESULTS

3.1 Phytoglobin1 expression influences waterlogging tolerance

The performance of wild type (WT) plants and plants over-expressing HvPgb1 [HvPgb1(OE)] was compared during waterlogging. Under control conditions, the values of gas exchange parameters: photosynthetic rate, stomatal conductance, and transpiration, as well as biomass of shoot and root were higher in WT plants compared to those over-expressing *HvPgb1* (Fig. 3 - 1). An opposite trend was observed in waterlogged conditions, when HvPgb1(OE) plants exhibited higher photosynthetic rate, stomatal conductance, and transpiration rate. Total chlorophyll remained unchanged in control and waterlogged HvPgb1(OE) (Fig 3 - 1D). At an anatomical level, waterlogging induced the formation of aerenchyma in HvPgb1(OE) plants, but not in WT plants (Fig. 3 - 2).

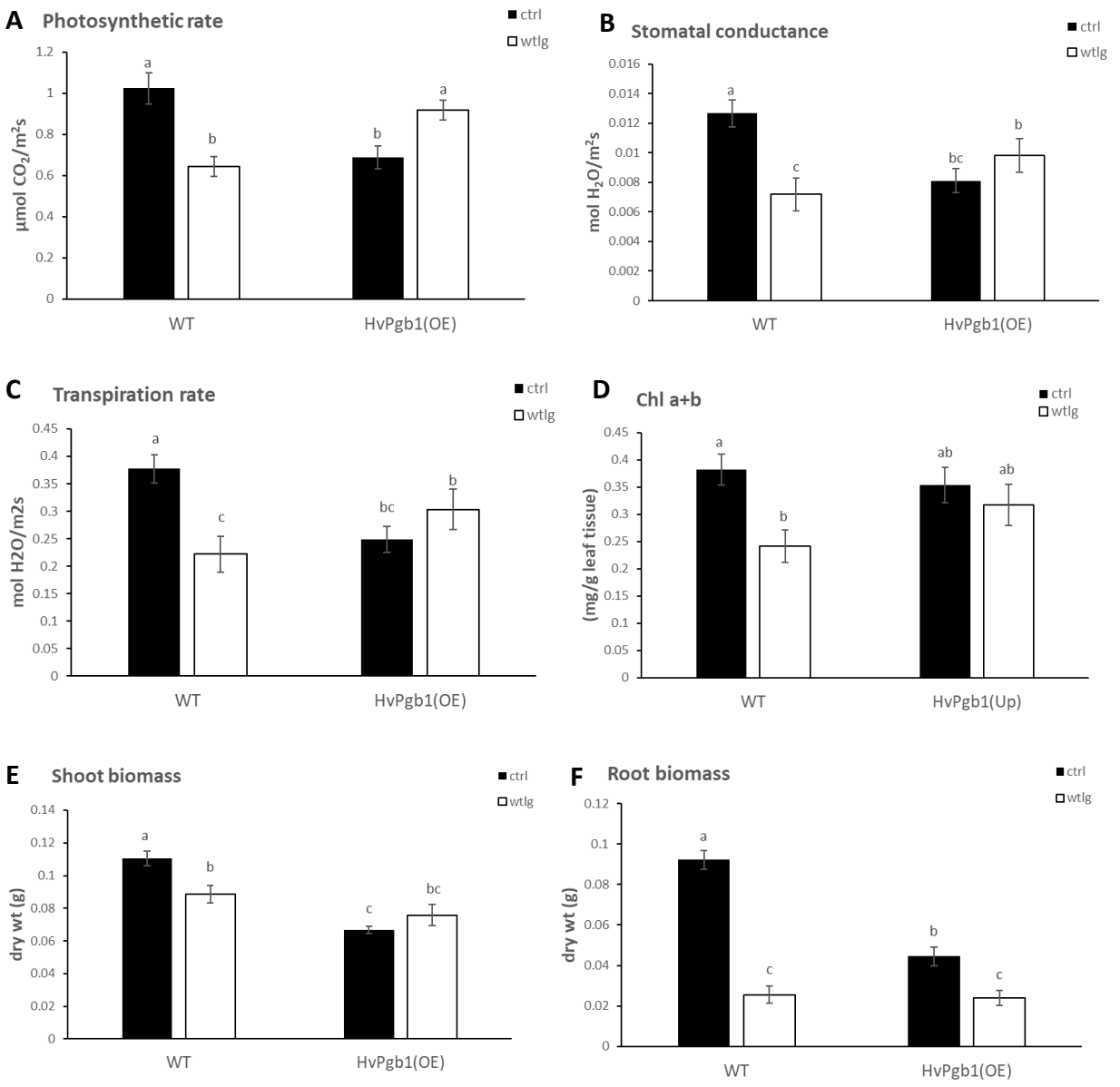


Figure 3 - 1. Photosynthetic rate (A), stomatal conductance (B), transpiration rate (C), total chlorophyll (D), and shoot (E) and root (F) biomass of wild type (WT) and the HvPgb1-overexpressing transgenic line [HvPgb1(OE)]. Measurements were conducted 7 days in control (ctrl) or waterlogged (wtlg) conditions. Values are means (n = 6; +/- SE); different letters denote statistical differences ($P < 0.10$).

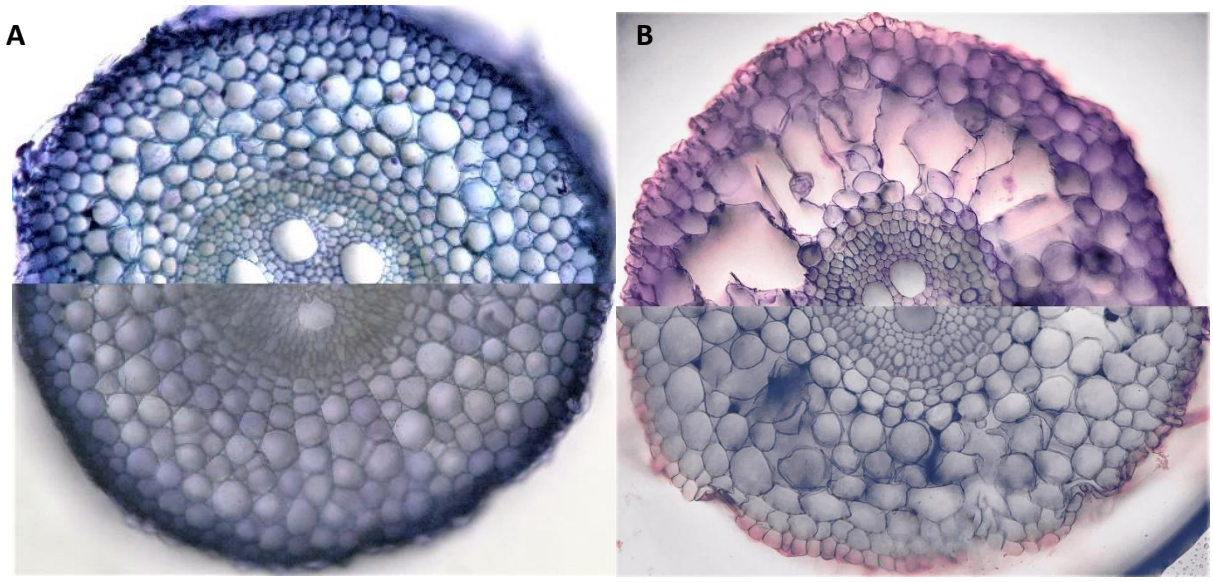


Figure 3 - 2. Cross sections of WT (A) and HvPgb1-overexpressor (B) roots. Waterlogged roots are shown on the upper half of each section, with their controls on the bottom half. Sections were taken 2.0 cm from root tip and wet-mounted with toluidine blue O. Images are representative of a total of 6 sections taken for each treatment. Roots were waterlogged for 3 days prior to sampling.

3.2 Nitrogen and ethylene content in shoots of waterlogged plants

The level of shoot nitrate, equal between the WT and HvPgb1(OE) plants under control conditions increased after 2 days of waterlogging in HvPgb1(OE) plants while it remained unaltered in WT plants (Fig 3 -3A). Foliar nitrogen levels were measured as well, and no significant differences were detected across all treatments (Fig 3 - 3B).

Under control conditions, similar levels of ethylene were observed between shoots of WT and HvPgb1(OE) plants (Fig 3 - 4). 24 hours of waterlogging did not change the amount of

ethylene in HvPgb1(OE) shoots but depressed that in WT shoots. The level of ethylene was further manipulated in leaves of waterlogged plants using pharmacological treatments with the ethylene receptor blocker silver nitrate ($AgNO_3$) and the ethylene-releasing agent Ethephon (Eth). $AgNO_3$ had a significant deleterious effect on waterlogged *HvPgb1*(OE)'s photosynthetic rate and stomatal conductance: retention (or % of waterlogged control) of photosynthetic rate was 6.70% and stomatal conductance was 25.0%. WT was not as severely affected, with photosynthetic rate retention at 51.5% and stomatal conductance at 67.5% (Fig 3 - 5A, B). In contrast, application of ethylene donor (ethephon) drastically improved the physiological performance under waterlogging, yielding values higher than their waterlogged controls. With ethephon applied, *HvPgb1*(OE) had a photosynthetic rate retention of 272.0% and stomatal conductance retention of 376.0%. Ethephon also had a positive, but even greater, effect in the WT: with photosynthetic rate retention of 350.0% and stomatal conductance retention at 550.0% (Fig 3 - 5 C, D).

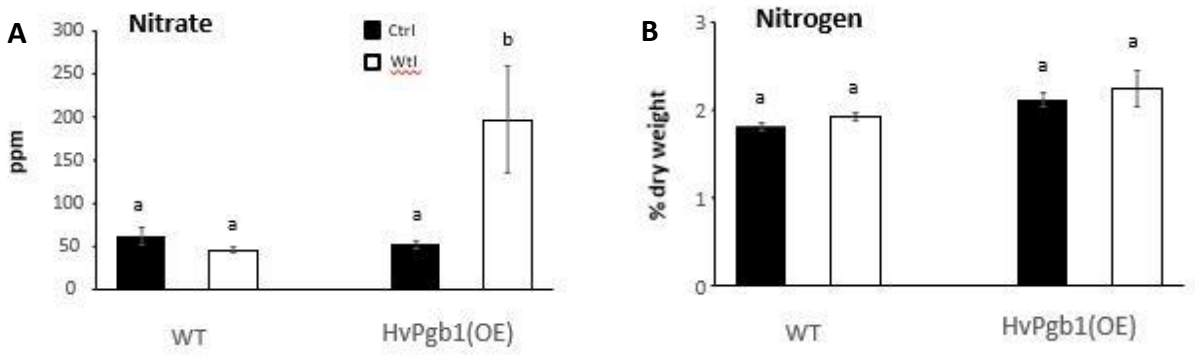


Figure 3 - 3. Nitrate (A) and nitrogen (B) levels in shoots of wild type (WT) and HvPgb1-overexpressing [HvPgb1(OE)] leaves. Measurements were conducted 2 days in control (ctrl) or waterlogged (wtlg) conditions. Bars are means (n = 3; +/- SE); different letters denote statistical differences ($P < 0.10$).

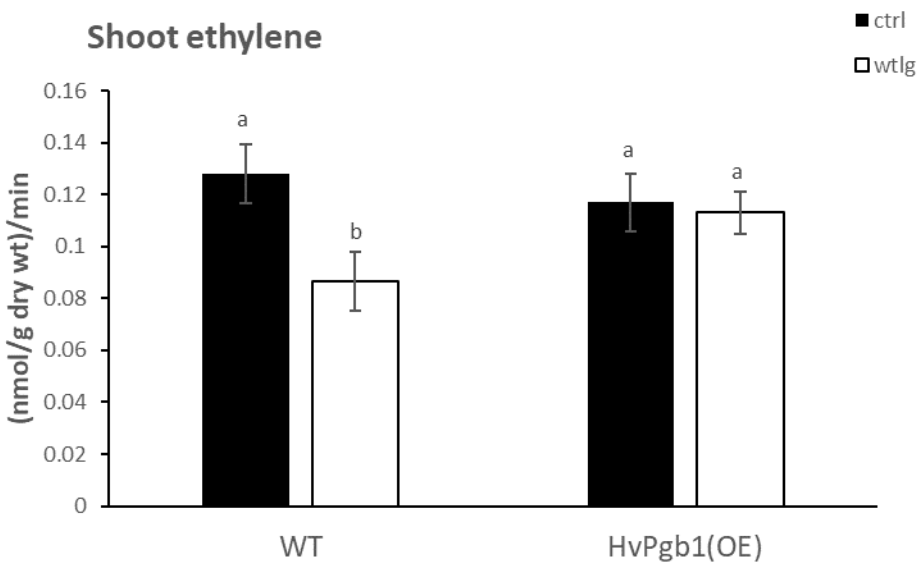


Figure 3 - 4. Ethylene gas quantification of control (ctrl) and waterlogged (wtlg) shoot tissue of wild type (WT) and its HvPgb1-overexpressing line [HvPgb1(OE)]. Measurements were taken after 24 hrs of waterlogging. Different letters denote significant differences. Bars are means (n = 9; +/- SE); different letters denote statistical differences ($P < 0.05$).

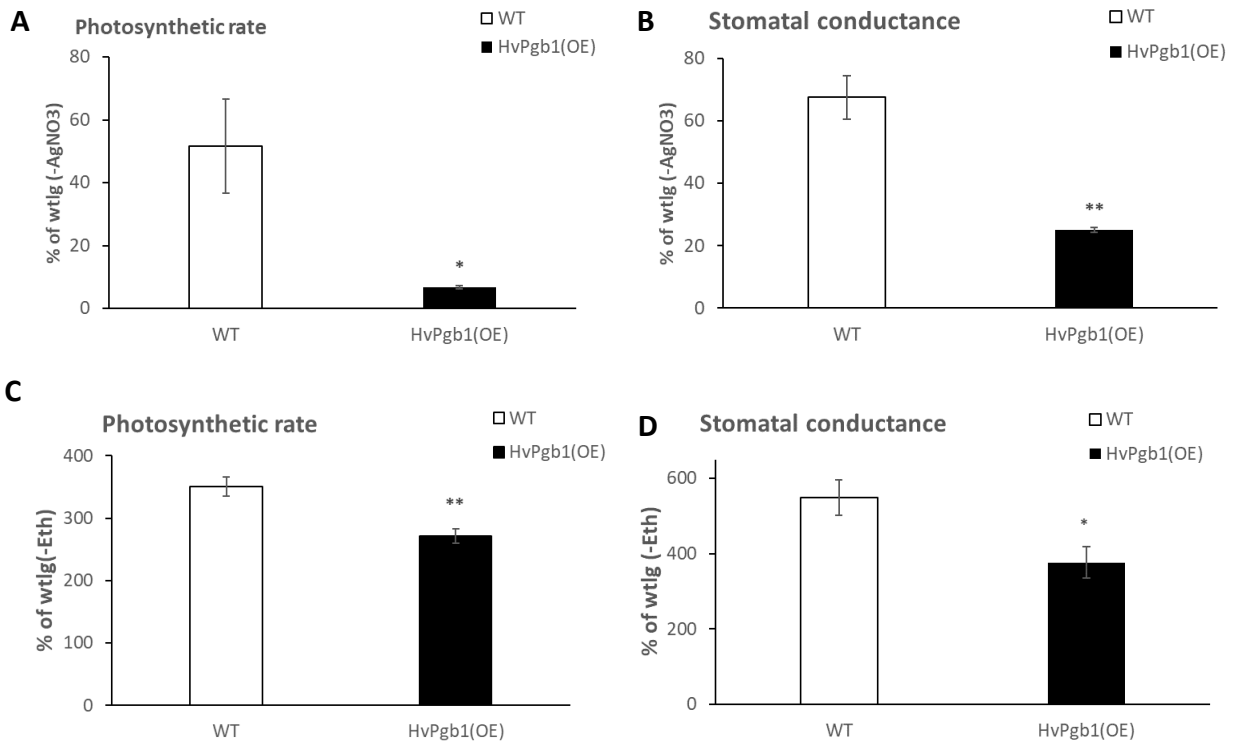


Figure 3 - 5. Retention (% of control) of photosynthetic rate and stomatal conductance of waterlogged WT and HvPgb1(OE) with foliar applied $AgNO_3$ (A, B respectively) and ethephon (Eth) (C, D respectively). Measurements were conducted 2 days in control (ctrl) or waterlogged (wtlg) conditions. Bars signify means (n = 6 ± SE) of % of controls; independent samples t-test shows all comparisons between WT and HvPgb1(OE) are significant. (* denotes $P < 0.01$, ** denotes $P < 0.001$)

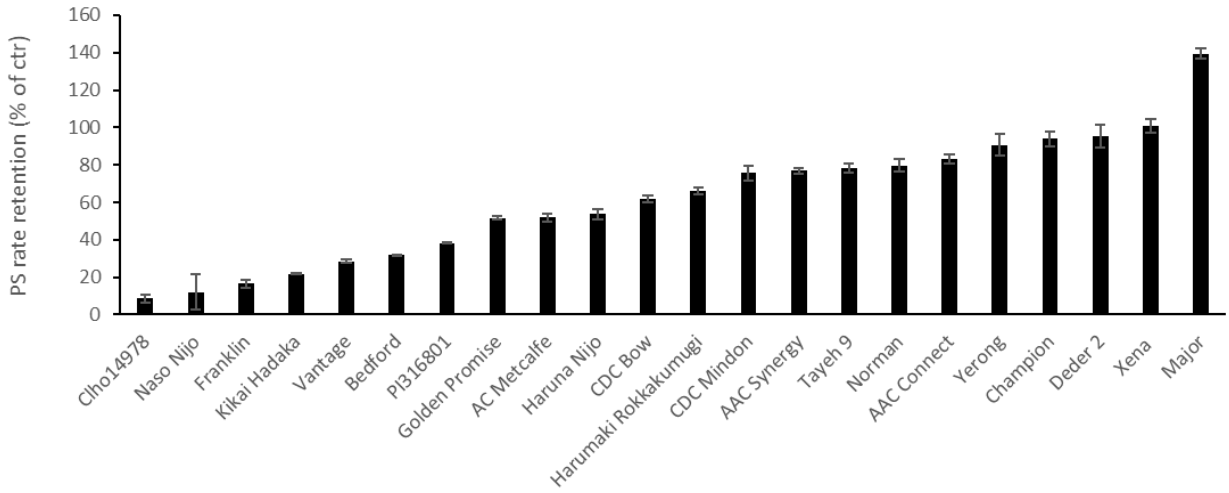
3.3 Expression of *HvPgb1* in barley genotypes of varying waterlogging stress tolerance

To examine the link between *HvPgb1* expression and tolerance to waterlogging, a total of 22 barley genotypes were screened using three physiological parameters: photosynthetic rate, stomatal conductance and total chlorophyll (Fig 3 - 6). These parameters

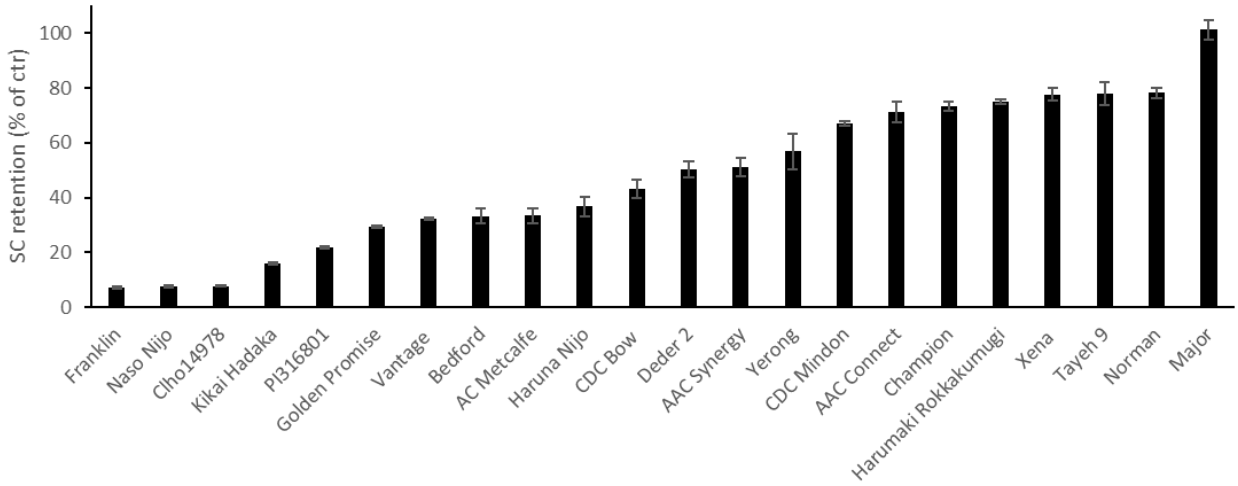
were selected according to Pang et al.'s (2004) correlation of these measurements to plant recovery post-waterlogging stress. Based on the combined analyses of the three parameters, six cultivars were selected for further studies: three tolerant (Deder 2, Major, Xena) and three susceptible (Nasjo Nijo, Golden Promise, Franklin). Tolerant lines were characterized by the formation of aerenchyma during waterlogging, while the root structure of the susceptible lines remained unaltered by the stress (Fig 3 - 7).

The expression of root *HvPgb1* slightly increased in all cultivars between 6-24 hours of waterlogging. In the shoot, a marked peak in *HvPgb1* expression occurred during the same timeframe in only the tolerant lines and not in the susceptible lines (Fig 3 - 8).

A


PS rate retention (% of ctr)
160
140
120
100
80
60
40
20
0
Clho14978
Naso Nijo
Franklin
Kikai Hadaka
Vantage
Bedford
PI316801
Golden Promise
AC Metcalfe
Haruna Nijo
CDC Bow
Harumaki Rokkakumugi
CDC Mindon
AAC Synergy
Tayeh 9
Norman
AAC Connect
Yerong
Champion
Deder 2
Xena
Major

B


SC retention (% of ctr)
100
80
60
40
20
0
Franklin
Naso Nijo
Clho14978
Kikai Hadaka
PI316801
Golden Promise
Vantage
Bedford
AC Metcalfe
Haruna Nijo
CDC Bow
Deder 2
AAC Synergy
Yerong
CDC Mindon
AAC Connect
Champion
Harumaki Rokkakumugi
Xena
Tayeh 9
Norman
Major

C

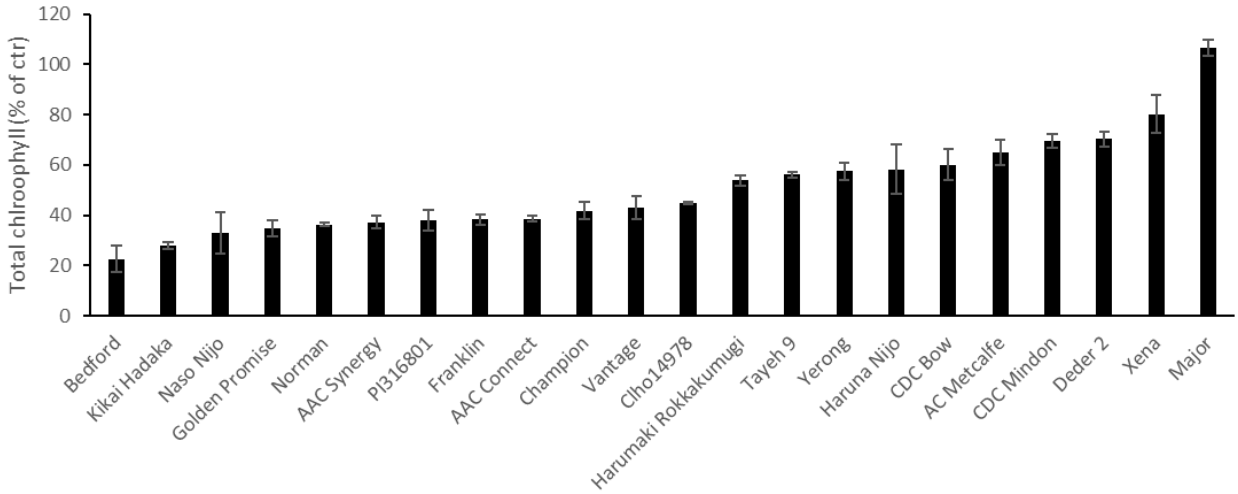

Total chlroophyll (% of ctr)
120
100
80
60
40
20
0
Bedford
Kikai Hadaka
Naso Nijo
Golden Promise
Norman
AAC Synergy
PI316801
Franklin
AAC Connect
Champion
Vantage
Clho14978
Harumaki Rokkakumugi
Tayeh 9
Yerong
Haruna Nijo
CDC Bow
AC Metcalfe
CDC Mindon
Deder 2
Xena
Major

Figure 3 - 6. Measurement of photosynthetic rate (A), stomatal conductance (B), and total chlorophyll content (C) of barley genotypes after 7 days of waterlogging. Retention values (% of control) were conducted after 7 days of waterlogging. Bars are means (n = 9) ± SE.

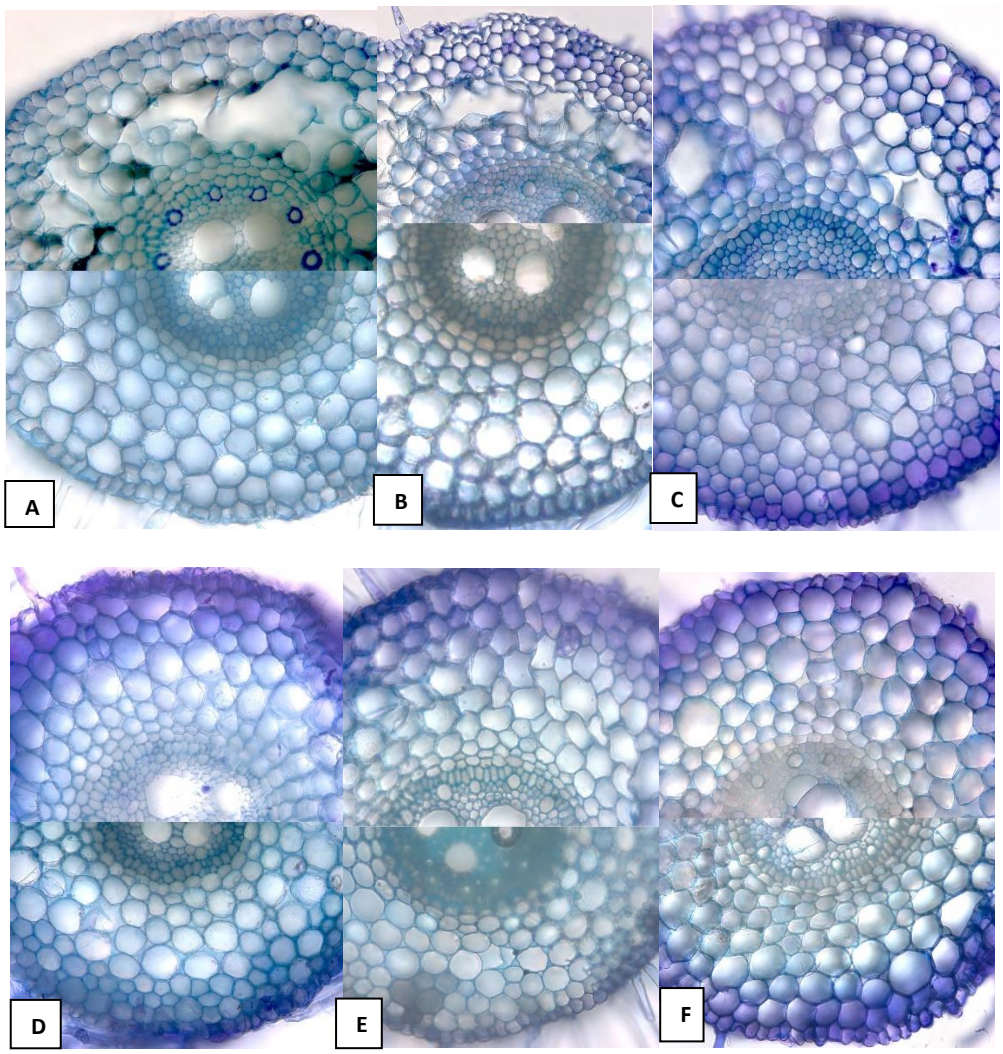
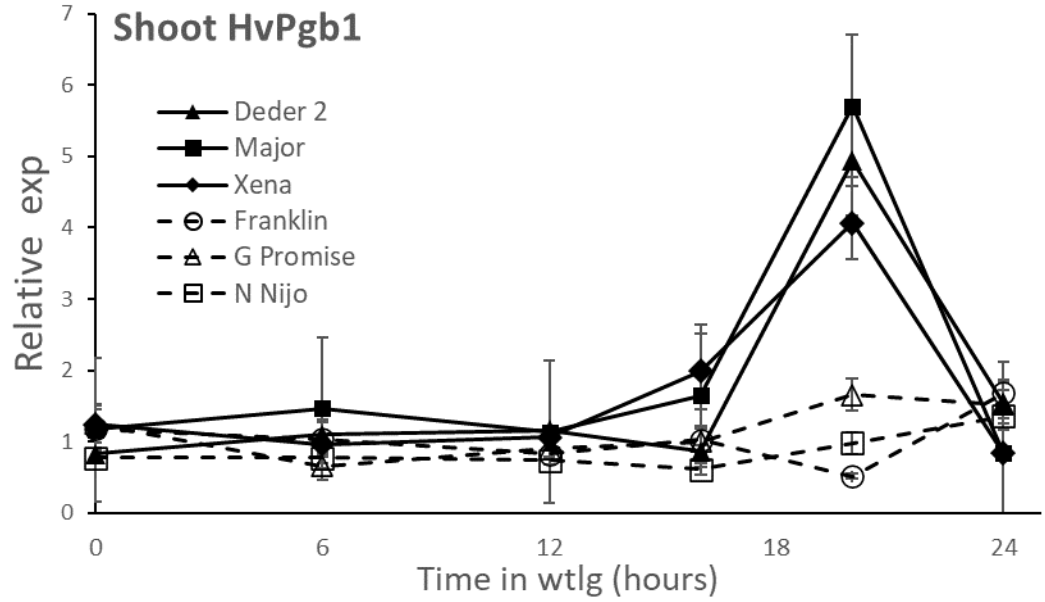


Figure 3 - 7. Cross section of control and waterlogged roots of selected tolerant and susceptible genotypes at day 3 post-waterlogging. Waterlogged roots are shown on the upper half of each section, with their controls on the bottom half. Deder 2 (A), Major (B), and Xena (C) are tolerant; Naso Nijo (D), Golden Promise (E) and Franklin (F) are susceptible.

A



B

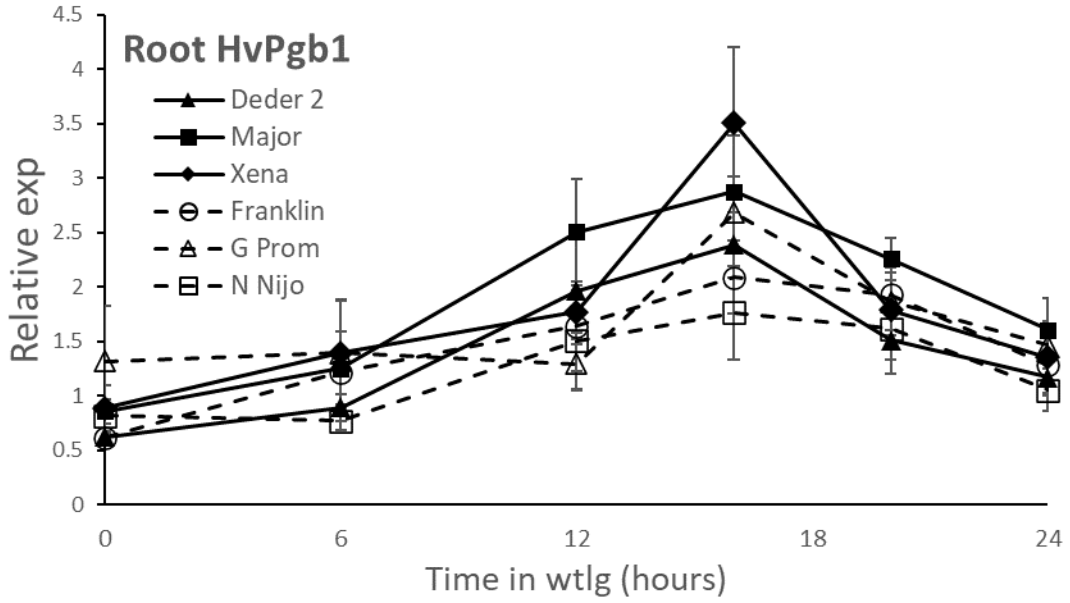


Figure 3 - 8. Progress curve of relative expression of shoot (A) and root (B) HvPgb1 from selected genotypes from 0 to 24 hours post-waterlogging. Solid lines represent tolerant genotypes, dashed lines represent susceptible. Values are means +/- SE (n = 3) with each replicate consisting of samples from at least 3 plants.

4. DISCUSSION

4.1 Overexpression of *HvPgb1* improves physiological response during waterlogging stress, but not under control conditions.

In our study, the performance of the waterlogging-susceptible wild type (WT) Golden Promise is being compared to its tolerant transgenic HvPgb1-overexpressing line [HvPgb1(OE)] to confirm if *Pgb1* expression does have mitigating effects on hypoxic stress, as it did in maize (Youssef et al., 2016), Arabidopsis (Hunt et al., 2002) and soybean (Mira et al., 2021b). The growth stage assessed was at the 3-leaf stage for two main reasons. First, in the prairies, barley is subjected to early waterlogging due to the melting of accumulated snow and the added early spring rainfalls (Buttle et al., 2016). Second, waterlogging susceptibility in barley is highest from leaf emergence to the booting stage (Borrego-Benjumea et al., 2019). As seedlings were used for the experiment, yield performance alone would not be reflective of the tolerance levels of the plants since shoot growth of early-waterlogged barley plants are not affected by early waterlogging (Ploschuk et al., 2018). Therefore, physiological parameters were more appropriate for this study.

During control (normoxic) conditions, WT plants performed better and had higher values of most of the parameters measured relative to HvPgb1(OE) plants, excluding total chlorophyll showing no significant difference (Fig 3 - 1). This observation was contrary to similar studies performed in other species documenting the absence of negative effects of Pgbs under control "unstressed" conditions (Hebelstrup and Jensen, 2008; Mira et al., 2021b, 2017). In barley,

however, Cochrane et al. (2017), and Hebelstrup et al. (2014) also reported penalties in biomass, plant height, and grain yield in plant over-expressing Pgb. These effects were attributed to the HvPgb1 reduction in NO causing a rise in respiratory processes, carbon oxidation, and ROS accumulation (Gupta et al., 2014), thus promoting oxidative stress and growth retardation (Sasidharan and Voesenek, 2015).

Imposition of waterlogging depresses gas exchange parameters and reduces shoot and root biomass in WT plants. This might reflect ROS damage to the photosynthetic apparatus caused by root hypoxia (Bai et al., 2013; Ploschuk et al., 2018), as well as limiting nitrogen translocation and use efficiency (Ren et al., 2017). A reduction in gas exchange parameters along with a decline in chlorophyll and biomass (Herzog et al., 2016; Visser and Voesenek, 2005) are typical of waterlogged barley plants (Mendiondo et al., 2016; Pang et al., 2004). The negative effects of waterlogging were attenuated in plant over-expressing HvPgb1. These plants had significantly higher photosynthetic rate, stomatal conductance, and transpiration rate relative to WT and were able to retain chlorophyll and biomass values comparable to those of control conditions (Fig 3 -1). To some extent, these results agree with those reported in barley by Andrzejczak et al. (2020) and reflect the well documented beneficial effect of Pgbs during hypoxia in other species (Kumari et al., 2022; Mira et al., 2021b; Silva-Cardenas et al., 2003; Youssef et al., 2016). These effects were attributed to a decline in NO, as a result of Pgb over-expression, and a subsequent attenuation of ROS accumulation and cell damage (Mira et al., 2021b; Youssef et al., 2016). At the root level, waterlogging induces formation of aerenchyma in HvPgb1(OE) roots, but not in WT plants. Aerenchyma formation is another trait strongly associated with waterlogging tolerance, as it enables O_2 diffusion in the hypoxic environment

(Armstrong, 1980; Bailey-Serres and Voesenek, 2008), thus sustaining energetic processes in the root (Dordas et al., 2003a; Igamberdiev et al., 2006; Silva-Cardenas et al., 2003). While this outcome is different from what Dordas et al. (2003a) observed in transgenic alfalfa cultures (where lines overexpressing *HvPgb1* had no aerenchyma formation under hypoxia), it does agree with other hypoxic barley studies (Luan et al., 2018a; Pang et al., 2004; Zhang et al., 2015).

Aerenchyma formation is controlled by ethylene (Yamauchi et al., 2016) and ROS (Steffens et al., 2011), both of which are influenced by NO through Pgbs. The development of aerenchyma is suggestive of elevated levels of ROS in the same tissue; an observation which is counterintuitive given the fact that Pgbs decrease ROS (Mira et al., 2017; Youssef et al., 2016). This suggests that HvPgb1 is only indirectly implicated in the formation of aerenchyma, consistent with Rajhi et al., (2011) who were not able to detect Pgb1 expression in cortical tissue undergoing PCD during hypoxia. The focus and interest on root Pgb1 expression studies are in meristematic tissue (Mira et al., 2017).

4.2 HvPgb1 expression influences ethylene accumulation during waterlogging.

Ethylene's role in hypoxic stress response – notably soil waterlogging – has mostly been tied to root-related mechanisms. In our study however, we targeted shoot ethylene changes to observe how ethylene signaling could partake in acclimating the rest of the plant tissue for prolonged periods in hypoxic soil, and if *HvPgb1* activity plays a part. The decrease in shoot ethylene in waterlogged WT (Fig 3 - 4) contradicts most of the results in literature where ethylene increase is typically found (Hartman et al., 2019; Ku et al., 1970; Voesenek and

Sasidharan, 2013). On the contrary, in HvPgb1(OE) plants the levels of ethylene did not vary as a result of hypoxia. The higher level of shoot ethylene in HvPgb1(OE) roots relative to WT plants is consistent with the higher levels of the same hormone observed in waterlogging tolerant barley lines relative to susceptible genotypes (Luan et al., 2018b). In our unpublished results, there is a downregulation of the ethylene biosynthesis gene *1-aminocyclopropane-1-carboxylase* (ACC) *oxidase* (*ACO*), and ethylene responsive factors (Supp. Fig 1) in waterlogged WT plants relative to HvPgb1(OE) plants that would account for the lower levels of ethylene in WT. During waterlogging, ACC is produced in hypoxic tissue (Zhou et al., 2001), translocated upward via xylem and converted to ethylene in oxygenated tissues (Bradford and Yang, 1980). In the shoot, ethylene induces several responses (English et al., 1995; Jackson, 1985), and it is required for the inhibition of abscisic acid (ABA)-induced stomatal closure (Tanaka et al., 2005). This would explain the lower stomatal conductance in waterlogged WT plants relative to HvPgb1(OE) plants, with the former accumulating less ethylene (Fig 3 - 1B). Furthermore, low levels of foliar ethylene have been associated to a decrease in total chlorophyll content (Tholen et al., 2004) and Rubisco (Tholen et al., 2007), thus reinforcing the idea that sustained levels of ethylene are required to support leaf functions during root waterlogging.

These observations are consistent with the pharmacological treatments performed in this study where an elevation of foliar ethylene increases photosynthetic rate and stomatal conductance, while the inhibition of ethylene signalling has contrasting results (Fig 3 - 5A, B). This agrees with the work of Shiono et al (2019) and Kim et al. (2018), observing similar patterns following manipulation of ethylene levels.

The action of ethylene is closely linked to NO and thus Pgbs through the N-end rule pathway regulating plant response to hypoxia (Gibbs et al., 2015). In normoxic conditions, the N-end rule pathway leads to the degradation of group VII ethylene responsive factors (ERVIIs) via the PRT6 proteasome (Gibbs et al., 2015) and requires both O_2 and NO. According to Hartman et al., (2019), the ethylene that accumulates during hypoxia induces the expression of *ETHYLENE-INSENSITIVE2*, which then triggers Pgb1 synthesis. Pgb1 then drives the Pgb-NO cycle and with O_2 utilized and NO scavenged, the N-end rule pathway cannot resume and ERVIIs are synthesized, allowing the transcription of hypoxia tolerance genes such as ADH, PDC and AlaAT (Hartman et al., 2021). In this study, the leaves are not submerged and still aerobic, so it is likely for the N-end rule pathway to resume. However, under *HvPgb1* overexpression, it can be speculated that the decrease in NO would impair the NO-dependent N-end rule pathway and permit a low level of ERVII stabilization, thus functioning as a shoot priming mechanism for the plant in the case of eventual shoot submergence. The idea that the N-end rule pathway's involvement in this system requires further study. The expression of these hypoxia tolerance genes was not measured in this study, but the maintenance of ethylene levels (Fig 3 – 4) and increase in nitrate (Fig 3 - 3A) from *HvPgb1*-overexpression during hypoxia places HvPgb1 activity as the potential connecting factor between nitrogen and ethylene-based shoot responses during root hypoxia.

4.3 *HvPgb1* expression increases nitrate transport in the shoot during waterlogging stress.

During waterlogging the level of nitrate, but not nitrogen, increase in HvPgb1(OE) plants, while they remain unchanged in WT plants (Fig. 4B). Nitrate is produced from Pgb1's

NO-scavenging activity but increased nitrate is also responsible for inducing nitrate reductase (NR) activity to generate nitrite and its subsequent reduction to NO (Wany et al., 2019). *Pgb1* expression and activity are then triggered, oxidizing NO back to nitrate (Igamberdiev et al., 2005; Igamberdiev and Hill, 2004). It is through this cycle that NAD^+ is generated to maintain glycolysis and generate ATP, thus maintaining energy status during hypoxic stress (Igamberdiev et al., 2004). In our results, there is a significant increase in foliar nitrate in the waterlogged HvPgb1(OE) plants (Fig 3 - 3A), denoting high Pgb1 activity. Although NO was not measured in this study, *HvPgb1* is able to scavenge NO (Andrzejczak et al., 2020; Cochrane et al., 2017; Gupta et al., 2014; Montilla-Bascón et al., 2017). It is also expected that increased foliar nitrate would induce NO production (Da-Silva et al., 2021), which then promotes Pgb1 activity (Igamberdiev and Hill, 2004). In soybean, short-term nitrate application alleviated fermentation and oxidative stress during waterlogging (da Silva and do Amarante, 2020) with Pgb1 being described as having a major role of Pgb1 in this reaction. Thus, the accumulation of nitrate in waterlogged HvPgb1(OE) plants might contribute to mitigate the stress. Specifically, nitrate availability has been linked to the synthesis of photosynthetic pigments (Da-Silva et al., 2021) and this could have been what occurred in waterlogged HvPgb1(OE) plants where we see increased photosynthetic rate and total chlorophyll (Fig 3 -1A, D).

Unpublished data from a similar study that compared the same two barley genotypes assessed leaf transcripts and found the expression nitrate transporters *HvNRT1.1* and *1.3* being downregulated in leaves of WT plants relative to HvPgb1(OE) plants (Supp. Fig 2). This suggests a higher ability to mobilize and translocate nitrates in leaves over-expressing HvPgb1 for adaptive metabolic processes. Additionally, the increase in nitrate transporters we see in the

aerobic leaves of waterlogged HvPgb1(OE) suggests larger quantities of nitrate are being stored in vacuoles for later, as preparation for nitrogen-deficient status brought on by hypoxia, or eventual mobilization to grain following recovery (Fan et al., 2017). The increase in nitrate reductase transcripts in HvPgb1(OE) relative to WT during hypoxia (Supp. Fig 2) indicates that the leaf is utilizing the increased nitrate levels in HvPgb1(OE) for amino acid synthesis. Furthermore, the induction of glutamate and glutamine synthetase transcripts in the same leaves also suggest that ornithine and arginine could be potential end products, an observation consistent with the pattern of arginine decarboxylase. This enzyme is one of the rate-controlling steps for the synthesis of polyamines, and its upregulation correlates to higher levels of polyamines (Chen et al., 2019). These observations point to higher levels of polyamines in HvPgb1(OE) leaves.

While nitrogen supply alters polyamine content (Garnica et al., 2009), polyamines increase NO levels in Arabidopsis seedlings (Tun et al., 2006). Thus, removal of NO by HvPgb1 could facilitate production of polyamines, as evidenced by an increase in their synthesis in drought stressed barley plants over-expressing HvPgb1, where they mitigate the effects of the stress (Montilla-Bascon et al., 2017). A similar protective mechanism could operate during waterlogging. Polyamines improve the functionality of photosystem II in chloroplast membranes and stimulate non-photochemical quenching (Ioannidis and Kotzabasis, 2007) reducing photooxidative damage from ROS (reviewed in (Müller et al., 2001). Interestingly, the Arabidopsis nitrate transporter, AtNRT1.3, has also been identified as a polyamine transporter (Tong et al., 2016).

Polyamine levels have not been measured in this study, but it is suggested that their increase in leaves of waterlogged HvPgb1(OE) plants contribute to the attenuation of the stress. Furthermore, polyamine synthesis is related to ethylene: In barley, HvPgb1(OE) plants exhibited increased tolerance to drought stress and, relative to WT, had higher levels of polyamines and lower ethylene content (Montilla-Bascón et al., 2017). This observation is not consistent with the finding of this thesis, as waterlogged HvPgb1(OE) plants accumulate more ethylene than their WT counterparts.

4.4 Can *HvPgb1* level be used as a marker associated to waterlogging tolerance?

It is commonplace in waterlogging stress to screen plants for their ability to recover after the imposition of the stress. Recovery was associated to the retention of photosynthetic parameters (Pang et al., 2004), which were therefore used in this study to screen the 22 genotypes (Fig 3 - 6). Formation of aerenchyma was also assessed (Fig 3 - 7), given the relevance of this morphological parameter in relation to waterlogging tolerance (Armstrong and Drew, 2002; Jackson and Colmer, 2005; Shiono et al., 2019). The tolerant genotypes, which compared to the susceptible genotyped formed aerenchyma and were able to better retain higher values for the gas exchanged parameters and chlorophyll, exhibited an induction of HvPgb1 in shoots after 20h in stress (Fig 3 - 8A). Surprisingly, no significant differences were found in root *HvPgb1* expression (Fig 3 - 8B), given the Pgb induction in this tissue was reported in similar studies (Mira et al., 2021b; Silva-Cardenas et al., 2003; Youssef et al., 2016). The significant difference in shoot *HvPgb1* expression in the tolerant genotypes suggests that *HvPgb1* activity, in regards to hypoxia tolerance, has greater relevance in shoot responses. Furthermore, the

observation that no differences in HvPgb1 levels between tolerant and susceptible genotypes were observed at 0h indicates that this gene cannot be used to predict the level of tolerance of the plant before the imposition of the stress.

5. CONCLUSIONS

This project aimed to assess the roles of Pgb1 in waterlogging stress tolerance in barley, but more specifically, its roles in the aboveground, aerobic tissue. Ethylene, as an important signaling hormone during waterlogging, was measured to investigate possible relationships with shoot Pgb1. As a by-product of its NO-scavenging properties, nitrate was also measured to test for Pgb1 activity, and consequently, for changes in nitrogen metabolism during the response.

One major result of higher *HvPgb1* expression found from this study was its ability to maintain photosynthetic functions during waterlogging-induced hypoxia. In *HvPgb1*-overexpressing plants, photosynthetic rate, stomatal conductance, transpiration rate, and total chlorophyll were either higher or unchanged relative to control. In this case where early waterlogging is being assessed in barley seedlings, photosynthetic parameters pose as a more useful phenotype to measure in selection protocols, rather than yield or vegetative biomass. One thing to note however is that much of the higher photosynthetic performance seen in HvPgb1(OE) plants is attributed to physiological penalties from their controls, which has been observed in previous research that use the same transgenic plant material.

Ethylene levels decreased in waterlogged WT but remained unchanged in waterlogged HvPgb1(OE), representing the continued mobilization of ACC from anaerobic root tissue to the aerobic shoot where it is converted to ethylene. The improvement in photosynthetic performance of both WT and HvPgb1(OE) in the ethephon-applied waterlogging treatments showed the important role of ethylene in shoot responses against waterlogging. But more importantly is the

display of ethylene's relationship with Pgb1 activity through the significant decrease in photosynthesis from decreasing ethylene perception (by way of the receptor blocker, $AgNO_3$) in the HvPgb1(OE) plants.

Pgb1 activity is also evidenced in the higher foliar nitrate seen from waterlogged HvPgb1(OE). Despite the lack of significant differences in root *HvPgb1* expression between genotypes, HvPgb1 is still active in roots and therefore nitrate levels would be increased. But it is unlikely that the nitrate generated in the root was translocated in the shoot to explain the nitrate increase we see, considering the nitrogen levels are unchanged. Therefore, it is more likely that ACC is the one mobilized to the shoot to be converted to ethylene, which then triggers Pgb1 activity and nitrate increase. This increase could contribute to the synthesis of polyamines, alleviators of the stress.

With altered *Pgb1* expression affecting ethylene and nitrate levels in shoot, we conclude that Pgb1 participates in the shoot signaling that follows root waterlogging stress in order to prepare the shoot for long-term stress. The ethylene-induced expression of hypoxia-tolerant genes and the nitrate-driven Pgb1-NO cycle are both beneficial to maintaining barley's photosynthetic performance during waterlogging-induced hypoxia. Despite a yield penalty brought on by constitutive expression of *HvPgb1*, a positive link was still found between the photosynthetic parameters measured and *HvPgb1*-expression, seen in both WT and HvPgb1(OE), as well as from the selected genotypes. We therefore cannot discount the potential of utilizing HvPgb1-expression as a marker for waterlogging tolerance. Alternatively, the results around foliar nitrate from the study also poses itself as another quantifiable tolerance trait, with

mineral analysis for nitrate measurements can be easily performed as an added test to phenotyping for breeding programs.

6. FUTURE DIRECTIONS

As mentioned previously, a connection between polyamine and NO activity in barley has been observed before, albeit in drought stress experiments (Montilla-Bascón et al., 2017). Supplementing this experimental design with polyamine quantification would confirm if they are involved in Pgb responses during waterlogging.

Despite the associations found between Pgb1 activity and waterlogging stress response, this study adds to several others (Cochrane et al., 2017; Gupta et al., 2014; Hebelstrup et al., 2014) that claim that the experimental use of the HvPgb1-overexpressing line adds complications by way of yield penalties in normoxic conditions. It would be more appropriate to move forward with plant material that has an induced or selective expression of HvPgb1, rather than one with constitutive expression.

7. REFERENCES

Ahammed, G.J., He, B.B., Qian, X.J., Zhou, Y.H., Shi, K., Zhou, J., Yu, J.Q., Xia, X.J., 2017. 24-Epibrassinolide alleviates organic pollutants-retarded root elongation by promoting redox homeostasis and secondary metabolism in Cucumis sativus L. Environmental Pollution 229, 922–931.

Ames, N., Storsley, J., 2015. Effects of barley on post - prandial glycemic response. Diabesity 1, 21–23.

Andrzejczak, O.A., Havelund, J.F., Wang, W.Q., Kovalchuk, S., Hagensen, C.E., Hasler-Sheetal, H., Jensen, O.N., Rogowska-Wrzesinska, A., Møller, I.M., Hebelstrup, K.H., 2020. The hypoxic proteome and metabolome of barley (Hordeum vulgare L.) with and without phytoglobin priming. International Journal of Molecular Sciences 21.

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55, 373–399.

Appleby, C.A., 1992. The origin and functions of haemoglobin in plants. Science Progress 76, 365–398.

Appleby, C.A., 1984. Leghemoglobin and rhizobium respiration. Annual Review of Plant Physiology 35, 443–478.

Armstrong, W., 1980. Aeration in higher plants. Advances in Botanical Research 7, 226–332.

Armstrong, W., Drew, M., 2002. Root growth and metabolism under oxygen deficiency, in: Waisel, Y; Eshel, A; Beeckman, T; Kafkafi, U. (Ed.), Plant Roots. CRC Press, Boca Raton, FL.

Arredondo-Peter, A.R., Hargrove, M.S., Sarath, G., Moran, J.F., Olson, J.S., Klucas, R. v, Physiology, S.P., Nov, N., Arredondo-peter, R., Hargrove, M.S., Sarath, G., Moran, J.F., Lohrman, J., Olson, J.S., Klucas, R. v, 1997. Rice hemoglobins : gene cloning , analysis , and O_2-binding kinetics of a recombinant protein synthesized in Escherichia coli. Plant Physiology 115, 1259–1266.

Bahmani, R., Kim, D.G., Na, J.D., Hwang, S., 2019. Expression of the tobacco non-symbiotic class 1 hemoglobin gene hb1 reduces cadmium levels by modulating cd transporter expression through decreasing nitric oxide and ROS level in Arabidopsis. Frontiers in Plant Science 10, 1–19.

Bai, T., Li, Cuiying, Li, Chao, Liang, D., Ma, F., 2013. Contrasting hypoxia tolerance and adaptation in Malus species is linked to differences in stomatal behavior and photosynthesis. Physiologia Plantarum 147, 514–523.

Bailey-Serres, J., Colmer, T.D., 2014. Plant tolerance of flooding stress - recent advances. Plant Cell and Environment 37, 2211–2215.

Bailey-Serres, J., Voesenek, L.A.C.J., 2008. Flooding stress acclimations and genetic diversity. Annual Review of Plant Biology 59, 313–339.

Barrett-Lennard, E.G., 2003. The interaction between waterlogging and salinity in higher plants: causes, consequences and implications. Plant and Soil 253, 35–54.

Baxter, A., Mittler, R., Suzuki, N., 2014. ROS as key players in plant stress signalling. Journal of Experimental Botany 65, 1229–1240.

Borrego-Benjumea, A., Carter, A., Glenn, A.J., Badea, A., 2019. Impact of excess moisture due to precipitation on barley grain yield in the Canadian Prairies. Canadian Journal of Plant Science 96, 93–96.

Borrego-Benjumea, A., Carter, A., Tucker, J.R., Yao, Z., Xu, W., Badea, A., 2020. Genome-wide analysis of gene expression provides new insights into waterlogging responses in barley. Plants 9.

Bradford, K.J., Yang, S.F.A., 1980. Xylem transport of 1-aminocyclopropane-1-carboxylic acid, an ethylene precursor, in waterlogged tomato plants. Plant Physiology 65, 322–326.

Buttle, J.M., Allen, D.M., Caissie, D., Davison, B., Hayashi, M., Peters, D.L., Pomeroy, J.W., Simonovic, S., st. Hilaire, A., Whitfield, P.H., 2016. Flood processes in Canada: regional and special aspects. Canadian Water Resources Journal.

Canadian Grain Commission (2019). https://www.grainscanada.gc.ca/en/grain-research/export-quality/cereals/malting-barley/2019/ (accessed 1.31.22).

Chen, D., Shao, Q., Yin, L., Younis, A., Zheng, B., 2019. Polyamine function in plants: metabolism, regulation on development, and roles in abiotic stress responses. Frontiers in Plant Science 9, 1–13.

Chen, Y., Chen, X., Wang, H., Bao, Y., Zhang, W., 2014. Examination of the leaf proteome during flooding stress and the induction of programmed cell death in maize. Proteome Science 12.

Chillo, S., Ranawana, D.V., Pratt, M., Henry, C.J.K., 2011. Glycemic response and glycemic index of semolina spaghetti enriched with barley b -glucan. Nutrition 27, 653–658.

Choudhury, S., Panda, P., Sahoo, L., Panda, S.K., 2013. Reactive oxygen species signaling in plants under abiotic stress. Plant Signaling and Behavior 8, e23681.

Clark, L.H., Harris, W.H., 1981. Observations on the root anatomy of rice (Oryza sativa L.). American Journal of Botany 68, 154–161.

Cochrane, D.W., Shah, J.K., Hebelstrup, K.H., Igamberdiev, A.U., 2017. Expression of phytoglobin affects nitric oxide metabolism and energy state of barley plants exposed to anoxia. Plant Science 265, 124–130.

Da Silva, C.J., do Amarante, L., 2020. Short-term nitrate supply decreases fermentation and oxidative stress caused by waterlogging in soybean plants. Environmental and Experimental Botany 176, 104078.

Da Silva, C.J., Shimoia, E.P., Posso, D.A., Cardoso, A.A., Batz, T.A., Claudia, A., Luciano, B.O., 2021. Nitrate nutrition increases foliar levels of nitric oxide and waterlogging tolerance in soybean. Acta Physiologiae Plantarum 43, 1–12.

de San Celedonio, R.P., Abeledo, L.G., Miralles, D.J., 2018. Physiological traits associated with reductions in grain number in wheat and barley under waterlogging. Plant and Soil 429, 469–481.

de San Celedonio, R.P., Abeledo, L.G., Miralles, D.J., 2014. Identifying the critical period for waterlogging on yield and its components in wheat and barley. Plant and Soil 378, 265–277.

Delledonne, M., 1998. Nitric oxide functions as a signal in plant disease resistance. Nature 394, 585–588.

Dordas, C., 2009. Nonsymbiotic hemoglobins and stress tolerance in plants. Plant Science 176, 433–440.

Dordas, C., Hasinoff, B.B., Igamberdiev, A.U., Manac'h, N., Rivoal, J., Hill, R.D., 2003a. Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. Plant Journal 35, 763–770.

Dordas, C., Rivoal, J., Hill, R.D., 2003b. Plant haemoglobins, nitric oxide, and hypoxic stress. Annals of Botany 91, 173–178.

Drew, M.C., 1997. Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. Annual Review of Plant Physiology and Plant Molecular Biology 48, 223–250.

Drew, M.C., Jackson, M.B., Giffard, S., 1979. Ethylene-promoted adventitious rooting and development to flooding in Zea mays L. Planta 88, 83–88.

Duff, S.M.G., Wittenberg, J.B., Hill, R.D., 1997. Expression, purification, and properties of recombinant barley (Hordeum sp.) hemoglobin. Optical spectra and reactions with gaseous ligands. Journal of Biological Chemistry 272, 16746–16752.

Elhiti, M., Hebelstrup, K.H., Wang, A., Li, C., Cui, Y., Hill, R.D., Stasolla, C., 2013. Function of type-2 Arabidopsis hemoglobin in the auxin-mediated formation of embryogenic cells during morphogenesis. Plant Journal 74, 946–958.

English, P.J., Lycett, G.W., Roberts, J.A., Jackson, M.B., 1995. Increased 1-aminocyclopropane-1-carboxylic acid oxidase activity in shoots of flooded tomato plants raises ethylene production to physiologically active levels. Plant Physiology 109, 1435–1440.

Evans, D.E., 2004. Aerenchyma formation. New Phytologist 161, 35–49. https://doi.org/10.1046/j.1469-8137.2003.00907.x

Fan, Xiaorong, Naz, M., Fan, Xiaoru, Xuan, W., Miller, A.J., Xu, G., 2017. Plant nitrate transporters: from gene function to application. Journal of Experimental Botany 68, 2463–2475.

FAOSTAT (2021). https://www.fao.org/faostat/en/#home

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D., Davies, J.M., Dolan, L., 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422, 442–446.

Garnica, M., Houdusse, F., Claude Yvin, J., Garcia-Mina, J.M., 2009. Nitrate supply induces changes in polyamine content and ethylene production in wheat plants grown with ammonium. Journal of Plant Physiology 166, 363–374.

Garrocho-Villegas, V., Gopalasubramaniam, S.K., Arredondo-Peter, R., 2007. Plant hemoglobins: what we know six decades after their discovery. Gene 398, 78–85.

Ghassemian, M., Nambara, E., Cutler, S., Kawaide, H., Kamiya, Y., McCourt, P., 2000. Regulation of abscisic acid signaling by the ethylene response pathway in arabidopsis. Plant Cell 12, 1117–1126.

Gibbs, D.J., Conde, J.V., Berckhan, S., Prasad, G., Mendiondo, G.M., Holdsworth, M.J., 2015. Group VII ethylene response factors coordinate oxygen and nitric oxide signal transduction and stress responses in plants. Plant Physiology 169, 23–31.

Gibbs, J., Greenway, H., 2003. Mechanisms of anoxia tolerance in plants. I. growth, survival and anaerobic catabolism. Functional Plant Biology 30, 1–47.

Gill, M.B., Zeng, F., Shabala, L., Zhang, G., Fan, Y., 2017. Cell-based phenotyping reveals QTL for membrane potential maintenance associated with hypoxia and salinity stress tolerance in barley. Frontiers in Plant Science 8, 1–9.

Gill, M.B., Zeng, F., Shabala, L., Zhang, G., Yu, M., Demidchik, V., Shabala, S., Zhou, M., 2019. Identification of QTL related to ROS formation under hypoxia and their association with waterlogging and salt tolerance in barley. International Journal of Molecular Sciences 20.

Giraldo, P., Benavente, E., Manzano-Agugliaro, F., Gimenez, E., 2019. Worldwide research trends on wheat and barley: A Bibliometric Comparative Analysis. Agronomy 9, 352.

Groß, F., Durner, J., Gaupels, F., 2013. Nitric oxide, antioxidants and prooxidants in plant defence responses. Frontiers in Plant Science 4, 1–15.

Guo, F.-Q., Okamoto, M., Crawford, N.M., 2003. Identification of a plant nitric oxide synthase gene involved in hormonal signalling. Science (1979) 302, 100–104.

Gupta, K.J., Hebelstrup, K.H., Kruger, N.J., Ratcliffe, R.G., 2014. Nitric oxide Is required for homeostasis of oxygen and reactive oxygen species in barley roots under aerobic conditions. Molecular Plant 7, 747–750.

Gupta, K.J., Hebelstrup, K.H., Mur, L.A.J., Igamberdiev, A.U., 2011. Plant hemoglobins: Important players at the crossroads between oxygen and nitric oxide. FEBS Letters 585, 3843–3849.

Guy, P.A., Sidaner, J.-P., Schroeder, S., Edney, M., MacGregor, A.W., Hill, R.D., 2002. Embryo phytoglobin gene expression as a measure of germination in cereals. Journal of Cereal Science 36, 147–156.

Haque, M.E., Abe, F., Mori, M., Oyanagi, A., Komatsu, S., Kawaguchi, K., 2014. Characterization of a wheat pathogenesis-related protein, TaBWPR-1.2, in seminal roots in response to waterlogging stress. Journal of Plant Physiology 171, 602–609.

Hartman, S., Liu, Z., Veen, H. van, Vicente, J., Reinen, E., Martopawiro, S., Zhang, H., Dongen, N. van, Bosman, F., Bassel, G.W., Visser, E.J.W., Bailey-serres, J., Theodoulou, F.L., Hebelstrup, K.H., Gibbs, D.J., Holdsworth, M.J., Sasidharan, R., Voesenek, L.A.C.J., 2019. Ethylene mediated NO depletion pre-adapts plants to hypoxia stress. Nature Communications 1–9.

Hartman, S., Sasidharan, R., Voesenek, L.A.C.J., 2021. The role of ethylene in metabolic acclimations to low oxygen. New Phytologist 229, 64–70.

Hebelstrup, K.H., Hunt, P., Dennis, E., Jensen, S.B., Jensen, E.Ø., 2006. Hemoglobin is essential for normal growth of Arabidopsis organs. Physiologia Plantarum 127, 157–166.

Hebelstrup, K.H., Jensen, E.Ø., 2008. Expression of NO scavenging hemoglobin is involved in the timing of bolting in Arabidopsis thaliana. Planta

Hebelstrup, K.H., Shah, J.K., Simpson, C., Schjoerring, J.K., Mandon, J., Cristescu, S.M., Harren, F.J.M., Christiansen, M.W., Mur, L.A.J., Igamberdiev, A.U., 2014. An assessment of the biotechnological use of hemoglobin modulation in cereals. Physiologia Plantarum 150, 593–603.

Herzog, M., Striker, G.G., Colmer, T.D., Pedersen, O., 2016. Mechanisms of waterlogging tolerance in wheat – a review of root and shoot physiology. Plant Cell and Environment 39, 1068–1086.

Hill, R., Hargrove, M., Arredondo-Peter, R., 2016. Phytoglobin: A novel nomenclature for plant globins accepted by the globin community at the 2014 XVIII conference on Oxygen-Binding and Sensing Proteins. F1000Res 5, 1–8.

Hill, R.D., 2012. Non-symbiotic haemoglobins-what's happening beyond nitric oxide scavenging? AoB Plants 12, 1–13.

Hirabayashi, Y., Mahendran, R., Koirala, S., Konoshima, L., Yamazaki, D., Watanabe, S., Kim, H., Kanae, S., 2013. Global flood risk under climate change. Nature Climate Change 3, 4–6.

Hsu, F., Chou, M., Peng, H., Chou, S., Shih, M., 2011. Insights into hypoxic systemic responses based on analyses of transcriptional regulation in arabidopsis. PLos 6, 14–16.

Huang, B., Johnson, J.W., Nesmith, S., Bridges, D.C., 1994. Growth, physiological and anatomical responses of two wheat genotypes to waterlogging and nutrient supply. Journal of Experimental Botany 45, 193–202.

Huang, S., Hill, R.D., Wally, O.S.D., Dionisio, G., Ayele, B.T., Jami, S.K., Stasolla, C., 2014. Hemoglobin control of cell survival/death decision regulates in vitro plant embryogenesis. Plant Physiology 165, 810–825.

Hufton, C.A., Besford, R.T., Wellburn, A.R., 1996. Effects of NO (+ NO2) pollution on growth, nitrate reductase activities and associated protein contents in glasshouse lettuce grown hydroponically in winter with CO2 enrichment. New Phytologist 133, 495–501.

Hunt, P.W., Klok, E.J., Trevaskis, B., Watts, R.A., Ellis, M.H., Peacock, W.J., Dennis, E.S., 2002. Increased level of hemoglobin 1 enhances survival of hypoxic stress and promotes early growth in arabidopsis thaliana. Proc Natl Acad Sci U S A 99, 17197–17202.

Igamberdiev, A.U., Baron, K., Manac'h-Little, N., Stoimenova, M., Hill, R.D., 2005. The haemoglobin/nitric oxide cycle: Involvement in flooding stress and effects on hormone signalling. Annals of Botany 96, 557–564.

Igamberdiev, A.U., Bykova, N. V., Hill, R.D., 2006. Nitric oxide scavenging by barley hemoglobin is facilitated by a monodehydroascorbate reductase-mediated ascorbate reduction of methemoglobin. Planta 223, 1033–1040.

Igamberdiev, A.U., Bykova, N. V., Shah, J.K., Hill, R.D., 2010. Anoxic nitric oxide cycling in plants: Participating reactions and possible mechanisms. Physiologia Plantarum 138, 393–404.

Igamberdiev, A.U., Hill, R.D., 2004. Nitrate, NO and haemoglobin in plant adaptation to hypoxia: An alternative to classic fermentation pathways. Journal of Experimental Botany 55, 2473–2482.

Ioannidis, N.E., Kotzabasis, K., 2007. Effects of polyamines on the functionality of photosynthetic membrane in vivo and in vitro. Biochimica et Biophysica Acta 1767, 1372–1382.

Ishizawa, K., Esashi, Y., 1984. Gaseous factors involved in the enhanced elongation of rice coleoptiles under water. Plant Cell and Environment 7, 239–245.

Jackson, M.B., Campbell, D.J., 1975. Movement of ethylene from roots to shoots, a factor in the responses of tomato plants to waterlogged soil conditions. New Phytologist 74, 397–406.

Jackson, M.B., Drew, M.C., 1984. Effects of flooding on growth and metabolism of herbaceous plants, in: Kozlowsky, T. (Ed.), Flooding and Plant Growth.

Jackson, M.B., 1985. Ethylene and responses of plants to soil waterlogging and submergence. Annual Review of Plant Physiology 36, 145–174.

Jackson, M.B., Colmer, T.D., 2005. Response and adaptation by plants to flooding stress. Annals of Botany 96, 501–505.

Jackson, M.B., Fenning, T.M., Drew, M.C., Saker, L.R., 1985. Stimulation of ethylene production and gas-space (aerenchyma) formation in adventitious roots of Zea mays L. by small partial pressures of oxygen. Planta 165, 486–492.

Ju, C., Chang, C., 2012. Advances in ethylene signalling: protein complexes at the endoplasmic reticulum membrane. AoB Plants 1–12.

Justin, S.H.F.W., Armstrong, W., 1987. The anatomical characteristics of roots and plant response to soil flooding. New Phytologist 106, 465–495.

Kamal, A.H.M., Komatsu, S., 2016. Jasmonic acid induced protein response to biophoton emissions and flooding stress in soybean. Journal of Proteomics 133, 33–47.

Kim, P.K.M., Zamora, R., Petrosko, P., Billiar, T.R., 2001. The regulatory role of nitric oxide in apoptosis. International Immunopharmacology 1, 1421–1441.

Kim, Y., Seo, C., Khan, A.L., Mun, B., Shahzad, R., Ko, J., Yun, B., Park, S., Lee, I., 2018. Exo-ethylene application mitigates waterlogging stress in soybean (Glycine max L). BMC Plant Biology 1–16.

Ku, H.S., Suge, H., Rappaport, L., Pratt, H.K., 1970. Stimulation of rice coleoptile growth by ethylene. Planta 90, 333–339.

Kubo, H., 1939. Hemeprotein from the root nodules of legumes. Acta Phytochim. 11, 195–200.

Kumari, A., Singh, P., Kaladhar, V.C., Manbir, Paul, D., Pathak, P.K., Gupta, K.J., 2022. Phytoglobin-NO cycle and AOX pathway play a role in anaerobic germination and growth of deepwater rice. Plant Cell and Environment 45, 178–190.

Labraña, X, Araus, JL, 1991. Effect of foliar applications of silver nitrate and ear removal on carbon dioxide assimilation in wheat flag leaves during grain-filling. Field Crops Research 28, 149–162

Landsmann, J., Dennis, E.S., Higgins, T.J. v., Appleby, C.A., Kortt, A.A., Peacock, W.J., 1986. Common evolutionary origin of legume and non-legume plant haemoglobins. Nature 324, 166–168.

Lee, S., Seo, P.J., Lee, H.J., Park, C.M., 2012. A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in Arabidopsis. Plant Journal 70, 831–844.

Leshem, Y.Y., Haramaty, E., 1996. The characterization and contrasting effects of the nitric oxide free radical in vegetative stress and senescence of Pisum sativum Linn. foliage. Journal of Plant Physiology 148, 258–263.

Levine, A., Tenhaken, R., Lamb, C., 2003. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79, 1–11.

Limami, A.M., Diab, H., Lothier, J., 2014. Nitrogen metabolism in plants under low oxygen stress. Planta 239, 531–541.

Liu, B., Sun, L., Ma, L., Hao, F.S., 2017. Both AtrbohD and AtrbohF are essential for mediating responses to oxygen deficiency in Arabidopsis. Plant Cell Reports 36, 947–957.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402-408.

Locke, J.M., Bryce, J.H., Morris, P.C., 2000. Contrasting effects of ethylene perception and biosynthesis inhibitors on germination and seedling growth of barley (Hordeum vulgare L.). Journal of Experimental Botany 51, 1843–1849.

Loreti, E., Poggi, A., Novi, G., Alpi, A., Perata, P., 2005. A genome-wide analysis of the effects of sucrose on gene expression in arabidopsis seedlings under anoxia. Plant Physiology 137, 1130–1138.

Luan, H., Guo, B., Pan, Y., Lv, C., Shen, H., Xu, R., 2018a. Morpho-anatomical and physiological responses to waterlogging stress in different barley (Hordeum vulgare L.) genotypes. Plant Growth Regulation 85, 399–409.

Luan, H., Shen, H., Pan, Y., Guo, B., Lv, C., Xu, R., 2018b. Elucidating the hypoxic stress response in barley (Hordeum vulgare L.) during waterlogging: A proteomics approach. Scientific Reports 8, 1–13.

Malt OEC - The Observatory of Economic Complexity (2019) https://oec.world/en/profile/hs/malt#:~:text=Malt%20are%20the%20world's%20588th,%2C%20and%20Canada%20(%24297M).

Manik, S.M.N., Pengilley, G., Dean, G., Field, B., Shabala, S., Zhou, M., 2019. Soil and crop management practices to minimize the impact of waterlogging on crop productivity. Frontiers in Plant Science 10, 1–23.

Manitoba Agricultural Services Corporation (MASC), 2019. https://www.masc.mb.ca/masc.nsf/index.html (accessed 1.31.22).

Mata, C.G., Lamattina, L., 2001. Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiology 126, 1196–1204.

Mcdonald, M.P., Galwey, N.W., Colmer, T.D., 2001. Waterlogging tolerance in the tribe Triticeae : the adventitious roots of Critesion marinum have a relatively high porosity and a barrier to radial oxygen loss. Plant Cell and Environment 24, 585–596.

Mendiondo, G.M., Gibbs, D.J., Szurman-zubrzycka, M., Korn, A., Marquez, J., Szarejko, I., Maluszynski, M., King, J., Axcell, B., Smart, K., Corbineau, F., Holdsworth, M.J., 2016. Enhanced waterlogging tolerance in barley by manipulation of expression of the N-end rule pathway E3 ligase PROTEOLYSIS6. Plant Biotechnology Journal 40–50.

Metzner, H., Rau, H., Senger, H., 1965. Studies on synchronization of some pigment-deficient *Chlorella* mutants. Planta (Berl.) 65, 186–194.

Mhamdi, A., van Breusegem, F., 2018. Reactive oxygen species in plant development. Development (Cambridge) 145.

Miller, A.J., Fan, X., Orsel, M., Smith, S.J., Wells, D.M., 2007. Nitrate transport and signalling. Journal of Experimental Botany 58, 2297–2306.

Miller, G., Schlauch, K., Tam, R., Cortes, D., Torres, M.A., Shulaev, V., Dangl, J.L., Mittler, R., 2009. The Plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. Science Signaling 2, 1–11.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., Mittler, R., 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant, Cell and Environment 33, 453–467.

Mira, M.M., Hill, R.D., Stasolla, C., 2016a. Regulation of programmed cell death by phytoglobins. Journal of Experimental Botany 67, 5901–5908.

Mira, M., Hill, R.D., Stasolla, C., 2016b. Phytoglobins improve hypoxic root growth by alleviating apical meristem cell death. Plant Physiology 172, 2044–2056.

Mira, M.M., Asmundson, B., Renault, S., Hill, R.D., Stasolla, C., 2021a. Suppression of the soybean (Glycine max) phytoglobin GmPgb1 improves tolerance to iron stress. Acta Physiologiae Plantarum 43, 1–14.

Mira, M.M., Huang, S., Hill, R.D., Stasolla, C., 2021b. Tolerance to excess moisture in soybean is enhanced by over-expression of the Glycine max Phytoglobin (GmPgb1). Plant Physiology and Biochemistry 159, 322–334.

Mira, M.M., Huang, S., Kapoor, K., Hammond, C., Hill, R.D., Stasolla, C., 2017. Expression of Arabidopsis class 1 phytoglobin (AtPgb1) delays death and degradation of the root apical meristem during severe PEG-induced water deficit. Journal of Experimental Botany 68, 5653–5668.

Misra, A.N., Vladkova, R., Singh, R., Misra, M., Dobrikova, A.G., Apostolova, E.L., 2014. Nitric oxide action and target sites of nitric oxide in chloroplasts. Nitric Oxide 39, 35–45.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Science 7, 405–410.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V.B., Vandepoele, K., Gollery, M., Shulaev, V., Breusegem, F. van, 2011. ROS signaling: the new wave? Trends in Plant Science 16, 300–309.

Montilla-Bascón, G., Rubiales, D., Hebelstrup, K.H., Mandon, J., Frans, J.M., Cristescu, S.M., Mur, L.A.J., Prats, E., 2017. Reduced nitric oxide levels during drought stress promote drought tolerance in barley and is associated with elevated polyamine biosynthesis. Scientific Reports 7, 1–15.

Müller, P., Li, X.P., Niyogi, K.K., 2001. Non-photochemical quenching. A response to excess light energy. Plant Physiology 125, 1558–1566.

Neill, S.J., Desikan, R., Hancock, J.T., 2003. Nitric oxide signalling in plants. New Phytologist 159, 11–35.

Pang, J., Zhou, M., Mendham, N., Shabala, S., 2004. Growth and physiological responses of six barley genotypes to waterlogging and subsequent recovery. Australian Journal of Agricultural Research 55, 895–906.

Ploschuk, R.A., Miralles, D.J., Colmer, T.D., Ploschuk, E.L., Striker, G.G., 2018. Waterlogging of winter crops at early and late stages: Impacts on leaf physiology, growth and yield. Frontiers in Plant Science 871, 1–15.

Pradet, A., Bomsel, J.L., 1978. Energy metabolism in plants under hypoxia and anoxia, in: Hook, DD; Crawford, R. (Ed.), Plant Life in Anaerobic Environments. pp. 89–118.

Province of Manitoba (2019) Climate Change in Manitoba. https://www.gov.mb.ca/climateandgreenplan/climatechange.html (accessed 1.31.22).

Pucciariello, C., Perata, P., 2017. New insights into reactive oxygen species and nitric oxide signalling under low oxygen in plants. Plant Cell and Environment 40, 473–482.

Qi, X., Li, Q., Ma, X., Qian, C., Wang, H., Ren, N., Shen, C., Huan, S., Xu, X., Xu, Q., Chen, X., 2017. Waterlogging-induced adventitious root formation in cucumber is regulated by ethylene and auxin through reactive oxygen species signalling. Plant Cell and Environment 42, 1458–1470.

Qu, Z.L., Zhong, N.Q., Wang, H.Y., Chen, A.P., Jian, G.L., Xia, G.X., 2006. Ectopic expression of the cotton non-symbiotic hemoglobin gene GhHbd1 triggers defense responses and increases disease tolerance in Arabidopsis. Plant and Cell Physiology 47, 1058–1068.

Rajhi, I., Yamauchi, T., Takahashi, H., Nishiuchi, S., Shiono, K., Watanabe, R., Mliki, A., Nagamura, Y., Tsutsumi, N., Nishizawa, N.K., Nakazono, M., 2011. Identification of genes expressed in maize root cortical cells during lysigenous aerenchyma formation using laser microdissection and microarray analyses. New Phytologist 190, 351–368.

Ren, B., Dong, S., Zhao, B., Liu, P., Zhang, J., 2017. Responses of nitrogen metabolism, uptake and translocation of maize to waterlogging at different growth stages. Frontiers in Plant Science 8, 1216.

Roblin, G, Perault, JM., 1985. Effects of ethephon, 1-aminocyclopropane-1-carboxylic acid, and inhibitors of ethylene synthesis on the gravitropically induced movement of *Mimosa pudica* Pulvinus 1. Plant Physiology 77, 922–925.

Romero, F.M., Maiale, S.J., Rossi, F.R., Marina, M., Ruı, O.A., Garriz, A., 2018. Polyamine metabolism responses to biotic and abiotic Stress, in: Polyamines. pp. 37–49.

Rosenzweig, C., Tubiello, F.N., Goldberg, R., Mills, E., Bloomfield, J., 2002. Increased crop damage in the US from excess precipitation under climate change. Global Environmental Change 12, 197–202.

Samad, A., Meisner, C.A., Saifuzzaman, M., van Ginkel, M., 2001. Waterlogging tolerance. *In* Reynolds, MP; Ortiz-Monastero, JI; McNab, A., eds, Application of physiology in Wheat Breeding. CIMMYT, Mexico, pp. 135–144.

Sasidharan, R., Bailey-Serres, J., Ashikari, M., Atwell, B.J., Colmer, T.D., Fagerstedt, K., Fukao, T., Geigenberger, P., Hebelstrup, K.H., Hill, R.D., Holdsworth, M.J., Ismail, A.M., Licausi, F., Mustroph, A., Nakazono, M., Pedersen, O., Perata, P., Sauter, M., Shih, M.-C., Sorrell, B.K., Striker, G.G., van Dongen, J.T., Whelan, J., Xiao, S., Visser, E.J.W., Voesenek, L.A.C.J., 2017. Community recommendations on terminology and procedures used in flooding and low oxygen stress research. The New Phytologist 214, 1403–1407.

Sasidharan, R., Voesenek, L.A.C.J., 2015. Ethylene-mediated acclimations to flooding stress. Plant Physiology 169, 3–12.

Saskatchewan Crop Insurance Corporation (2019). https://www.scic.ca/

Sauter, M., 2013. Root responses to flooding. Current Opinion in Plant Biology 16, 282–286.

Setter, T.L., Burguess, P., Waters, I., Kuo, J., 1999. Genetic diversity of barley and wheat for waterlogging tolerance in Western Australia., in: Australian Barley Technical Symposium. Melbourne.

Setter, T.L., Waters, I., 2003. Review of prospects for germplasm improvement for waterlogging tolerance in wheat, barley and oats. Plant and Soil 253, 1–34.

Shiono, K., Ejiri, M., Shimizu, K., Yamada, S., 2019. Improved waterlogging tolerance of barley (Hordeum vulgare) by pretreatment with ethephon. Plant Production Science 22, 285–295.

Silva-Cardenas, R.I., Ricard, B., Saglio, P., Hill, R.D., 2003. Hemoglobin and hypoxic acclimation in maize root tips. Russian Journal of Plant Physiology 50, 821–826.

Smagghe, B.J., Hoy, J.A., Percifield, R., Kundu, S., Hargrove, M.S., Sarath, G., Hilbert, J., Watts, R.A., Dennis, E.S., Peacock, W.J., Dewilde, S., Moens, L., Blouin, G.C., Olson, J.S., Appleby, C.A., 2009. Correlations between oxygen affinity and sequence classifications of plant hemoglobins. Biopolymers 91.

Sørensen, C.K., Carciofi, M., Hasler-Sheetal, H., Zafari, S., Andrzejczak, O., Hovmøller, M.S., Møller, I.M., Hebelstrup, K.H., 2019. Overexpression of phytoglobin in barley alters both compatible and incompatible interactions with the mildew pathogen Blumeria graminis. Plant Pathology 68, 152–162.

Sowa, A.W., Duff, S.M.G., Guy, P.A., Hill, R.D., Sowa, A.W., Duff, S.M.G., Guy, P., Hill, R.D., 1998. Altering hemoglobin levels changes energy status in maize cells under hypoxia. Proceedings of the National Academy of Sciences 95, 10317–10321.

Steffens, B., Geske, T., Sauter, M., 2011. Aerenchyma formation in the rice stem and its promotion by H_2O_2. New Phytologist 190, 369–378.

Stoimenova, M., Igamberdiev, A.U., Gupta, K.J., Hill, R.D., 2007. Nitrite-driven anaerobic ATP synthesis in barley and rice root mitochondria. Planta 226, 465–474.

Suzuki, N., Miller, G., Morales, J., Shulaev, V., Torres, M.A., Mittler, R., 2011. Respiratory burst oxidases: the engines of ROS signaling. Current Opinion in Plant Biology 14, 691–699.

Tan, X., Xu, H., Khan, S., Equiza, M.A., Lee, S.H., Vaziriyeganeh, M., Zwiazek, J.J., 2018. Plant water transport and aquaporins in oxygen-deprived environments. Journal of Plant Physiology 227, 20–30.

Tanaka, Y., Sano, T., Tamaoki, M., Nakajima, N., Kondo, N., 2005. Ethylene inhibits abscisic acid-induced stomatal closure in arabidopsis. Plant Physiology 138, 2337–2343.

Taylor, E.R., Nie, X.Z., MacGregor, A.W., Hill, R.D., 1994. A cereal haemoglobin gene is expressed in seed and root tissues under anaerobic conditions. Plant Molecular Biology 24, 853–862.

Tholen, D., Voesenek, L.A.C.J., Poorter, H., 2004. Ethylene insensitivity does not increase leaf area or relative growth rate in arabidopsis, Nicotiana tabacum, and Petunia x hybrida 1. Plant Physiology 134, 1803–1812.

Tholen, D., Pons, T.L., Voesenek, L.A.C.J., Poorter, H., 2007. Ethylene insensitivity results in down-regulation of rubisco expression and photosynthetic capacity in tobacco. Plant Physiology 144, 1305–1315

Tong, C., Hill, C.B., Zhou, G., Zhang, X., Jia, Y., 2021. Opportunities for mproving waterlogging tolerance in cereal crops — physiological traits and genetic mechanisms. Plants 10, 1–22.

Tong, W., Imai, A., Tabata, R., Shigenobu, S., Yamaguchi, K., 2016. Polyamine resistance is increased by mutations in a nitrate transporter Arabidopsis thaliana. Frontiers in Plant Science 7, 1–10.

Torres, M.A., Dangl, J.L., 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Current Opinion in Plant Biology 8, 397–403.

Triantaphylidès, C., Havaux, M., 2009. Singlet oxygen in plants: production, detoxification and signaling. Trends in Plant Science 14, 219–228.

Trump, B.F., Smuckler, E.A., Benditt, E. P., 1961. A method for staining epoxy sections for light microscopy. Journal of Ultrastructure Research 5, 343–348.

Tun, N.N., Santa-Catarina, C., Begum, T., Silveira, V., Handro, W., Segal Floh, E.I., Scherer, G.F.E., 2006. Polyamines induce rapid biosynthesis of nitric oxide (NO) in Arabidopsis thaliana seedlings. Plant and Cell Physiology 47, 346–354.

Vanhoudt, N., Vandenhove, H., Horemans, N., Wannijin, J., Bujanic, A., Vangronsveld, J., Cuypers, A., 2010. Study of oxidative stress related responses induced in Arabidopsis thaliana following mixed exposure to uranium and cadmium. Plant Physiology and Biochemistry 48, 879–886

Vigeolas, H., Hühn, D.H., Geigenberger, P., 2011. Nonsymbiotic hemoglobin-2 leads to an elevated energy state and to a combined increase in polyunsaturated fatty acids and total oil content when overexpressed in developing seeds of transgenic arabidopsis plants. Plant Physiology 155, 1435–1444.

Visser, E.J.W., Voesenek, L.A.C.J., 2005. Acclimation to soil flooding sensing and signal transduction. Plant and Soil 254, 197–214.

Voesenek, L.A.C.J., Bailey-Serres, J., 2015. Flood adaptive traits and processes: An overview. New Phytologist 206, 57–73.

Voesenek, L.A.C.J., Sasidharan, R., 2013. Ethylene - and oxygen signalling - drive plant survival during flooding. Plant Biology 15, 426–435.

Vriezen, W.H., Hulzink, R., Mariani, C., Voesenek, L.A.C.J., 1999. 1-aminocyclopropane-1-carboxylate oxidase activity limits ethylene biosynthesis in Rumex palustris during submergence. Plant Physiology 121, 189–195.

Vriezen, W.H., Zhou, Z., van der Straeten, D., 2003. Regulation of submergence-induced enhanced shoot elongation in Oryza sativa L. Annals of Botany 91, 263–270.

Wany, A., Gupta, A.K., Kumari, A., Mishra, S., Singh, N., Pandey, S., Vanvari, R., Igamberdiev, A.U., Fernie, A.R., Gupta, K.J., 2019. Nitrate nutrition influences multiple factors in order to increase energy efficiency under hypoxia in Arabidopsis. Annals of Botany 123, 691–705.

Watts, R.A., Hunt, P.W., Hvitved, A.N., Hargrove, M.S., Peacock, W.J., Dennis, E.S., 2001. A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. Proc Natl Acad Sci U S A 98, 10119–10124.

Westra, S., Fowler, H.J., Evans, J.P., Alexander, L. v., Berg, P., Johnson, F., Kendon, E.J., Lenderink, G., Roberts, N.M., 2014. Future changes to the intensity and frequency of short-duration extreme rainfall. American Geophysical Union 522–555.

Wittenberg, J.B., Bolognesi, M., Wittenberg, B.A., Guertin, M., 2002. Truncated hemoglobins: A new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. Journal of Biological Chemistry 277, 871–874.

Yaduvanshi, N.P.S., Setter, T.L., Sharma, S.K., Singh, K.N., Kulshreshtha, N., 2012. Influence of waterlogging on yield of wheat (Triticum aestivum), redox potentials, and concentrations of microelements in different soils in India. Soil Research 50, 489–499.

Yamauchi, T., Rajhi, I., Nakazono, M., 2011. Lysigenous aerenchyma formation in maize root is confined to cortical cells by regulation of genes related to generation and scavenging of reactive oxygen species. Plant Signaling and Behavior 6, 759–761.

Yamauchi, T., Tanaka, A., Mori, H., Takamure, I., Kato, K., Nakazono, M., 2016. Ethylene-dependent aerenchyma formation in adventitious roots is regulated differently in rice and maize. Plant Cell and Environment 39, 2145–2157.

Yamauchi, T., Watanabe, K., Fukazawa, A., Mori, H., Abe, F., Kawaguchi, K., Oyanagi, A., Nakazono, M., 2014. Ethylene and reactive oxygen species are involved in root aerenchyma formation and adaptation of wheat seedlings to oxygen-deficient conditions. Journal of Experimental Botany 65, 261–273.

Yamauchi, T., Yoshioka, M., Fukazawa, A., Mori, H., Nishizawa, N.K., Tsutsumi, N., Yoshioka, H., Nakazono, M., 2017. An NADPH oxidase RBOH functions in rice roots during lysigenous aerenchyma formation under oxygen-deficient conditions. Plant Cell 29, 775–790.

Yang, C., Li, W., Cao, J., Meng, F., Yu, Y., Huang, J., Jiang, L., Liu, M., Zhang, Z., Chen, X., Miyamoto, K., Yamane, H., Zhang, J., Chen, S., Liu, J., 2017. Activation of ethylene signaling pathways enhances disease resistance by regulating ROS and phytoalexin production in rice. Plant Journal 89, 338–353.

Yordanova, R.Y., Christov, K.N., Popova, L.P., 2004. Antioxidative enzymes in barley plants subjected to soil flooding. Environmental and Experimental Botany 51, 93–101.

Youssef, M.S., Mira, M.M., Renault, S., Hill, R.D., Stasolla, C., 2016. Phytoglobin expression influences soil flooding response of corn plants. Annals of Botany 118, 919–931.

Yu, F., Han, X., Geng, C., Zhao, Y., Zhang, Z., Qiu, F., 2015. Comparative proteomic analysis revealing the complex network associated with waterlogging stress in maize (Zea mays L) seedling root cells. Proteomics 15, 135–147.

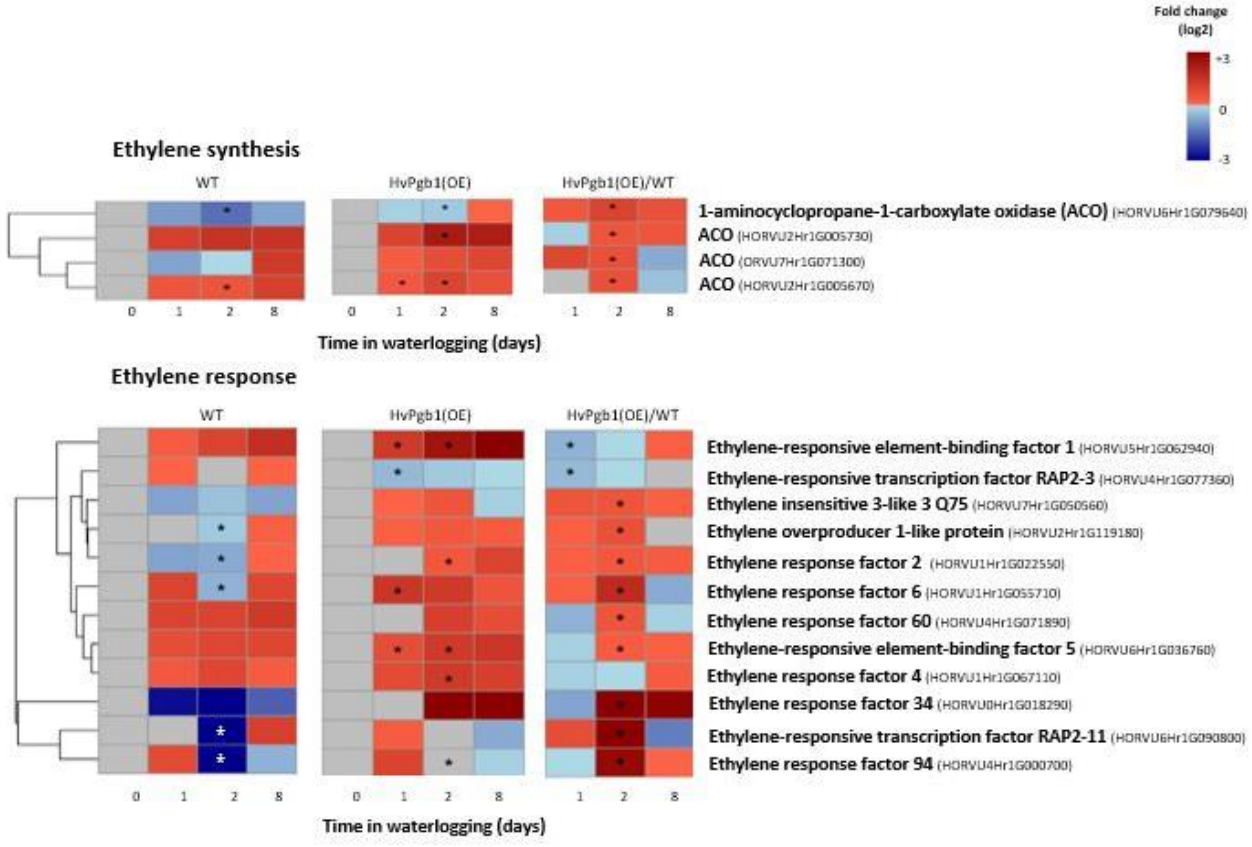
Zhang, X., Shabala, S., Koutoulis, A., Shabala, L., 2017. Meta-analysis of major QTL for abiotic stress tolerance in barley and implications for barley breeding. Planta 245, 283–295.

Zhang, X., Shabala, S., Koutoulis, A., Shabala, L., Johnson, P., Hayes, D., Nichols, D.S., Zhou, M., 2015. Waterlogging tolerance in barley is associated with faster aerenchyma formation in adventitious roots. Plant and Soil 394, 355–372.

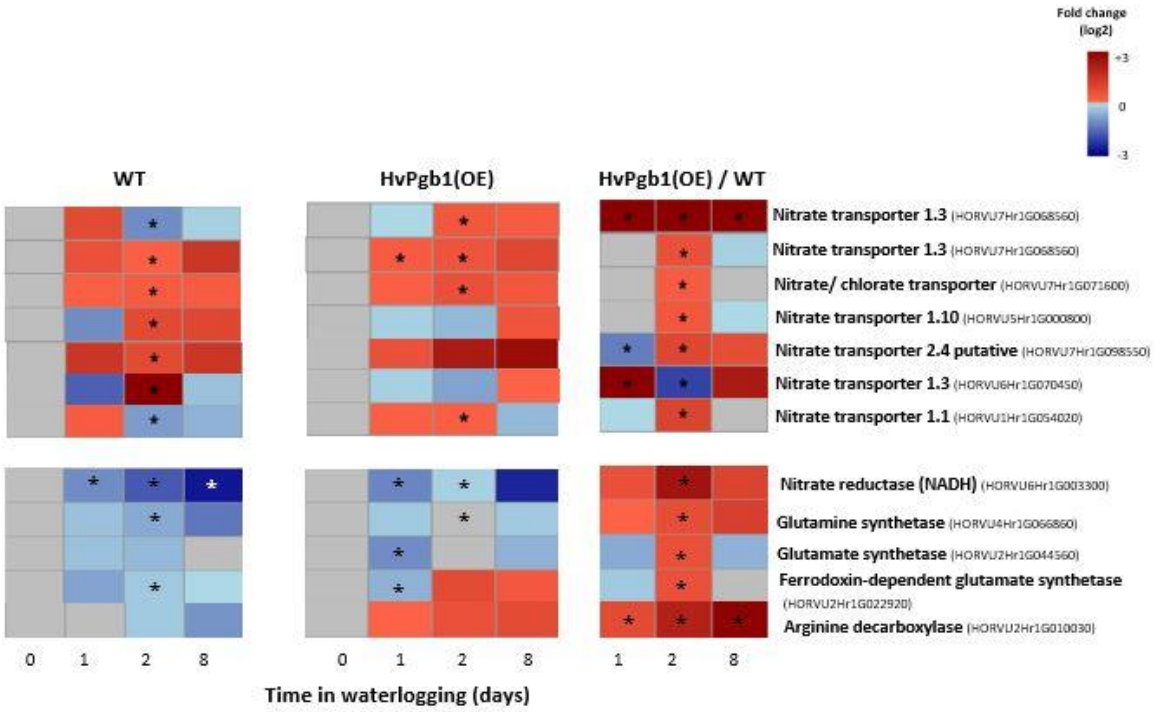
Zhang, X., Zhou, G., Shabala, S., Koutoulis, A., 2016. Identification of aerenchyma formation - related QTL in barley that can be effective in breeding for waterlogging tolerance. Theoretical and Applied Genetics 129, 1167–1177.

Zhou, Z., Vriezen, W., Caeneghem, W. van, Montagu, M. van, Straeten, D. van der, 2001. Rapid induction of a novel ACC synthase gene in deepwater rice seedlings upon complete submergence. Euphytica 121, 137–143.

SUPPLEMENTARY FIGURES



Supplementary figure 1. Time course and pairwise comparison of ethylene synthesis and response transcripts in wild type (WT) and HvPgb1-overexpressing [HvPgb1(OE)] lines. Asterisks denote significant difference (adjusted $p > 0.05$). Colours reflect fold change (log2) with red at +3 and blue at -3.



Supplementary figure 2. Time course and pairwise comparison of nitrogen metabolism transcripts in wild type (WT) and HvPgb1-overexpressing [HvPgb1(OE)] lines. Asterisks denote significant difference (adjusted $p > 0.05$). Colours reflect fold change (log2) with red at +3 and blue at -3.