Over-expression of Phytoglobin alleviates PEG-induced water stress in maize shoots

by

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ABBREVIATIONS

ABA, Abscisic acid

ACC, 1-aminocyclopropane-1-carboxylic acid

ACO, 1-aminocyclopropane-1-carboxylic acid oxidase

ACS, 1-aminocyclopropane-1-carboxylic acid synthase

ADP, Adenosine diphosphate

AOA, Aminooxyacetic acid

Ca^{2+}, Calcium

cGMP, Cyclic GMP

CO_2, Carbon dioxide

cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide

CTR, Constitutive triple response

DEPC, Diethyl pyrocarbonate

EBF, EIN3 binding F-box protein

EIN, Ethylene insensitive

ERF, Ethylene response factor

ETH, Ethephon

K^+, Potassium

Mg, Magnesium

NADPH, Nicotinamide adenine dinucleotide phosphate

NBT, Nitroblue tetrazolium

NO, Nitric oxide

NOS, Nitric oxide synthase

NR, Nitrite reductase

PCD, Programmed cell death

PEG, Polyethylene glycol

PEP, Phosphoenolpyruvate

Pgb, Phytoglobin

RBOH, Respiratory burst oxidase homologue

ROS, Reactive oxygen species

sGC, Soluble guanylyl cyclase

SNP, Sodium nitroprusside

WT, Wild type

XOR, Xanthanine oxioreductase

ZmPgb, *Zea mays* phytoglobin

ABSTRACT

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Over-expression of Phytoglobin alleviates PEG-induced water stress in maize shoots

Supervisor: Dr. Claudio Stasolla

Phytoglobins are heme-containing proteins found in plants that are upregulated during stress conditions. The effect of drought stress on 3-leaf stage (V2) maize (*Zea mays* L.) seedlings over-expressing or down-regulating the *Zea mays phytoglobin 1* gene (*ZmPgb1*) was investigated using applications of 25% w/v polyethylene glycol (PEG) to mimic drought. Over-expression of *ZmPgb1* increased drought tolerance, decreased wilting, and decreased the accumulation of ethylene and reactive oxygen species (ROS), compared to wild type (WT) plants and plants in which the level of *ZmPgb1* was down-regulated. Gene expression studies conducted during the first 16 hours of water stress revealed a transcriptional induction of ethylene synthesis and response, as well as ROS production, in the *ZmPgb1* down-regulating plants relative to WT plants. This was in contrast to the *ZmPgb1* over-expressing plants where genes participating in ethylene synthesis and response exhibited the lowest expression levels, and ROS production was also limited. Ethylene measurements supported the expression studies; relative to WT, ethylene levels were higher in plants down-regulating *ZmPgb1* and lower in those up-regulating the same gene. Pharmacological treatments with the NO donor sodium nitroprusside (SNP) and the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) showed that *ZmPgb1* effects were modulated by NO. Ethylene and ROS increased in environments

enriched with NO, and decreased in those depleted in NO. Furthermore, while an experimental rise in ethylene activated the expression of *RBOH*s (*Respiratory Burst Oxidase Homologs*), encoding subunits of the ROS-producing NADPH oxidase machinery, a reduction in ethylene decreased the expression of the same genes. Based on these results, a model is proposed in which suppression of NO signalling by *ZmPgb1* reduces ethylene accumulation and response, and production of ROS, which are conditions alleviating drought stress.

1 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 recommended by the Journal of Experimental Botany. This thesis is presented as a single manuscript, which contains an abstract, introduction, materials and methods, results, and a discussion section. Supplementary tables are placed following the results section of the manuscript. A literature review precedes the manuscript and general discussion and conclusions follows the manuscript.

2 LITERATURE REVIEW

2.1 Introduction

One of the major uncertainties of the future due to climate change is the amount and distribution of rainfall. Currently, one of the most significant threats to global food security is drought (Farooq et al., 2009). Drought-induced yield loss surpasses losses from all other causes, due to the severity and extent of drought stress (Farooq et al., 2009). Minimizing the yield gap between optimal and drought stress conditions is one of the main concerns to plant breeders today (Cattivelli et al., 2008). Water deficit affects plants in a number of ways. Nearly every plant process is affected by water availability, which is not surprising considering water accounts for 80-90% of fresh weight of most herbaceous plants (Bhattacharjee and Saha, 2014). During vegetative growth, drought stress affects plants by reducing plant height, leaf area and overall growth (Farooq et al., 2009). Even more critical for yield is the amount of water available to plants during reproductive development. In maize (*Zea mays*), for example, restriction of water during flowering delays silking, which increases the anthesis-to-silking interval (Cattivelli et al., 2008). A longer interval between anthesis and silking means that by the time the silks have emerged, the pollen is no longer viable to pollinate them. This often results in barren tips on the ears of corn. Drought also often leads to embryo abortion, which has a major effect on grain yield (Kakumanu et al., 2012). It is therefore critical for future world food security to select plants able to tolerate drought stress conditions.

2.2 Water Stress Physiology

2.2.1 Agronomic classification of drought

Drought stress in the field happens in a number of discrete stages. Agronomically, the soil in the field is designated as saturated when the water can drain freely, due to gravity. This stage would occur directly after a sustained rainfall where the field has more water than the soil is able to maintain. When all the water has drained from the field, the soil reaches field capacity. Loss of water from evaporation and uptake from plant roots occur until the water is depleted, which leads to the wilting point, the point where no more water can be taken from the field. At the wilting point, plants are unable to meet their physiological needs and will wilt and die (Bhattacharjee and Saha, 2014).

2.2.2 Effects of drought stress on plant physiology

2.2.2.1 Plant growth reduction

One of the main effects of plant drought stress is the reduction in plant growth. Cell growth is one of the plant processes most responsive to drought (Farooq et al., 2009). Both root elongation and leaf area expansion are drastically decreased under drought stress, but there are also large reductions in plant height, leaf number, fresh weight, and dry weight (Bhattacharjee and Saha, 2014). There are two main reasons why this reduction occurs. The first is the loss of turgor pressure in the plant cells. Turgor pressure in plants is the main driver of cell elongation. Due to the rigidity of cell walls, the water inside the cytoplasm of the cell exerts a mechanical pressure on the walls of the cell. When the cell is elongating, new cell wall material will be added, which makes the cell wall soft (Kroeger et al., 2011). The cell wall is differentially softened, which allows for directional growth. The walls perpendicular to the direction of growth

have reduced tensile strength, and when turgor pressure is exerted on the cell, the cell expands and elongates to help accommodate the pressure (Kroeger et al., 2011). In this way, all growth and enlargement in the plant occurs and high levels of turgor pressure are required. When there are low levels of water available, the plant cannot maintain the turgor pressure needed for elongation, and growth slows (Farooq et al., 2009).

2.2.2.2 Photosynthetic rate reduction

The second reason for the arrest of growth during drought stress conditions is the reduction in photosynthesis. Photosynthesis is the process through which plants fix carbon to produce organic and inorganic components to synthesize new protoplasm and cell walls (Bhattacharjee & Saha 2014). A reduced rate of photosynthesis can compromise plant growth and normal functions. Drought can reduce photosynthesis through several factors, including stomatal closure, the reduction in leaf expansion, diminished photosynthetic machinery, early senescence of leaves, and the reduction of sugar synthesis (Farooq et al., 2009). Under drought conditions, one of the first reactions of the plant is to close the stomata to limit the amount of water lost through transpiration. Stomatal closure is accepted as being the main reason for decreased photosynthesis in a drought stressed plant (Cornic, 2000). The behavior of stomata is more sensitive to the water status of the soil than that of leaves, suggesting that stomata respond quickly to chemical signals, such as those from abscisic acid, produced by the root (Sharp et al., 1994). The influence of abscisic acid on drought stress response will be elucidated further in this review. Photosynthetic enzymes, such as Rubisco, are also affected by drought stress, and photosynthesis is limited by a decline in Rubisco activity. Under severe drought stress conditions, the oxygenase activity of Rubisco is increased due to the higher level of oxygen

accumulating in the mesophyll tissue following the closure of the stomata (Parry et al., 2002). Thus, rather than performing photosynthesis and building up carbon reserves, the plant breaks down photosynthate. Continued photosynthetic reactions under limited CO_2 concentrations can result in the production of reactive oxygen species (ROS), which are very damaging to the cell (Basu et al., 2016) and will be discussed later in this review. C4 plants, like maize, are better adapted to minimize this costly reaction as they spatially segregate Rubisco from oxygen, and funnel CO_2 to the active site (Kellogg, 2013). They also utilize a carbon compound in the mesophyll called phosphoenolpyruvate (PEP) that binds with bicarbonate via PEP carboxylase to form malate. This 4-carbon compound travels to Rubisco, is decarboxylated, and Rubisco uses the freed CO_2, where regular C3 photosynthesis cycle takes over (Kellogg, 2013). This dual-action efficiency is the reason why C4 plants are more productive in warm climates (with higher transpiration) than C3 plants.

2.2.2.3 Changes in resource allocation

One major effect caused by drought is a change in resource allocation. Plant growth rates are reduced under drought stress as discussed above, but the ratio of root growth to shoot growth is also affected. Leaf growth is usually decreased more severely than root growth, thus affecting photosynthesis, and photosynthates are redirected to favour the growth of roots (Bhattacharjee and Saha, 2014). By prioritizing the growth of the roots over the shoot, the plant may increase its survival by reaching moist soil in the lowered water table (Farooq et al., 2009).

Prolonged drought stress, however, does result in root death (Duan et al., 2010). Change in plant biomass as a result of resource reallocation can increase water use efficiency, which is

the ratio between dry matter production and water consumption. Plants with high water use efficiency are better able to make use of limited water, and use less to perform their basic functions. It has been found that drought stressed plants have a higher water use efficiency (Bhattacharjee and Saha, 2014), which is likely due to the reduced amount of water lost through the stomata (Farooq et al., 2009).

2.2.2.4 Changes in molecular uptake/synthesis

Osmotic adjustment is a major strategy used by plants to cope with conditions of water stress. This process is achieved through the cytoplasmic accumulation of low molecular weight compounds to reduce the water potential inside the cell, thereby ensuring the flow of water from the soil (Farooq et al., 2009; Chen and Jiang, 2010). Plant cells amass a number of osmotic molecules including sugars, sugar alcohols, proline, glycine betaine, organic acids, calcium, potassium, and chloride ions (Farooq et al., 2009). Osmotic adjustment is not only important for the maintenance of turgor pressure, which allows a plant to delay wilting, it is also important for the maintenance of crucial cell processes and metabolic activity that allows the plant to continue growth when water becomes available (Chen and Jiang, 2010). Osmotic adjustment is also involved in the maintenance of stomatal conductance, photosynthesis, leaf water volume, and growth under drought stress conditions (Bhattacharjee and Saha, 2014). Another important benefit of osmotic adjustment is limiting the toxic amounts of Na^+ accumulating in the cell (Chen and Jiang, 2010). Compatible solutes in osmotic adjustment are not only tied to decreasing salinity, they also help with other drought stress effects such as detoxification of reactive oxygen species (ROS), protection of membrane integrity, and stabilization of enzymes and proteins (Basu et al., 2016).

Uptake of nutrients is also affected by water stress. Prolonged drought interferes with nutrient uptake and unloading mechanisms, as well as reduced transpirational flow due to lack of water (Farooq et al., 2009). In maize, the uptake of K^+ and Ca^{2+} is increased under drought stress conditions as a result of osmotic adjustment. Exogenous application of K^+ has been found to increase tolerance to drought stress in sunflower (*Helianthus* spp.), and it is one of the primary osmolytes in plants (Farooq et al., 2009). It is also important to note that the uptake of nutrients is generally reduced under drought stress conditions. This is the result of decreasing uptake efficiency of older roots under drying conditions, combined with the decrease in availability of nutrients in the soil. The decrease in root uptake efficiency is tied to the fact that there is limited ATP in drought stressed plants and active transport systems in the roots are impaired or destroyed (Bhattacharjee and Saha, 2014).

There are a number of other molecules that are implicated in drought stress responses, as shown in Figure 2.1. Reactive nitrogen species like nitric oxide (NO) and ROS serve as signalling molecules. Hormones like abscisic acid and ethylene are also upregulated under drought stress conditions, which aid in the control of plant growth and metabolism under adverse conditions (Chaves and Oliveira, 2004). These other factors will be discussed in detail in this review.

2.2.3 Drought stress adaptation

Plant adaptations to drought stress can be grouped into two categories. The first is long-term adaptations, where plants develop morphological characteristics through the process of

natural selection to help cope with low water levels. Such plants are called xerophytes, and exhibit a number of physical characteristics that help to retain water and scavenge water from their surroundings. These adaptations include an increase in the ratio of leaf vein tissue to leaf surface, increased stomatal density, smaller sized stomata, smaller epidermal and mesophyll cells, denser trichomes (leaf hairs), smaller trichomes, deeper root systems, thicker epidermal walls, and thicker outer cuticles (Bhattacharjee and Saha, 2014).

The second type of adaptation is short-term and involves rapid changes often observed during conditions of moderate water supply, and will be discussed later. Although there are a number of physiological responses to drought stress, individual plant survivability also depends upon factors such as species, developmental stage, seed provenance, intensity and duration of drought stress (Tátrai et al., 2016).

2.2.4 Aquaporins

Aquaporins are water channel proteins, and are essential for the movement of water in a plant. It is estimated that aquaporins facilitate 20-85% of all plant water transport, depending on the species (Parent et al., 2009). Plants also contain a large number of aquaporin genes, ranging from 30 to greater than 70, depending on species (Aroca et al., 2012). The movement of water by aquaporins flows in response to stresses such as cold, drought, salinity, anoxia, and mineral starvation through reversible phosphorylation (Luu and Maurel, 2005). It also has been shown that aquaporins are able to move from internal membranes to the plasma membrane by translocating the aquaporin proteins in vesicles (Aroca et al., 2012). Some aquaporins may also transport small solutes and gases instead of water (Liu et al., 2007).

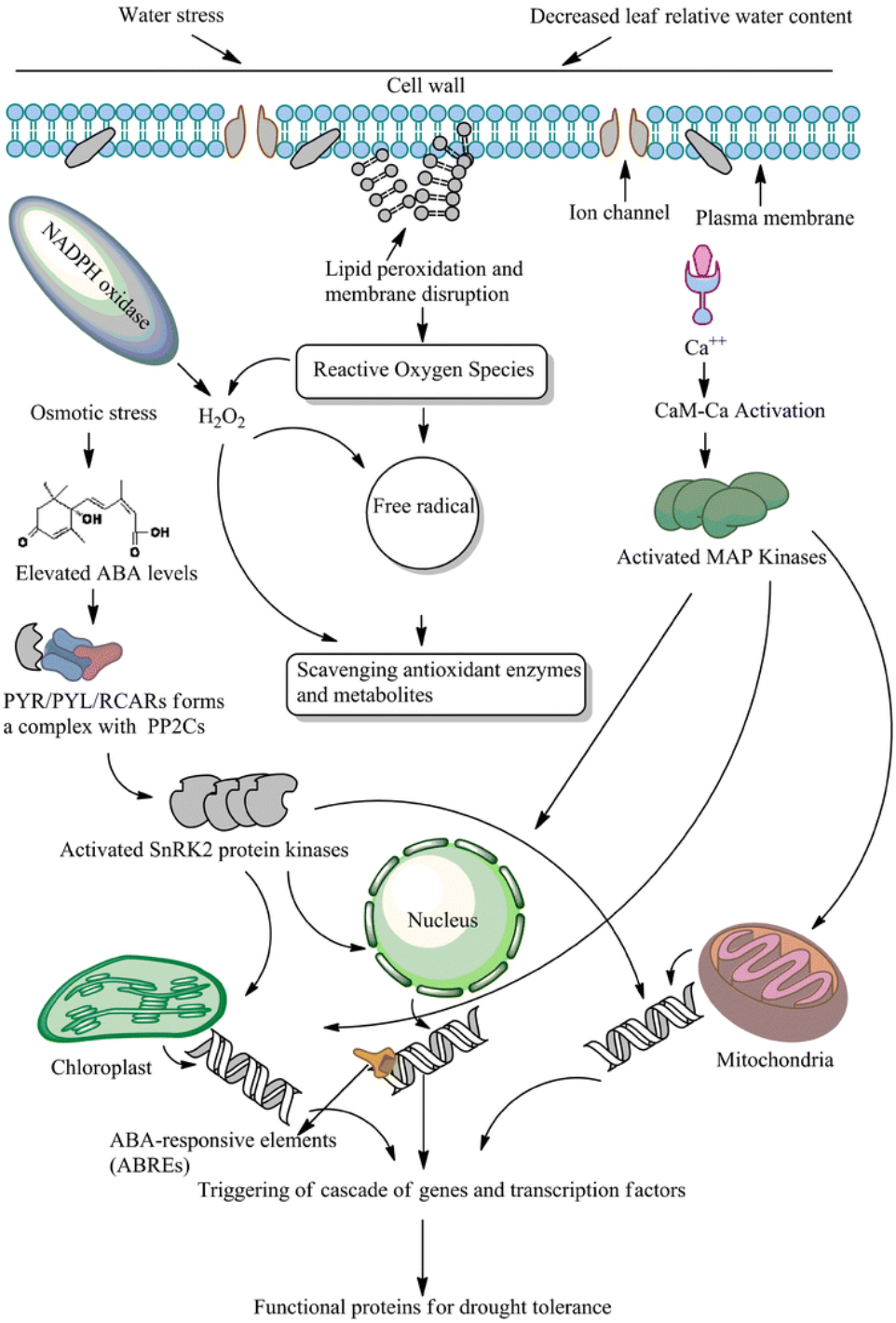


Figure 2.1. Early signalling in the drought stress response. Figure shows the multiple levels of effect in the plant cell with the earliest responses depicted at the top and move downward with time. Figure is from Shanker et al. (2014) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

The opening and closure, or “gating”, of aquaporin channels is regulated by a number of hormones and signalling molecules. The application of ABA to maize plants showed an upregulation in the production of aquaporin proteins (Parent et al., 2009). Reactive oxygen species (ROS) have also been implicated in the gating of aquaporins, through oxidation of the aquaporins, or through oxidation of the lipid membrane (Luu and Maurel, 2005). One study found that application of the ROS hydrogen peroxide closed the aquaporin channels and reduced water permeability in maize roots (Ye and Steudle, 2006). Other studies have found that nitric oxide (NO), another signalling molecule, may have an effect on aquaporins. It was observed that NO is part of a signalling pathway involving aquaporins and germination, and that an increase in the amount of NO with sodium nitroprusside (SNP) increased the aquaporin transcript level 5.8-fold (Liu et al., 2007).

2.3 Nitric Oxide

Nitric oxide (NO) is an important signalling molecule regulating a diverse array of events during plant growth and development. Nitric oxide is a free radical in its gaseous state, and can diffuse easily through the lipid membrane, where it is found in both cytoplasm and membranes (Wilson et al., 2008). Nitric oxide in animals is produced via NO synthase (NOS) enzymes, however similar NO synthesizing enzymes have not yet been discovered in plants (Wilson et al., 2008).

2.3.1 Synthesis of nitric oxide

2.3.1.1 Nitric oxide synthase (NOS) enzymes

Nitric oxide production in animals is mediated by nitric oxide synthase (NOS), the enzyme which catalyzes the reaction to form the end products NO and citrulline (Palavan-Unsal and Arisan, 2009). There is increasing evidence for a NOS-like reaction in plants (Wilson et al., 2008; Palavan-Unsal and Arisan, 2009) but an analog of the mammalian NOS gene in Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) genomes has remained elusive. The search for a NOS enzyme in plants is difficult because NO production is localized in different areas of the cell. In animals, NOS is present in the mitochondria, whereas NOS-like enzymes have been found to be produced in the cytoplasm of plant cells and then transported into the nucleus. In plant cells, NOS-like activity has also been found in the matrix of peroxisomes and chloroplasts (Palavan-Unsal and Arisan, 2009).

A gene called *AtNOS1* was suggested as a plant NOS gene due to the enzyme's ability to convert L-arginine to L-citrulline. A knockout mutant of the gene was also found to have reduced NO content in guard cells and roots. *AtNOS1* has since been accepted as an important component of NO production/accumulation even if it is not a direct homolog to mammalian NOS (Wilson et al., 2008).

2.3.1.2 Nitrite reductase (NR) enzymes

Along with production by the NOS-like enzyme, NO can also be produced in plant cells by the activity of an enzyme called nitrite reductase (NR). This enzyme can produce NO from nitrite in a NADPH-dependant reaction (Wilson et al., 2008; Palavan-Unsal and Arisan, 2009).

The production of NO by NR increases under stress conditions such as hypoxia (Wilson et al., 2008). There has also been evidence for pathogen-induced NO production by NR (Palavan-Unsal and Arisan, 2009).

2.3.2 Nitric oxide signalling

As a signalling molecule, NO acts to modify numerous plant proteins in response to various intrinsic and extrinsic stimuli. As shown in Figure 2.2, NO has been found to affect numerous plant hormones and processes. In animals, the main method of NO signal transmission is through the heme group in soluble guanylyl cyclase (sGC) (Wilson et al., 2008). The binding of NO to sGC leads to an increase in cyclic GMP (cGMP) which is downstream of another signalling intermediary: cyclic ADP-ribose. This in turn stimulates the release of Ca^{2+} which is a factor in many plant signalling cascades (Wilson et al., 2008), and it is postulated that a similar mechanism is used in plants. Another minor NO signalling pathway is through direct S-nitrosylation and indirect trans-nitrosylation of cysteine residues and glutathione (Wilson et al., 2008).

Nitric oxide, in its role as a signalling molecule, has many different effects on plant growth and development. Nitric oxide effects vary on different processes depending on the concentration and other hormones/signals involved. For example, high concentrations of NO were shown to inhibit tomato (*Solanum lycopersicum*) growth, while low concentrations enhanced growth (Palavan-Unsal and Arisan, 2009). Other effects of increased NO content include the inhibition of hypocotyl growth, de-etiolation, increased chlorophyll content in pea (*Pisum sativum*) leaf guard cells, slowed chlorophyll loss in pathogen-infected potato (*Solanum*

tuberosum) leaves, stimulated root growth, and increased iron availability (Palavan-Unsal and Arisan, 2009).

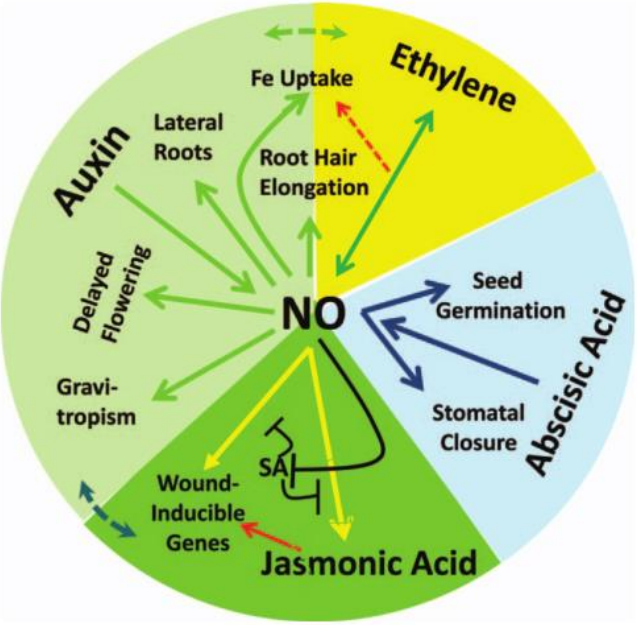


Figure 2.2. The relationship between NO, hormones, and biological function. NO is implicated in interactions with ethylene, auxin, abscisic acid, jasmonic acid, and others, and these hormones have a profound effect on various processes within the plant. Figure is from Hill (2012) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

2.3.3 Nitric oxide and abscisic acid (ABA)

One of the most well-studied interactions of NO is with ABA during stomatal closure. ABA causes an increase in NO, which is required for ABA-mediated guard cell closure (León et al., 2014). Nitric oxide can also reciprocally affect the levels of ABA in the plant cell. One study found that brassinosteroid-induced ABA production in corn is due to the induction of NO, and that NO upregulates *vp14*, an ABA biosynthetic gene (Zhang et al., 2011). It has also been observed that NO increases or decreases antioxidant systems depending on its concentration and development of the plant (Zhang et al., 2007).

2.3.4 Nitric oxide and ethylene

Nitric oxide and ethylene signalling is complex but the two seem to interact, especially under stress conditions. Low concentrations of NO inhibit ethylene and promote growth, which is reversed by high NO concentrations (Palavan-Unsal and Arisan, 2009). Treatments with NO induce the production of ethylene in Arabidopsis and tobacco (*Nicotiana tabacum*). One study found that the application of the NO-donor sodium nitroprusside (SNP) increased the production of ethylene, as well as the expression of the ethylene biosynthesis genes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (*ACS*) and ACC oxidase (*ACO*) in Arabidopsis (Ahlfors et al., 2009). Imposition of magnesium deficiency stress in Arabidopsis seedlings increased both NO and ethylene concentrations, as well as *ACS* and *ACO* expression. Sodium nitroprusside (SNP) was also shown to increase ethylene levels, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) decreased ethylene levels in the Mg-stressed Arabidopsis plants, clearly linking the upregulation of NO to ethylene under stress conditions

(Liu et al., 2017). The same study also showed a role for NO in the ethylene-mediated root hair development under Mg deficiency.

Ethylene and NO also may have reciprocal effects on one another. A study of cucumber (*Cucumis sativus*) explants showed an increase in the production of NO in plants treated with ethylene during adventitious rooting (Xu et al., 2017). It has also been found that the application of the ethylene precursor ACC upregulates the NO synthesis enzymes nitrite reductase and the NOS-like enzyme in Arabidopsis. As well, the signal of a NO-binding fluorescent dye in Arabidopsis roots was significantly increased by the application of ACC (Liu et al., 2017).

2.3.5 Nitric oxide and reactive oxygen species (ROS)

Nitric oxide is intrinsically linked with ROS, which are also involved in a variety of signal transduction pathways (Wilson et al., 2008). One additional NO producing enzyme, xanthanine oxidoreductase (XOR), is able to produce both ROS and NO. Reactive oxygen species and NO interact with each other under different conditions. The effect of ROS on NO is both stimulatory and repressive. Superoxide anions can reduce the amount of NO in a cell through a reaction that produces peroxynitrite, which is also involved in plant signalling. However, ROS can also increase the cell levels of NO by increasing NO-generating enzyme activity (Wilson et al., 2008). Reactive oxygen species molecules are part of a pathway that directly produces NO: horseradish (*Armoracia rusticana*) peroxidases facilitate an interaction between hydroxyurea and H_2O_2 which forms NO. Nitric oxide and ROS may also interact to induce ABA biosynthesis (Palavan-Unsal and Arisan, 2009).

There is recent evidence for the role of NO signalling during stress responses. Recent genetic transcriptional analysis has shown thousands of NO response genes, the majority of which are related to stress (Shi et al., 2014). As discussed, one of the main interactions during drought stress responses is the closure of the stomata via ABA (León et al., 2014). Proper regulation of stomata is an important factor in the ability of plants to tolerate reduced soil water availability (Patakas et al., 2010).

2.4 Phytoglobins (Pgbs)

Hemoglobins are globular proteins found in animals, plants, algae, and bacteria (Dordas, 2009), that possess a heme prosthetic group capable of reversibly binding with oxygen. Other ligands such as NO, carbon monoxide, and hydrogen sulfide can also bind to hemoglobins (Gupta et al., 2011). Associated with the ability to transport ligands like oxygen (Hoy and Hargrove, 2008), hemoglobins can have different sizes, functions, and structures from monomers to multisubunit complexes (Dordas, 2009). Although there is no rational argument for the division, other than historical, plant hemoglobins have traditionally been subdivided into two types: those involved in symbiotic relationships, such as leghemoglobin, and those having a different function. As a result, plant hemoglobins were renamed ‘Phytoglobins’ (Pgbs) to help differentiate them from hemoglobins in other species and to facilitate proper discussion of their function in plants (Hill et al., 2016).

The first plant hemoglobin, leghemoglobin, was isolated from soybean (*Glycine max*) root nodules (Kubo, 1939). Leghemoglobin and related phytoglobins act in a symbiotic relationship with *Rhizobium* spp. and other symbiotic bacteria to facilitate nitrogen fixation (Hoy

& Hargrove 2008). The leghemoglobin molecules assist the *Rhizobium* by helping to diffuse atmospheric oxygen to the bacteria that are fixing nitrogen in the cell. This is important because the main enzyme in the nitrogen fixation reaction, nitrogenase, requires very low oxygen levels as oxygen can also serve as an alternative electron acceptor in the reaction (Hoy and Hargrove, 2008). Leghemoglobin is vital for nitrogen fixation in legumes, and helps to improve soil fertility. It is also important to note that expression of all Pgbs is upregulated during symbiosis which may suggest a redundant role of all types of Pgbs during symbiotic legume root nodule reactions (Gupta et al. 2011). The structure of leghemoglobins is pentacoordinate, with one histidine side chain coordinating the fifth site of the heme iron, leaving the sixth site open for ligand binding (Gupta et al. 2011). See Figure 2.3 for differences in structure between pentacoordinate and hexacoordinate Pgbs.

From a phylogenetic perspective, phytoglobins can be divided into four classes (Vinogradov et al., 2011), a symbiotic type, and three classes that have functional roles in plant growth and development (Stasolla and Hill, 2017). Three of them have slight changes in amino acid sequence that influence their biochemistry while the fourth is a truncated form that has a two on two alpha helical fold, as compared to the three on three fold common in other hemoglobins (Hoy and Hargrove, 2008).

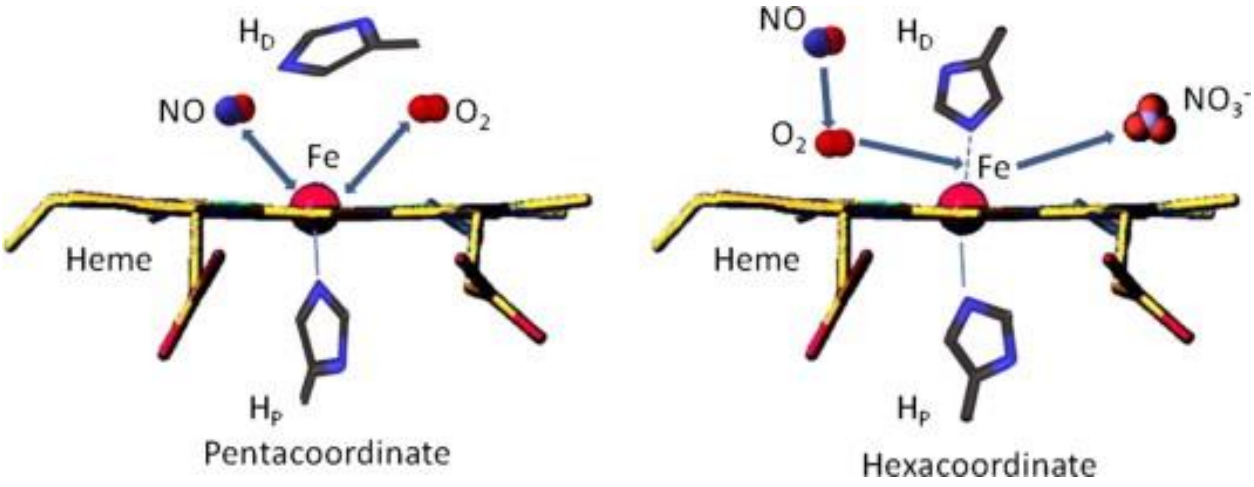


Figure 2.3. Diagram of pentacoordinate and hexacoordinate hemoglobins, showing the difference in orientation of the proximal (H_p) and distal (H_d) histidine. Pentacoordinate hemoglobin orientation is mainly used for reversible ligand binding of O_2 and NO, while hexacoordinate is used for tightly binding O_2. Figure is from Gupta et al. (2011) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

2.4.1.1 Class 1 non-symbiotic Pgbs

Class 1 Pgbs are weakly hexacoordinate, allowing an equilibrium between the pentacoordinated and hexacoordinated states (Smagghe et al., 2009). Class 1 Pgbs have very high oxygen affinities and low oxygen dissociation rate constants. They also have low cellular concentrations, and thus are unlikely to function in oxygen transport, oxygen sensing, or oxygen storage capacities (Hoy and Hargrove, 2008; Dordas, 2009). High redox potential combined with the high oxygen affinity additionally indicates that Pgbs are not likely to function in electron transport (Hoy and Hargrove, 2008). The extremely high oxygen affinity of class 1 Pgbs allows them to remain oxygenated even under very low oxygen concentrations, lower than those found under biological conditions (Hill, 1998; Sowa et al., 1998; Dordas, 2009). Oxygen-dependant NO scavenging is the main role for class 1 Pgbs (Hill, 2012). Class 1 Pgbs oxygenate NO to

nitrate, forming methemoglobin, which is reduced by an associated reductase (Igamberdiev et al., 2005). A detailed mechanism for the scavenging of NO by Pgbs is shown in Figure 2.4. The reduction of NO to non-toxic nitrate is implicated in stress responses such as hypoxia (Igamberdiev and Hill, 2004; Gupta et al., 2011).

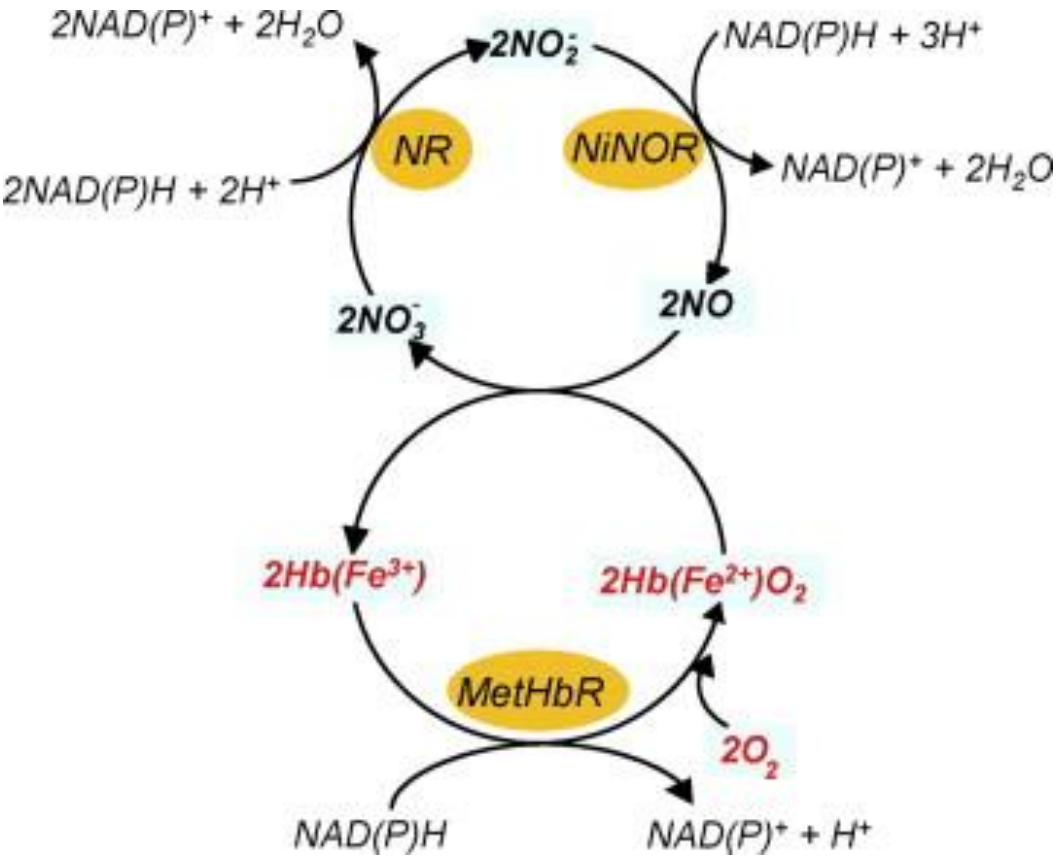


Figure 2.4. The reaction mechanism of class 1 Pgb scavenging nitric oxide (NO). NO is converted to nitrate through an interaction with oxygenated ferrous hemoglobin. Figure is from Gupta et al. (2011) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

The expression of class 1 Pgb is induced by a number of stresses, including osmotic stress, cold stress, P, K, and Fe deficiencies, darkness, exposure to nitrate, respiratory inhibitors, rhizobial infection, superoxide, NO, and plant hormones such as ABA, cytokinins, ethylene, and jasmonic acid (Dordas, 2009). It has also been found that overexpression of class 1 Pgbs enhances tolerance to stress, such as hypoxia, disease, and NO (Hill, 2012). This is likely due to increased antioxidant status in the cell (Hill, 2012), a concept that will be discussed in detail further in this review. One study also found that Arabidopsis plants over-expressing class 1 Pgbs have greater shoot and root weights than the control after two weeks of growth (Hunt et al., 2002). Class 1 Pgbs have been found in seed tissue, roots, leaves, flowers, and meristematic tissue in Arabidopsis, rice, barley (*Hordeum vulgare*), corn, lotus (*Nelumbo nucifera*) and many other species (Hill, 2012).

2.4.1.2 Class 2 Pgbs

Class 2 Pgbs have a hexacoordinate structure rather than the partially pentacoordinate structure found in class 1 Pgbs (Smagghe et al., 2009). They have lower oxygen affinity than class 1, but have been postulated to also help with scavenging NO (Dordas, 2009). One review pointed out the possibility of class 2 Pgbs having a role in facilitating oxygen diffusion instead of NO scavenging, due to the presence in the protein of only one docking site for ligands, making it impossible for both NO and oxygen to bind, which is essential for the reduction of NO to nitrate (Gupta et al., 2011). Class 2 Pgbs are induced by some environmental stresses such as chilling or pharmacological treatment with cytokinins, but other stresses such as nutrient deprivation and hypoxia have no effect (Hill, 2012). Overexpression of class 2 Pgbs also increases tolerance to

hypoxic stress (Dordas, 2009). Class 2 Pgbs are expressed in roots, the xylem and phloem of young leaves, and at the junction of the pedicel and stem (Hill, 2012).

2.4.1.3 Class 3 Pgbs

Class 3 Pgbs, or truncated Pgbs, are so-called because of their different folding structure. Rather than the typical '3 on 3' alpha-helical 'sandwich fold', truncated Pgbs have a '2 on 2' fold, even though their nucleotide sequence is not necessarily shorter (Hoy and Hargrove, 2008). Truncated Pgbs are the most recently discovered, and not much is known about them. They are expressed throughout the plant, and are not upregulated by stress factors that cause the upregulation of class 1 and class 2 Pgbs (Hill, 2012). The structure of truncated Pgb is pentacoordinate in an oxygenated state, which forms a hexacoordinate structure after reduction (Gupta et al., 2011). The truncated Pgbs also have a lower oxygen affinity than the class 1 Pgbs, and have been proposed to act as a possible oxygen carriers (Gupta et al., 2011). It is interesting to note that the expression of truncated Pgbs is supressed rather than induced under hypoxic conditions. Truncated Pgbs could also assist with mycorrhizal symbiotic associations, and again may be as a result of NO scavenging in the plant (Gupta et al., 2011).

2.4.1.4 Phytoglobin signalling

It has been postulated that Pgb gene expression is altered only by stress conditions as it is the interference with the hormonal signal transduction by NO that is modulated by the Pgb (Igamberdiev et al., 2005). The hypothesis that Pgbs alter hormone response through NO modulation is derived from Pgbs being found throughout the plant kingdom, that Pgb and NO

expression fluctuate throughout plant development, and both are altered by stress conditions (Hill, 2012).

2.5 Ethylene

2.5.1 Ethylene biosynthesis and perception

Ethylene is a two-carbon alkene and a gaseous plant hormone. Despite its simple chemical structure, it has a complex role and is involved in many plant processes including germination, seedling growth in the dark, sex determination, fruit ripening, abscission, leaf and flower senescence, root growth rate, adventitious root development, root hair growth, and gravitropism (Geisler-Lee et al. 2010). Ethylene is also notably involved in stress responses such as hypoxia, mechanical impedance, pathogen attack, pollution stress, injury, and osmotic stress (Ke and Sun, 2004; Geisler-Lee et al., 2010). Ethylene is routinely used by plants as a signal molecule as it can diffuse quickly from cell to cell across membranes. Ethylene is even able to transmit signals to target cells in neighboring plants (Wang et al., 2016).

Ethylene is biosynthesized from methionine. Methionine is converted into S-adenosylmethionine (AdoMet) by AdoMet synthetase, which is converted to ACC by ACC synthase (ACS). This is then oxidized by ACO to produce ethylene, CO_2 and HCN (Geisler-Lee et al., 2010). This synthesis cycle is shown in Figure 2.5.

In Arabidopsis, the ACS multigene family contains nine members, while in maize there are only three: *ZmACS2*, *ZmACS6*, and *ZmACS7*. There are four members of the maize ACO family: *ZmACO15, ZmACO20, ZmACO31,* and *ZmACO35*. *ZmACS6* is responsible for the

majority of foliar ethylene production (Geisler-Lee et al., 2010). The conversion of AdoMet to ACC by ACS is often the slowest step during ethylene synthesis. The spatial and temporal regulation of ethylene biosynthesis is also likely due to ACS expression and activation (Trobacher, 2009).

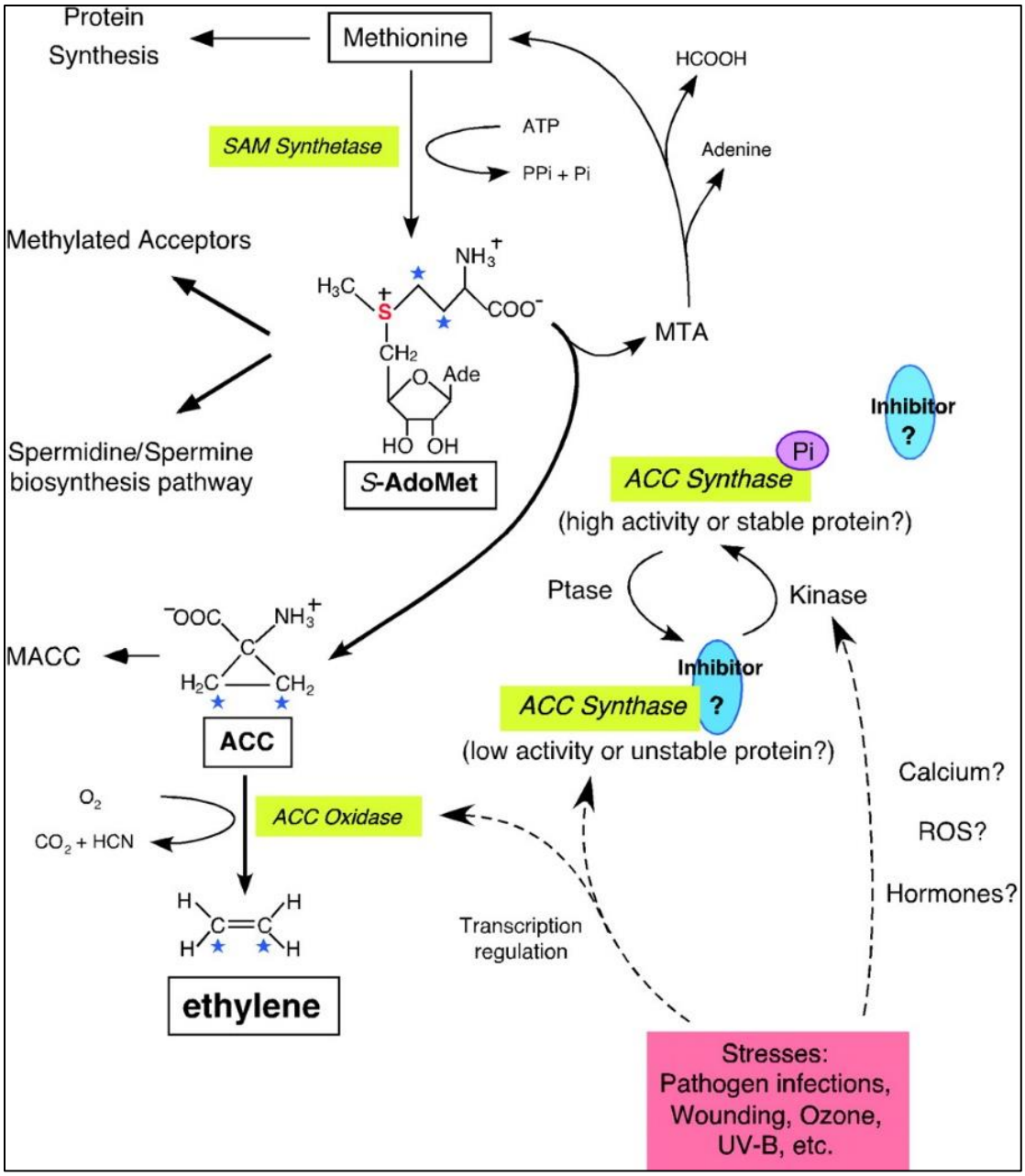


Figure 2.5. Ethylene biosynthesis pathway. This figure shows the multiple precursors (methionine, AdoMet, and ACC) of ethylene, as well as the enzymes involved (ACC synthase, ACC oxidase). It also gives a possible explanation for the increase in ethylene biosynthesis during stress conditions. Figure is from Wang et al. (2002) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

2.5.2 Ethylene signalling responses

Ethylene interferes with many signalling pathways. Mutation of the ethylene insensitive gene (*EIN*), as well as mutation of other ethylene biosynthetic and response genes produce the "Triple Response" (Potuschak et al., 2003). The Triple Response was characterized in 1932 (Crocker, 1932) and has three distinctive phenotypic characters, used to screen for ethylene mutants. In many plant species, Triple Response consists of horizontal growth, inhibition of stem elongation, and thickening of the stem. In Arabidopsis, the response causes the inhibition of hypocotyl and root elongation, an exaggerated apical hook, and a thickened hypocotyl (Potuschak et al., 2003). Ethylene receptors (*ETR1, ETR2, EIN4, ERS1*, and *ERS2*) act upstream of the *constitutive triple response* (*CTR*) gene, whose mutants show the Triple Response phenotype. *CTR* activates *EIN*, which is a component of a signalling cascade that activates an ethylene-response protein, encoded by the gene *EIN3 binding f-box protein* or *EBF* (Potuschak et al., 2003).

Other signalling molecules having interactional cross-talk with ethylene include gibberellin, jasmonic acid, auxin, and light signals. Jasmonic acid interacts with ethylene during defense mechanisms (particularly pathogen attack) by affecting the expression of ethylene

response factors (*ERF*s). If either the ethylene or jasmonic acid pathway is blocked, *ERF1* is not expressed (Trobacher, 2009).

2.5.2.1 Ethylene signalling during drought stress

Similar to other abiotic stresses such as cold stress, ethylene is able to produce both positive and negative effects in a plant undergoing drought stress. A recent study found that ethylene-insensitive mutants had lower water loss rates under drought-stressed conditions due to hastened stomatal closure. The plants had improved water retention and reduced leaf transpiration. The researchers hypothesized that ethylene likely plays an antagonistic role with ABA during drought stress conditions. Ethylene may impede ABA's ability to close stomata under stress conditions (Wang et al., 2016).

However, it has also been found that ethylene may have a role during stomatal closure by inducing the production of ROS in plant stomatal guard cells, causing them to close (Kazan, 2015). Ethylene may also have a protective role during drought stress, as its upregulation causes leaf abscission. With fewer leaves, a drought stressed plant may lose less water by transpiration (Arraes et al., 2015). One study observed that a knockout in the maize *ACS* gene, *ZmACS6*, reduced foliar ethylene by up to 90%, and delayed leaf senescence under water stress. These ethylene-deficient leaves also maintained their viability for an extended period, retaining their chlorophyll, protein, and Rubisco for more time than wild-type leaves (Young et al., 2004). Drought stressed plants lose their foliar chlorophyll as part of the senescence process, but the inhibition of ethylene synthesis is able to reduce drought-induced chlorophyll loss in leaves (Young et al., 2004). Foliar chlorophyll and ethylene levels have an inverse correlation during

drought stress. *ZmACS2* mutants, which have slightly decreased contents of ethylene, had a slightly increased level of foliar chlorophyll compared to wild type. *ZmACS6* mutants, with a large reduction in the level of ethylene, had much higher level of chlorophyll in their leaves than the wild type plants – up to a 20-fold increase (Young et al., 2004).

Drought stress in the field has a number of extenuating stressors beyond a lack of water, such as heat stress and soil compaction. One study found that the mechanical impedance of roots in dry soil caused an increase in the amount of both ACC and ethylene, resulting in root growth inhibition (Sharp and LeNoble, 2001). Additional research shows that the ethylene signal transmission mutants *ein2* had increased tolerance to heat stress, which may point to ethylene (or its signalling cascades) having a negative effect on thermotolerance (Kazan, 2015). Conversely, the ethylene response genes may help protect the plant during environmental stress. Ethylene response factor (*ERF*) genes are induced by both drought stress and salinity, as salinity naturally increases in the soil during drought events. Arabidopsis lines over-expressing the *ERF1* gene were more tolerant to drought, heat and salinity stress (Cheng et al., 2013).

Regardless of its possible positive or negative effect during abiotic stress conditions, ethylene is very important to cell signalling. Accumulation of ethylene as well as ethylene precursors (such as ACC) occur in a wide range of stress conditions such as salinity, flooding and drought (Klay et al., 2014)

2.6 ABA

One phytohormone with a close interaction with ROS during drought stress is abscisic acid (ABA). An immediate response to drought stress is the closing of the stomata, which is induced by ABA. Impairing the NADPH oxidase machinery that produces hydrogen peroxide inhibited stomatal closing and this phenotype was reversed by hydrogen peroxide application (Desikan et al., 2004). Abscisic acid gene expression has been found to be strongly upregulated in response to water stress (Jiang and Zhang, 2002). Abscisic acid is often applied to ornamental flowers as an 'antitranspirant' during shipping to induce the closing of stomata and prevent drought stress (Waterland et al., 2010). The closing of stomata is one of the first physiological responses to drought stress, and the signal from the root to the shoot is mediated by the ABA signal pathway involving sulphate that travels through the xylem sap of the plant (Ernst et al., 2010). Under limited water conditions, accumulation of ABA in the stomata triggers an efflux of ions, lowering the water potential of the guard cells, thus closing the stomata (Desikan et al., 2004).

ABA, as a stress hormone, is normally associated with the production of antioxidants in response signalling pathways, but it has also been found to precede the generation of ROS. Antioxidants and ROS have antagonistic effects on one another, with the ability of antioxidants to accept electrons from the ROS. This possibly indicates a two-way interaction between ABA and ROS where one induces the production of the other (Jiang and Zhang, 2002). However, the exact mechanism of this interaction remains unclear.

ABA is also able to upregulate antioxidant systems in the plant, which aid in stress responses by neutralizing the toxic activity of the ROS (Zhang et al., 2007). The change in leaf water potential due to drought stress is a trigger that induces the biosynthesis of ABA. One study found that the ABA biosynthesis machinery was induced under -10 – 12 bars for *Ambrosia* and -8 bars for maize (Wright, 1977).

2.7 Reactive oxygen species

Reactive oxygen species (ROS) are molecules containing oxygen in which the oxygen residue has an unpaired electron; this free radical state results in a more reactive molecule. There are four main types of ROS molecules. Singlet oxygen is formed when excess energy is transferred to the ground state oxygen molecule. Superoxide is formed when one unpaired electron is added to the oxygen molecule. Hydrogen peroxide is created when two electrons are added to the oxygen molecule, and the hydroxyl radical is generated when three electrons are added to the oxygen molecule (Apel and Hirt, 2004). The differences between these ROS and details on their formation are depicted in Figure 2.6.

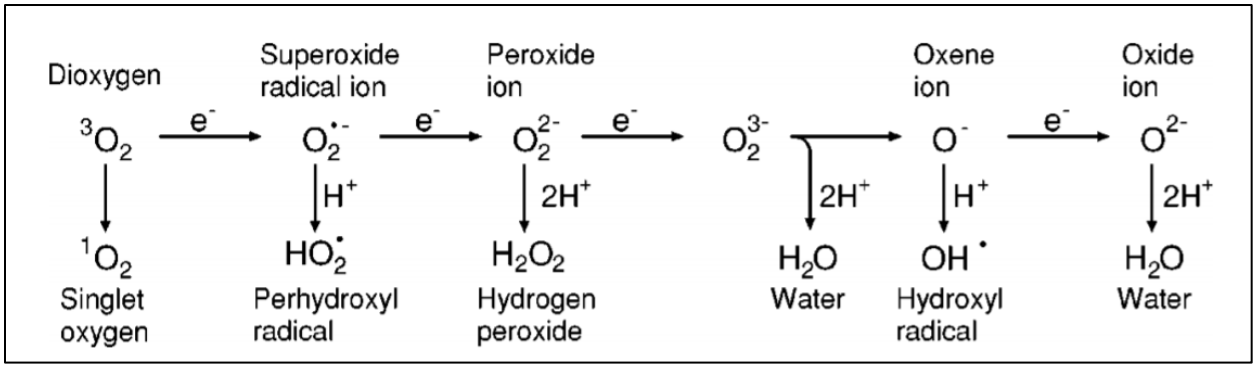


Figure 2.6. Production of different types of reactive oxygen species (ROS) through energy transfer and addition of unpaired electrons. Electron transfer reactions lead to sequential reduction into various ROS from left to right. Figure is from Apel and Hirt (2004) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

Reactive oxygen species are very damaging to the plant cell, and can cause disruptive responses like lipid peroxidation, protein unfolding, and DNA damage (Miller et al., 2010). Under normal cellular conditions, ROS are produced in small amounts in the chloroplast, mitochondria and peroxisomes (Overmyer et al., 2003; Apel and Hirt, 2004; Miller et al., 2010). However, under stress conditions, the delicate equilibrium of ROS production and scavenging is disturbed, which causes the rapid rise of intracellular levels of ROS (Apel and Hirt, 2004; Kar, 2011). The balance between ROS production and scavenging depends on changes in growth conditions and water availability, as well as the severity and duration of stress and the plant's ability to acclimate to the stress condition (Miller et al., 2010).

Antioxidants such as ascorbic acid, glutathione, as well as ROS scavenging enzymes such as superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase, and peroxiredoxin are essential for ROS detoxification (Miller et al., 2010). The ability of plant cells

to detoxify ROS is essential for their survival, as ROS deactivation mechanisms have been found in almost all components of the cell (Miller et al., 2010). Overproduction of ROS leads to oxidative damage including membrane disruption, protein degradation and enzyme inactivation (Farooq et al., 2009). All of these types of damage are extremely deleterious for normal cellular functioning, eventually resulting in the death of the cell. If left unchecked, it can cause the death of the entire plant (You and Chan, 2015). Peroxisomes are also involved in ROS production. Reduced CO_2 to O_2 ratio due to stomatal closure leads to an increase in photorespiration and the production of glycolate in chloroplasts. The glycolate is oxidized in the peroxisome by the glycolate-oxidase enzymes which accounts for most of the hydrogen peroxide production during photorespiration (Miller et al., 2010).

In animal cells, the mitochondria are key producers of ROS. However, in plants, the amount of ROS produced by mitochondria is relatively low. This is postulated to be due to the presence of the alternative oxidase enzyme, which helps to catalyze the reduction of oxygen by ubiquinone. Transgenic cells that contained antisense alternative oxidase genes had five times more ROS than the control cells (Apel and Hirt, 2004). In mitochondria, complex I and complex III are the main sites of ROS production. Ubisemiquinone transfers electrons to oxygen to generate superoxide which is reduced to hydrogen peroxide (Miller et al., 2010). Production of ROS in the mitochondria increases under drought stress conditions, which may be due to the increased need for mitochondrial ATP to help compensate for decreased chloroplast ATP synthesis (Miller et al., 2010).

Besides their negative effects on plant behaviour when over-produced, ROS also act as signalling molecules during stress responses (Apel and Hirt, 2004; Miller et al., 2010; You and Chan, 2015). Thus, plants must find a delicate balance between upregulating a toxic compound and a beneficial signal transduction molecule. Special ROS produced by a plant homolog of NADPH oxidase called respiratory burst oxidase homologs (RBOH) have been implicated in assisting in drought stress tolerance (Sagi and Fluhr, 2006; Miller et al., 2010). This NADPH oxidase enzyme complex has been found in animals, plants, and fungi indicating a common ancestor, and its importance in the cell. The most common inducers of the RBOH-produced superoxide are conditions of anoxia or hypoxia, as well as nitrogen stress (Sagi and Fluhr, 2006). Respiratory burst oxidase homologs siphon electrons from NADH or NADPH and transfer them to O_2 to produce superoxide (Jiang and Zhang, 2002).

Reactive oxygen species produced by NADPH oxidase have recently been found to be involved in a number of stress responses in plant cells. There is some evidence to suggest that ROS are essential signals in the mediation of ABA-induced stomatal closure. Reactive oxygen species are synthesized in guard cells under stress conditions, and a number of mutants have demonstrated that ABA induces ROS synthesis which causes the closure of stomata via the calcium channels in the plasma membrane (Apel and Hirt, 2004). Abscisic acid has also been linked to increasing ROS, which triggers the antioxidant system in plants, helping to maintain the redox state and limit damage under stress conditions (You and Chan, 2015). Although this complex mechanism is not yet well understood, it is important to underline the fragile equilibrium in plant cells between an increase in ROS, to help with plant cell stress signalling, and an unchecked increase in ROS under stress conditions that causes cellular damage.

OBJECTIVES

In light of the relevant literature on the various signalling molecules and hormones, and their role in drought stress responses, the objectives of this study are as follows:

- To assess if experimental manipulations in *Pgb1* expression alters maize plant response to drought stress
- To examine the interaction of NO, ethylene, ROS, and ABA in the Pgb response to PEG-induced water stress in maize shoots, and to propose a mechanistic model

3 CHAPTER ONE: Over-expression of Phytoglobin alleviates PEG-induced water stress in maize shoots

3.1 Abstract

Phytoglobins are heme-containing proteins found in plants that are upregulated during stress conditions. The effect of drought stress on 3-leaf stage (V2) maize (*Zea mays* L.) seedlings over-expressing or down-regulating the *Zea mays phytoglobin 1* gene (*ZmPgb1*) was investigated using applications of 25% w/v polyethylene glycol (PEG) to mimic drought. Over-expression of *ZmPgb1* increased drought tolerance, decreased wilting, and decreased the accumulation of ethylene and reactive oxygen species (ROS), compared to wild type (WT) plants and plants in which the level of *ZmPgb1* was down-regulated. Gene expression studies conducted during the first 16 hours of water stress revealed a transcriptional induction of ethylene synthesis and response, as well as ROS production, in the *ZmPgb1* down-regulating plants relative to WT plants. This was in contrast to the *ZmPgb1* over-expressing plants where genes participating in ethylene synthesis and response exhibited the lowest expression levels, and ROS production was also limited. Ethylene measurements supported the expression studies; relative to WT, ethylene levels were higher in plants down-regulating *ZmPgb1* and lower in those up-regulating the same gene. Pharmacological treatments with the NO donor sodium nitroprusside (SNP) and the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) showed that *ZmPgb1* effects were modulated by NO. Ethylene and ROS increased in environments enriched with NO, and decreased in those depleted in NO. Furthermore, while an experimental rise in ethylene activated the expression of *RBOH*s (*Respiratory Burst Oxidase Homologs*), encoding subunits of the ROS-producing NADPH oxidase machinery, a reduction in ethylene decreased the expression of the same genes. Based on these results a model is proposed in which

suppression of NO signalling by *ZmPgb1* reduces ethylene accumulation and response, and production of ROS, which are conditions alleviating drought stress.

3.2 Introduction

Drought stress is a major threat to world food security, due to uncertainty in the amount and distribution of rainfall (Farooq et al., 2009). Drought-induced yield loss is the greatest source of reduced yield, thus minimizing this reduction in yield is a main concern of plant breeders today (Cattivelli et al., 2008). Drought stress can be simulated in laboratory conditions using polyethylene glycol (PEG), which is a high molecular weight compound that draws water into the apoplastic space of a cell in the same way as natural drought stress (Steuter et al., 1981; Joshi et al., 2011). A number of physiological mechanisms are used by plants to cope with drought stress. Some of these mechanisms include decreasing root elongation and leaf area expansion, reducing the ratio of shoots to roots to favour root growth, and closing stomata to prevent water loss through transpiration (Bhattacharjee and Saha, 2014). These responses are triggered by signalling molecules such as nitric oxide (NO), a gaseous free radicle molecule that is able to easily diffuse through the lipid membrane (Wilson et al., 2008). Nitric oxide is an integral component to drought stress signalling (Shi et al., 2014). It has been observed that signalling of NO is concentration-dependent, as opposing effects can be produced depending on the concentration in NO in the cell (Hebelstrup and Jensen, 2008). Nitric oxide is involved in the ABA-mediated stomatal closure response during drought stress signalling, and some studies have evidenced that an elevation in NO levels increase drought stress tolerance (Garcia-Mata and Lamattina, 2001). This is in contrast to plants exposed to drought, where decreased levels of NO alleviate the inhibition of root growth (Mira et al., 2017).

Another component of plant signalling that is affected by drought stress is ethylene. The levels of ethylene and its precursors tend to increase during drought stress (Klay et al., 2014). Several independent studies show an intimate relationship between NO and ethylene. For example, high levels of NO induce production of ethylene, specifically through the upregulation of the ethylene biosynthetic enzymes *ACC Synthase* (*ACS*) and *ACC Oxidase* (*ACO*) (Ahlfors et al., 2009). Ethylene response as well as ethylene biosynthesis is also linked to drought stress responses, where *Ethylene Response Factor* (*ERF*) genes are upregulated during drought stress (Cheng et al., 2013). *EIN3 Binding F-Box Protein* (*EBF*), another gene that is sensitive to plant ethylene levels, is upregulated during abiotic stress, and is induced by both ethylene and its precursors (Potuschak et al., 2003; Zhang et al., 2010).

Ethylene has been shown to be involved in the regulation of reactive oxygen species (ROS) levels in the plant during drought stress conditions (Kazan, 2015). Reactive oxygen species are involved in stress signalling and ABA-mediated stomatal closure, and production of ROS in the guard cells causes them to close (McAinsh et al., 1996). A role of ROS in the early stages of programmed cell death (PCD) has also been suggested (Overmyer et al., 2003). A rapid increase in cellular ROS can be toxic to cells, and results in damaged membranes, DNA, and proteins (Farooq et al., 2009). Plants with high levels of NO have increased expression of the *Respiratory Burst Oxidase Homolog* (*RBOH*) genes, which encode NADPH oxidase enzymes responsible for ROS generation during stress conditions (Mira et al., 2017).

During stress responses, factors influencing NO homeostasis, as well as ethylene and ROS levels, are therefore determinant for plant survival. Phytoglobins, animal-like hemoglobins found in plants, are known to scavenge NO (Igamberdiev et al., 2004) and alter ethylene and ROS content (Huang et al., 2014; Mira et al., 2017; Montilla-Bascón et al., 2017). Phytoglobins are divided into two main classes: symbiotic phytoglobins, which are involved regulating oxygen levels in the root nodules of soybeans, and non-symbiotic phytoglobins, which have an oxygen affinity that is too high to function as an oxygen carrier (Hill, 2012). Non-symbiotic phytoglobins are further divided into three sub-classes. Class 1 phytoglobins, which are examined in this study, and function to oxygenate NO to nitrate, forming methemoglobin (Gupta et al., 2011). Plant NO in *Pgb* transgenic plants has been measured experimentally, and it is demonstrated that plants over-expressing *Pgb* have reduced levels of NO, while plants down-regulating *Pgb* expression have increased levels of NO (Dordas et al., 2003; Igamberdiev et al., 2004). It has also been found that over-expressing *Pgb1* increases tolerance to stress (Dordas, 2009), which is supported by the results of this study. Class 2 Pgbs may also be involved in stress responses, and class 3 phytoglobins are not upregulated by stress factors in the same way that class 1 and 2 Pgbs are (Hill, 2012).

Modulation of NO and ethylene in this study was performed in a similar way to recent work, where maize plants were excised at the base of the stem, placed in pre-treatment solutions, then treated with PEG (Zhang et al., 2011). The authors investigated the role of brassinosteroids on PEG-induced water stress in maize seedlings, and found that inhibiting brassinosteroid biosynthesis aggravated oxidative stress, while application of ABA alleviated it. They also pre-treated their maize seedlings with the ABA biosynthesis inhibitor fluoridone, and found that this

also aggravated water stress. This shows that their method of pharmacological treatment, used as a basis for this study, is appropriate for determining water-stress response pathways in maize seedlings. The use of 25% w/v PEG was found to be an adequate concentration for resolving differences between lines. The purpose of this study was to examine the role of class 1 phytoglobins during the drought stress response in maize seedlings, their involvement in modulating nitric oxide, and their effect on the stress-related components ethylene, abscisic acid, and ROS. A model is proposed explaining the results.

3.3 Materials and Methods

3.3.1 Plant Material

Zea mays (Hi II Type II) lines over-expressing [*ZmPgb1* (S)] or downregulating [*ZmPgb1* (A)] phytoglobin were those previously described (Huang et al., 2014). Seeds were surface sterilized with a 10% bleach solution on a rocking platform in a weigh boat for 3 minutes, then washed and soaked in sterile distilled H_2O in the dark overnight. Plants were grown in a growth chamber at 22°C for 16h in the light/18°C for 8h in the dark, at a light intensity of 242 μE m^{-2} s^{-1}. Maize plants at the three leaf stage (two collar) were excised at the base of the stem with a razor blade and placed in 100 mL of distilled water in a beaker for one hour to recover from wound stress, as previously described in a paper by Zhang et al., 2011. They were then placed in 500 mL 25% (w/v) polyethylene glycol (PEG-8000) solution for up to 16 hours, with three replicates per line (Steuter et al., 1981; Joshi et al., 2011). See Figure 3.1 for a schematic diagram of this process.

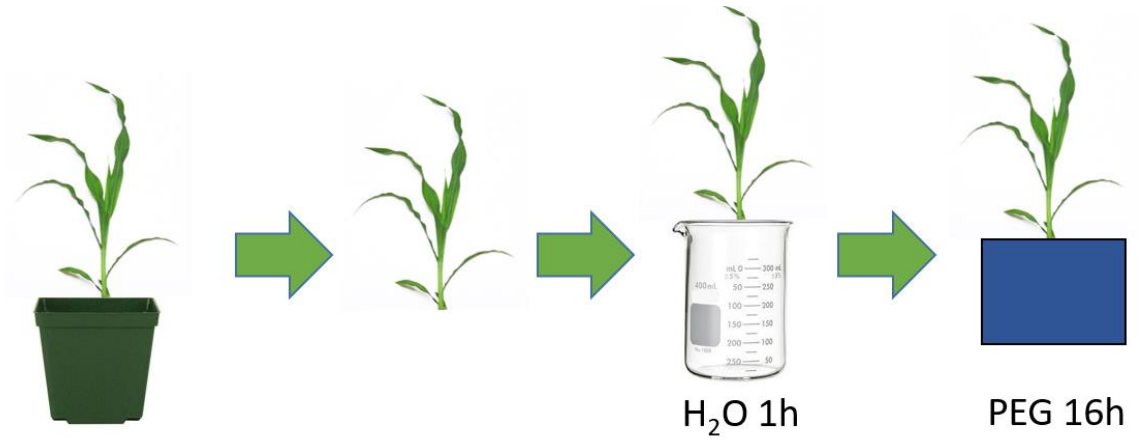


Figure 3.1. Schematic diagram showing treatment of plants in the main experiment. Plants were excised at the base of the stem at the 2-collar stage, placed in distilled water for one hour, then transferred to water or PEG for up to 16 hours.

3.3.2 Chemical pre-treatments

When administering chemical pre-treatments, the plants were grown as before, excised and placed in 100 mL distilled water for 1 hour. Plants were then placed in foil-wrapped beakers containing 100 mL of pre-treatment for 4 hours. Pre-treatments were prepared as 100mM/200mM stocks then diluted as needed. The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) and the NO donor sodium nitroprusside (SNP) were applied at concentrations of 200 µM and 100 µM, respectively (Huang et al., 2014). The ethylene donor ethephon (ETH) and the ethylene biosynthesis inhibitor aminooxyacetic acid (AOA) were both applied at a concentration of 100 µM (Sharp, 2002; Zhang et al., 2010). After the pre-treatment, plants were placed in foil-wrapped containers with 500 mL 25% w/v PEG solution with the addition of the same concentration of pre-treatment for 8 hours.

3.3.3 RNA Extraction and Gene Expression

TRI-Reagent solution (Invitrogen) was used to extract RNA, and the cDNA Reverse Transcription Kit (Applied Biosystems) was used for the synthesis of cDNA (Huang et al., 2014). The cDNA was prepared as follows:

Fresh treated maize shoot tissue was ground with liquid nitrogen in a mortar and pestle. 1 mL of TRI-Reagent was added, and the solution was pipetted into a microcentrifuge tube and incubated at room temperature for ten minutes. Samples were then centrifuged at 12000xG at 4°C for 10 minutes. The supernatant was pipetted into a clean tube, then 200 µL chloroform was added. The solution was gently inverted and incubated at room temperature for 10 minutes. The tubes were centrifuged at 12000xG, then the supernatant was pipetted into a clean tube. 500 µL of 2-propanol was added, and samples were gently inverted and left to incubate for another 10 minutes. Samples were centrifuged again at 12000xG 4°C. The RNA pelleted to the bottom, and the supernatant was discarded. Pellets were washed with 1 mL 70% ethanol/DEPC water, vortexed, and then centrifuged at 7500xG 4°C for 5 minutes. The supernatant was poured off, and the pellet was air dried. The RNA pellet was dissolved in 88 µL DEPC water.

The DNase I recombinant RNase-free kit from Roche (Sigma) was used. With the samples on ice, 2 µL of DNase I and 10 µL of buffer was added to each sample, and the tubes were vortexed. The samples were incubated for 30 minutes at 37°C, 5 minutes at 10°C, then 10 minutes at 75°C. During the 10°C step, 4 µL of EDTA per 100 µL sample was added. After incubation, 10 µL 3M NaOAc and 200 µL 95% EtOH were added to each sample tube, then the tubes were vortexed. Samples were then placed at -20°C for a minimum of 30 minutes. Samples

were centrifuged at 21000xG for 30 minutes at 4°C. The supernatant was poured out, then the pellet was washed with 500 µL ethanol. The pellets were then dissolved in 100 µL DEPC water.

The concentration of RNA was determined using a Nanodrop spectrophotometer. The samples were then diluted such that there was 2 µg nucleic acid in each 10 µL sample, then the samples were placed on ice. The cDNA Reverse Transcription Kit (Applied Biosystems) was used. From this kit, 2 µL buffer, 2 µL primer, 0.8 µL dNPTs, 4.2 µL DEPC water, and 1 µL enzyme was added to each sample. Tubes were incubated for 15 minutes at 25°C, 2 hours at 37°C, then 85°C for 5 minutes.

All primers used in gene expression studies are listed in Supplementary Table 1. Relative gene expression levels were measured using the $2^{-\Delta\Delta CT}$ method, with actin as the reference gene (Livak and Schmittgen 2001).

3.3.4 ROS Staining

To visualize the production of reactive oxygen species in the maize leaf, a stain for superoxide was used. Plants were subjected to PEG treatment for 8 hours as described above, and then placed in a 0.5mg/mL nitroblue tetrazolium (NBT) solution in 10mM potassium phosphate buffer (pH 7.6) in a 50 mL foil-wrapped falcon tube and were incubated in darkness at 25°C for three hours (Campbell et al., 2015). Chlorophyll was removed from the leaves by boiling in 95% ethanol in a water bath, and the leaves were rehydrated in a 10% glycerol solution for 24 hours. Leaves were then gently spread out and photographed (Campbell et al., 2015).

3.3.5 Ethylene Gas Chromatography

Excised plant shoots were treated, as above, in a PEG or water solution for 8h. Excised plants were then incubated in a sealed, foil-wrapped 15 mL test tube for 3 hours in the dark at 22°C. The gas in the headspace (1 mL) was extracted using a syringe, and analyzed with a Bruker 450-GC gas chromatograph (Mira et al., 2016). Data analysis conducted using the Bruker Compass Data analysis 3.0 software.

3.3.6 Statistical Analysis

One-way analysis of variance of the data was conducted using the GLMMIX procedure (SAS Institute, 2005) of SAS University Edition Version 9.04.01. Treatment means were compared using the Tukey test ($\alpha = 0.05$).

3.3.7 ABA Analysis

Freeze dried maize shoot tissue was ground with liquid nitrogen in a mortar and pestle into a powder, then extracted with 80% v/v acetonitrile containing 1% v/v acetic acid and internal standard. ABA was extracted and purified as per Son et al. (2016). ABA quantification was performed with liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS, Agilent 1260-6430) as per Yoshimoto et al. (2009). ABA extraction and quantification was performed on three replicates of each line, from each treatment.

3.4 Results

3.4.1 Expression of the *ZmPgb1* gene affects the wilted phenotype in PEG-treated shoots

As shown in Figure 3.2, the *ZmPgb1* gene is quickly upregulated under conditions of PEG-induced water stress in WT shoots, reaching a peak within 4 hours. One of the most visible symptoms of a drought stressed plant is wilting. Wilted plants are unable to meet their physiological needs (Bhattacharjee and Saha, 2014) and have reduced cell turgor pressure (Kroeger et al., 2011). The maize lines studied, a wild type (WT), a *ZmPgb1* over-expressing [*ZmPgb1* (S)] and a *ZmPgb1* down-regulating [*ZmPgb1* (A)] line, exhibited remarkable differences in wilting when exposed to drought conditions simulated with 25% PEG. As shown in Figure 3.3, shoots subjected to the PEG treatment are more wilted than shoots under control (-PEG) conditions. Additionally, the overexpressor line appeared less wilted under PEG-induced stress conditions than the WT and down-regulator lines. Phenotypically, the WT and *ZmPgb1* (A) lines had more severely drooping leaves, the appearance of lost turgor pressure, and the leaves were darker and wrinkled in comparison to *ZmPgb1* (S) leaves after the PEG treatment.

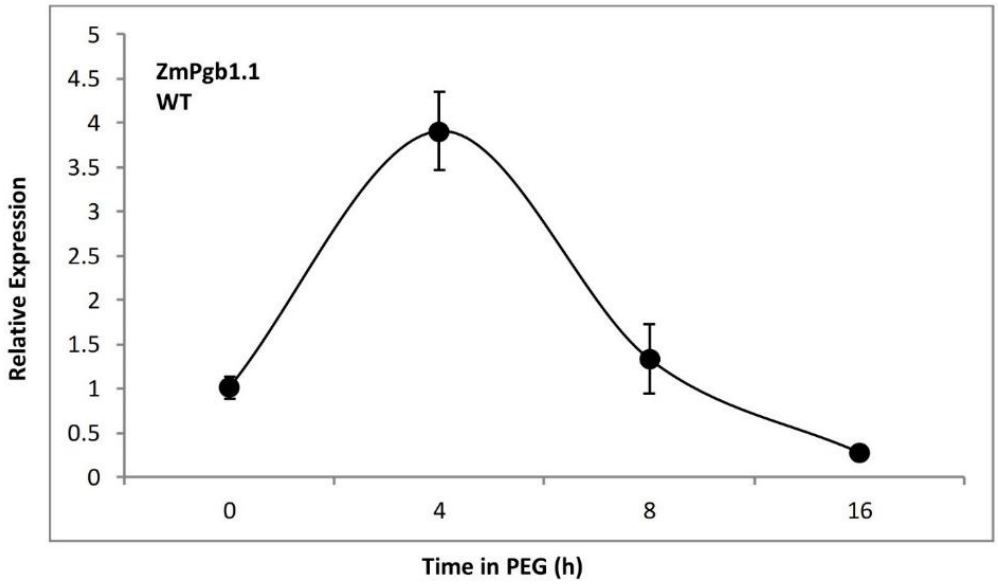


Figure 3.2. Gene expression of the *Zea mays Phytoglobin 1.1 (ZmPgb1.1)* gene over time when treated with 25% w/v PEG in the shoot tissue of the wild type (WT) line. Values are normalized to the WT at 0 hours (set at 1) and the means are ±SE of three biological replicates.

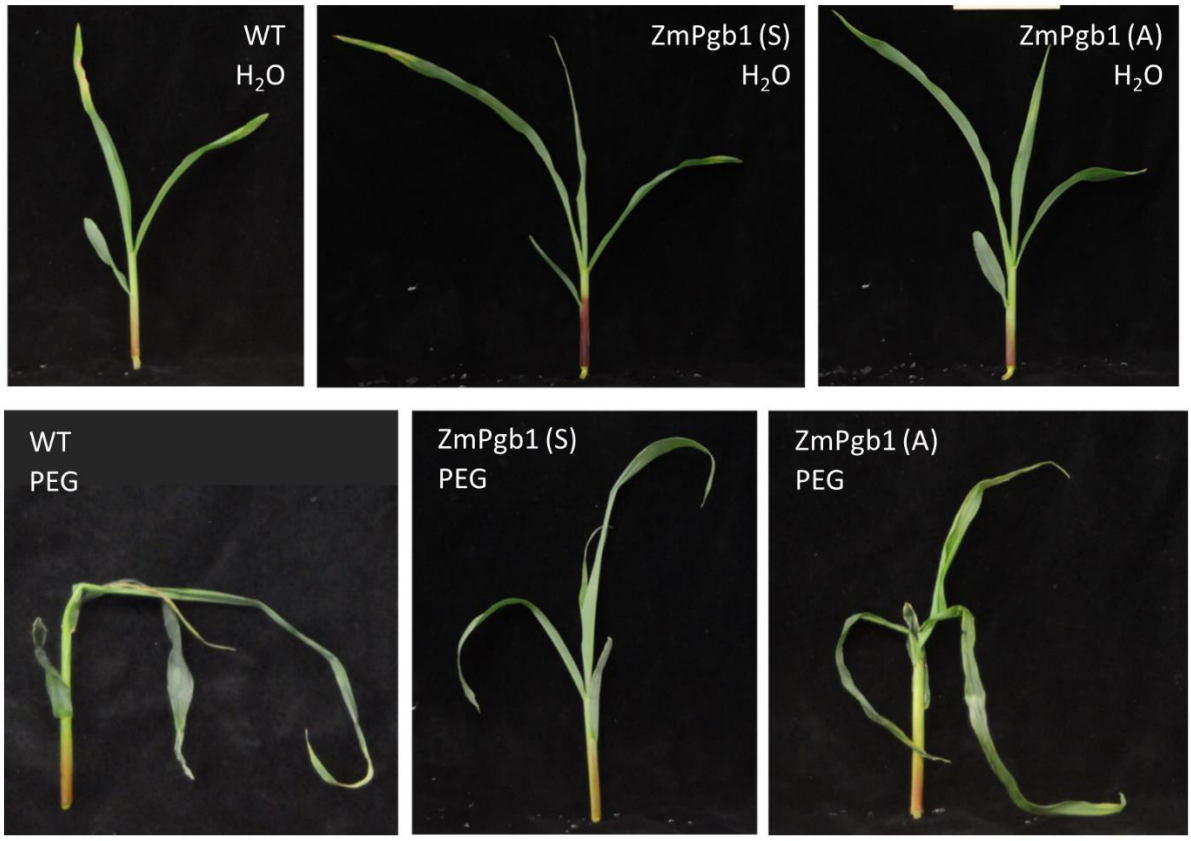


Figure 3.3. Effect of 16 hours in 25% w/v PEG treatment or water control on maize shoots. The *ZmPgb1* overexpressor [*ZmPgb1* (S)] line was able to maintain turgidity during PEG-induced stress compared to the *ZmPgb1* down-regulator [*ZmPgb1* (A)] and WT line.

3.4.2 Overproduction of *ZmPgb1* increases ABA response

Given that ABA is an important factor in plant drought stress responses (Ernst et al., 2010), and that Pgbs scavenge NO which is involved in ABA signalling (León et al., 2014) the effect of *ZmPgb1* expression on ABA level and ABA-related gene expression was studied. The level of ABA was not significantly different among lines after 16 hours in PEG (Figure 3.4). *Abscisic Acid Insensitive 5 (ABI5)*, a main ABA response gene (Lopez-Molina et al., 2001), was measured in maize seedlings treated with PEG over the course of 0, 4, 8, and 16 hours (Figure 3.5). An increase in the expression of this ABA signalling gene in the overexpressor [*ZmPgb1* (S)] line, compared to the WT and down-regulator [*ZmPgb1* (A)] lines was observed. A peak in expression in the *ZmPgb1* (S) line was observed within 8 hours of exposure to the stress.

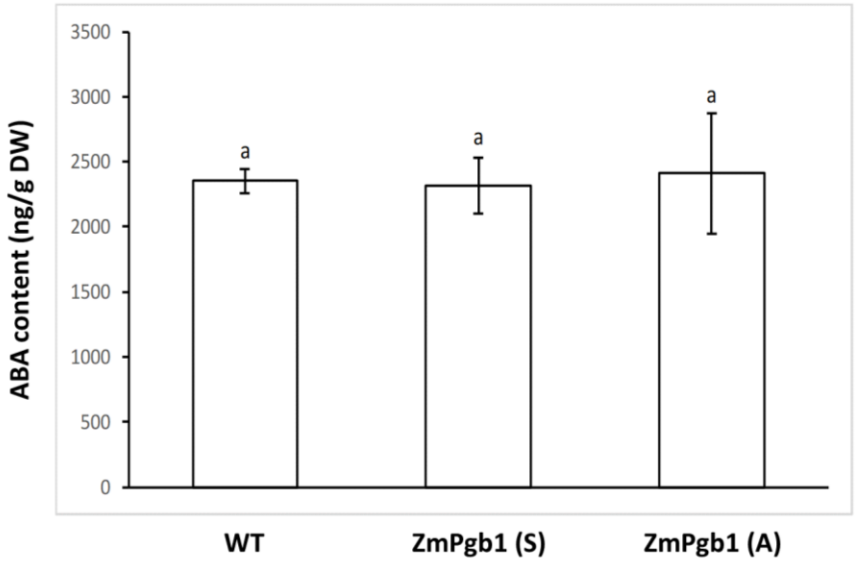


Figure 3.4. Abscisic acid (ABA) quantification in maize shoots treated with PEG for 16 hours. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are means ±SE of at least 3 biological replicates. Letters indicate statistically significant differences ($P<0.05$).

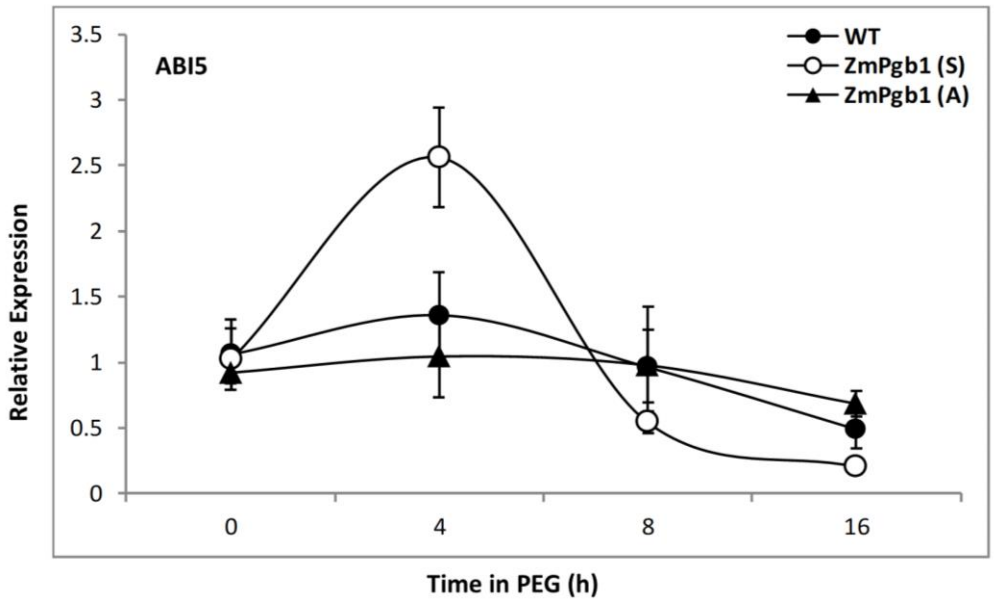


Figure 3.5. Relative expression level of the *Abscisic Acid Insensitive 5 (ABI5)* gene in maize shoots treated with 25% w/v PEG. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates.

3.4.3 *ZmPgb1* alters ethylene biosynthesis and response

There have been a number of studies that link NO and ethylene under stress conditions (Palavan-Unsal and Arisan, 2009). Several genes encoding for the ethylene biosynthetic enzymes *ACC synthase* (*ACS2, 6,* and *7*), and *ACC oxidase* (*ACO15, 20, 31,* and *35*), as well as the ethylene response factors *EIN3 Binding F-Box Protein 1* (*EBF1*) and *Ethylene Response Factor 2* (*ERF2*) were measured. Relative to WT, downregulation of *Pgb1* [*ZmPgb1* (A) line] increased the expression of all *ACS* genes (Figure 3.6) and several *ACO* genes (*ACO15*, *20*, and *31*) (Figure 3.7). A peak in expression was observed within 8h in PEG. The *ZmPgb1* overexpressor [*ZmPgb1* (S)] line had reduced expression of many of the *ACS* and *ACO* genes measured (Figure 3.6 and 3.7). A similar expression profile was also observed for the two ethylene responsive genes *EBF1* and *ERF2* which also peaked within 8 hours in the *ZmPgb1* (A) line treated with PEG (Figure 3.8).

Ethylene accumulation during the PEG-induced drought was also measured using gas chromatography (Figure 3.9). Measurements for ethylene were conducted at 8 hours, corresponding to an approximate expression peak that occurred within that timeframe exhibited by the majority of genes measured. Following a similar pattern as the relative gene expression, the highest levels of ethylene were observed in the *ZmPgb1* (A) line treated with PEG (Figure 3.9). Intermediate values were observed for the WT line, while the *ZmPgb1* (S) line had the lowest levels of ethylene (Figure 3.9). All lines, regardless of the genotype, showed a statistically significant increase in the levels of ethylene when subjected to the PEG treatment (denoted as ‘PEG’).

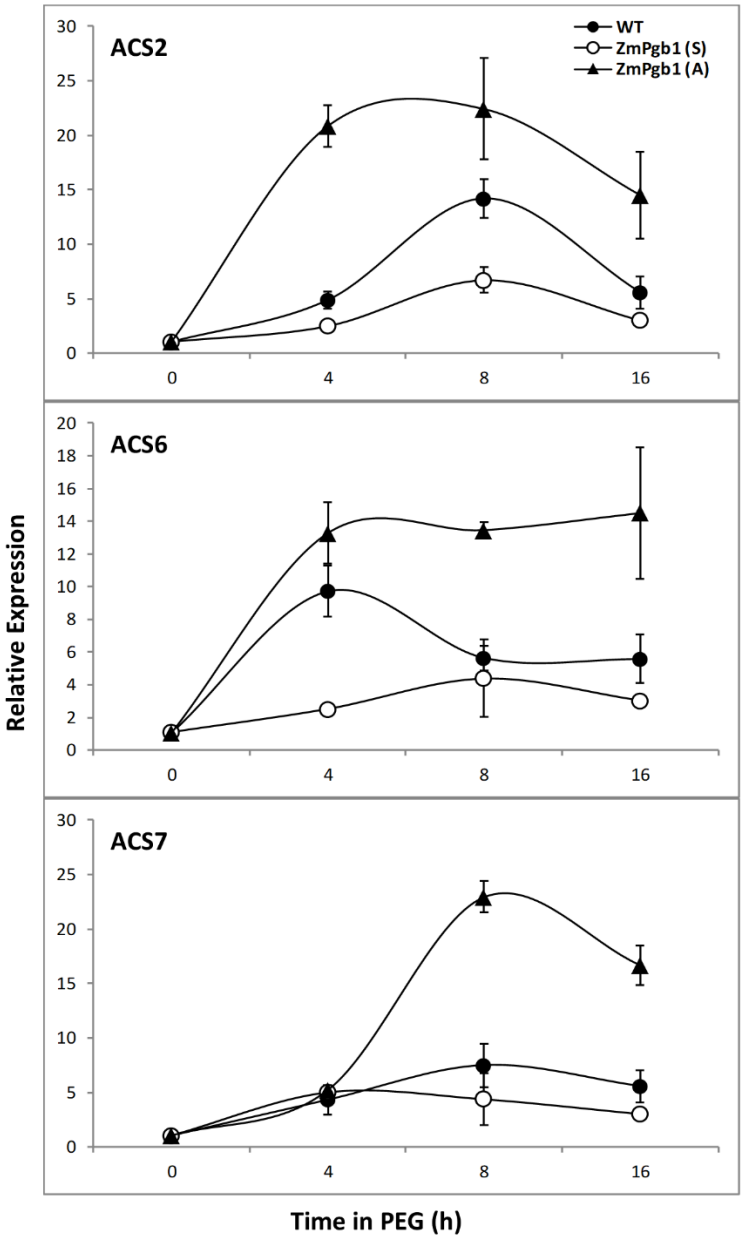


Figure 3.6. Relative expression levels of the *ACC Synthase (ACS2, 6,* and *7)* genes in maize shoots treated with 25% w/v PEG. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates.

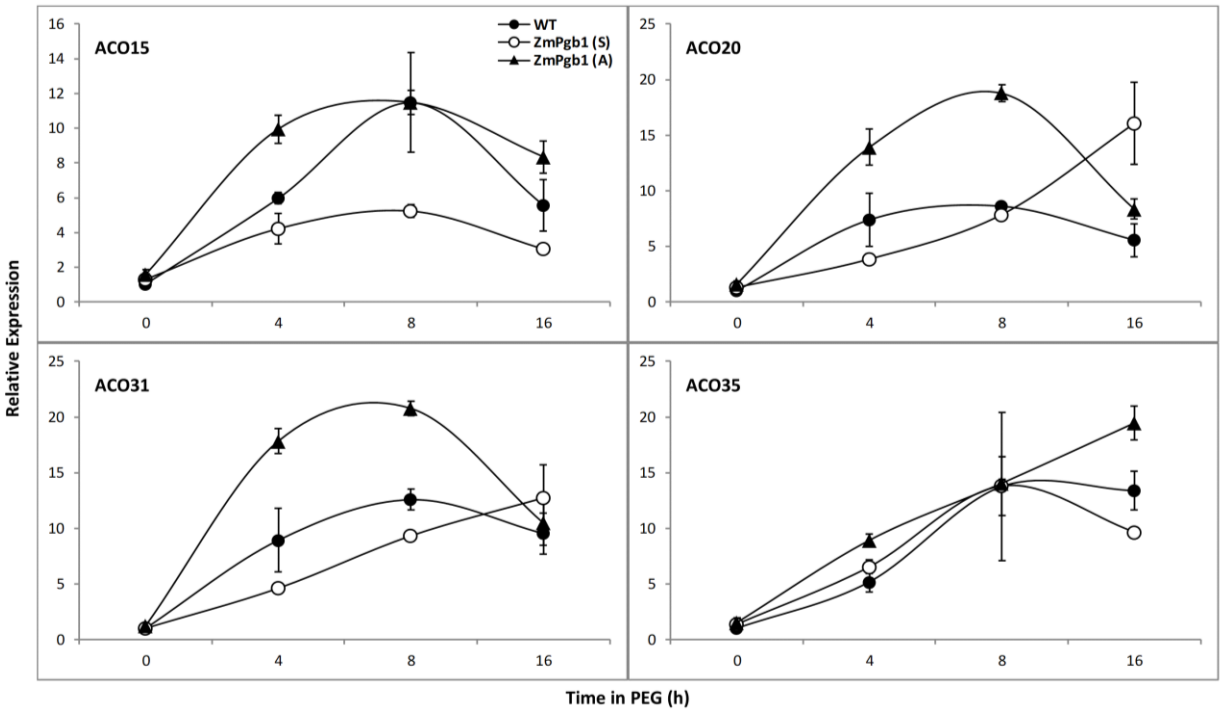


Figure 3.7. Relative expression levels of the *ACC Oxidase (ACO15, 20, 31* and *35)* genes in maize shoots treated with 25% w/v PEG. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates.

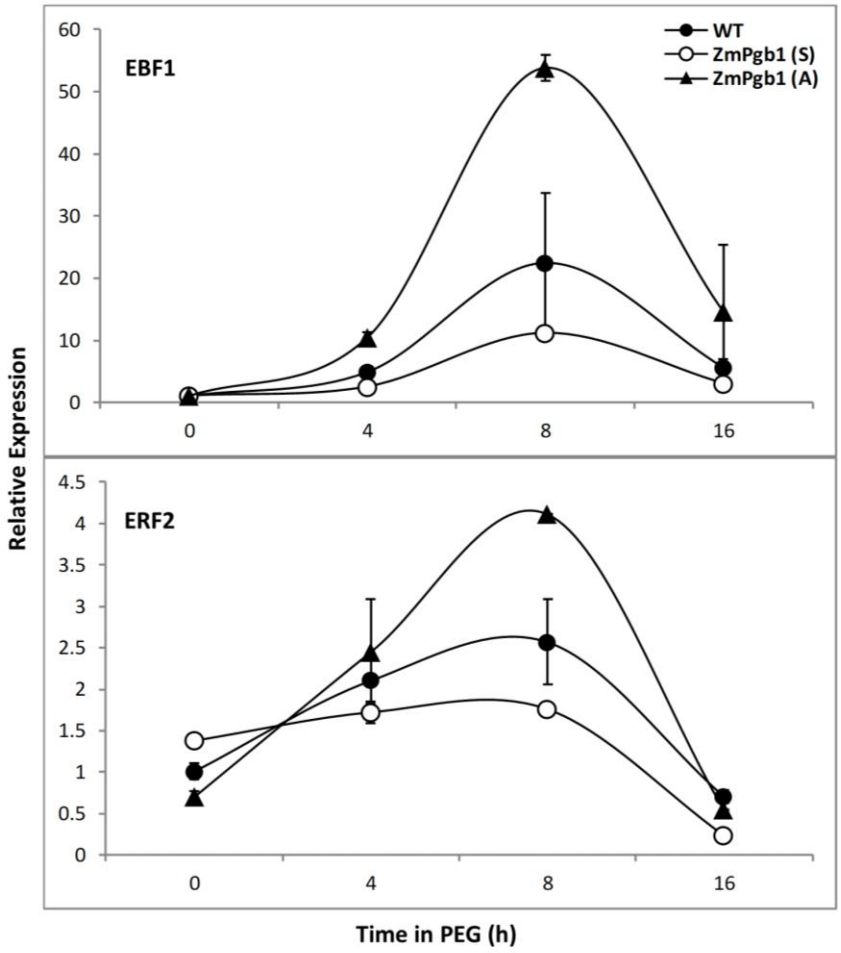


Figure 3.8. Relative expression levels of the *EIN3 Binding F-Box Protein 1* (*EBF1*) *and Ethylene Response Factor 2* (*ERF2*) genes in maize shoots treated with 25% w/v PEG. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates.

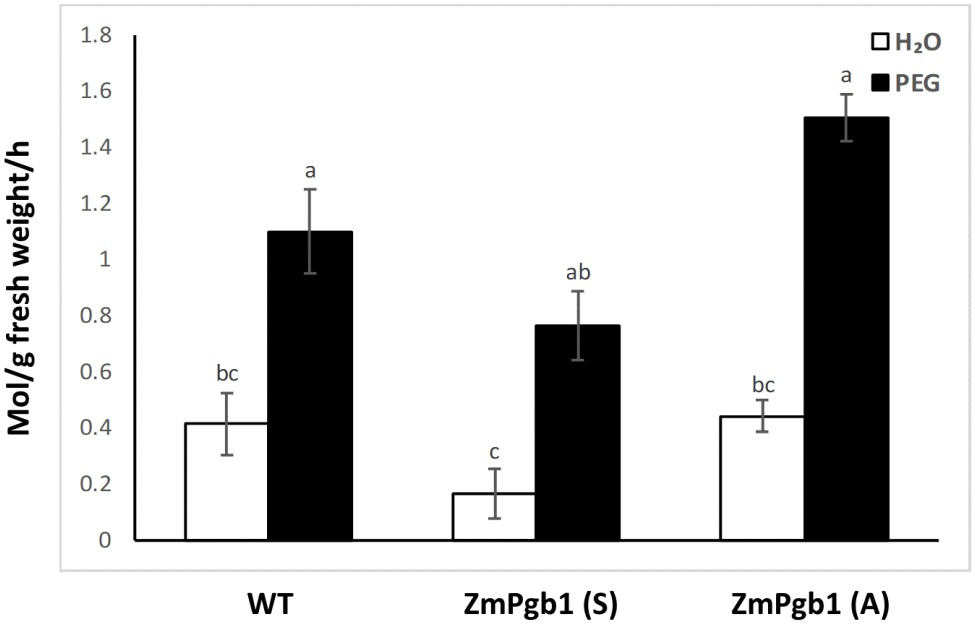


Figure 3.9. Ethylene quantification (mol/g fresh weight/h) in maize shoots treated with PEG for 8h (PEG) or water for 8h as a control (H_2O). Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are means ±SE of at least 3 biological replicates. Letters indicate statistically significant differences ($P<0.05$).

3.4.4 Reactive oxygen species (ROS) production is affected by *ZmPgb1*

Reactive oxygen species (ROS) are known to increase under stress conditions (Apel and Hirt, 2004). In plants, the major source of ROS is the enzyme complex NADPH oxidase composed of several sub-units encoded by the *Respiratory Burst Oxidase Homolog* (*RBOH*) genes (Sagi and Fluhr, 2006). This enzyme complex has been implicated in drought stress responses (Miller et al., 2010). In maize, four *RBOH* genes (named *RBOHA, RBOHB, RBOHC,* and *RBOHD*) have been identified (Lin et al., 2009) and their relative expression was measured during the course of the stress treatment (Figure 3.10). The expression of *RBOHA* and *RBOHB* increased during the PEG treatment, especially in the *ZmPgb1* (A) line, reaching a maximum level within 8 hours. Relative to WT, the *ZmPgb1* (S) line showed a decrease in expression for both genes. A very similar expression pattern was also observed with *RBOHC* and *RBOHD*, which peaked within 4h in the *ZmPgb1* down-regulating line [*ZmPgb1* (A)] (Figure 3.10).

Localization of ROS was also measured using nitroblue tetrazolium (NBT) which produces blue deposits when reacting with superoxide (Campbell et al., 2015). Supporting the *RBOH* gene expression results, shoots (regardless of the genotype) exposed to the water control had the lowest amount of stain (Figure 3.11). Application of PEG increased the ROS staining pattern in the first (Figure 3.11A) and second (Figure 3.11B) leaf. In both leaves, a more pronounced blue coloration was observed in the *ZmPgb1* (A) line, while a lighter staining pattern was observed in the *ZmPgb1* overexpressing line [*ZmPgb1* (S)] relative to WT (Figure 3.11).

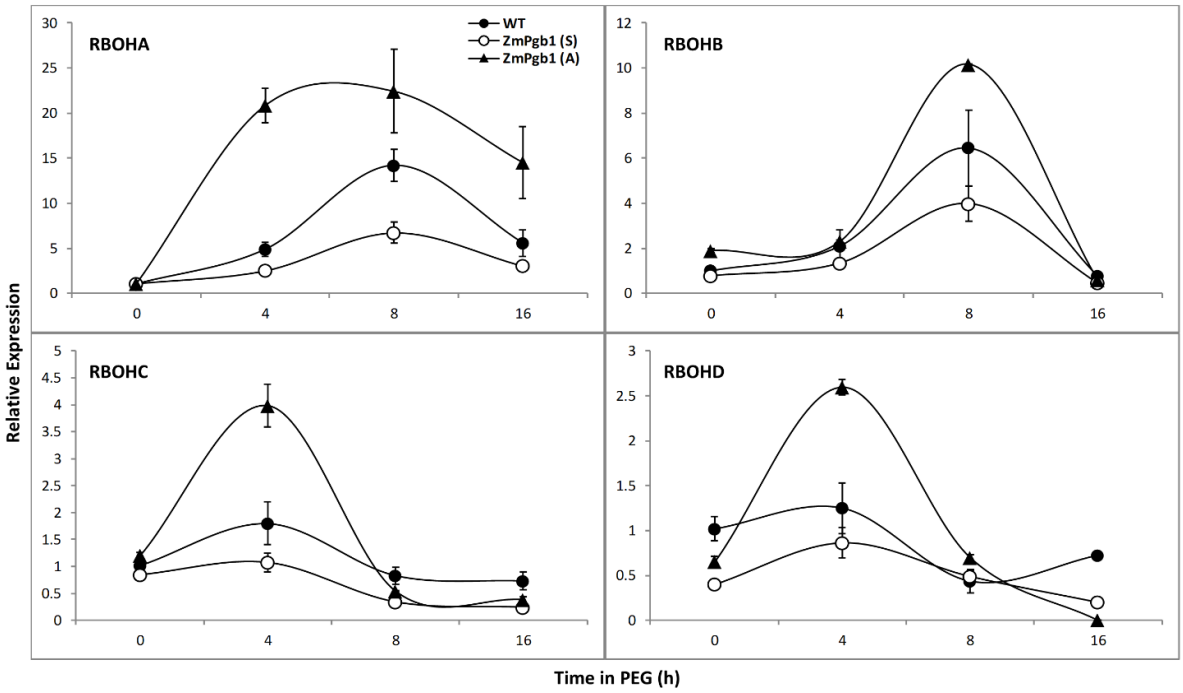


Figure 3.10. Relative expression levels of the *Respiratory Burst Oxidase Homolog (RBOHA, B C,* and *D)* genes in maize shoots treated with 25% w/v PEG. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates.

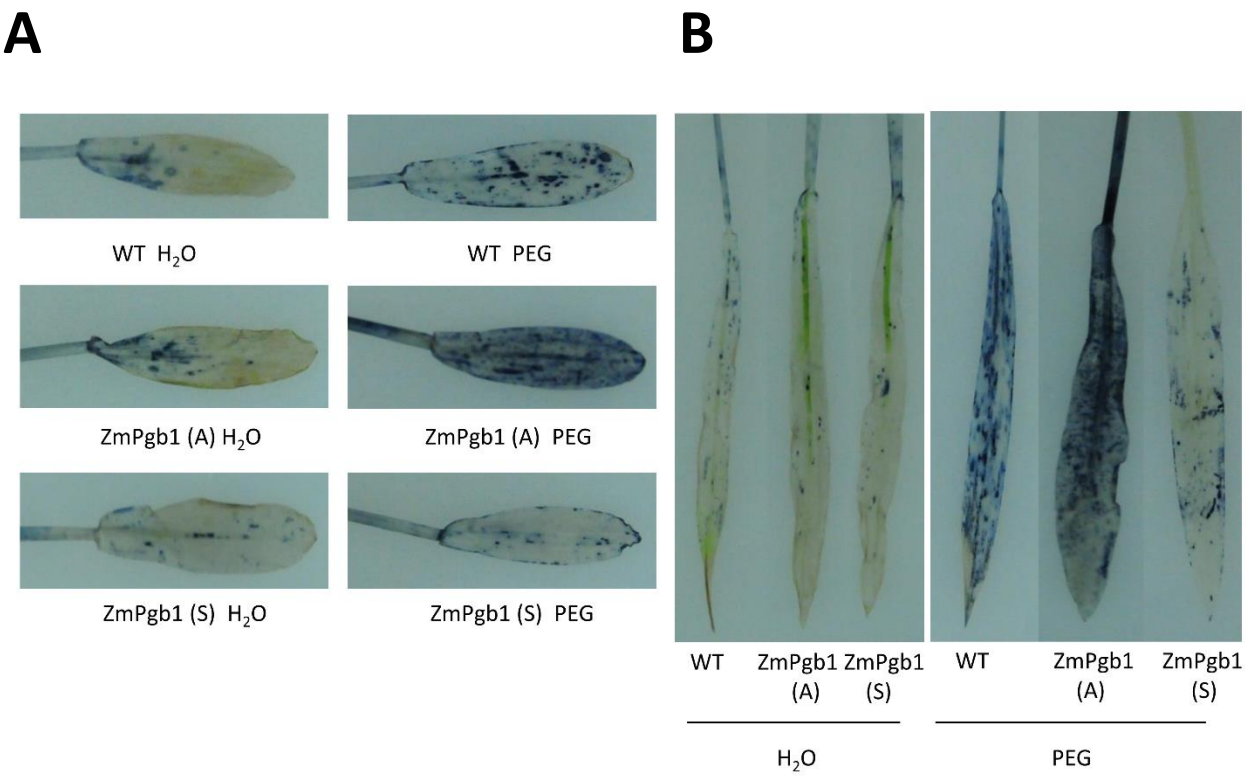


Figure 3.11. Nitroblue tetrazolium (NBT) staining of maize leaves after 8 hours in PEG or water treatment. Blue stain indicates the presence of the ROS superoxide. Staining was conducted on first **(A)** and second **(B)** leaves of WT plants, plants overexpressing *ZmPgb1* [*ZmPgb1* (S)], and plants downregulating *ZmPgb1* [*ZmPgb1* (A)].

3.4.5 Nitric oxide signalling induces ethylene and ROS production

Phytoglobins are effective scavengers of nitric oxide (NO) (Hebelstrup and Jensen, 2008), and NO is linked to ethylene in the regulation of several stress responses, including drought (Palavan-Unsal and Arisan, 2009). To examine the link between *ZmPgb1*, NO, and ethylene on the observed phenotype, pharmacological treatments were conducted to manipulate the level of NO and/or ethylene.

NO was modulated using the NO donor SNP, and the NO scavenger cPTIO (Huang et al., 2014). While the WT maize seedlings were pretreated with both SNP and cPTIO, the *ZmPgb1* overexpressor, exhibiting the lowest level of NO (Dordas et al., 2003; Igamberdiev et al., 2004) was pretreated with SNP to increase NO. In contrast, the *ZmPgb1* down-regulator, over-accumulating NO (Dordas et al., 2003; Igamberdiev et al., 2004) was pre-treated with cPTIO to decrease NO.

These treatments were used to measure the transcript levels of the ethylene biosynthetic and response genes, as well as genes encoding for the NADPH enzymes complexes. All measurements were conducted at 8h in PEG. A general increased expression in the ethylene biosynthesis genes *ACS* and *ACO* (Figure 3.12 and 3.13), ethylene responsive genes *EBF* and *ERF* (Figure 3.14), and *RBOH* genes (Figure 3.15) were observed when the level of NO was elevated by SNP. Treating plants with the NO scavenger cPTIO decreased expression of many genes analyzed (Figures 3.12 – 3.15).

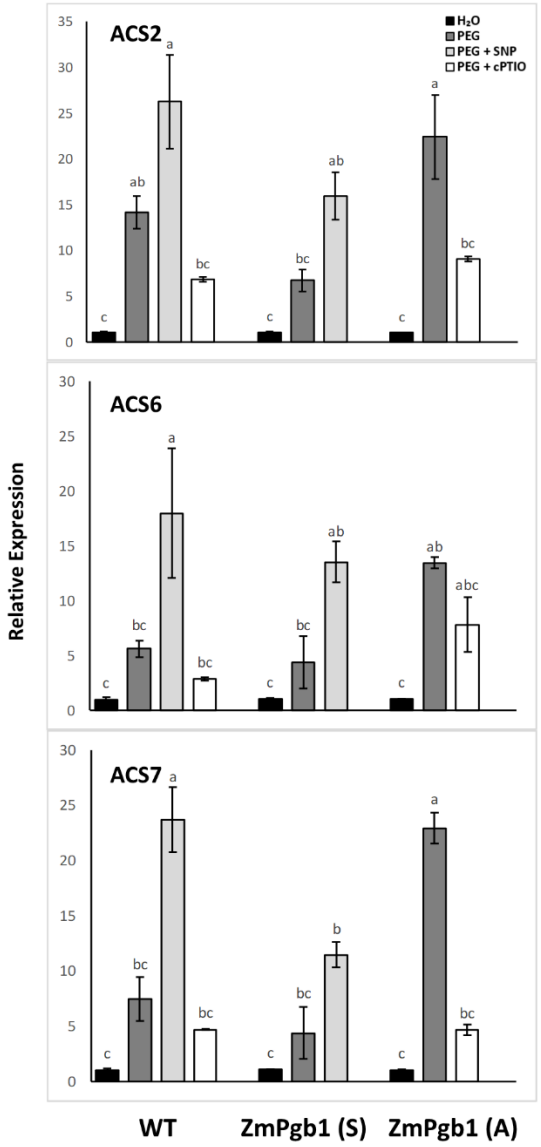


Figure 3.12. Relative expression levels of the *ACC Synthase (ACS2, 6* and *7)* genes in maize shoots pre-treated with SNP and cPTIO, and treated for 8 hours with 25% w/v PEG. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates. Letters indicate statistically significant differences ($P<0.05$). Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO).

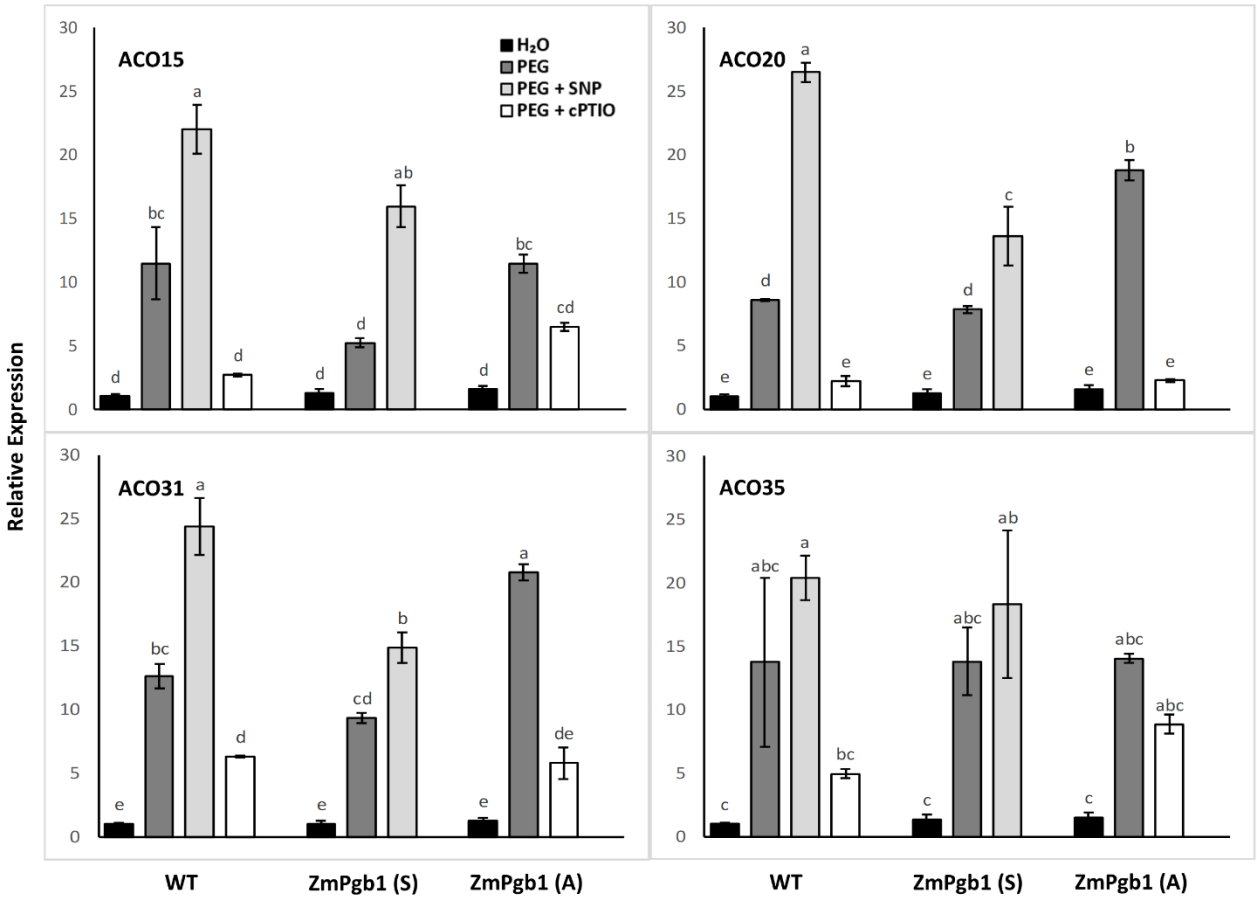


Figure 3.13. Relative expression levels of the *ACC Oxidase (ACO15, 20, 31* and *35)* genes in maize shoots pre-treated with SNP and cPTIO, and treated for 8 hours with 25% w/v PEG. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates. Letters indicate statistically significant differences ($P<0.05$). Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO).

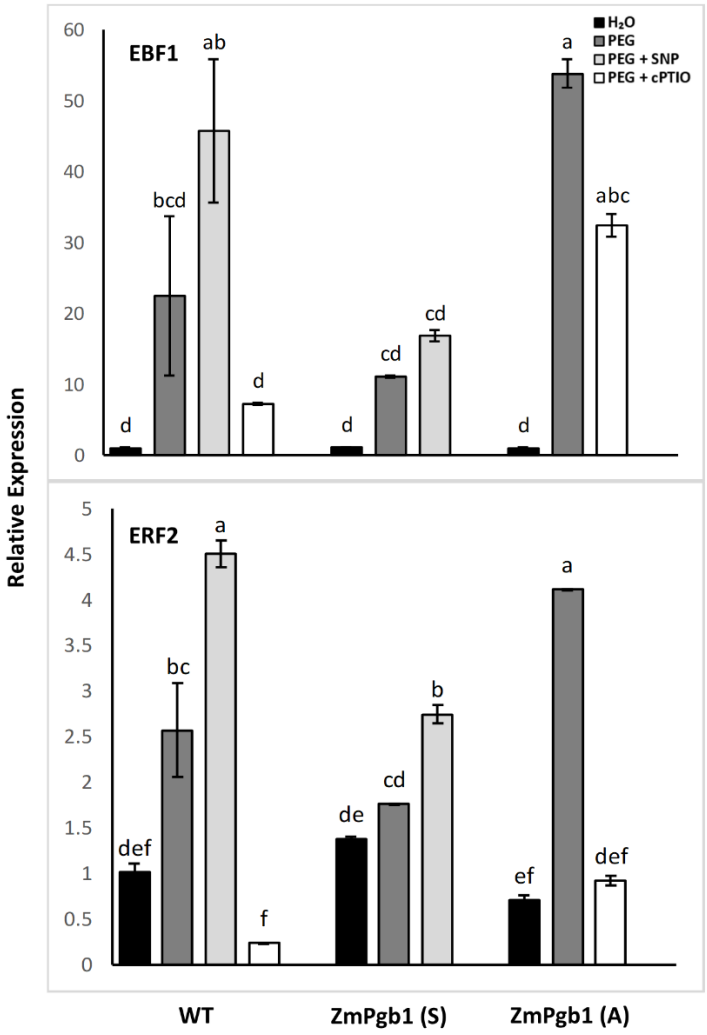


Figure 3.14. Relative expression levels of the *EIN3 Binding F-Box Protein 1* (*EBF1*) and *Ethylene Response Factor 2* (*ERF2*) genes in maize shoots pre-treated with SNP and cPTIO, and shoots treated for 8 hours with 25% w/v PEG. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates. Letters indicate statistically significant differences ($P<0.05$). Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO).

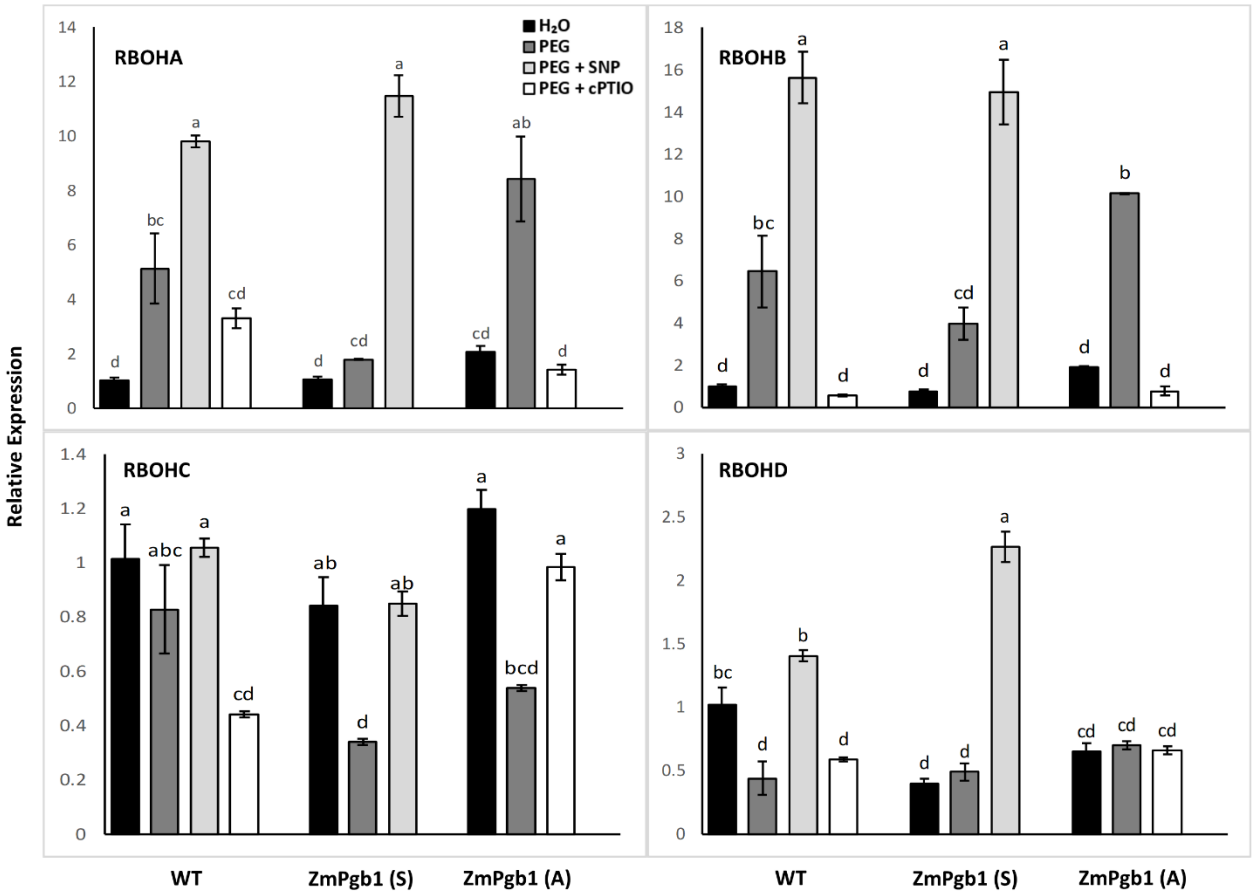
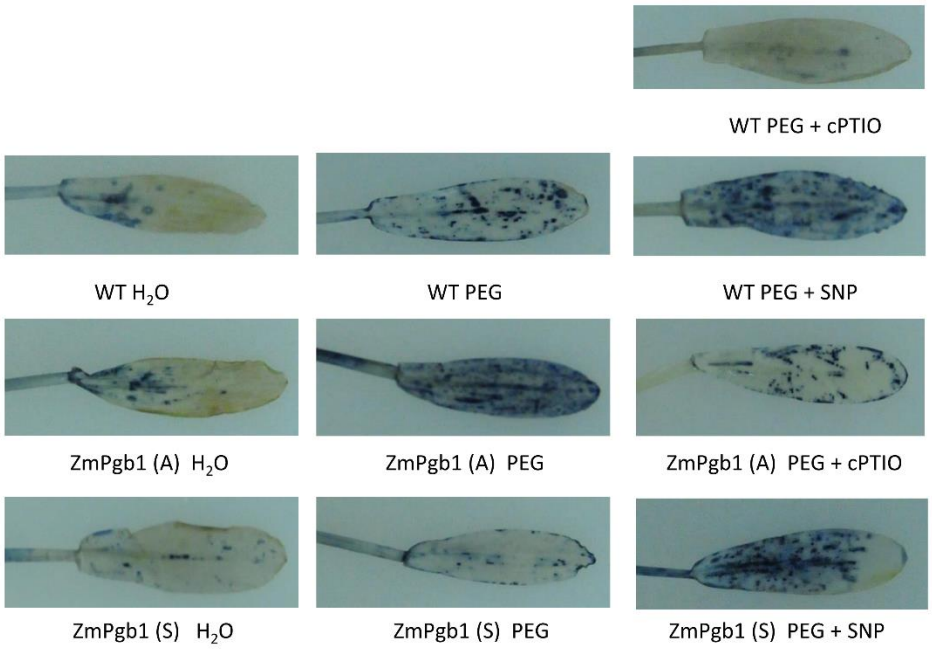


Figure 3.15. Relative expression levels of the *Respiratory Burst Oxidase Homolog (RBOHA, B, C* and *D)* genes in maize shoots pre-treated with SNP and cPTIO, and treated for 8 hours with 25% w/v PEG. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates. Letters indicate statistically significant differences ($P<0.05$). Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO).

The effect of pre-treatments with SNP and cPTIO on ROS accumulation was also measured using nitroblue tetrazolium (NBT). In agreement with the behavior of the *RBOH* genes, elevation of the NO level with SNP increased NBT staining in the WT and *ZmPgb1* (S) lines (Figure 3.16). In contrast, a decrease in the amount of NO with cPTIO reduced the staining pattern in the WT and *ZmPgb1* (A) lines (Figure 3.16). This pattern was consistent in the first (Figure 3.16A) and the second leaf (Figure 3.16B) of the maize shoots. Collectively, these results support a model in which the effects of *ZmPgb1* on the transcriptional regulation of ethylene and ROS are mediated by NO.

A


WT PEG + cPTIO
WT H2O
WT PEG
WT PEG + SNP
ZmPgb1 (A) H2O
ZmPgb1 (A) PEG
ZmPgb1 (A) PEG + cPTIO
ZmPgb1 (S) H2O
ZmPgb1 (S) PEG
ZmPgb1 (S) PEG + SNP

B

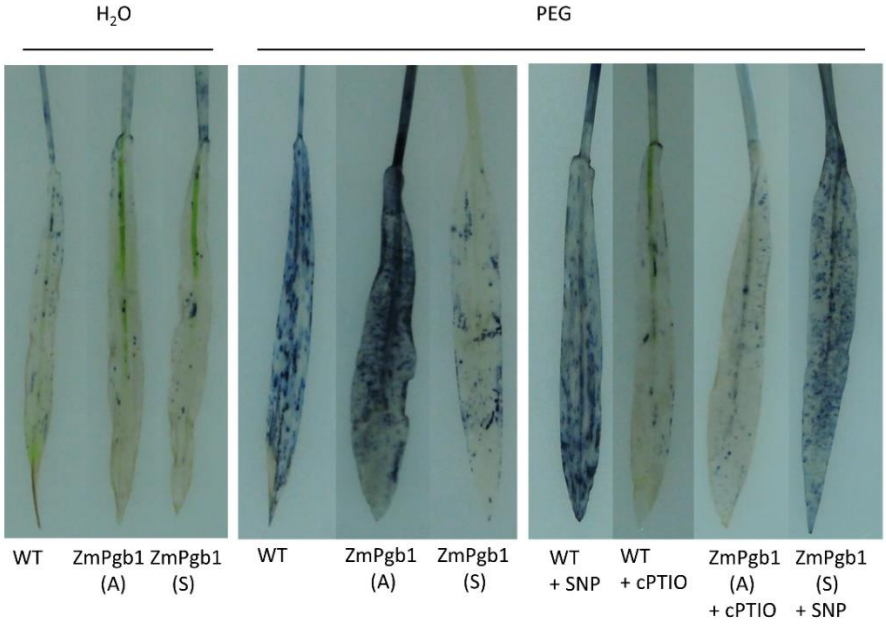

H2O
PEG
WT
ZmPgb1 (A)
ZmPgb1 (S)
WT
ZmPgb1 (A)
ZmPgb1 (S)
WT + SNP
WT + cPTIO
ZmPgb1 (A) + cPTIO
ZmPgb1 (S) + SNP

Figure 3.16. Nitroblue tetrazolium (NBT) staining of maize shoots after 8 hours in PEG, with pre-treatment of SNP or cPTIO, or a water control treatment. Blue stain indicates the presence of the ROS superoxide. Staining was conducted on first **(A)** and second **(B)** leaves of WT plants, plants overexpressing *ZmPgb1* [*ZmPgb1* (S)], and plants downregulating *ZmPgb1* [*ZmPgb1* (A)]. Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO).

3.4.6 Ethylene modulation alters production of ROS

Ethylene has been shown to induce the production of ROS in plants during drought stress (Kazan, 2015). To elucidate the connection between ethylene and ROS in *ZmPgb1* response, pharmacological treatments were performed to elevate ethylene using ethephon (ETH) (Zhang et al., 2010), or inhibit ethylene biosynthesis with aminooxyacetic acid (AOA) (Sharp, 2002). While both compounds were used in the WT line, ETH was used to elevate ethylene in the *ZmPgb1* (S) line (exhibiting the lowest level of ethylene in PEG, Figure 3.9), and AOA was applied to decrease ethylene synthesis in the *ZmPgb1* (A) line (exhibiting the highest level of ethylene in PEG, Figure 3.9).

An experimental increase in ethylene with ETH led to a general rise in the expression of the *RBOH* genes (with the exception of *RBOHC* in WT plants) in PEG-induced water stressed plants (Figure 3.17). A decrease in ethylene with AOA caused a reduction in *RBOHA* and *RBOHB* expression in both WT and *ZmPgb1* (A) plants relative to their untreated control, and an incremental increase in *RBOHC* expression (albeit very small; note scale in Figure 3.17). No differences in expression pattern were observed for *RBOHD* (Figure 3.17).

The effect of ETH and AOA modulation on ROS localization in the maize lines was also investigated using NBT staining. Water stressed (denoted by 'PEG') wild type and *ZmPgb1* (S) shoots pre-treated with ETH showed an increase in ROS staining while WT and *ZmPgb1* (A) pre-treated with AOA exhibited a reduced staining pattern (Figure 3.18). These results suggest that the effects of *ZmPgb1* on ROS production are mediated by ethylene.

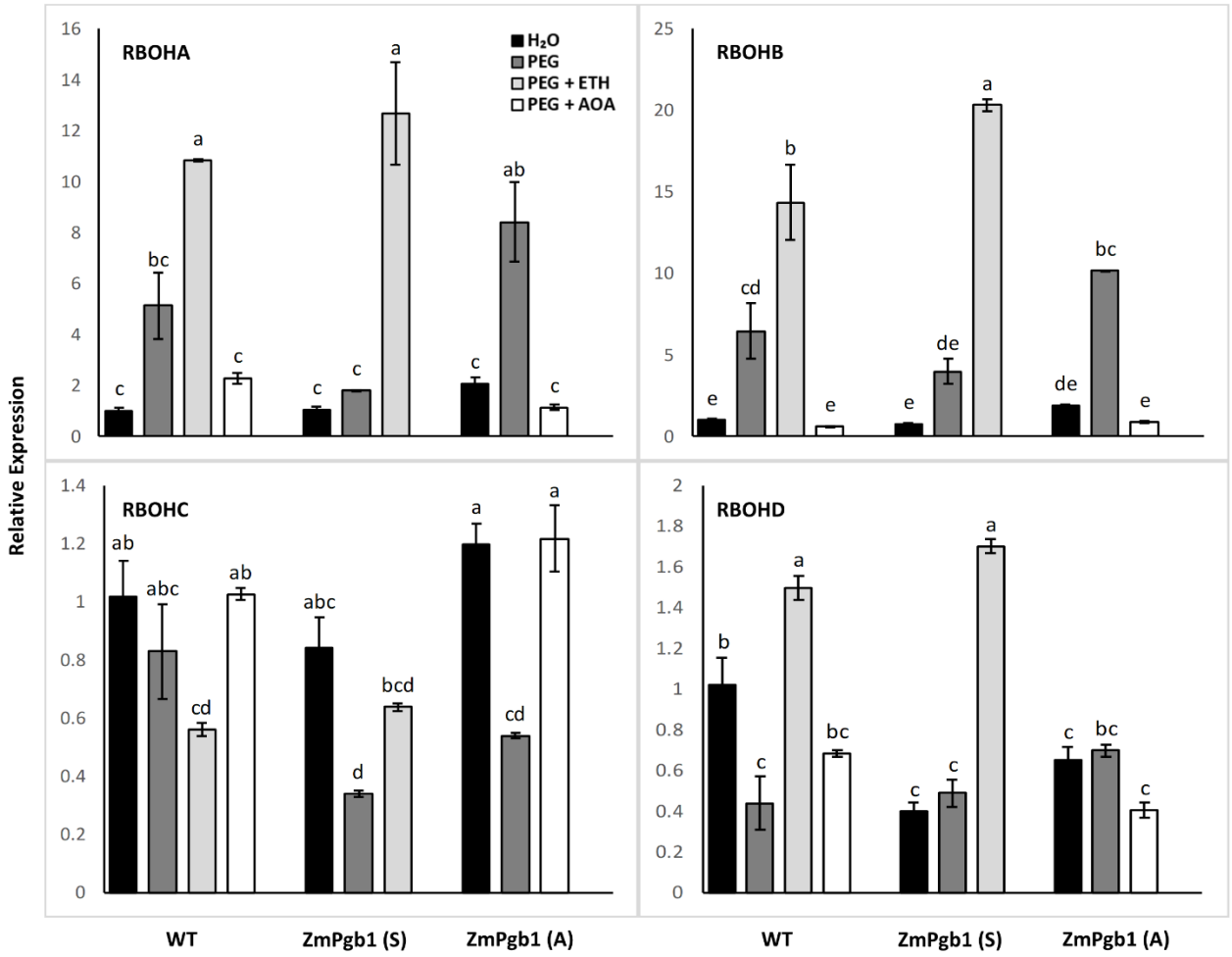
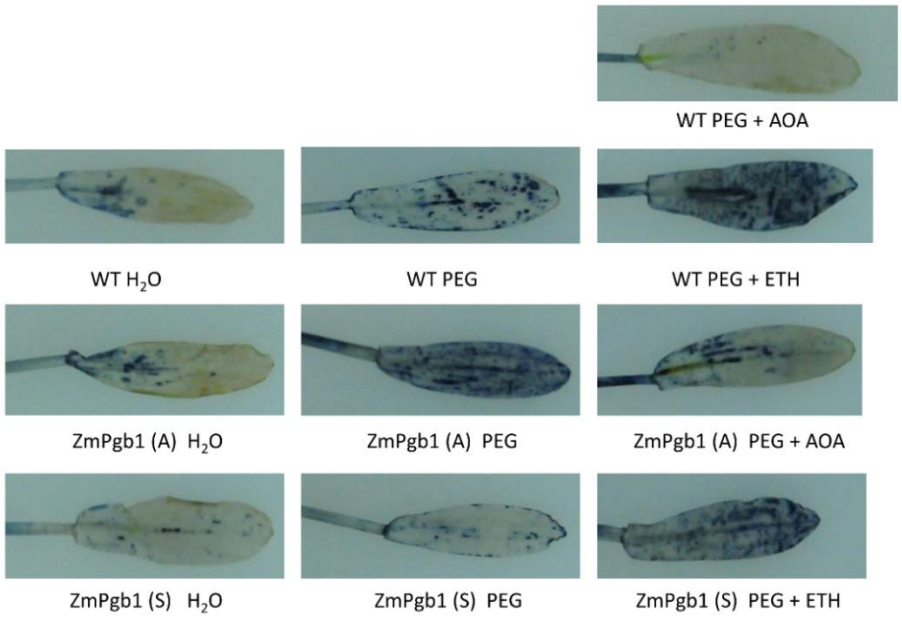


Figure 3.17. Relative expression levels of the *Respiratory Burst Oxidase Homolog (RBOHA, B, C* and *D)* genes in maize shoots pre-treated with ETH or AOA, and treated for 8 hours with 25% w/v PEG. Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates. Letters indicate statistically significant differences ($P<0.05$). Ethephon (ETH), aminooxyacetic acid (AOA).

A


WT PEG + AOA
WT H2O
WT PEG
WT PEG + ETH
ZmPgb1 (A) H2O
ZmPgb1 (A) PEG
ZmPgb1 (A) PEG + AOA
ZmPgb1 (S) H2O
ZmPgb1 (S) PEG
ZmPgb1 (S) PEG + ETH

B

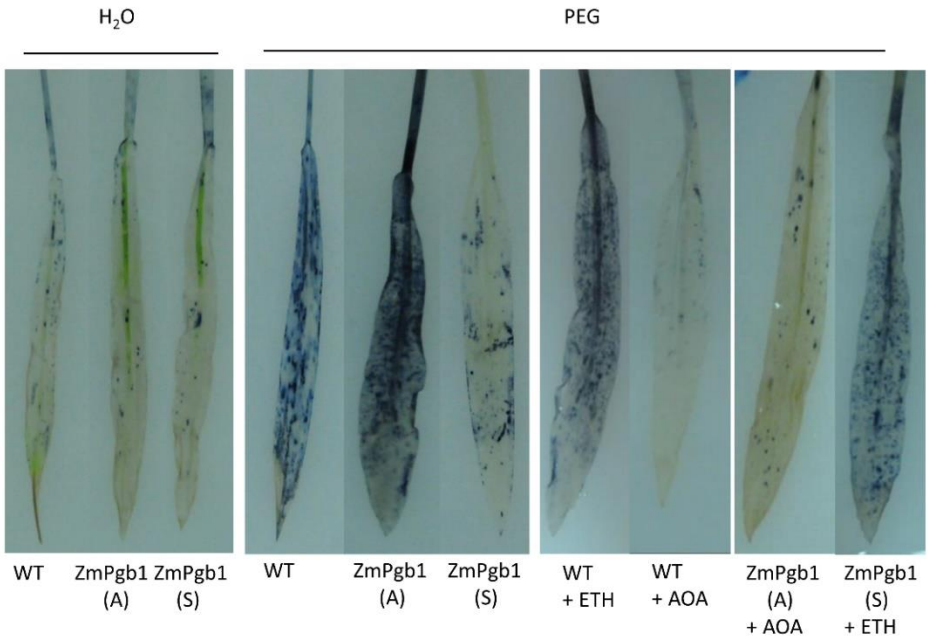

H2O
PEG
WT
ZmPgb1 (A)
ZmPgb1 (S)
WT
ZmPgb1 (A)
ZmPgb1 (S)
WT + ETH
WT + AOA
ZmPgb1 (A) + AOA
ZmPgb1 (S) + ETH

Figure 3.18. Nitroblue tetrazolium (NBT) staining of maize shoots after 8 hours in PEG, with pre-treatment of ETH or AOA, or a water control treatment. Blue stain indicates the presence of the ROS superoxide. Staining was conducted on first **(A)** and second **(B)** leaves of WT plants, plants overexpressing *ZmPgb1* [*ZmPgb1* (S)], and plants downregulating *ZmPgb1* [*ZmPgb1* (A)]. Ethephon (ETH), aminooxyacetic acid (AOA).

3.4.7 *ZmPgb1*, NO, ethylene, and ROS are involved in drought stress signalling

NO and ethylene have been shown to be intrinsically linked with the *ZmPgb1* drought stress response and to be able to modulate ROS. As shown above, the level of ROS is in fact influenced by either applications of SNP or cPTIO (altering NO) (Figures 3.12 – 3.16), or ETH and AOA (altering ethylene) (Figure 3.17 and 3.18). To investigate the signalling order of NO and ethylene in the *ZmPgb1* response, the levels of both compounds were altered simultaneously during the pre-treatment stage, prior to the 8h PEG application. An elevation in ethylene level by ETH reversed the effect of cPTIO in reducing the expression of *RBOH* (compare the cPTIO treatment with the cPTIO + ETH treatment), while a decrease in ethylene by AOA was sufficient to reverse the effect of SNP in increasing the expression of *RBOH* (Figure 3.19). These results support the conclusion that NO acts upstream of ethylene in the *ZmPgb1* response.

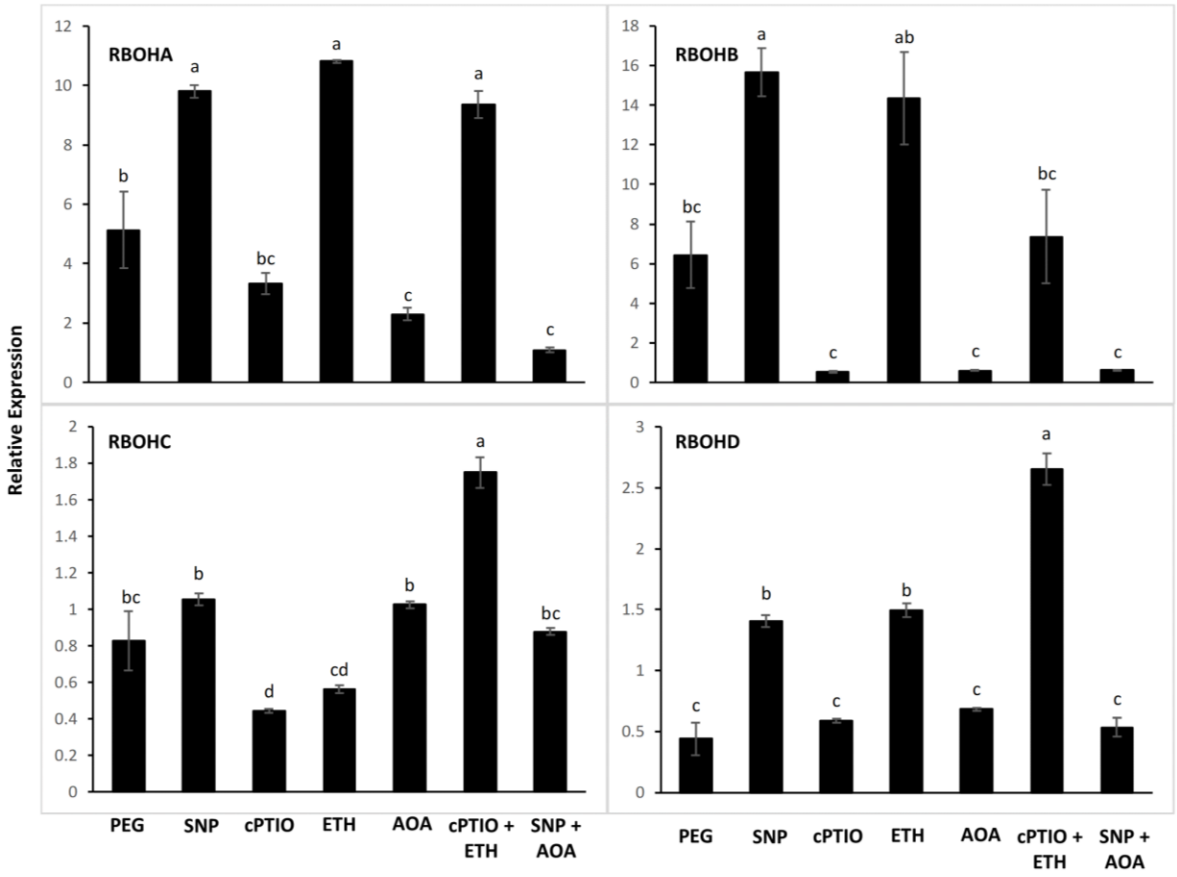
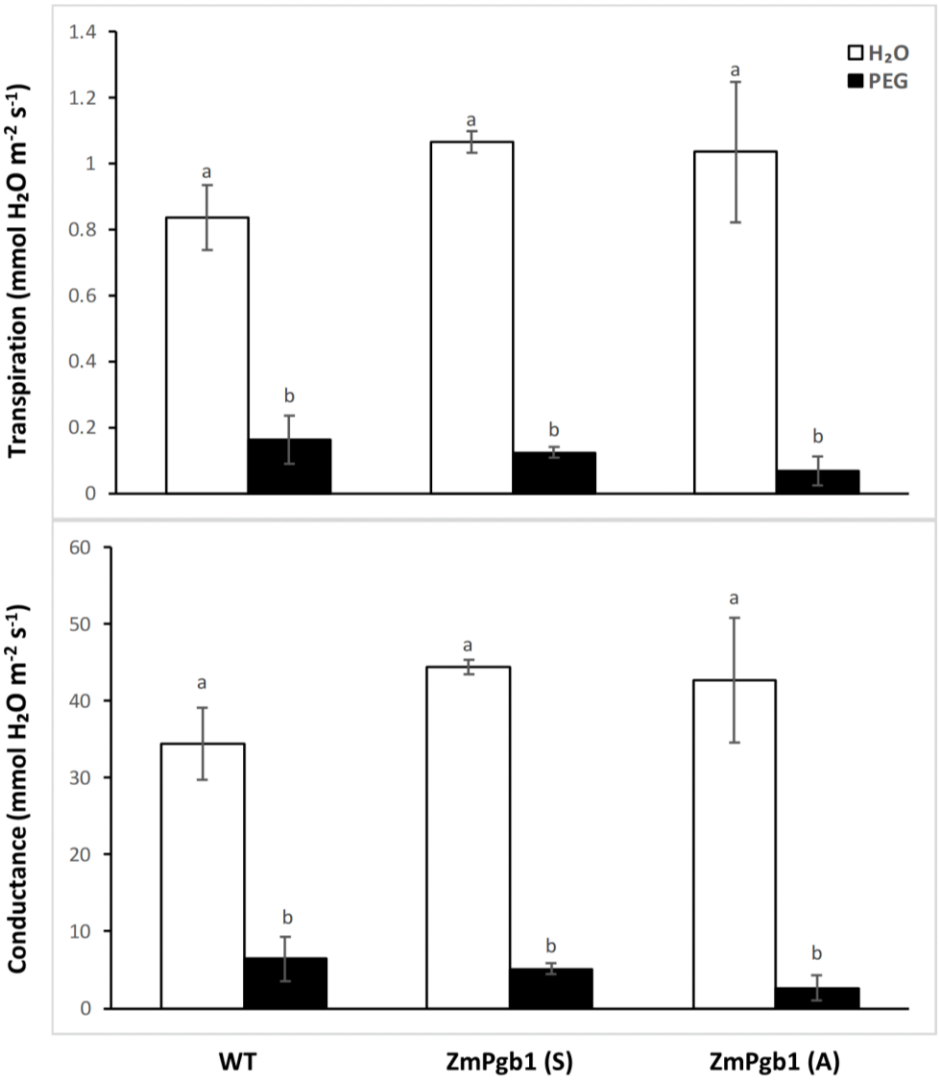


Figure 3.19. Relative expression levels of the *Respiratory Burst Oxidase Homolog (RBOHA, B, C* and *D)* genes in maize shoots pre-treated with SNP, cPTIO, ETH, and/or AOA, and treated for 8 hours with 25% w/v PEG. Plants utilized were of the wild type (WT) line. Values are normalized to the WT value at 0 hours (set at 1) and the means ±SE were determined from three biological replicates. Letters indicate statistically significant differences ($P<0.05$). Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), ethephon (ETH), aminooxyacetic acid (AOA).

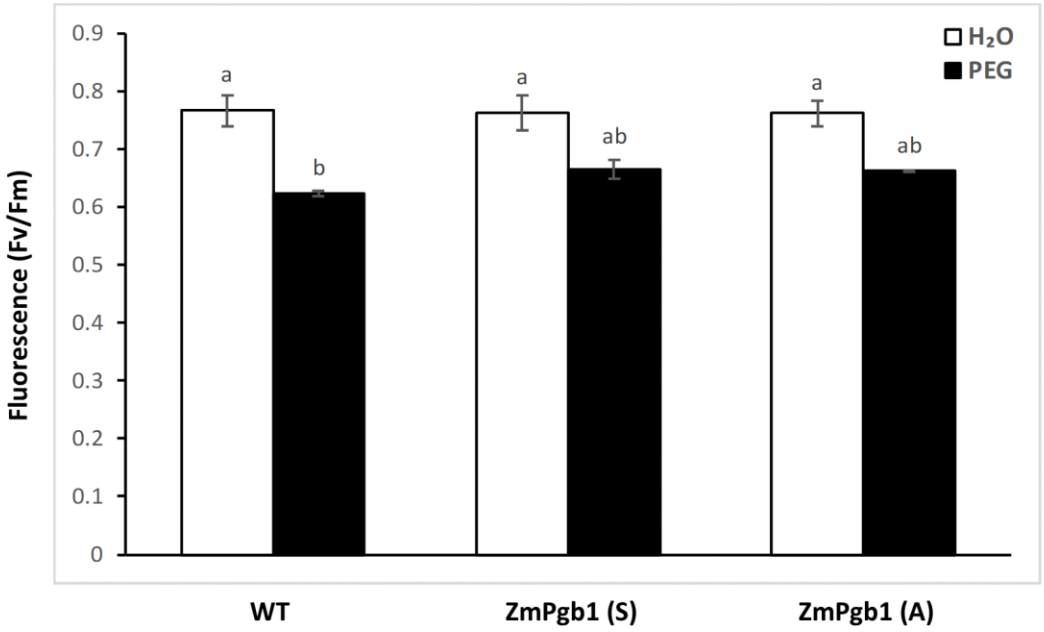
3.5 Supplementary Data

Supplementary Table 1. Primers used in gene expression

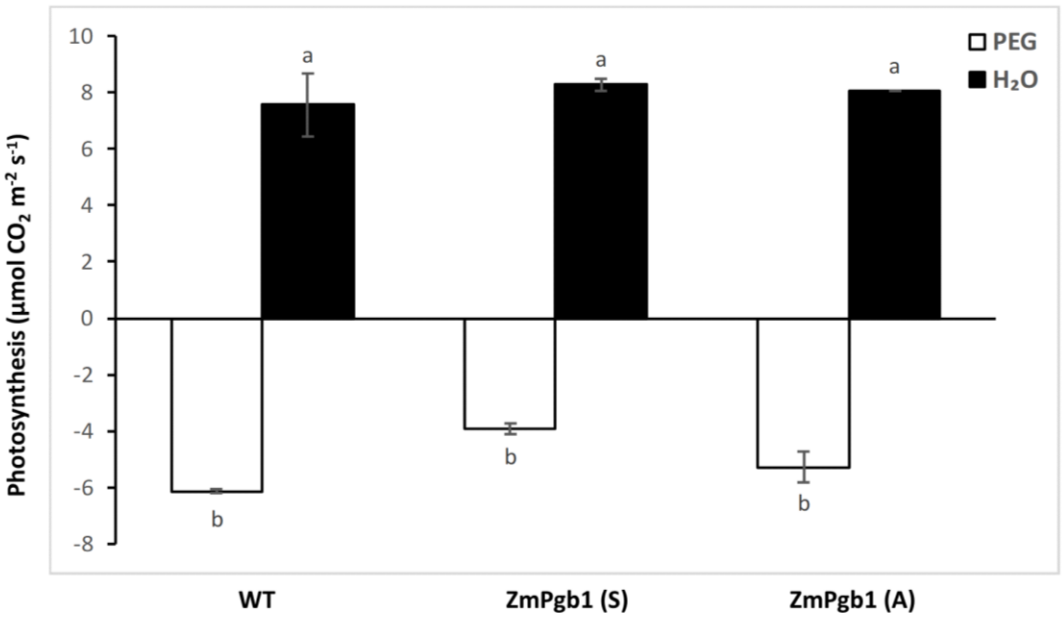
Name	Sequence
ZmABI5 – F	GGAAGCAAATGGACAGGAGTAG
ZmABI5 – R	CGTAGATGCCACTAGGTTTGAC
ZmEBF1 – F	CTGTCCGGCTGTATGAAGGT
ZmEBF1 – R	TGGTTGCCAATGAAGTTGAA
ZmERF2 – F	AGACAATGAGGCGTGCAAGT
ZmERF2 – R	AATTGCTCCCGAGCTTATCG
ZmACS2 – F	ATCGCGTACAGCCTCTCCAAGGA
ZmACS2 – R	GGCCATGAACTCCGCGTCC
ZmACS6 – F	CGCGCCGCCACGGACGACG
ZmACS6 – R	ATCTTGGTGGCCGCGGAGAC
ZmACS7 – F	ATCGCGTACAGCCTCTCCAAGGA
ZmACS7 – R	TGCCATGAACTCCGCGTCGG
ZmACO15 – F	AGCGGCGGCGACGCATACC
ZmACO15 – R	GGAGATGACTTGGGCGCTGCAA
ZmACO20 – F	CGTTCGGCACCAAGGTGAGC
ZmACO20 – R	ACGTCCACCCACTCCCCGC
ZmACO31 – F	AGCGGCGGCGACGCATACC
ZmACO31 – R	GGAGATGACTTTGGCGCCCC
ZmACO35 – F	CGTTCGGCACCAAGGTGAGC
ZmACO35 – R	CACGTCCACCCACTCCCCG
ZmRBOHA – F	CACACGTGACCTGCGACTTC
ZmRBOHA – R	CCCCAAGGTGGCCATGA
ZmRBOHB – F	GGCCAGTACTTCGGTGAAACA
ZmRBOHB – R	ATTACACCAGTGATGCCTTCCA
ZmRBOHC – F	TTCTCTTGCCTGTATGCCGC
ZmRBOHC – R	CTTTCGTATTCCGCAGCCA
ZmRBOHD – F	CCGGCTGCAGACGTTCTT
ZmRBOHD – R	CCTGATCCGTGATCTTCGAAA
ZmPgb1 – F	TCCGCTTCTTTCTCAAGGTCTTCG
ZmPgb1 – R	AGGTCATGACGAAGACGGACAT
Actin – F	GATGGTCAGGTCATCACCATTG
Actin – R	AACAAGGGATGGTTGGAACAAC



Supplementary Figure 1. Stomatal conductance and transpiration (mmol H_2O m^{-2} s^{-1}) in maize shoots treated with PEG for 8h (PEG) or water for 8h as a control (H_2O). Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are means ±SE of at least 3 biological replicates. Letters indicate statistically significant differences ($P<0.05$).



Supplementary Figure 2. Fluorescence (Fv/Fm) in maize shoots treated with PEG for 8h (PEG) or water for 8h as a control (H_2O). Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are means ±SE of at least 3 biological replicates. Letters indicate statistically significant differences ($P<0.05$).



Supplementary Figure 3. Photosynthesis (µmol CO_2 m^{-2} s^{-1}) in maize shoots treated with PEG for 8h (PEG) or water for 8h as a control (H_2O). Plants utilized were a wild type (WT) line, a *ZmPgb1* overexpressing line [*ZmPgb1* (S)], and a *ZmPgb1* downregulating line [*ZmPgb1* (A)]. Values are means ±SE of at least 3 biological replicates. Letters indicate statistically significant differences (P<0.05).

3.6 Discussion

Due to their sessile nature, and the need to withstand their environment to survive, plants have developed a number of physiological mechanisms to cope with drought stress. Reduced water slows plant growth by drastically decreasing root elongation and leaf area expansion (Bhattacharjee and Saha, 2014). The ratio of root to shoot growth is also increased, as photosynthates are redirected to favour the growth of roots, in order to reach areas of higher moisture levels (Farooq et al., 2009). Plants also close their stomata to limit the amount of water loss through transpiration, which causes a reduction in the photosynthetic rate (Farooq et al., 2009). All of these changes are evoked by unique and complex transduction pathways involving several signalling molecules, including NO (Palavan-Unsal and Arisan, 2009). Nitric oxide is able to diffuse through the lipid membrane of cells (Wilson et al., 2008). This property permits its participation in a variety of plant responses, where it has been found to alter growth and development, but also suggests that regulation of NO levels may be an important aspect of physiological processes. For example, increased NO levels inhibit hypocotyl growth, increase chlorophyll content and iron availability, and interact with a number of hormones that also have important effects on plants (Palavan-Unsal and Arisan, 2009; Hill, 2012).

Several factors contribute to maintain and regulate NO homeostasis (Wilson et al., 2008), and the ability of Pgbs to scavenge NO has been well-characterized experimentally (Hebelstrup et al., 2006). Specifically, oxygenated ferrous Pgb converts NO to nitrate, and forms methemoglobin (Igamberdiev et al., 2005). Due to this property, it is has been demonstrated that plants overexpressing *Pgb* have reduced levels of NO compared to the wild type, while plants that down-regulate *Pgb* have increased levels of NO (Dordas et al., 2003; Igamberdiev and Hill,

2004). This function is important during drought stress responses, as NO is an integral component of plant signalling under conditions of limited water (Shi et al., 2014).

Here it is shown that suppression of the maize *ZmPgb1*, reducing NO levels (Dordas et al., 2003), decreases tolerance to PEG-induced water stress relative to the wild type and *ZmPgb1* overexpressing plants (see Figure 3.3 for a phenotypic comparison). The requirement of Pgbs for drought stress tolerance has been demonstrated in other studies. In barley, over-expression of *Pgb* enhances tolerance to limited water conditions (Montilla-Bascón et al., 2017). In agreement with these results, Arabidopsis *Pgb1* over-expressing plants, with decreased NO levels, exhibited a reduced root growth inhibition when exposed to increasing levels of PEG (Mira et al., 2017). Specifically, it was demonstrated that *Pgb1* is required to maintain an integral and functional root apical meristem under conditions of reduced water which inhibits root growth in wild type plants. Consistent with the results presented here, suppression of the Arabidopsis *Pgb1* aggravates drought-induced root growth inhibition causing a premature degradation of the root apical meristem (Mira et al. 2017).

The role of NO during drought stress is dichotomous, and may be related to the concentration of NO supplied (Hebelstrup and Jensen, 2008). Some studies showed that increasing NO levels with SNP confers drought stress tolerance (Garcia-Mata and Lamattina, 2001). Nitric oxide is also involved in ABA-mediated stomatal closure during drought stress (León et al., 2014). Abscisic acid is a major hormone involved in plant drought stress responses, and accumulation of ABA in the guard cells of plants triggers stomatal closure (Desikan et al., 2004). Abscisic acid has been connected with a number of hormones and signalling molecules,

such as the production of ethylene increasing in ABA deficient plants (Sharp, 2002). ABA also may have an antagonistic interaction with ROS (Jiang and Zhang, 2002; Zhang et al., 2007). These results show that a decrease in NO in the *ZmPgb1* (S) plants have increased expression of the ABA response gene *ABA Insensitive 5* (*ABI5*) during drought stress (Figure 3.5). However, ABA quantification shows no statistically significant difference in ABA content between lines after 16 hours in PEG (Figure 3.4), which leads to the conclusion that differences in ABA signalling may be occurring through changes in signalling molecule expression, not endogenous levels of ABA. This is in contrast to a recent study that found that ABA content decreased in the *ZmPgb1* (A) lines (Kapoor et al., 2018). However, these results may differ due to ABA having a separate role during maize embryogenesis as compared to drought stress in seedlings.

Drought stress has a significant effect on plant ethylene levels. Accumulation of ethylene as well as its precursors (such as ACC) during drought stress has been demonstrated (Klay et al., 2014). Nitric oxide and ethylene are closely linked, and the induction of ethylene production has been previously documented in a number of pharmacological studies looking at the effect of NO modulation on ethylene levels in the plant (Ahlfors et al., 2009; Liu et al., 2017). In this regard, Ahlfors (2009) found that treating Arabidopsis with SNP induced production of ethylene, through the transcriptional induction of *ACS* and *ACO*. This agrees with the results of this study showing that application of SNP increases the relative expression of *ACS* and *ACO* (Figures 3.12 and 3.13). Another study found that ethylene levels, as well as *ACS* and *ACO* transcripts, increased during water stress in soybeans (Arraes et al., 2015), which is true for all lines in this study (Figures 3.6 and 3.7). A more recent study on barley found that WT plants had higher ethylene levels and increased abundance of *ACS1*, *ACS2* and *ACS5*, relative to the *Pgb1* over-

expressing plants exhibiting higher tolerance to water stress (Montilla-Bascón et al., 2017). Increased tolerance to drought stress with decreased ethylene levels may be due to a delay in ethylene-mediated senescence. This seems to be in agreement with this work showing a decreased expression of *ACS* and *ACO* in maize over-expressing *ZmPgb1* (Figures 3.6 and 3.7). Conversely, the down-regulating [*ZmPgb1* (A)] line showed opposing results, that is an elevation in the abundance of ethylene biosynthesis transcripts (Figures 3.6 and 3.7). The link between *ZmPgb1* and ethylene was further strengthened by measuring the amount of ethylene present in the tissue. As shown in Figure 3.9, ethylene level was lowest in the *ZmPgb1* (S) line. This observation directly supports the results found in the recent study where increased drought stress tolerance in the barley *Pgb* over-expressing line was linked to lower levels of ethylene (Montilla-Bascón et al., 2017).

Ethylene response genes are also linked to drought stress, with the *Ethylene Response Factor* (*ERF*) family of genes being induced under drought stress (Cheng et al., 2013). Arabidopsis *Pgb1* down-regulators, most susceptible to water stress, show the highest levels of *ERF1*, *2*, and *10* when exposed to PEG (Mira et al., 2017), an observation which agrees with the present study (Figure 3.8). The genes *EIN3 Binding F-Box Protein 1* (*EBF1*) and *Ethylene Response Factor 2* (*ERF2*) are involved in ethylene response, interacting with the *Ethylene Insensitive 3 (EIN3)* transcription factor (Guo and Ecker, 2003). The ethylene response genes *EBF1* and *ERF2* have been found to have increased expression under abiotic stress such as hypoxia and waterlogging (Takahashi et al., 2015; Mira et al., 2016). Expression of *EBF* and *ERF* is also directly induced by ethylene and its precursor ACC (Potuschak et al., 2003; Zhang et al., 2010). *EBF1* is a negative regulator of *EIN3*, and is involved in its proteolysis (Guo and

Ecker, 2003; Kazan, 2015), whereas *ERF2* is downstream of *EIN3* in a signalling pathway (Cheng et al., 2013; Kazan, 2015), but both are very sensitive to ethylene levels and are quickly upregulated when ethylene is detected (Zhang et al., 2010). The two ethylene response genes *EBF1* and *ERF2* showed a consistent pattern mimicking that of the ethylene biosynthetic genes. Both sets of genes are suppressed under conditions of low NO levels [*ZmPgb1* (S)], and induced in environments characterized by high levels of NO [*ZmPgb1* (A)] (Figure 3.8).

Pharmacological treatments were used to reinforce the correlation between NO and ethylene in drought stressed maize plants. Increase of NO with SNP induced the expression of *ACS*, *ACO*, and ethylene response genes *EBF* and *ERF* (Figures 3.12 – 3.14). This was in contrast to plants treated with the NO scavenger cPTIO, which showed a decreased expression of the same genes. Based on these results, it is suggested that NO acts upstream to ethylene in the *ZmPgb1* response.

Reactive oxygen species generated through NADPH oxidases (encoded by the *RBOH* family of genes) have been implicated in drought stress responses due to their involvement in stress signalling and ABA-induced stomatal closure (Sagi and Fluhr, 2006; Miller et al., 2010). Production of ROS in the guard cells may be part of a signalling cascade to induce closure, and one study found that application of ROS to stomata caused them to close (McAinsh et al., 1996). Generation of ROS in response to stress is also extremely damaging to plant cells, and can cause detrimental effects such as lipid peroxidation, protein unfolding and DNA damage (Miller et al., 2010). This disruption has been implicated in the early stages of PCD (Hill et al., 2013). Therefore, plants must maintain a fragile equilibrium between upregulation of ROS as a

signalling molecule to aid in stress response, and its unchecked increase to toxic levels due to stress. Regardless of the dual role, ROS levels rise rapidly during stress conditions (Apel and Hirt, 2004). Production of was examined by staining for superoxide with NBT and measuring the expression of *RBOHA, B, C* and *D*. Plants downregulating *ZmPgb1*, and accumulating NO (Dordas et al., 2003; Igamberdiev and Hill, 2004), showed increased ROS staining and expression of the *RBOH* genes. This was in contrast to plants overexpressing *ZmPgb1*, and depleted in NO (Dordas et al., 2003; Igamberdiev and Hill, 2004), which had decreased ROS staining and lower expression of the *RBOH* genes (Figures 3.10 and 3.11). This pattern agrees with recent studies in Arabidopsis during PEG-induced drought stress, where *Pgb1* down-regulators had increased expression of the *RBOH* genes (Mira et al., 2017). A similar result was also demonstrated by the same authors in maize plants exposed to low oxygen levels (Mira et al., 2016). Thus, it is suggested that one key function of Pgbs during conditions of stress is to prevent the damaging over-accumulation of ROS through NO. The link between NO production and ROS was further demonstrated through pharmacological modulations of NO. Increasing NO levels with SNP increased ROS staining, while decreasing NO reduced the ROS staining in maize leaves (Figure 3.14).

In addition to NO, ethylene is also involved in the regulation of ROS homeostasis during conditions of drought stress (Kazan, 2015). Modulation of ethylene using ETH and AOA was used to measure the production of ROS (Sharp and LeNoble, 2001; Zhang et al., 2010). A reduction in the production of ROS, estimated by decreased expression of *RBOH* genes and decreased NBT staining, was achieved by reducing ethylene with AOA (Figures 3.17 and 3.18). Conversely, an increase in *RBOH* gene expression and NBT staining was observed when

ethephon was applied to elevate ethylene (Figures 3.17 and 3.18). Thus, both manipulations of NO or ethylene are sufficient to influence ROS levels.

One tenuous link in this pathway may be to aquaporins and should be explored. Water channel proteins are vital to inter- and intra-cellular water movement. Their control has been found to be influenced by NO, and application of NO through SNP caused increased levels of aquaporin gene expression (Liu et al., 2007). Further study of how the expression of these genes change during drought stress, and their link with Pgbs under stress conditions should be conducted in the future.

It appears the results presented in this study reinforce a model for maize seedlings under drought stress conditions where overexpression of *Pgb1* protects plants. During drought stress, Pgbs scavenge nitric oxide, thus preventing the production of ethylene, and reducing the amount of damaging ROS. Down-regulation of *Pgb1* shows an extremely wilted phenotype compared to *ZmPgb1* (S) (Figure 3.3), which may be due to increased ROS levels in the plant. A figure showing this model is presented in Figure 3.20.

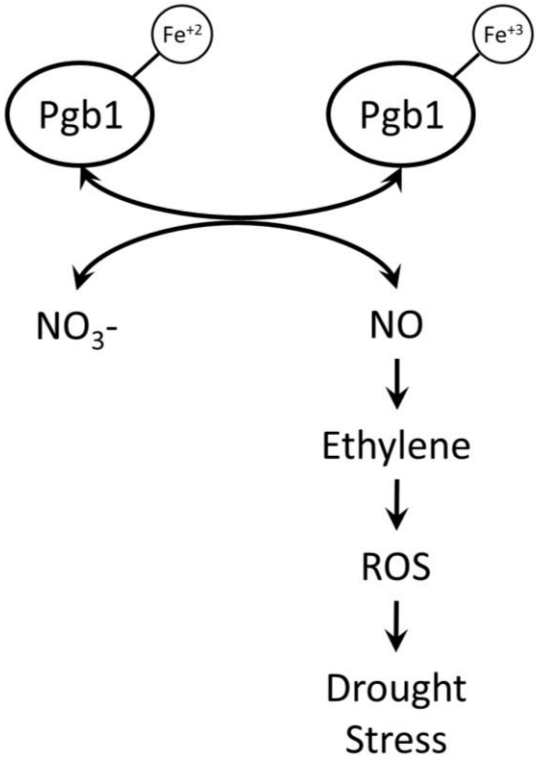


Figure 3.20. Proposed model of the involvement of *Pgb1* during drought stress responses. Overexpression of *ZmPgb1* reduces the level of active NO in the cell, thus reducing ethylene levels, resulting in decreased ROS production, reduced plant damage, and decreased amounts of plant stress.

4 GENERAL DISCUSSION AND CONCLUSIONS

Drought stress tolerance is extremely important for plants as they need to cope with unpredictable changes in environmental conditions. Development of plants that are able to withstand water stress is vital, as a rising world population calls for increased food security. Climate change causes not only drought stress, but a general unpredictability in weather patterns, causing severe environmental effects such as flooding and heat stress. Breeding plants that are not only drought-resistant but also generally stress tolerant is therefore of utmost importance. Plants with increased levels of phytoglobins (Pgbs), that scavenge NO, supress ethylene and ROS, as well as limit cell death during adverse conditions have been shown to tolerate diverse types of stress.

In this study, it was observed that over-expressing the maize *Pgb1* gene increased drought tolerance and reduced wilting when PEG was used to reproduce drought stress. This was in contrast to *Pgb1* down-regulators and WT lines which exhibited the highest sensitivity to PEG. *Pgb1* overexpressors also showed a decrease in the transcription level of ethylene biosynthesis and response genes, as well as ROS-producing NADPH oxidase genes (*RBOH*s), compared to the WT. Opposite results were observed in lines in which *Pgb1* was suppressed. Relative to WT, the expression of the same genes were induced by the *Pgb1* down-regulators. Gas chromatography analyses confirmed the transcriptional differences in ethylene biosynthesis: relative to WT, the *Pgb1* over-expressing lines had lower levels of ethylene while the *Pgb1*-downregulating lines accumulated the highest amount. A similar trend was also observed when ROS were localized through staining techniques. The heaviest staining pattern was observed in

lines suppressing *Pgb1*, while intermediate and lowest staining was noted in the WT and *Pgb1*-overexpressing lines respectively.

Modulation of NO and ethylene with pharmacological treatments showed that the effects of *Pgb1* on PEG-induced drought stress tolerance were modulated by NO. Specifically, increasing NO with SNP elevated ethylene and ROS, while application of the NO scavenger cPTIO decreased both. An experimental rise in ethylene with ethephon increased *RBOH* gene expression, as well as ROS staining in WT and *Pgb1* up-regulating plants. Decreasing ethylene synthesis with AOA showed opposing results: *RBOH* expression decreased, and ROS staining diminished in both WT and *Pgb1* down-regulating lines. These results led to the proposed model in which the NO scavenging activity of *Pgb1* reduces ethylene biosynthesis and signalling, as well as diminishing production of ROS. These effects enhance drought stress tolerance, as observed in the over-expressing lines.

These results and the proposed mechanistic model are consistent with some previous work utilizing the same lines to study flooding tolerance. It must be noted, however, that contrary to the established participation of ABA in drought stress tolerance, the level of ABA does not change among lines during the imposition of drought. The increased expression of *ABI5* measured in the *Pgb1* over-expressing plants suggests that ABA response, rather than accumulation, might participate in the enhanced drought stress tolerance under conditions of high *Pgb1* levels.

One limitation of this study is using excised shoots as compared to whole plants. When the shoots were excised the stomata may have closed. This is evidenced by the very low photosynthesis and stomatal conductance rates (Supplementary Figures 1-3). An advantage, however, of excising the shoots is in the ease of delivery of pharmacological treatments to the maize shoot, and has been performed in this way in previous studies (Zhang et al., 2011). Pharmacological treatments were the basis for the creation of the model for this study, and were very important in understanding the underlying mechanism involving Pgbs during drought stress responses in maize.

Future directions for this study would include a closer examination at some of the possible peripheral members of this pathway, such as additional ABA response genes and aquaporins, and the influence of Pgbs on their levels. Abscisic acid and ethylene signalling have been connected to the gating of aquaporins, which affects root water potential, so aquaporin gene expression and the root hydraulic conductivity should be examined over time, in a study where the plants are not excised. Some drought stress mechanisms also involve the sequestration of osmolytes into the plant cell, and an analysis of the levels of these molecules in the different lines may reveal a difference. Additional ROS staining could be used to examine how the combination of treatments modulating both ethylene and NO affects ROS production. In the farther future, phytoglobin over-expressing plants would be useful as water stress-tolerant varieties. The phytoglobin gene could also be used as a potential marker for stress tolerance to enhance breeding decision-making.

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