Effect of phytoglobin1 (Pgb1) expression on the regeneration of maize (*Zea mays L.*) root quiescent centers (QCs) during hypoxia

by

Rathnayaka Pathiranage Rajitha Gayan Lakmini

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

MASTER OF SCIENCE

Department of Plant Science

University of Manitoba

Winnipeg

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ACKNOWLEDGEMENTS

I want to start by saying how grateful I am to my graduate supervisor, Dr. Claudio Stasolla. I'm super lucky to have a well-experienced, responsible, determined, and focused mentor like you. The past two years have been very challenging for me personally and I am thankful with my whole heart for being patient and kind to me and keeping guiding me. Grazie!. I am also deeply grateful to my committee members for their valuable feedback and contributions. Thank you very much, Dr. Hill, for taking the time for discussions on my project and writing. Your appreciation motivates me very much. Merci beaucoup! Dr. Sylvie, for giving me valuable insight into my project and improving the quality of my work. I very much enjoyed your stress physiology class and thank you for your dedication to improving our writing skills. My heartiest gratitude goes to Martha, who was with me from the beginning to the end, giving me positive energy every step of the way. Wherever you are, family is home. I cannot thank my parents enough, my mom Chandra Pathiranage and my dad Ranjith Pathiranage for always loving me no matter what. Thank you for enduring this 'first time without me', but I hope did justice to the selfless sacrifices you made for me throughout my academic journey. Thank you, my loving husband Sanjaya, for taking very good care of me throughout this journey and always being my best friend. I would like to express my gratitude to Dr. Mira, for always being patient with me and offering help whenever needed and to Shimaa for her enormous help and positivity all the time. I would like to give a big shout-out to my lab mates Samadhi, Ehsanul, James, Sonia, Wisdom, Eman, Dr. Youssef and Rafael who have been so supportive, caring and believing in me constantly.

I express my heartfelt gratitude to each one of you for your unwavering support, encouragement, and faith in me. This thesis would not have become a reality without you.

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LIST OF ABBREVIATIONS

QC – quiescent center

Pgb – phytoglobin

NO – nitric oxide

NR - nitrate reductases

ROS – Reactive oxygen species

PCD – programmed cell death

WT – wild type

ZmPgb1.1(S) – maize Pgb1 overexpressed line

ZmPgb1.1(A) – maize Pgb1 downregulated line

NADPH - nicotinamide adenine dinucleotide phosphate

RBOH - respiratory burst oxidase homologs

SNAP - S-nitroso-N-acetylpenicillamine

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

ABSTRACT

Rathnayaka Pathiranage, Rajitha Gayan Lakmini. M.Sc., The University of Manitoba, March 2023. **Effect of phytoglobin (Pgb) expression on the regeneration of maize (*Zea mays* L.) root quiescent centers (QCs) during hypoxia**. Supervisor: Dr. Claudio Stasolla.

Maize (*Zea mays*) root quiescent center (QC) cells deteriorate during hypoxic treatment, halting root growth. Over-expression of the Phytoglobin1 *ZmPgb1.1* is known to alleviate these effects through the retention of the auxin flow along the root profile required for QC specification. To elucidate QC-specific hypoxia responses and whether *ZmPgb1.1* exercises a direct role on QC stem cells, we performed a QC functionality test. This was done by estimating the ability of QCs to regenerate a root *in-vitro* in a hypoxic environment. Hypoxia decreased the functionality of the QCs by depressing the expression of several genes participating in the synthesis and response of auxin. This was accompanied by a decrease in DR5 signal in oxygen deprived QCs and a consequent suppression of QC markers and genes of jasmonic acid (JA) synthesis and signaling. Over-expression of *ZmPgb1.1* mitigated all these responses. Through pharmacological alterations of auxin and JA (through 1-Naphthaleneacetic acid and methyl jasmonate, respectively), it is demonstrated that both compounds are required for QC functionality under hypoxia, and that JA acts downstream of auxin during QC regeneration. A model is proposed whereby the *ZmPgb1.1* maintenance of auxin in hypoxic QCs is determinant for the retention of their functionality, with JA supporting the regeneration of roots from the QCs.

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 Plant Physiology. This thesis is presented as a single manuscript, containing an abstract, introduction, materials and methods, results, discussion, conclusions, and future perspectives section. A literature review replaces the introduction of the manuscript, covering the broad background of research question, while supplemental figures and table are positioned after the body of the manuscript.

1. LITERATURE REVIEW

1.1. Introduction

1.1.1. History and production of maize (*Zea mays* L.)

Zea mays, commonly known as maize/corn is the third most important cereal crop in the world and is grown worldwide across a broad range of climatic conditions. Its origin is subjected to several theories, but most generally it is believed to be a wild grass that was developed into a food staple by Native Americans in central Mexico over 7000 years ago (Ranum *et al.,* 2014). Explorers of indigenous tribes in Central America and Mexico brought the plant to Latin America, the Caribbean and then the United States and Canada (Brown *et al.,* 1985). The distribution of maize to Europe was by European explorers, and later traders introduced maize to Asia and Africa (Ranum *et al.,* 2014).

Maize is a C4 grass which has adapted to a wide range of soil types, climatic conditions, and slightly acidic to neutral soil pHs (5.5-7.0) (Nafziger, 2016). Maize is a popular staple food for a substantial percentage of the world's population (Nuss & Tanumihardjo, 2010). The United States, China and Brazil are the leading producers of maize. With the rapid increase in production between 2000 and 2019, global maize production was 1.1 billion tons, becoming the most produced (dry grain: annual) crop worldwide in the 2017-2019 triennium period, accounting for 12% of world primary crops (FAO, 2021).

Currently, maize is grown in every Canadian province. Ontario, Quebec, and Manitoba produce approximately 63%, 26%, and 9% of the total national yield, respectively. Due to

increases in temperature and the development of new varieties, the potential to expand maize production in northern regions is being considered (Government of Canada, 2021; Statistics Canada, 2019). Canada exported a total of 1.41 million tonnes of maize in 2020-21, with an increase of 0.68 million tonnes from the previous year. Of this total export, around 97% was from Eastern Canada while the remaining 3% was from Western Canada (AAFC, 2021).

Maize is a popular food crop owing to its diverse functionality as a food source for both humans and animals (Nuss & Tanumihardjo, 2010). It can be processed into a variety of food sources such as starch, sweeteners, oil, and various industrial products including alcohol and ethanol for pharmaceutical, textile and cosmetic industries. The usage of maize for fuel ethanol has increased substantially over the past decade, at the expense of other uses including livestock feeding and human consumption (Ranum *et al.*, 2014). Maize kernels contain around 80% carbohydrate, 1.74% lipid and 6.2% protein, delivering about 365kcal/100g (USDA National Nutrient Database, 2020). Yellow maize contains many important vitamins except vitamin B-12. Vitamin A and E are the prevalent fat-soluble vitamins found in maize which are important antioxidants (Nuss & Tanumihardjo, 2010).

1.1.2. Climatic challenges for maize production

Changes in global weather patterns have affected crop production worldwide, posing a serious challenge to food security (Mbow *et al*., 2019). Plants are subjected to various abiotic stresses including drought, salinity, and excess moisture (Pardo, 2010). The average global surface temperature is predicted to increase, causing the water cycle to “accelerate” due to the high rate of evaporation; as a result, more extreme heavy precipitation is expected. According to climate

predictions, by 2100 rainfall will increase by more than 1% with a possible increase of up to 12%. This trend will increase flooding which has caused more than 650 USD billion in economic damage and nearly 1,420,000 causalities between 1998 and 2017 (Wallemacq *et al.*, 2018).

In Canada, and in particular Manitoba, flooding events are becoming more prominent (Babaei, 2017), and excess moisture stress is a serious threat to both national and local agriculture (Crop Report, Manitoba Agriculture, 2022). Among crops, maize is very susceptible to excess moisture; 75% of maize lost in the US between 2014-2019 was due to precipitations (Berardi *et al.*, 2019). Factors aggravating flooding damage in maize are higher temperatures and a young developmental stage of the plant limiting the growth above the water surface (Fausey *et al*., 1985; Zaidi *et al*., 2004).

Excess moisture can result in the full submergence of the plant, or soil saturation, i.e. waterlogging (Visser *et al.,* 2003). Both events, depending on their duration and severity, can compromise the growth and development of the plant, as they limit the availability of oxygen (Bailey-Serres & Voesenek, 2008). Specific terminology is utilized to define the availability of O_2. Normoxia is often described as the O_2 level close to 21%, while hypoxia refers to lowers levels of O_2 still able to sustain important biological processes, like aerobic respiration (Armstrong *et al.*, 2009; Sasidharan *et al.,* 2017). However, for experimental purposes, oxygen concentrations between 1%-5% are usually utilized to generate hypoxia (Loreti & Perata, 2020). Anoxia is referred to the complete absence of O_2 in the environment. Nevertheless, true anoxic conditions are highly unlikely in plant tissues due to O_2 generation from photosynthesis (Sasidharan *et al.*, 2017).

1.2. Excess moisture and crops

1.2.1. Hypoxia-induced damage during conditions of excess moisture

While the reduced level of O_2 in the soil could be considered hypoxic, the actual availability and requirement of O_2 in the tissue might vary considerably from cell to cell. For example, meristematic cells are by nature hypoxic due to their location and structure; thus, they rely on lower levels of oxygen to carry basal functions (Loreti *et al.*, 2016). In contrast, the high metabolic activity of proliferating cells necessitates a higher concentration of O_2; therefore, it is not surprising that within the plant body oxygen gradients form (Weits *et al.*, 2021). The diffusion of O_2 within these gradients is further influenced by the anatomical characteristics of the tissue, such that higher diffusion is for example facilitated in tissue with large intercellular spaces (Sairam *et al.*, 2008). For these reasons, O_2 availability in these tissues mainly depends on the balance maintained among its consumption, production, and diffusion (Loreti & Perata, 2020).

Excess moisture in the form of waterlogging depletes O_2 at the root surface, as O_2 diffusion is 10^4-fold slower in water relative to air (Sand-Jensen *et al.*, 2022). This condition can be further exacerbated by the consumption of available O_2 by soil microorganisms in the rhizosphere (Chérif *et al.*, 1997). Besides depleting O_2, excess moisture in the soil can result in the accumulation of CO_2 resulting from its lower diffusion in water (Greenway *et al.*, 2006). Waterlogged soils have higher CO_2 partial pressure to a maximum between 10-40 kPa. Solubility of CO_2 in aqueous media is approximately 34-fold higher than O_2 (Armstrong, 1979). This high solubility along with the substantial conversion of CO_2 to HCO_3^- further alters the soil pH (Greenway *et al.*, 2006). Soil pH has profound influences on the biological, chemical, and physical properties that affect plant

growth and biomass yield (Brady & Weil, 2008). Excess moisture also activates a sequence of changes in soil chemical and electro-chemical properties, that can result in the accumulation of ions such as Fe^{2+} and Mn^{2+} in toxic levels which is detrimental to the plant roots (Manik *et al.,* 2019). Levels of secondary metabolites such as volatile fatty acids and phenolics also become harmful to the roots in the low-pH rhizosphere. The activity of nitrifying microbe communities can be inhibited in waterlogged soils due to this low pH condition and hypoxic/anoxic condition, resulting in depleted N availability. This reduction of nitrification negatively impacts N-dependent crop productivity (Manik *et al.,* 2019). Based on this information, it is therefore apparent that the effects of excess moisture are not solely restricted to a limitation in O_2, but extend to other compounding factors influencing root growth and plant productivity.

Waterlogging effects on crops, as found in maize, would depend on waterlogging duration and growth stage. In both spring and summer maize waterlogging caused most damage at V3 stage followed by V6 stage, and grain yield was also affected (Ren *et al*., 2016; Tian *et al*., 2019). Excess moisture causes reduction in photosynthetic activity through restricted stomatal conductance (Castonguay *et al.,* 1993). Another noticeable sign of waterlogging stress is leaf chlorosis, which contributes to the early leaf senescence to facilitate the remobilization of nitrogen to new leaves. This nutrient competitiveness in the shoots is caused because of the lower nutrient uptake and transfer from the affected root system (Herzog *et al.,* 2016). Furthermore, chlorosis contributes to the decline in photosynthetic activity (Fiallo-Olivé & Navas-Castillo, 2019).

The gaseous hormone ethylene is a key signaling agent in abiotic stresses that activates key responses (Grichko & Glick 2001). Ethylene is involved in root growth and regulates the elongation of root cells in coordination with auxin (Růžička *et al*., 2007). Typically, the concentration of ethylene is a determinant for promoting or inhibiting root growth (Konings & Jackson, 1979). Ethylene accumulates in plant tissues during submergence, due to both entrapment and low gas diffusion through the water. Excessive ethylene accumulation can inhibit root elongation (Visser *et al*., 1997), and under prolonged conditions trigger cellular death compromising plant survival (Mira *et al.,* 2017). Ethylene-driven aerenchyma formation is a major morphological adaptation in plants to withstand hypoxia caused by flooding (Fukao *et al.,* 2019). The biological synthesis of ethylene precursors is also induced under low O_2 conditions (Fukao & Bailey-Serres, 2008; Grichko & Glick 2001). However, since the final step of ethylene biosynthesis is O_2 dependent, hypoxic roots are not capable of synthesizing much ethylene. therefore it is hypothesized that the intermediates produced in hypoxic roots will be transported to aerated shoots in xylem sap and works as a signal from the hypoxic root to the shoot (Jackson, 2002).

Another undesirable consequence of hypoxia is the accumulation of reactive oxygen species (ROS), which result from oxygen being reduced to different degrees. Reactive oxygen species (ROS) include free radicals such as hydroxyl ($HO^{\cdot}$) and superoxide ($O_2^{\cdot -}$) radicals, or molecules such as singlet oxygen ($^1O_2$), ozone (O_3) and hydrogen peroxide (H_2O_2) (Hancock *et al.,* 2001). Redox reactions in chloroplasts and mitochondria, and the activity of several peroxidases and oxidases such as NADPH (Nicotinamide adenine dinucleotide phosphate) oxidase and NADH oxidase are main producers of ROS in plant tissues (Saed-Moucheshi *et al.,* 2014). While ROS are considered to be a key secondary messengers of various hormone-mediated responses, if exceeding

physiological levels, they can cause protein oxidation, nucleic acid damage, and enzyme inhibition, and trigger responses leading to cell death. A typical example of ROS-induced damage is lipid peroxidation, affecting cellular membranes (Sharma *et al.,* 2012). Therefore, elevated levels of ROS in hypoxic tissue can become a significant threat to plants, disrupting their development and endangering their survival (Yordanova *et al.,* 2004).

1.2.2. Plant responses to hypoxia

Plants respond to hypoxia through a variety of adaptive responses at cellular, tissue and organism level (Loreti & Perata, 2020). Some plants adopt a low oxygen quiescence strategy by slowing down metabolic activities, and thus their growth. This is commonly observed where field flood depth is higher and the duration of the stress is short (Bailey-Serres & Voesenek, 2008). Some plants adopt the low oxygen escape strategy, usually observed in shallow flood depth conditions and under prolonged stress. It allows the shoot to grow and reach the atmosphere, facilitating gas diffusion and photosynthesis (Voesenek *et al.,* 2004). However, this strategy costs the plant a great amount of energy because it involves rapid growth of tissues (Bailey-Serres & Voesenek, 2008).

Oxygen is central for energy production, fueling metabolic activities (Dennis *et al.,* 2000). It is the final electron acceptor of the mitochondrial electron transport chain during cellular respiration. Therefore, O_2 deficiencies during hypoxia lead to low ATP: ADP ratios (Bailey-Serres & Voesenek, 2008; Perata & Alpi, 1993). In response to oxygen deficiency, plants shift from aerobic respiration to anaerobic fermentation as an adaptation, despite the lower ATP yield (36 ATP from aerobic metabolism vs. 2 ATP only from glycolysis) (Dennis *et al.,* 2000). Alcohol

dehydrogenase (ADH) and pyruvate decarboxylase (PDC) are fermentation enzymes, and plants with reduced activity of both enzymes show less hypoxic tolerance (Loreti *et al.,* 2016). Pyruvate molecules generated through glycolysis are converted to acetaldehyde by PDC and then reduced to ethanol (Perata & Alpi, 1993). Conversion of pyruvate molecules to ethanol is of utmost importance because NADH is oxidized back into NAD^+ which can sustain glycolysis. Because of these reasons, the availability of sugars and fermentation enzymes to carry out glycolysis and ethanol fermentation respectively, are major requirements for plant survival during submergence (Loreti *et al.,* 2018). Starchless *A. thaliana* mutant plants are very susceptible to waterlogging and incapable of inducing fermentation-related genes when exposed to excess moisture (Loreti *et al.,* 2018).

As indicated in the previous sections, ROS accumulate in hypoxic environments, causing oxidative damage (Hasanuzzaman *et al.,* 2017). A strategy adopted by plants to reduce the level of ROS below toxic levels is through the activation of the antioxidant system consisting of ROS scavenging enzymes such as catalase, superoxide dismutase, peroxidase, and glutathione peroxidase, as well as low-molecular-weight antioxidant metabolites such as ascorbate, glutathione, phenolic compounds and tocopherols (Garg & Manchanda, 2009; Saed-Moucheshi *et al.,* 2014). Antioxidant responses are generally depressed by oxygen deficit, and plants tolerant to stress often exhibit higher antioxidant capabilities (Arbona *et al.*, 2008; Yordanova *et al.*, 2004).

At a structural level, the most characterized responses of plants to hypoxia are the formation of aerenchyma and adventitious roots: two major adaptations of plants to waterlogging (Fukao *et al.,* 2019). Aerenchyma are empty channels generated by cellular degeneration which facilitate oxygen supplies to the hypoxic tissues (Kozlowski, 1984). In major crops such as wheat, maize

and barley, aerenchyma formation is induced by both endogenous and exogenously supplied ethylene, which triggers programmed cell death (PCD) in cortical cells of the root (Drew *et al.,* 1979). Formation of aerenchyma is often concurrent with the protrusion of adventitious roots, specialized roots that arise at the base of the submerged shoot, often growing above the water level (Fukao *et al.,* 2019). Adventitious roots, common in hypoxic maize, *Oryza sativa* (rice) and *Triticum aestivum* (wheat) are characterized by highly porous cortical tissue and the presence of schizogenous or lysigenous aerenchyma (Visser & Voesenek, 2005). Schizogenous aerenchyma formation involves differentiated development of cells to generate spaces compared to the cell death involved in lysigenous aerenchyma formation (Evans, 2004). Both ethylene and auxin contribute to the initiation of adventitious roots, even though the detailed mechanisms are still not fully understood (Zhang *et al.,* 2018).

The O_2 delivered to the root apex by the aerenchyma is prone to radial diffusion and is often lost into the rhizosphere. To avert this loss, some plants develop radial O_2 loss (ROL) barriers, anatomical root structures consisting of oxygen-impermeable layers due to the deposition of lignin and suberin (Armstrong & Armstrong, 1988). The relevance of ROL barriers for waterlogging tolerance is best exemplified in *Zea nicaraguensis* (teosinte), a wild relative of maize, which unlike maize can grow in water-saturated soil due to the formation of extensive ROL barriers (Abiko *et al*., 2012). Additional adaptations to hypoxia include the thinning of leaf cuticle, the elongation of shoot and petioles, and nastic movements to better optimize gas exchanges between plant and atmosphere (Bailey-Serres *et al.,* 2012).

1.2.3. Nitric oxide in hypoxic responses

As indicated in the previous section, many of the plant responses to hypoxia, whether they involve morphological or physiological modifications, are controlled by plant hormones. Hormone synthesis and responses in plants are mediated by signal molecules, the most relevant of which in the context of hypoxia is nitric oxide (NO) (Hill, 2012). In animals, NO is produced during hypoxic stress and is involved in blood vessel capillary dilation (Fago & Jensen, 2015). Nitric oxide accumulates in tissues of oxygen-stressed plants (Wany *et al.,* 2017) where it can have noxious effects if generated at high levels (Dordas *et al.,* 2003). Unlike the animal system, synthesis and signaling of NO in plants are not fully understood (Astier *et al*., 2018).

In plants, NO can be produced in several ways; the most prominent of which occurs through the action of nitrate reductases (NR), nitrite reductases and other molybdenum cofactor-dependent enzymes (Leon & Costa-Broseta, 2020). It has been hypothesized that the mitochondrial electron transport chain can also act as an alternative reducing agent (Gupta *et al.,* 2011), where nitrite (NO_2^-) is reduced to NO at complex III and IV (Igamberdiev *et al*., 2010). This latter process is believed to be highly influenced by hypoxia due to the reduced availability of oxygen (Leon & Costa-Broseta, 2020). Production of NO occurs in several cellular compartments: in the cytoplasm, through the nitrate-dependent NO synthesis catalyzed by cytosolic NRs, in the peroxisomes through the arginine-dependent pathway catalyzed by NO synthase (NOS) like enzymes (Leon & Costa-Broseta, 2020), and in the mitochondria (Gupta *et al.,* 2011). Additionally, with the help of *in-vitro* exposure assays, Jasid et al. (2006) demonstrated two NO producing pathways functioning in chloroplasts, one dependent on NOS-like enzyme activity and the other one dependent on nitrite.

The primary action of NO on plant metabolism is through post-translational modifications (S-nytrosylations) of proteins. This alters the structure, function, activity, and subcellular location of proteins (Leon & Costa-Broseta, 2020). In *Arabidopsis* the action of NO donors affected about 700 transcription factors involved in various functions such as protein degradation, hormone signaling and abiotic and biotic stress responses (Imran *et al.*, 2018; Leon & Costa-Broseta, 2020). Thus, the effects of varying levels of NO are very extensive and alter a wide variety of processes ranging from pollination, flowering, leaf senescence, stomatal closure, root growth, seed dormancy and seed germination. Many of these NO-controlled processes are mediated by hormones. For example, NO is a negative regulator in the ABA signaling pathway by promoting either inactivation of the ABA receptors or the degradation of positive regulators (Leon & Costa-Broseta, 2020). Nitric oxide also participates in root development, in some cases as a downstream component of auxin responses (Chen *et al.*, 2010), while in others as a regulator of auxin synthesis and transport (Sanz *et al.*, 2014). Many NO-responses are dependent upon its concentration. For example, overall plant growth in several species including *Solanum lycopersicum* (tomato), *Pisum Sativum* (pea) and *Lactuca sativa* (lettuce), is induced by low levels of NO, but inhibited at high concentrations (Sami *et al.*, 2018).

Plant responses linked to excess moisture are also modulated to some extent by NO. Exogenous application of NO induces adventitious roots in cucumber and plays a key role in the formation of lateral roots in tomato (Correa-Aragunde *et al.*, 2006). Similarly, aerenchyma formation is influenced by the level of NO. Nitric oxide induces programmed cell death (PCD) leading to aerenchyma formation by promoting cortical cell death, cell wall disintegration, cell shrinkage and dismantling, causing a radial assembly of aerenchyma. Wheat seedlings supplemented with 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO: a NO scavenger)

showed a reduction in aerenchyma formation, indicating the involvement of NO in hypoxia adaptation (Wany *et al.,* 2017). Ethylene is closely involved in aerenchyma formation (Zhang *et al.,* 2018) and NO induces the expression of ethylene biosynthetic genes l-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase also during hypoxia. Supplementation of cPTIO reduced the ethylene levels in hypoxic wheat seedlings showing the direct influence of NO on ethylene synthesis. Another essential component of PCD that modulates aerenchyma formation is ROS (Steffens *et al.,* 2011). A major source of ROS is NADPH oxidase and NO induces the expression of the ROS-encoding gene - respiratory burst oxidase homologs (*RBOHs*), suggesting a strong relationship between NO and ROS during hypoxia (Wany *et al.,* 2017).

The detrimental role of excessive levels of NO, accumulating during hypoxia, has been extensively documented in relation to the degradation of the meristematic cells at the maize root pole (Mira *et al.,* 2016). In the same study, retention of meristematic integrity was re-established following a depletion of NO by cPTIO. The meristem deterioration in hypoxic roots was in part explained by the NO-reduction of auxin transport at the root tip (Mira *et al.,* 2016), which is a requirement for the proper function of meristematic cells (Fernández-Marcos *et al.,* 2011). Root degradation was observed in hypoxic barley roots accumulating NO (Dordas *et al.,* 2003), and also in other instances when NO accumulates as a result of other types of abiotic stress (Desikan *et al.,* 2002; Mira *et al.,* 2017; Zhao *et al.,* 2009). Altogether, it appears that the root apical meristem (RAM) is vulnerable to hypoxia, and the ability to modulate NO in meristematic cells can be employed as a strategy to diminish the negative effects of low oxygen on root growth.

1.3. Root apical meristem (RAM)

1.3.1. General structure and function

Plant development largely depends on the pluripotent stem cells in meristems (Strotmann & Stahl, 2021). In both animals and plants, stem cells are characterized as undifferentiated cells with self-renewing capabilities. They generate two daughter cells, one of which will replace the mother cell and the other will differentiate (Alvarado & Yamanaka, 2014). The two main apical meristems in plants are the shoot apical meristem (SAM) generating the aerial organs, and the root apical meristem (RAM) at the root apex (Strotmann & Stahl, 2021).

In the root, the RAM is subtended by the root cap, and occupies the most apical domain of the root, below the elongation and maturation zones (Desvoyes *et al.,* 2021). Cells produced by the RAM are displaced along the root profile; after a few rounds of cell division, they elongate and differentiate. In *A. thaliana,* apical meristem formation begins with the very first zygotic division. The first asymmetrical division of the zygote forms two daughter cells that diverge in size and fate, forming the primary apical–basal axis of the embryo. The hypophysis, the uppermost cell that is derived from the basal daughter cell, gives rise to the root meristem organizer: quiescent center (QC) and the columella initials (Yamoune *et al.,* 2021). The stem cells in the root are in a unique microenvironment referred to as the stem cell niche (SCN) (Aida *et al.,* 2004), providing support and stimuli essential to maintain stemness (Smith, 2006). The SCN is composed of a quiescent center (QC) and surrounding initials. The model of a multicellular “cytogenerative center” located within the RAM, which generates all the root cells while functioning as a source of cell regeneration reservoir included in the RAM, was first proposed by Clowes in 1953, based on his

experiments and observations on *Fagus sylvatica* (European beech) and *Vicia faba* (fava bean) roots. Clowes later renamed it as “quiescent center” due to its slower proliferative nature, which he observed in the RAM of maize (Clowes, 1953, 1956a). Quiescent center is comprised of mitotically inert cells that are weakly stained and lack vacuoles. This group of cells is surrounded by mitotically active, densely stained stem cells (Ubogoeva *et al.*, 2021). One of the key roles of the QC is to maintain the surrounding initial cells in an undifferentiated state (van den Berg *et al*., 1997). The characteristics of the QC are very different from that of the surrounding initials. Quiescent cells are characterized by low RNA and protein content, as well as by reduced RNA polymerase activity (Kerk & Feldman, 1995), and lower DNA synthesis rate compared to the adjacent initials (Clowes, 1956a). Cells within the QC spend a considerable amount of time in the G1/S phase and undergo divisions every 180-200 hours, which is 3–10 times slower than the other mitotically active root cells (Jiang *et al.*, 2003).

The number of root quiescent cells varies among species, ranging from 4 in *Arabidopsis* to about 800-1000 cells in maize (Jiang *et al.*, 2003). Due to the large number of QC cells, maize roots are more amenable to studies investigating the characteristics of these unique groups of cells. The overall root architecture of maize has some peculiar characteristics. One of the striking features is the distinct boundary that distinguishes the root cap from the rest of the root apical tissue. This appearance is determined by thick cell walls in the region, forming the closed-type RAM (Barlow, 1974). The walls of the quiescent center cells of maize are also quite thick while the cell wall thickness of root cap initials and the other meristematic sections change with age. Cytoplasmic organelles differ in number, size, shape, and quantity along the root axis. Mitochondria and plastids per unit volume of cytoplasm decrease while the density of mitochondria and golgi bodies increase along the root (Clowes & Juniper, 1964). Cells in the QCs

divide approximately every 170 hours, while the surrounding initials anywhere between 10-25 hours (Kerk & Feldman, 1995).

1.3.2. Maintenance of the QC: the role of auxin

Auxin is one of the best-studied substances that has been shown to affect root meristem characteristics. It is known to guide the patterning of the embryonic root from the very first mitotic divisions of the zygote (Jiang & Feldman, 2005). Establishment of an auxin maximum in the root apex is an early obligatory step in SCN (and QC) specification. Mutation of some genes mediating the auxin responses such as *BODENLOS, MONOPTEROS* and *AUXIN RESISTANT* results in the inability to specify the SCN and form a functional QC and RAM (Jiang & Feldman, 2005). Moreover, extended exposure of *Arabidopsis* roots to auxin transport inhibitors causes variations in QC location (Friml *et al.,*2002; Sabatini *et al.,*1999). This evidence confirms the significant role of auxin, and in particular its basipetal transport along the root profile, in SCN specification.

In *Arabidopsis* embryogenesis, an auxin maximum is initially detected in the hypophysis, the precursor of the QC, coincidentally with the expression of QC-specific marker genes (Sabatini *et al.,* 1999). The establishment of this auxin maximum is the consequence of a relocation in the auxin efflux proteins “PIN-FORMED” (PIN), regulating its transport. In particular, expression and localization of PINs are closely related to root specification, as their dysfunction evokes aberrations in the QC (Jiang & Feldman, 2005). Mutant analyses further support the role of auxin. Development of the QC in mutants of the *HOBBIT* gene (*hbt*) is disrupted by inaccurate hypophysial development. This structural defect is also linked to auxin responsiveness as DR5::GUS auxin reporter gene expression is diminished in the *hobbit* mutant, while the repressors

of auxin responses AXR3/IAA17 are induced (Willemsen *et al.*, 1998). The basipetal auxin movement regulated by PIN1 in cortical cells is determinant for the formation and maintenance of cells in the QC; *pin1* mutants fail to accumulate auxin at the root tip and form abnormal QCs (Blilou *et al.*, 2005). Similarly, defects in PIN4 result in altered auxin gradient in the root and defects in both embryonic and post-embryonic RAMs (Friml *et al.*, 2002). The role of PIN7 has been studied in relation to the early stages of root specification, and its mutation causes disordered divisions of the hypophysis during embryo development (Jiang & Feldman, 2005). In *pin1pin4pin7* triple mutants, the auxin maximum and the starch granules that denote the differentiated columella cells are shifted proximally, resulting in abnormal root patterning (Blilou *et al.*, 2005). From these studies it is therefore apparent that auxin is not only required for the formation of the embryonic QC, but also for QC maintenance during post-embryonic development.

One of the functions of auxin in QC cells is to generate a highly oxidized environment which is necessary to maintain the state of quiescence (Jiang & Feldman, 2005). In the QC, auxin reduces the level of ascorbic acid by elevating the activity of ascorbate oxidase (Kerk & Feldman, 1995). As ascorbic acid is essential in the transition from the G1 to S phase of the cell cycle (Liso *et al.*, 1988), its depletion represses cell proliferation. In the context of low oxygen stress, Mira *et al*. (2020) demonstrated that the hypoxic deterioration of the QC is a consequence of the abolishment of the oxidized environment (and accumulation of ascorbic acid), as a result of impaired auxin supply to the root. Regeneration of an oxidized environment through supplementations of either auxin or dehydroascorbate was sufficient to maintain functional QCs in hypoxic roots (Mira *et al.*, 2020). The action of auxin is intimately linked to jasmonic acid (JA), another important plant growth regulator which is discussed in the next section.

1.3.3. Specific roles of jasmonic acid in root development

Jasmonic acid is a key hormone involved in responses to biotic and abiotic stresses of plants. The *opr7opr8* double mutant showed high susceptibility to "damping-off" disease caused by the soil-borne oomycete *Pythium* spp. in maize seedlings (Yan *et al.,* 2012), JA action is closely related to the function of the RAM. Specifically, JA is implicated in the restoration of root tissue following physical damage. The level of JA increased upon laser ablation of QC and a delay in forming new cells was observed in JA-receptor mutants (*coi1*) (Zhou *et al.,* 2019). The root "restorative" effect of JA is mediated by the *RETINOBLASTOMA-RELATED-SCARECROW-ETHYLENE RESPONSE FACTOR115* (*RBR-SCR-ERF115*) protein network, with ERF115 playing a central role (Heyman *et al.,* 2016). When the QC was laser ablated, mutants of JA reception (*coi1*) showed lower induction of *ERF115* compared to WT, while overexpression of *ERF115* accelerated the regeneration of new cells and formation of ablated tissues. Furthermore, following disruption of the RBR-SCR protein network the newly formed cells occupying the region of the ablated QC failed to express the QC marker genes *WUSCHEL-RELATED HOMEOBOX 5 (WOX5)* and *SCARECROW (SCR),* the function of which will be described in the next section (Zhou *et al.,* 2019).

The JA regeneration of root tissue through *ERF115* is mediated by *MYC2,* a well characterized intermediate of JA signaling (Chen *et al.,* 2011). Following an increase in JA, MYC2 accumulates and binds to the promoter of *ERF109.* Induction of *ERF109* by *MYC2* elevates the expression of *ERF115* triggering the cell regeneration steps (Zhou *et al.,* 2019).

The role of JA in the maintenance of root cells is related to auxin, with two observations reinforcing this notion (Blilou *et al.*, 2005). First, it is suggested that auxin, with JA, contributes to the elevation of ERF115, as evidenced by the enhanced *ERF115* promoter activity by IAA, and by the initial increment in *ERF115* expression in JA-treated protoxylem cells, known to be particularly enriched in auxin (Zhou *et al.*, 2019). Second, auxin and JA regulate stem cell quiescence and maintenance by acting on the QC marker *PLETHORA (PLT)* in an antagonistic fashion (Chen *et al.*, 2011). A preliminary mechanistic model integrating auxin with the JA - mediated regenerative response following physical damage of the RAM has been proposed by Zhou *et al*. (2019) (Fig. 1).

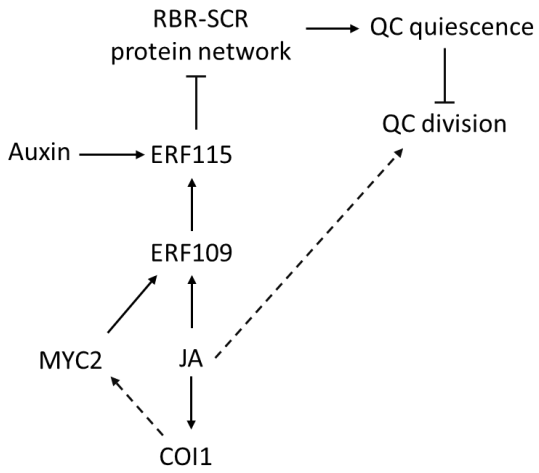


Figure 1. Induction of QC division by JA. The figure is adapted from Zhou *et al.* (2019).

1.3.4. Molecular regulation of root apical meristem (RAM)

The formation and regulation of the RAM by auxin and JA are underpinned by a complex genetic network, involving *WUSCHEL-RELATED HOMEOBOX 5* (WOX5), *SHORT-ROOT*

(SHR), SCARECROW (SCR), PLETHORA (PLT), and *AUXIN RESPONSE FACTORS (ARF).* The role of these genes will be described in more detail.

The *WUSCHEL-RELATED HOMEOBOX 5* (*WOX5*) is a marker for QC formation during embryogenesis, and its specification during post-embryonic growth (Pi *et al.,* 2015). The gene is initially expressed in the hypophysis of early globular embryos; as the hypophysis divides, *WOX5* transcripts become restricted to the upper lens-shaped cell, which is the direct progenitor of the QC (Haecker *et al.,* 2004). In *Arabidopsis*, WOX5 mRNA can be detected in the 4-cell QC throughout the heart stage and bent cotyledon stage embryos. One of the primary roles of WOX5 is the autonomous prevention of QC divisions, maintaining the cells in their quiescent nature so that the characteristics of stem cells are maintained (Forzani *et al.,* 2014). Differentiation of columella initials, as a result of QC proliferation, is a characteristic of *wox5-1* mutants (Pardal & Heidstra, 2021). Moreover, ectopic expression of *WOX5* causes the development of multiple undifferentiated columella cell layers (Pi *et al.,* 2015). The function of WOX5 in maintaining a state of quiescence is mediated by cyclin activity. Plant cell cycle is regulated by the *RETINOBLASTOMA-RELATED* (*RBR*) gene, which is inactivated when phosphorylated by cyclin–cyclin-dependent kinase (CYC–CDKs), allowing the progression of the cell cycle (Boniotti & Gutierrez, 2001). It has been shown that CYCLIN activity is inhibited in the QC by WOX5. The ectopic expression of *CYCLIN D3;3* (*CYCD3;3*) regulated by the *WOX5* promoter induces transverse divisions in the QC and may result in potential loss of QC identity (Forzani *et al.,* 2014; Sabatini, 2003). This suggests that the proper expression of *WOX5* in the QC is essential in maintaining both quiescence and the correct cellular identity of the cells in the QC (Forzani *et al.,* 2014). Despite evidence suggesting that *WOX5* expression is limited to the QC, WOX5 protein has shown intercellular movement to the adjacent columella initials, regulating the distal stem cells

and conferring an undifferentiated state (Pi *et al.,* 2015; Sarkar *et al.,* 2007). However, the mobility of WOX5 protein was recently questioned by Berckmans *et al.,* (2020). Regardless, WOX5 is a significant regulator in root SCN maintenance, by conferring a quiescent state to the QC cells (Strotmann & Stahl, 2021).

Stem cell patterning at the root pole is also linked to the expression of several transcription factors. *SHORT-ROOT* and *SCARECROW*, included in the GRAS family, are two important transcription factors required for QC specification and especially linked to the correct positioning of the QC (Sabatini *et al.,* 2003). They regulate the asymmetric division of the ground tissue, generating cortex and endodermis. During *Arabidopsis* embryogenesis, *SCR* is initially expressed in QC progenitor cells and later moved to the ground tissue initials (Wysocka-Diller *et al*., 2000). *SHORT-ROOT* is initially expressed in the vascular tissue, but moves as a protein to the QC, endodermis, and ground tissue initials (Nakajima *et al.,* 2001). *SHORT-ROOT* stimulates the expression of *SCR*, confining it to the QC, endodermis, and ground tissue initials, thereby specifying a single layer of endodermis (Cui *et al.,* 2007). Thus, failure to express either transcription factor results in a single layer of ground tissue. The single mutants of these genes have dysfunctional QCs, characterized by a disorganized SCN, starch accumulation in the columella initials, and expression of QC identity markers such as *QC25* and *QC46* at unusual sites (Pardal & Heidstra, 2021). The combined activity of SHR and SCR specifies the complete layer encompassing the pro-vascular tissue of the root and is essential for QC specification (Aida *et al.,* 2004).

Another essential transcriptional factor for QC specification and maintenance is the *PLETHORA* (*PLT*). The gene is first expressed in the basal embryo region, then in the embryonic

root primordium, and later in SCN of the root apex (Aida *et al.,* 2004). Single *PLT* mutations normally do not affect RAM structure, but *plt1:plt2* double mutation reduces the size of the meristem through terminal differentiation. Consistently, the expression of QC identity markers *QC25*, *QC46*, and *QC184* are impaired in these double mutants, and starch accumulation in the columella initials can be observed (Aida *et al.,* 2004). Moreover, development of a primary root is completely inhibited in *plt1:plt2:plt3* triple mutants (Aida *et al.,* 2004; Galinha *et al.,* 2007). Even though *plt1:plt2* double mutants show defects in the QCs similar to those observed in *shr* and *scr* mutants, the expression of *SHR* and *SCR* are unaltered in *plt1:plt2* double mutants. This suggests that *PLT* operates through mechanisms different from those of *SHR* and *SCR* (Aida *et al.,* 2004; Pardal & Heidstra, 2021; Santuari *et al.,* 2016). The PLT regulation of the RAM is closely linked to the PIN-mediated auxin transport, as *PLT* transcripts distribution is regulated by the auxin maximum at the root pole (Aida *et al.,* 2004). Specifically, *PLT* transcript accumulation can be increased through application of auxin and is dependent on auxin response factors (ARF) (Scheres *et al.,* 1994). Furthermore, PLT fine-tunes auxin maximum by regulating the transcription of *PIN1* through feedback mechanisms (Jiang and Feldman, 2005). These observations support a model in which PLT fine tunes auxin action at the QC.

Given the involvement of auxin in root development and QC specification, it is not surprising that ARFs contribute to the regulation of in the QC. AUXIN RESPONSIVE FACTORS (ARFs) bind to auxin response elements (AuxRE) in promoters of target genes, modulating auxin response (Teotia *et al.,* 2008). The *Arabidopsis* genome comprises 23 ARFs and their mutants have revealed their corresponding regulatory developmental processes (Li *et al.,* 2016). For example, ARF5 is essential for lateral root formation and the initiation of primary root in early embryogenesis (Goh *et al.,* 2012), while *ARF7* and *ARF19* activate early auxin response genes in root tissues. In

arf7:arf19 double mutants hypocotyl and root development is compromised (Wilmoth *et al.*, 2005). Among ARF members directly related to QC functioning is *ARF10*, which reduces the SCN domains and promotes the differentiation of columella initials (Wang *et al.*, 2005). Expression studies of ARFs have also been conducted in maize, which contains 35 ARFs (Liu *et al.*, 2011). Of these, *ZmARF5* and *ZmARF7a* have shown overlapping enhanced expression in QC alongside *ZmAUX1* (an auxin influx carrier gene) and *ZmPLT*. The expression levels of *ZmARF2, ZmARF6, ZmARF8* and *ZmARF10* were found to be similar in QCs and proximal meristems (Jiang *et al.*, 2010). In the same study, it was reported that decapping of QC substantially increases the expression of *ZmAUX1* and *ZmPLT* but decreases the expression of *ZmARF7a*. All together, these studies suggest that formation and maintenance of the QC are regulated by coordinated gene expression involving many components, with auxin playing a central role. Expression domains of the major genes discussed in RAM are shown in Figure 2.

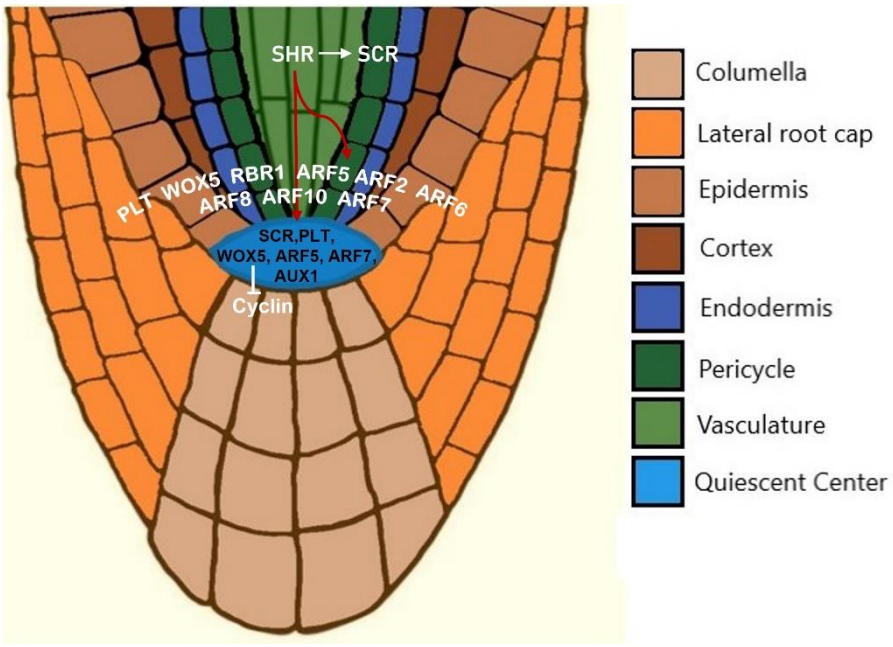


Figure 2. Structure of the root apical meristem, showing the quiescent center and surrounding tissue types. Expression domains of relevant genes is also shown.

1.4. Phytoglobin

1.4.1. Phytoglobins: key regulators in development and stress

Phytoglobins (Pgbs) are plant proteins related to globins, found in archaea, bacteria and eukarya, all characterized by a structure containing a heme (Fe) group (Freitas *et al.,* 2004). Vertebrate red blood cells have hemoglobin, a heme-containing tetramer globin protein that binds O_2 molecules and facilitates respiration (Virtanen & Laine, 1946), while muscles contain myoglobin, a monomer globin protein involved in O_2 storage rather than O_2 transport (Appleby, 1992). Leghemoglobin, which is stimulated and produced in legume plants during the symbiotic relationship with nitrogen-fixing bacteria, was the only known hemoglobins-like protein reported in plants for a long time. Later scientists documented that hemoglobin-like proteins exist in non-legumes as well (Appleby *et al.,* 1983); they were termed "nonsymbiotic hemoglobins (nsHbs)" to distinguish them from leghemoglobin. The nonsymbiotic hemoglobins are ancestral to the symbiotic hemoglobins and are more prevalent throughout the plant kingdom. They have a higher affinity for O_2 than symbiotic hemoglobins (Arredondo-Peter *et al.,* 1998). Later the term "Phytoglobins" was proposed by Hill *et al*. (2016) for these "nonsymbiotic hemoglobins", considering the need for a definitive logical name for these proteins, differentiating them from Hbs, but preserving the correspondence to their origin and characteristics. Based on phylogenetic studies, 6 groups of plant hemoglobins have been initially identified (Garrocho-Villegas & Arredondo-Peter, 2008). They were subsequently changed with the new phytoglobin nomenclature introduced in Hill *et al*. (2016) as follows (Table 1).

Table 1: Current nomenclature for plant hemoglobins (Hill *et al.,* 2016)

Previous name and abbreviation	New nomenclature
Nonsymbiotic hemoglobin (nsHb)	Phytogb0
Class/type 1 nonsymbiotic hemoglobin (nsHb-1)	Phytogb1
Class/type 2 nonsymbiotic hemoglobin (nsHb-2)	Phytogb2
Symbiotic hemoglobin (symHb)	SymPhytogb
Leghemoglobin (Lb)	Lb
Class/type 3 nonsymbiotic hemoglobin/ Truncated hemoglobin (tHb)	Phytogb3

Of these 6 types, Pgb1 (Phytogb1), Pgb2 (Phytogb2) and Pgb3 (Phytogb3) (class1/class2/class3, respectively) will be described in more detail.

Both monocots and dicots contain Pgb1, while Pgb2 is exclusive to dicots. Phytoglobin 3 is found in algae and land plants (Hill *et al.,* 2016). Among the three classes, class1 Pgbs have the highest affinity for O_2, while class 3 has the lowest. Because of this high affinity, Pgbs1 are capable of binding to O_2 in very low concentrations and are not considered oxygen transporters (Stasolla & Hill, 2017). Rather, their major role is to scavenge NO (Hill, 2012), especially during conditions of stress, as demonstrated in maize roots (Mira, *et al.,* 2016). The regulation of NO by Pgb occurs through the Pgb/No cycle (Fig. 3). Phytoglobin binds O_2 becoming oxygenated-Pgb (Fe^{2+}) which, in the presence of NO, will rapidly react to form nitrate (NO_3^-) and metphytoglobin (Fe^{3+}). The conversion of metphytoglobin back to Pgb is catalyzed by a metphytoglobin reductase, oxidizing NADH to NAD^+ (Dordas, 2009; Gupta *et al.*, 2011; Igamberdiev & Hill, 2004).

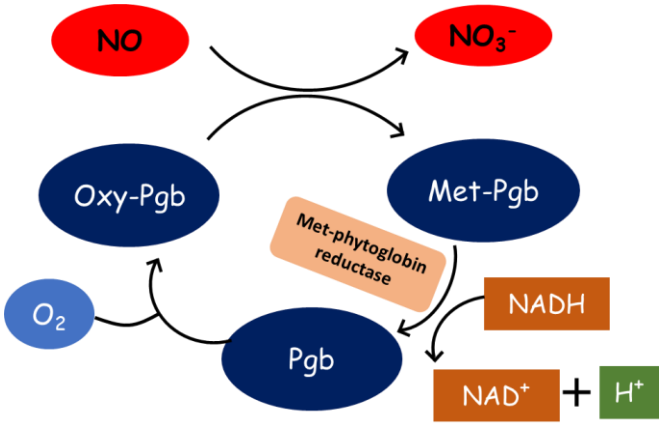


Figure 3. Pgb/NO cycle. The figure is adapted from Dordas, 2009; Gupta *et al.*, 2011; Igamberdiev & Hill, 2004. Oxy-Pgb- oxygenated Pgb; Met-Pgb- metphytoglobin

1.4.2. Phytoglobins roles in growth and development

Even though Pgbs are widely studied for their participation in stress responses, it is suggested that they play a role in ordinary plant growth and development and are expressed in various plant tissues. Bogusz *et al*. (1990) reported that Pgbs are expressed in root meristems of *Parasponia andersonii* and *Trema tomentosa* (Poison peach), and in the vascular cylinder of transgenic *Nicotiana tabacum*. In both *A. thaliana* and rice, class 2 Pgb genes (*ahb2 and rhb2*, respectively) are expressed in leaves while the class1 Pgb genes (*ahb1 and rhb1*, respectively) are expressed both in roots and leaves (Arredondo-Peter *et al.*, 1997a; Trevaskis *et al.*, 1997). Class1 Pgb in *A. thaliana* (*Glb1*) is strongly expressed in root tips, while in germinating seeds it is expressed at the base of the hypocotyl from where root system develops. In the shoot, it is expressed in leaf hydathodes and the apical and auxiliary meristems. The strong *Glb1* expression in dormant and immature meristems tends to disappear as differentiation occurs (Hebelstrup *et al.*, 2013;

Heckmann *et al.,* 2006). Furthermore, mutation of *Glb1* evoked phenotypic deviations which are correlated to the accumulation of NO (Hebelstrup *et al.,* 2006).

More recent studies suggest the participation of Pgbs in cell fate acquisition, as documented for example during *in-vitro* embryogenesis. Suppression of *Pgb2* induces the somatic-embryogenic transition in *Arabidopsis* through changes in auxin synthesis and transport (Elhiti *et al.,* 2013). These effects were mediated by jasmonic acid (JA), with NO increasing the expression of JA biosynthetic genes suppressing *MYC2* and inducing *JAZ1* (Mira *et al.*,2016). During maize somatic embryogenesis, suppression of either *Pgb1* or *Pgb2* triggers program cell death (PCD) and ultimately determines the number of somatic embryos produced in culture (Huang *et al.,* 2014). Based on these results, it was suggested that Pgbs might have an important function in cell fate, and specifically determine whether cells remain undifferentiated, or initiate the differentiation process.

1.4.3. Phytoglobins roles in stress responses

The function of Pgbs was initially studied in relation to stress, as their expression is highly induced by biotic and abiotic stressors. These include rhizobial infection, osmotic stress, hypoxia, nutrient deficiencies, low temperature stress and NO exposure (Dordas, 2009; Qu *et al.,* 2006; Sasakura *et al.,* 2006; Wang *et al.,* 2003). The role of Pgbs is however mostly documented in relation to hypoxic stress, where the proteins play a protective role (Hunt *et al.,* 2002; De Castro *et al.*, 2022; Mira *et al.*, 2016, 2020, 2021). This concept has been demonstrated in several species. In *A. thaliana*, overexpression of *Pgb1* increased seedling survival during 48h hypoxia treatment. The same seedlings had higher after-treatment survival rates (up to 63–77%) relative to WT (8–

27%) (Hunt *et al.*, 2002). Sustained growth and retention of photosynthetic parameters were also reported in waterlogged barley (De Castro *et al*., 2022) and maize (Youssef *et al.*, 2016), as well as submerged soybean plants (Mira *et al.*, 2021). These effects were attributed to the reduced ROS-induced damage (through activation of the antioxidant machinery) in photosynthetic tissue (Youssef *et al.*, 2016).

The protective function of Pgb1 has been described in more detail in roots, the first organs to perceive waterlogging (and hypoxic stress). In maize, one of the first events triggered by low oxygen is the deterioration of the QC, with the subsequent differentiation of the surrounding initials, followed by the arrest of root growth (Mira *et al.*, 2016). These phenotypic defects are underlined by reduced auxin transport (as a result of PIN miss-expression), depletion of the auxin maximum at the root tip causing the activation of the QC, and ROS-induced cell death (Mira *et al.*, 2016). Over-expression of *Pgb1* alleviated these effects; the retention of PIN1-auxin transport, auxin activity at the RAM, and a reduction in ROS (through activation of the antioxidant system) contributed to the maintenance of a functional QC and increased root survival (Mira *et al.*, 2016; 2021). These results agree with the role of NO in suppressing auxin transport to the root tips, as documented in *Arabidopsis* (Fernández-Marcos *et al.*, 2011), and the observation that Pgb1 protein is preferentially localized in mature tissue above the QC, in regions overlapping with PIN1 (our lab, unpublished data). The viability of the maize QC during hypoxia was further estimated by the ability of QCs, dissected from hypoxic roots, to regenerate and form roots under normoxia conditions. As expected, the higher regeneration occurred in QCs over-expressing *Pgb1* (Mira *et al*., 2020).

1.5. Aims of the thesis

The only study on the effect of QC function during hypoxia is that of Mira *et al*. (2020). The study demonstrated that QCs dissected from hypoxic roots lose their functionality, estimated by their ability to regenerate roots under normoxic conditions. The QC reduction in viability was mitigated when Pgb1 was over-expressed, and exacerbated when the same gene was suppressed (Mira *et al.*, 2020). This experimental design, however, is not suitable to determine if the viability of the QC during low oxygen stress depends on the presence of mature tissue, as the QCs were dissected only after the hypoxic treatment was imposed to the whole root. Can QC stem cells respond to hypoxia independently from the more mature cells? Can the level of Pgb1 in QC cells influence their response to the stress? Are these responses mediated by auxin, JA, and NO?

To address these questions, in this thesis the QCs were first isolated from the root grown under normoxic conditions, and then allowed to regenerate roots under hypoxia. This approach allows to examine the response of the QC to low oxygen in the absence of the mature root tissue. Furthermore, the role of auxin, JA and NO during the regeneration process in collaboration with the Pgb1 was analyzed through gene expression and pharmacological studies.

2. MATERIAL AND METHODS

2.1. Plant material

Maize (*Zea mays* L.) plants overexpressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1* were previously characterized in Mira *et al*. (2016) and Youssef *et al*. (2016). Relative to the WT, *ZmPgb1.1* expression was about 10 times higher in roots of the ZmPgb1.1(S) line, and about half in those of the ZmPgb1.1(A) line (Youssef *et al*., 2016).

2.2. QC functionality test

Maize seeds were sterilized with 50% bleach and germinated on moistened filter papers in petri dishes in dark for 2 days under ambient air (normoxia). Dissection of the QCs and culture conditions were those used by Feldman (1976). Briefly, QCs were rapidly dissected from the roots and cultured on Murashige and Skoog medium with Gamborg vitamins supplemented with kinetin (0.1 mg/L). The QCs were allowed to regenerate a root either under normoxia (ambient air), or hypoxia (4% oxygen), as reported by Mira *et al*. (2020). The number of QCs able to regenerate roots was recoded after 5 days in culture. Experiments were conducted in three biological replicates, each containing a minimum of 10 QCs.

2.3. Pharmacological treatments

For pharmacological treatments, the following chemicals were added in the QC regeneration medium: the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (10 μM), the NO donor S-nitroso-N-acetylpenicillamine (SNAP) (10 μM), 1-Naphthaleneacetic acid (NAA) (2 μM), the auxin biosynthetic inhibitor (2S)-2-Amino-4-(2-aminophenyl)-4-oxo-butanoic acid (L-kynurenine, LK) (2 μM), Jasmonic acid (JA) (1 μM), and the JA biosynthesis inhibitor 1-phenyl-3-pyrazolidinone (Phenidone, Phe) (100 μM). The concentration of these chemicals was selected empirically based on preliminary experiments, and on the results reported by Mira *et al*. (2020).

2.4. Auxin activity and localization of NO

Auxin activity was assayed by monitoring the auxin-induced DR5 in the maize DR5:RFP reporter line (Mira *et al*., 2020). Nitric oxide was localized with the DAF-2DA specific fluorescent dye using the procedure outlined in Mira *et al*. (2020). Images were visualized under an epi-fluorescent LEICA microscope and the fluorescence level was measured using the Image J software. Measurements were conducted after 24h of treatment (normoxia or hypoxia).

2.5. Gene expression studies

Quiescent centers (QCs) that were dissected and treated with normoxia and hypoxia for 0, 3, 6, 12, and 24h were collected in liquid nitrogen and stored at -80 ˚C. RNA was extracted using the RNeasy Plant mini kit (Qiagen). RNA concentration was checked using a NanoDrop spectrophotometer (ThermoScientific). Synthesis of cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The levels of transcripts were measured by quantitative (q) real time PCR (qRT-PCR) and analyzed using the $2^{-\Delta\Delta}$CT method (Livak & Schmittgen, 2001). The *ZmActin* was selected as the housekeeping gene (Jiang *et al*., 2010). The list of primers is provided in Supplemental Table 1. Expression levels were presented with heatmaps generated using the ggplot package of the R software (version 2022.07.2 Build 576).

2.6. Statistical analysis

Data were analyzed by two-way ANOVA and the treatment means were compared by Duncan's test ($\alpha = 0.05$) to differentiate the significance of differences between parameters. All the statistical analysis was done using R (version 2022.07.2 Build 576). Heatmaps for hypoxic gene expression data were generated using R software using ggplot package. All experiments presented in this study were conducted using three biological replicates, each consisting of a minimum of 10 QCs for QC functionality tests and 30 QCs for expression studies.

3. RESULTS

3.1. Over-expression of *ZmPgb1.1* enhances QC functionality during hypoxia

To determine if dysregulation of *ZmPgb1.1* influences the viability of hypoxic QCs in the absence of mature tissue, we performed a "QC functionality test" (Supplemental Fig. S1 and Mira *et al*., 2020). This was done by counting the percentage of QCs, dissected from normoxic (ambient air) roots, able to regenerate a root in culture either under 4% oxygen (hypoxia) or normoxia (control). Relative to control, hypoxia depressed QC functionality; and these effects were attenuated by the over-expression of *ZmPgb1.1* [ZmPgb1.1(S)] and exacerbated by the suppression of the same gene [ZmPgb1.1(A)] [(i) in Fig. 4A)]. This trend phenocopied that obtained by Mira et al. (2020) in which QCs experienced hypoxia while in the presence of mature tissue, i.e. within the intact root, before being isolated and allowed to regenerate roots under normoxia [(ii) in Fig. 4A]. Similarities in the results suggest that *ZmPgb1.1* has a direct effect on QC cells during low oxygen stress.

Changes in gene expression during the QC functionality test occur rapidly (within hours) (Mira *et al*., 2020). Therefore, transcriptome studies were conducted within the first 24h in QCs isolated from normoxic roots and allowed to regenerate a root either under normoxia (control) or hypoxia. Under normoxia the expression of *ZmPgb1.1* peaked between 3-6h in WT and ZmPgb1.1(A) QCs (Fig. 4B). Relative to WT, the expression of *ZmPgb1.1* was greater in ZmPgb1.1(S) QCs and lower in those where the gene was down-regulated [ZmPgb1.1(A)] QCs. The induction in gene expression caused by hypoxia was also measured. Under hypoxia the expression of *ZmPgb1.1*

increased in all genotypes, especially in ZmPgb1.1(S) QCs within the first 12h of treatment (Fig. 4B).

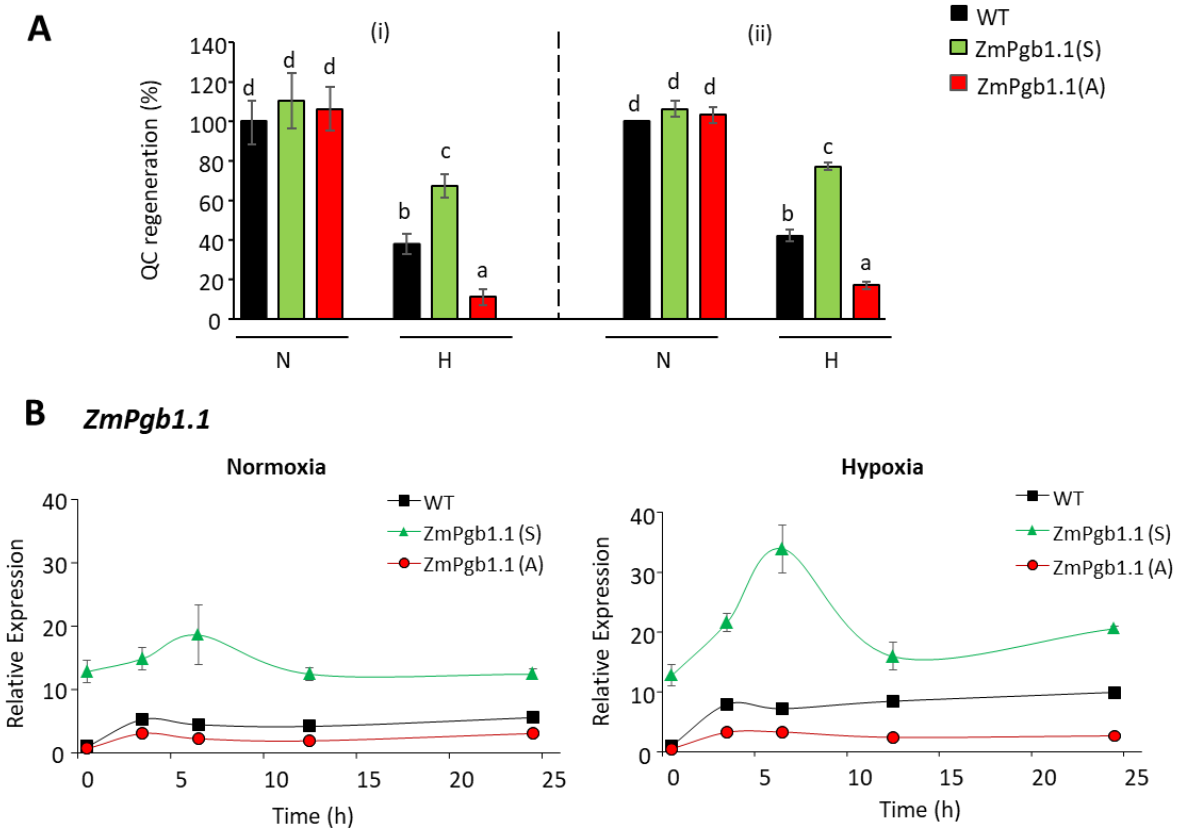


Figure 4. QC functionality test and *ZmPgb1.1* expression in QC stem cells. **(A)** QC functionality test in WT QCs and QCs over-expressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1*, measuring the percentage of QCs able to generate a root in culture. **(i)** QCs dissected from aerobic roots were allowed to regenerate roots in air (normoxia, N) or 4% oxygen (hypoxia, H). **(ii)** QC dissected from aerobic (N) or hypoxic (H) roots were allowed to regenerate roots in air. Values are normalized to the respective WT value (N) set at 100%. Letters indicate statistically significant differences ($P<0.05$). **(B)** Relative expression of *ZmPgb1.1* during the first 24h of root regeneration in QCs isolated from aerobic roots and cultured under normoxia (ambient air) or hypoxia (4% oxygen). Values are means $\pm$ SE normalized to the WT at 0h set at 1. Values were generated using a minimum of three biological replicates each containing at least 10 QCs.

3.2. *ZmPgb1.1* affects the expression of QC marker genes during hypoxia

Specification of cell fate at the QC is regulated by the two marker genes *PLETHORA* (*ZmPLT*) and *WUSCHEL-RELATED HOMEOBOX 5* (*ZmWOX5*) (Aida *et al*., 2004; Forzani *et al*., 2014). No pronounced differences in the expression of both genes were observed in QCs of the three genotypes cultured in normoxia (Fig. 5). Low oxygen depressed both *ZmPLT* and *ZmWOX5* in WT and ZmPgb1.1(A) QCs especially between 0-3h, but not in ZmPgb1.1(S) QCs. These latter QCs exhibited induction especially for *ZmWOX5*, which relative to normoxia exhibited a higher expression during the initial 12h of treatment.

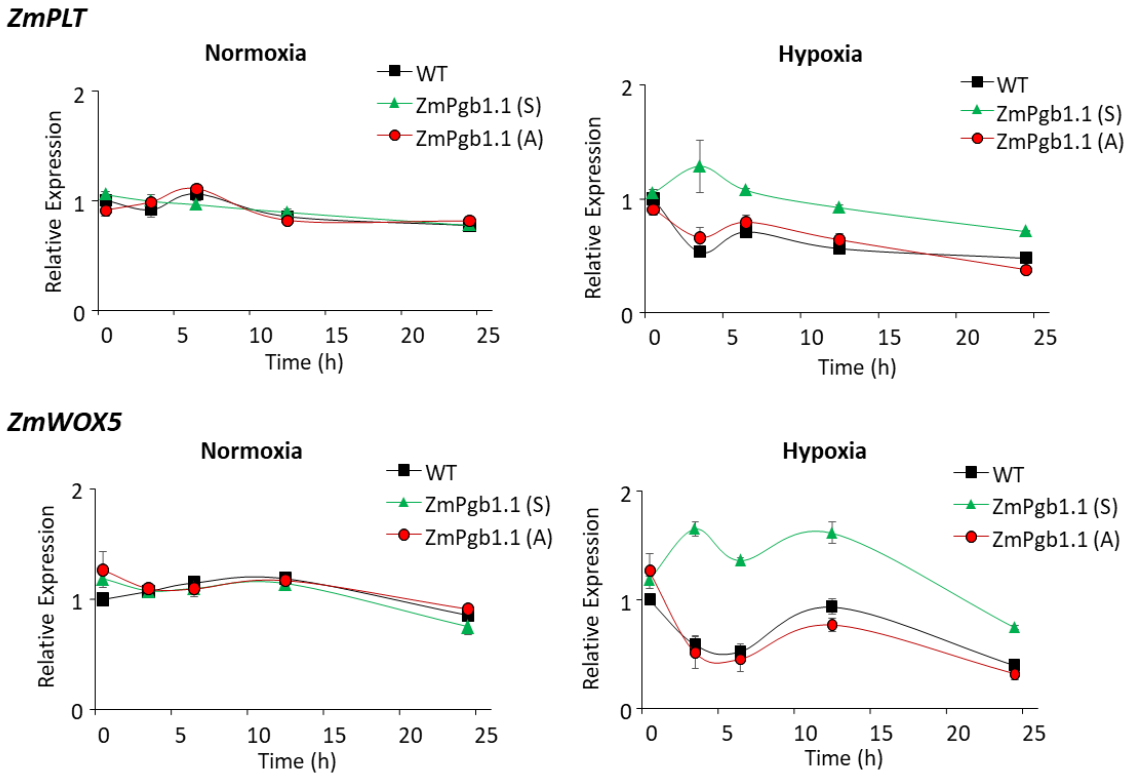


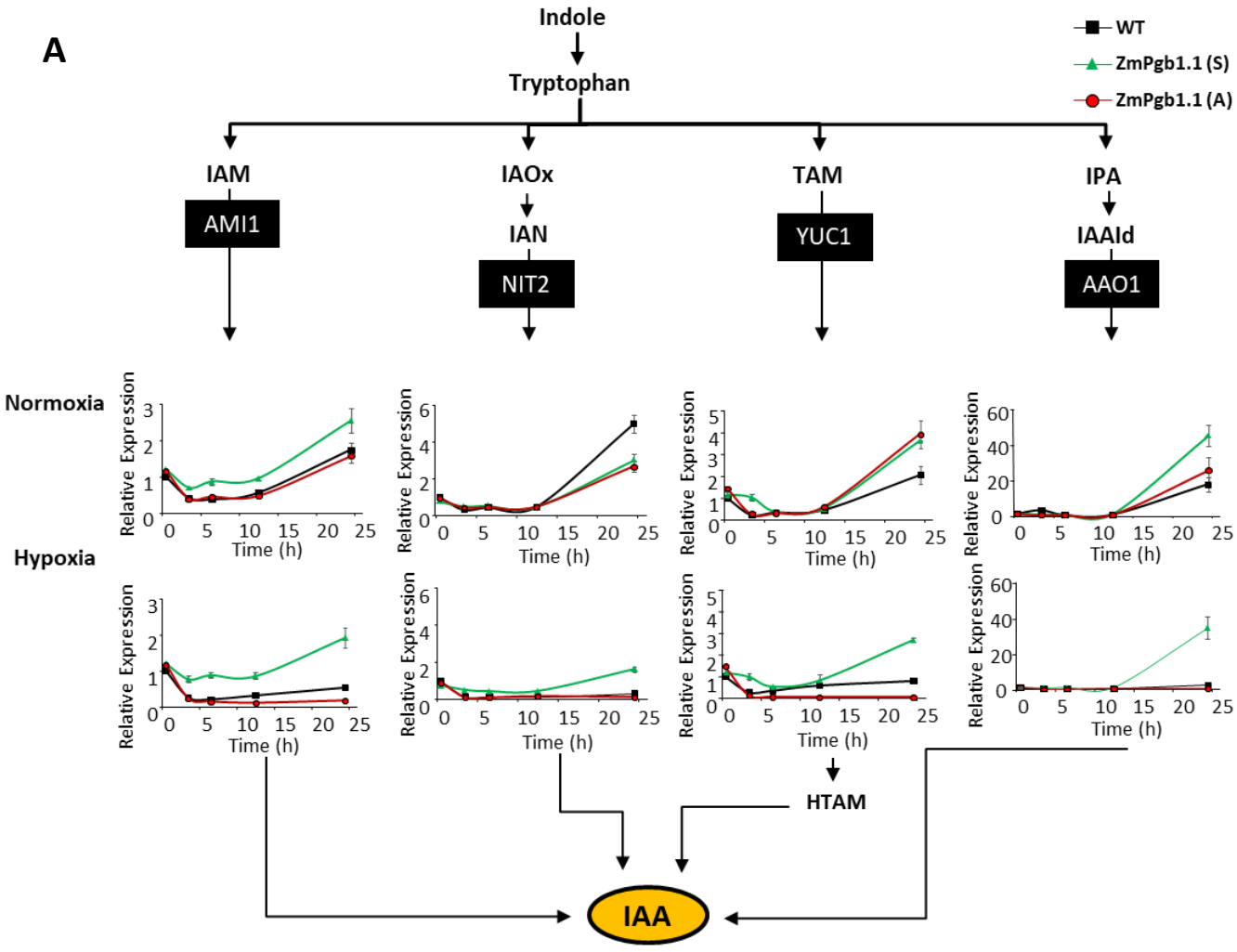
Figure 5. Relative expression of *PLT* and *WOX5* during the first 24h of root regeneration in QCs isolated from aerobic roots and cultured under normoxia (ambient air) or hypoxia (4% oxygen). QCs were isolated from WT roots and roots over-expressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1*. Values are means ± SE normalized to the WT at 0h set at 1. Values were generated using a minimum of three biological replicates each containing at least 10 QCs.

3.3. Auxin synthesis and activity in hypoxic QCs are influenced by *ZmPgb1.1* dysregulation

Functionality of QC cells is regulated by auxin (Kerk & Feldman, 1995); thus, expression of auxin biosynthetic and responsive genes was analyzed during the QC functionality test under normoxia and hypoxia. In maize, four tryprophan-dependent pathways have been hypothesized: the indole-3-acetamide (IAM), the indole-3-acetaldoxime (IAOx), the tryptamine (TAM), and the indole-3-pyruvic acid (IPA) pathways (Fig. 6A) (Phillips *et al*., 2011). The expression of the key respective enzymes, *AMIDASE1* (*AMI1*), *NITRILASE2 (NIT2), YUCCA 1 (YUC1)* and *ALDEHYDE OXIDASE1* (*AAO1*) increased between 12-24h in normoxic QCs of all genotypes. Hypoxia repressed the expression of all genes in WT and ZmPgb1.1(A) QCs especially during the first hours of stress. This was in contrast to ZmPgb1.1(S) QCs, where the expression of the genes remained high under low oxygen (Fig. 6A).

AUXIN RESPONSIVE FACTORS (ARF2, 5-8, and *10*), are expressed at the QC niche and correlate to auxin maxima (Jiang *et al*., 2010). In oxygen sufficient environments the transcript levels of *ARF2, 7* and *10* rose in all genotypes at different times in culture, while those of *ARF5* declined between 3-6h (Fig. 6B). The expression of *ARF6* and *8* increased during the first hours in normoxia before plateauing (*ARF6*) or declining (*ARF8*) during the following hours. Except for *ARF8*, hypoxia caused an overall depression in *ARF*s in WT and ZmPgb1.1(A) QCs. This overall depression was mitigated in ZmPgb1.1(S) QCs, exhibiting higher expression of *ARF2, 6*, *7*, and *10* at different time in culture relative to the other genotypes. The auxin DR5:RFP reporter

line was also used to monitor auxin activity in WT QCs. Compared to normoxia, exposure to low oxygen for 24h decreased the DR5 fluorescent signal (Fig. 6C).



Abbreviations: Indole-3-acetamide (IAM), Indole-3-acetaldoxime (IAOx), Tryptamine (TAM), N-hydroxyl tryptamine (HTAM), Indole-3-pyruvic aciD (IPA), Indole-3-acetaldehyde (IAAld)

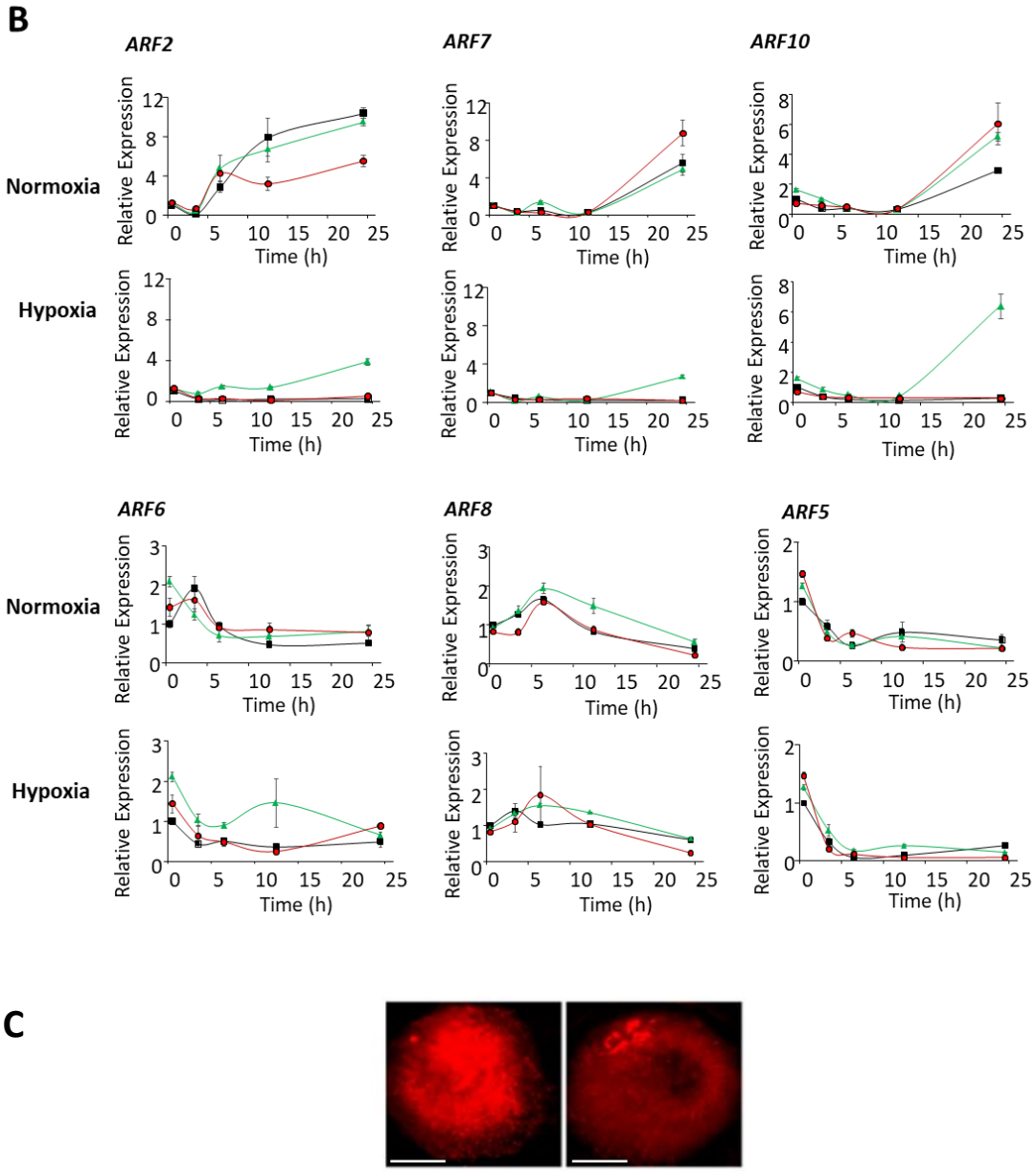
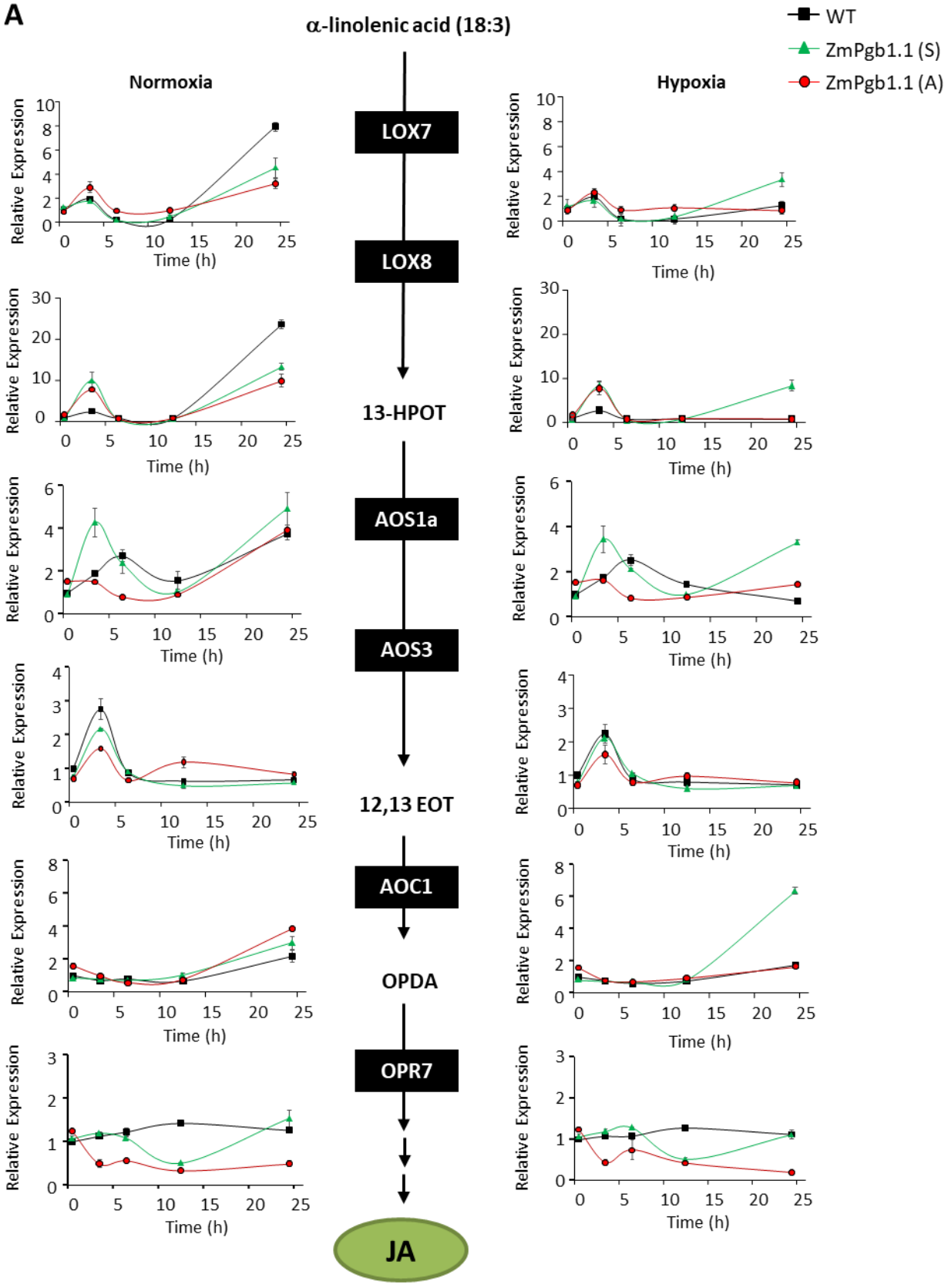


Figure 6. Auxin synthesis and response**.** Relative expression of: **(A)** auxin biosynthetic genes *AMI1*, *NIT2*, *YUC1*, and *AAO1* and **(B)** responsive genes *ARF2*, *5-8*, and *10* during the first 24h of root regeneration in QCs isolated from aerobic roots and cultured under normoxia (ambient air) or hypoxia (4% oxygen). QCs were isolated from WT roots and roots over-expressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1*. Values are means $\pm$ SE normalized to the WT at 0h set at 1. Values were generated using a minimum of three biological replicates each containing at least 10 QCs. **(C)** Signal of DR5:RFP in WT QCs after 24h in air (left panel) and 4% oxygen (right panel). Scale bar = 300 μm.

3.4. Jasmonic acid synthesis and response are altered by hypoxia and *ZmPgb1.1*

Given the participation of JA in the regulation (Drisch & Stahl, 2015) and regeneration ability (Zhou *et al*., 2019) of QC cells, the transcripts of several JA biosynthetic genes were measured. With the exception of *ALLENE OXIDE SYNTHASE* (*AOS*) *3*, all biosynthetic genes were induced in normoxic QCs between 12-24h in culture (Fig. 7A). In WT and ZmPgb1.1(A) QCs, hypoxia decreased the expression of *LOX7*, *8*, *AOS1a* from 12h to 24h. This depression was attenuated in hypoxic ZmPgb1.1(S) QCs, which also exhibited an induction of *AOC1* (relative to normoxia). No pronounced changes between normoxic and hypoxic conditions were observed for *OXO-PHYTODIENOIC ACID REDUCTASE7* (*OPR7*).

Jasmonic acid responses are mediated by MYC2 (Liu *et al*., 2019), which is induced in normoxic QC after 12h. Hypoxia decreases the levels of *MYC2* transcripts in WT and ZmPgb1.1(A) QCs especially later in culture, without evoking major changes in ZmPgb1.1(S) QCs (Fig. 7B).


A
α-linolenic acid (18:3)
WT
ZmPgb1.1 (S)
ZmPgb1.1 (A)
Normoxia
Hypoxia
LOX7
LOX8
13-HPOT
AOS1a
AOS3
12,13 EOT
AOC1
OPDA
OPR7
JA
Relative Expression
Time (h)

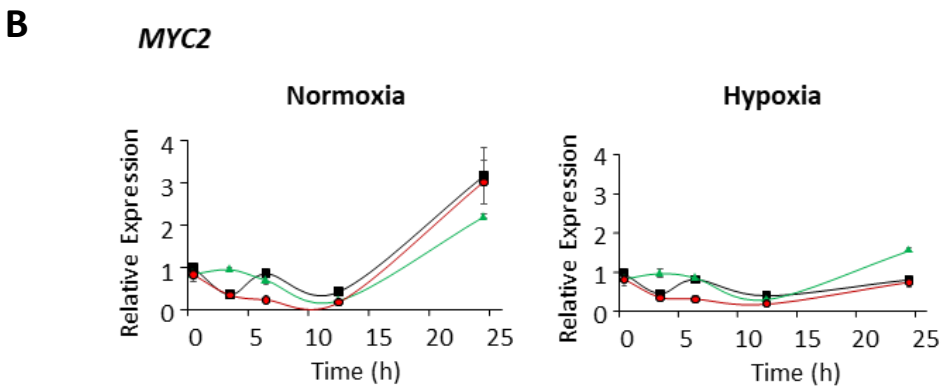


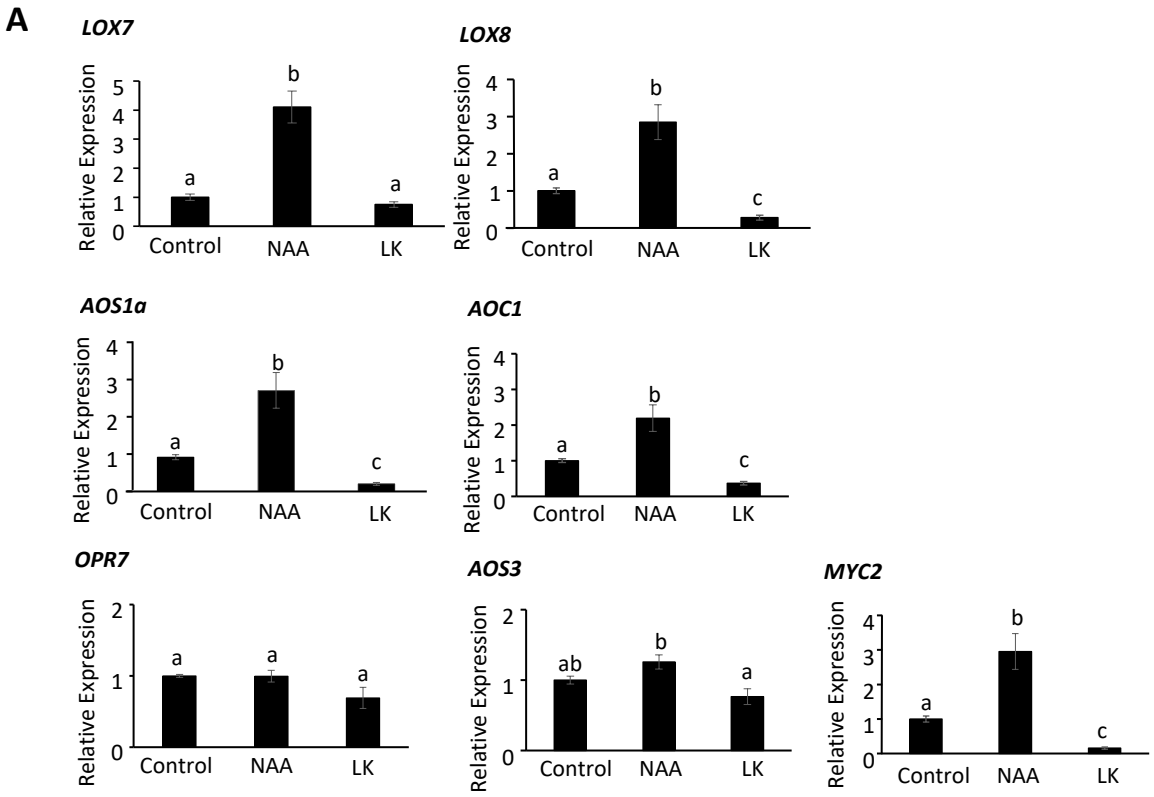
Figure 7. Jasmonic acid (JA) synthesis and response**.** Relative expression of the (A) JA biosynthetic genes *LOX7, 8, AOS1a, AOS3, AOC1,* and *OPR7,* as well as (B) the JA responsive *MYC2* during the first 24h of root regeneration in QCs isolated from aerobic roots and cultured under normoxia (ambient air) or hypoxia (4% oxygen). QCs were isolated from WT roots and roots over-expressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] *ZmPgb1.1*. Values are means ± SE normalized to the WT at 0h set at 1. Values were generated using a minimum of three biological replicates each containing at least 10 QCs.

3.5. Jasmonic acid synthesis and response are modulated by auxin during root regeneration from hypoxic QCs

To further the relationship between JA and auxin in the QC functionality test under low oxygen, the expression of JA- and auxin-related genes was measured after respective manipulations of auxin and JA in hypoxic WT QCs for 24h. Exogenous application of NAA elevated the expression of *LOX7, LOX8, AOS1a, AOC1* and *MYC2*, while a suppression of the same genes (with the exception of *LOX7*) occurred in the presence of the auxin biosynthetic inhibitor L-Kynurenine (LK) (He *et al*., 2011) (Fig. 8A). Changes in JA amount by supplementations of either JA or the

JA-biosynthetic inhibitor Phenidone (Phe) (Engelberth, 2011) had no effects auxin biosynthetic genes (Fig. 8B).

The effect of similar pharmacological treatments were evaluated during the QC functionality test under low oxygen. Treatments elevating NAA and JA increased the QC regeneration frequency, while those depleting NAA (by LK) or JA (by Phe) had inhibitory effects (Fig. 8C). The observation that JA partially reverts the inhibition of LK (compare LK with JA+LK), while NAA does not revert the inhibition of Phe (compare Phe with NAA+Phe), suggests that JA action is downstream of auxin. Furthermore, NAA and JA respectively abolish the inhibition by LK and Phe, confirming the specificity of the two inhibitors.



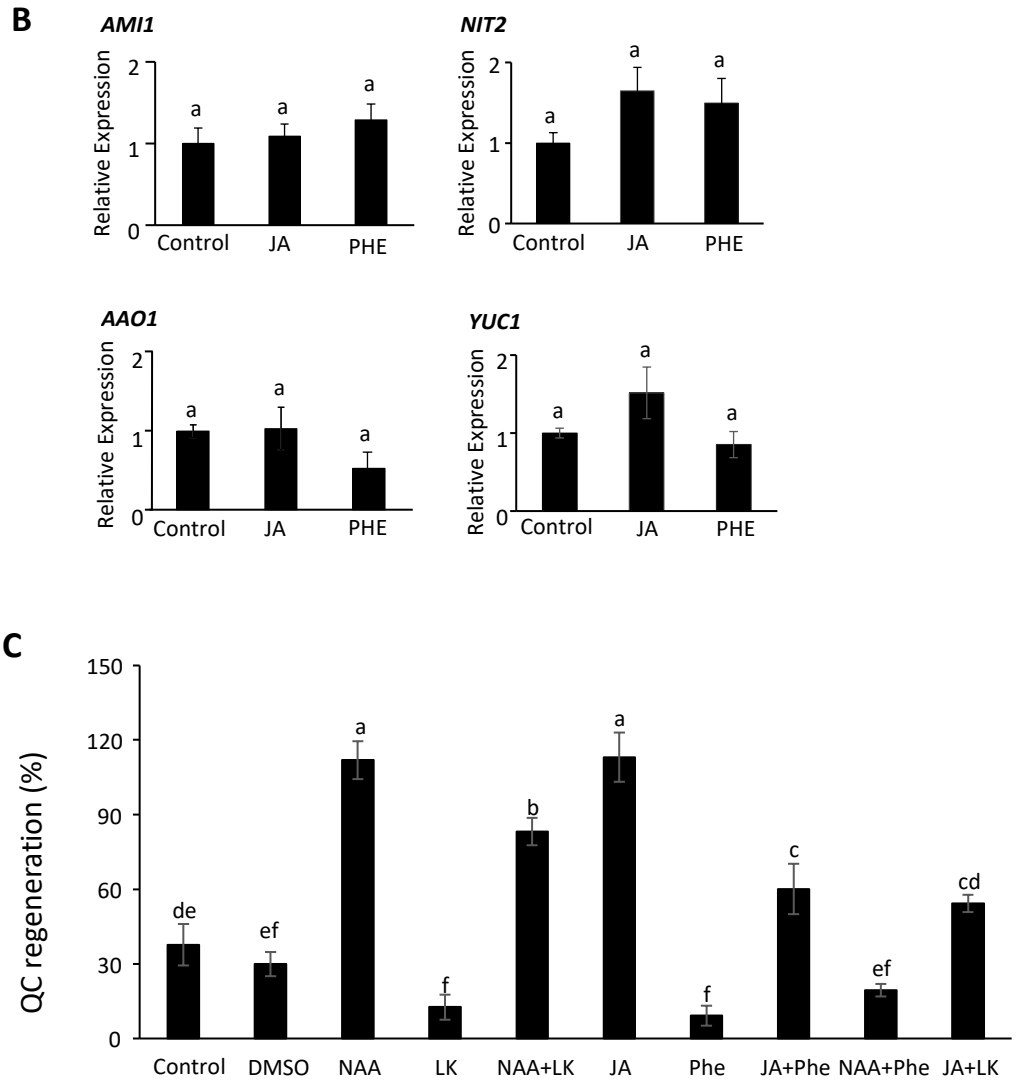


Figure 8. Interplay between auxin and JA**. (A)** Relative expression of the JA biosynthetic genes *LOX7, 8, AOS1a, AOS3, AOC1,* and *OPR7,* as well as the JA responsive *MYC2* after 24h of root regeneration in hypoxic WT QCs treated with the auxin NAA or the biosynthetic auxin inhibitor LK. **(B)** Relative expression of the IAA biosynthetic genes *AMI1, NIT2, YUC1*, and *AAO1* after 24h of root regeneration in hypoxic QCs treated with the auxin JA or the biosynthetic JA inhibitor Phe. Values $\pm$ SE are normalized to the control value set at 1. Letters indicate statistically significant differences ($P<0.05$). Values were generated using a minimum of three biological replicates each containing at least 10 QCs. **(C)** QC functionality test in hypoxic WT tissue treated with NAA, LK, JA, and /or Phe. QCs dissected from normoxic WT roots were allowed to regenerate under hypoxia in the presence of the chemicals listed above. Values are means $\pm$ SE generated using a minimum of three biological replicates each containing at least 10 QCs.

4. DISCUSSION

Understanding how stem cells respond to environmental stimuli, and whether these responses occur autonomously, or rely on extrinsic cues provided by the mature tissue, is one of the most important, and yet poorly understood questions in biology. What is apparent in plants is that root stem cells are vulnerable to diverse conditions of stress (Mira *et al*., 2016, 2017, 2020), and their ability to remain functional when exposed to sub-optimal environmental conditions is a determinant factor for the growth of the root, and possibly the survival of the organism. It has been previously demonstrated that hypoxia deteriorates the maize QC stem cells through physiological changes occurring in mature tissue, outside of the stem cell niche (Mira *et al*., 2020). Specifically, the accumulation of NO in poorly oxygenated roots alters the root-ward movement of auxin in the elongation zone, dissipating the auxin maximum at the root tip required for the specification of the QC, causing the QC cells to deteriorate. These effects were alleviated by a reduction of NO resulting from the over-expression of *ZmPgb.1.1* (Mira *et al*., 2020). This model was developed using a system in which intact roots were exposed to hypoxia, and then QC stem cells were surgically removed and allowed to regenerate a root under normoxia. From that study however, it is unclear whether 1) hypoxia can have a direct effect on the QC stem cells, and 2) *ZmPgb.1.1* acts within the QC, affecting survival. By eliminating the influence of mature tissue through the precise dissection of QCs before the imposition of hypoxia (Kerk & Feldman, 1995; Mira *et al*., 2020), here it was revealed that low oxygen decreases the functionality of the stem cells, and that over-expression of *ZmPgb1.1* in the same cells is sufficient to enhance their functionality.

The low regeneration ability of QCs dissected from normoxic roots and then exposed to low oxygen suggests that hypoxia can reduce stem cell functionality through mechanisms unrelated to

auxin supply from mature cells (Fig. 4A-ii). One of these mechanisms is the reduction in auxin biosynthesis. Root stem cells are able to synthesize auxin (Ljung *et al*., 2005), a process which is impaired during the first hours in hypoxia, as denoted by the depression of several auxin biosynthetic enzymes (*AMI1*, *NIT2*, and *AAO1*). The transcript levels of these three genes correlate to the amount of auxin present in the tissue, as best exemplified for *AMI1* and *NIT2*, where their mutation reduces free auxin while their constitutive expression elevates cellular auxin (Lehmann *et al*., 2017; Pérez-Alonso *et al*., 2021). The hypoxia depression of auxin biosynthesis might explain the reduction in DR5:RFP signal and the down-regulation of the *ARFs* (with the exception of *ARF8*) after 6h, suggesting impaired auxin responses (Fig. 6). This observation agrees not only with the suppression of auxin-induced genes during lateral root development by ERF-VII, a mediator of hypoxic responses (Shukla *et al*., 2019), but also with the requirement of auxin for stem cell functionality under hypoxia. The frequency of QC regeneration under low oxygen is in fact increased by NAA and decreased by the auxin biosynthetic inhibitor (LK). L-kynurenine inhibits the ethylene-induced auxin biosynthesis in roots (Fig. 8C) (He *et al*., 2011, Watanabe *et al*., 2021).

Auxin defines the activity of the QC cells (Kerk et al., 2000), and plays a regulatory role in root regeneration (Matosevich & Efroni, 2021). QC stem cell activity is regulated by the interaction of the two QC-expressed transcription factors *PLT* and *WOX5* (Aida *et al*., 2004; Forzani *et al*., 2014) the expression of which requires auxin (Burkart *et al*., 2022). The rapid auxin-depletion in hypoxic QCs is possibly related to their downregulation (Fig. 5B), which is known to cause the consumption of the QC (Galinha *et al*., 2007; Forzani *et al*., 2014). Thus, it is expected that the suppression of both *PLT* and *WOX5* in oxygen insufficient environments impairs the activity of

the QCs, and ultimately their inherent capability to function as the “organizer center” in the maintenance of the RAM.

Another plausible requirement for auxin is to support the QC-root regeneration by elevating JA responses. Like other stress hormones, JA controls the division rate of organizer cells (Chen *et al*., 2011), and interplays with auxin in the regulation of RAM (Xu *et al*., 2020). Relevant to our studies, JA and its signaling-intermediate MYC2 have been implicated in the regeneration of root cells following mechanical damage or excision (Zhou *et al*., 2019). Our findings suggest that JA also plays an important role in QC regeneration, possibly at later hours. Many JA biosynthetic genes and *MYC2* are induced in normoxic QCs after 12h in culture but repressed by hypoxia from 12-24h (Fig. 7). Furthermore, the QC root regeneration is promoted by exogenous JA but inhibited by the JA biosynthetic inhibitor Phe. Phenidone reduces JA by inhibiting lipoxygenases involved in the first step of JA biosynthesis (Cucurou *et al*., 1991). We also suggest that JA acts downstream of auxin, with auxin required for its activity. First, pharmacological manipulations of auxin content (by NAA and LK) alter the expression of JA biosynthetic genes and *MYC2* in a pattern consistent with the requirement of auxin, while changes in JA content (by JA and Phe) do not alter the expression of auxin biosynthetic genes (Fig 8C). Lastly, the reduction in JA abolishes the promotive effect of NAA on QC regeneration (compare NAA and NAA+Phe), while an increase in JA reverts the inhibition of auxin depletion (compare LK and JA+LK).

Besides describing a QC-specific effect of hypoxia through changes in auxin, this study also expands on the role of Pgbs during low oxygen stress. In addition to regulating QC activity by interfering with events in mature root tissue (Mira *et al*., 2020), we show that *ZmPgb1.1* has a direct role on isolated QCs. Functionality of the QCs is enhanced by the over-expression

of *ZmPgb1.1* and repressed by its down-regulation. Furthermore, in agreement with the NO scavenging properties of the protein (Hill, 2012), ZmPgb1.1 effects during hypoxia are mediated by NO. Two pieces of evidence support this notion: the presence of *ZmPgb1.1* decreases NO, and QC regeneration is inhibited by a rise in NO (by SNAP) and promoted by a decrease in NO (by cPTIO) (Supplemental Figure S2). Our transcriptome analyses further suggest that the protective effect exercised by *ZmPgb1.1*, likely through NO, is linked to the retention of auxin synthesis and response in hypoxic QCs, with the consequent maintenance of activity (through *PLT* and *WOX5*), and regeneration capacity (through JA) of the hypoxic QCs. The interaction between NO and auxin is complex to define, as in addition of acting downstream of auxin in morphogenic responses (reviewed in Domingos *et al*., 2015), NO influences auxin synthesis and responses (Elhiti *et al*., 2013; Sánchez-Vicente *et al*., 2021). Consistent with the proposed role of regulating QC stem viability during hypoxia, *ZmPgb1.1* influences cell fate survival during maize somatic embryogenesis. Cells targeted to die by suppression of *ZmPgb1.1* (and accumulation of NO) were depleted in auxin, while those which survived were enriched in *ZmPgb1.1* (and depleted in NO) and accumulated auxin (Huang *et al*., 2014). Among the similarities shared between embryogenic cells and stem cells (Elhiti *et al*., 2010), both characterized by an undifferentiated state, *ZmPgb1.1* might be pivotal in regulating cell fate in both.

5. CONCLUSIONS

Collectively, this study demonstrates that hypoxic stress has direct negative effects on the functionality of isolated root QC stem cells, which are influenced by the expression of *ZmPgb1.1*. Hypoxia depresses QC functionality by suppressing synthesis of and response to auxin, required for the activity (through *PLT* and *WOX5*) and regeneration ability (through JA) of the QC stem cells. Through the retention of auxin synthesis in the QCs, *ZmPgb1.1* mitigates the hypoxia inhibition. By integrating these results to a previous model on intact roots (Mira *et al*., 2020) we propose that deterioration of QC stem cells in hypoxic roots is the result of auxin deficiency in the same cells. This is attributable to two separate but equally important events: impaired auxin supply flowing from mature tissues (Mira *et al*., 2020), and reduced auxin production within the QCs (this study). By alleviating both events, *ZmPgb1.1* contributes to the retention of functional stem cells, ultimately ensuring the survival of the root in low oxygen conditions.

6. FUTURE PERSPECTIVES

The described link between hypoxic QC responses and the role of Pgbs in modulating hormone synthesis and responses protecting the QCs is fundamental for understanding how stem cells function during conditions of stress. In addition, this information can be valuable in breeding programs where the levels of Pgbs present in the tissue could be used to predict how plants cope with excess moisture. Excess moisture is predicted to become a critical abiotic challenge in the future. Further transcriptome analyses of auxin response, auxin transport, and QC marker genes (eg. *QC25*, *QC46*) can also further shed light into the mechanisms through which auxin mediates QC characteristics. In parallel studies, the use of Pgb1 antibodies would define where the protein is expressed within the QC cells, and how its levels vary during hypoxia.

Another exciting path to be explored will be the involvement of Ca^{2+} in activating the transcription of *Pgbs* and genes coding for anaerobic fermentation enzymes in QC, two processes that contribute to maintaining glycolysis during hypoxia (Igamberdiev & Hill, 2018), and how pH levels are involved in these changes. Furthermore, studies on ethylene response factors also could deepen our understanding on the cross-hormonal regulation between ethylene (produced under oxygen deficit) and auxin in the stem cells. Finally, it might be interesting to see how the transgenic lines dysregulating *ZmPgb.1.1* perform under field conditions and in response to excess moisture. These avenues may play major steps in trait-to-gene-to-field approaches (Loreti & Striker, 2020) in identifying and integrating the effective levels of Pgbs into economic crops.

7. REFERENCES

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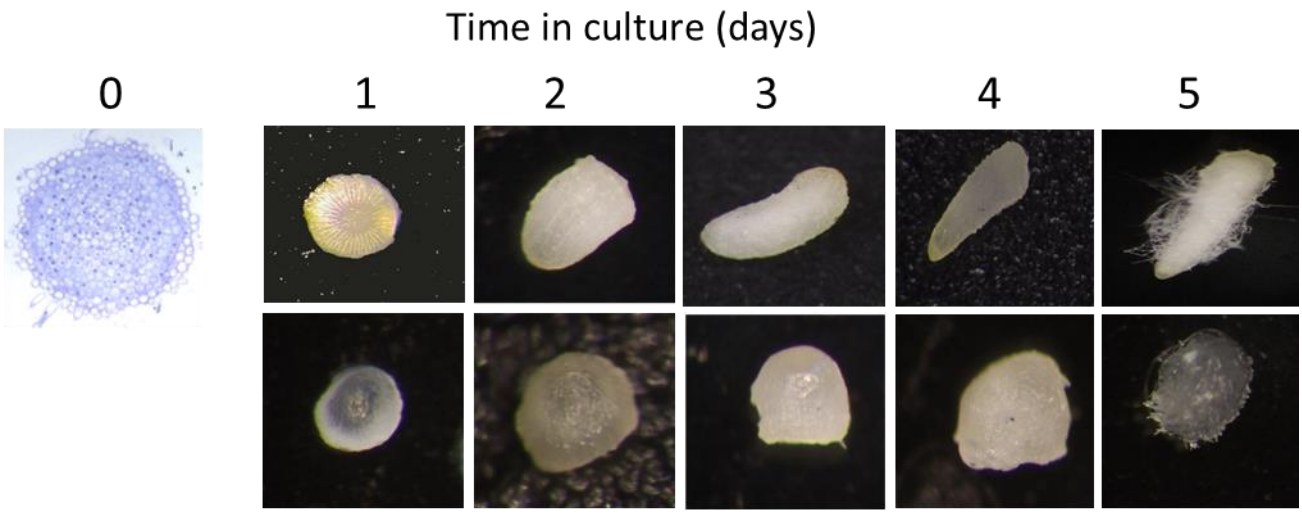
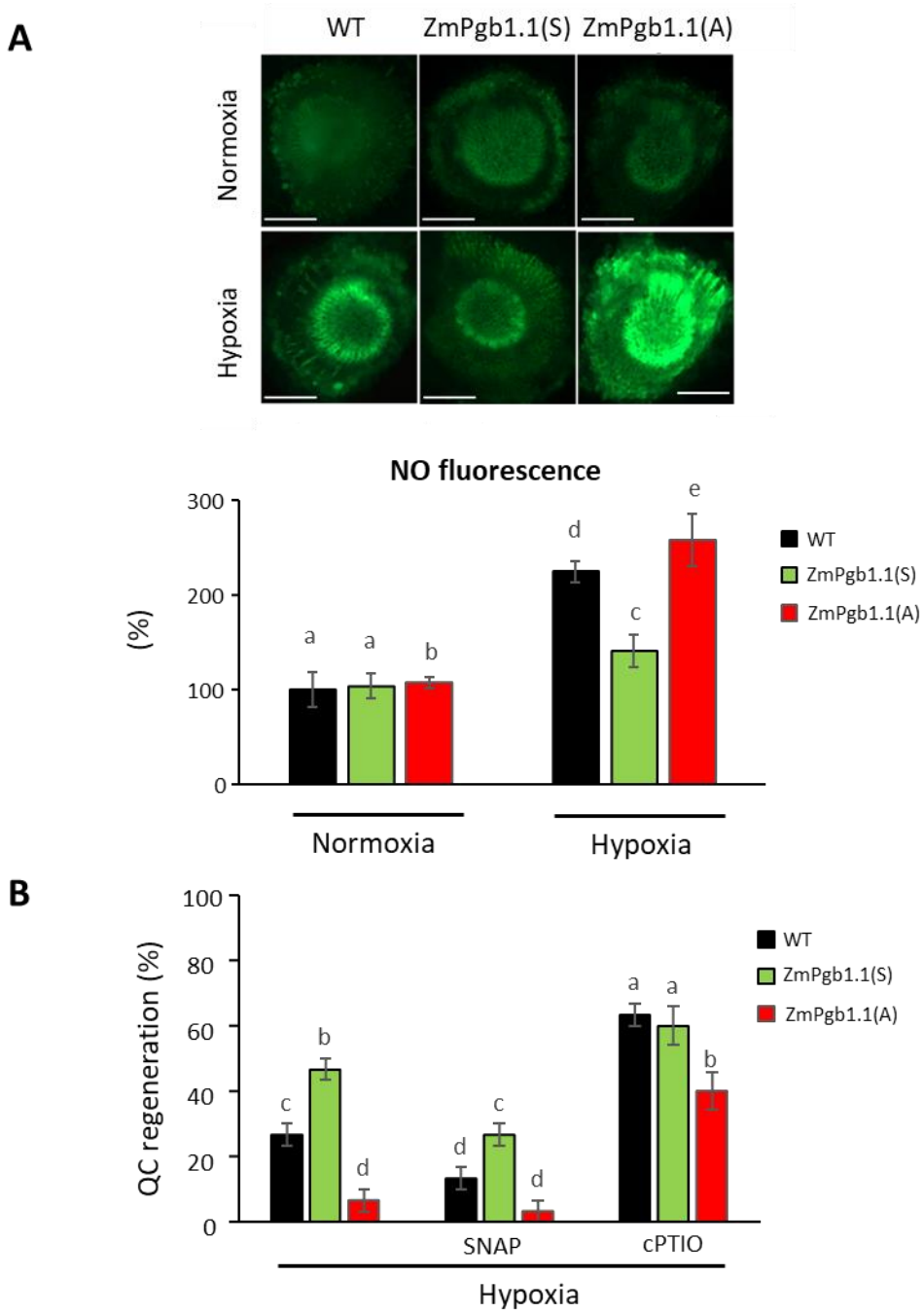
SUPPLEMENTARY FIGURES



Supplemental Figure S1. Example of a QC functionality test under hypoxia. Dissected QCs (day 0) consisted of about 800 cells (Jiang *et al*., 2013). Quiescent centers (QCs) retaining their functionality under hypoxia were able to regenerate a root after a few days in culture (top panel). Root formation did not occur for those QCs losing their functionality under low oxygen (bottom panel).



Supplemental Fig. S2. (A) Localization of nitric oxide (NO) using the NO-specific fluorescent probe. DAF-2DA in WT QCs and QCs over-expressing [ZmPgb1.1(S)] or down-regulating [ZmPgb1.1(A)] Zmpgb1.1. Measurements were taken after 24 hours in normoxia or hypoxia. Magnification bar = 300 µm. The relative changes in NO fluorescence signal was also measured. Values, means ± SE of three biological replicates each containing10 QCs, are percentage changes relative to the WT (normoxia) value set at 100%. **(B)** QC functionality test in the presence of the NO releasing agent S-nitroso-N-acetylpenicillamine (SNAP) and the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Values are means ± SE of three biological replicates each containing 10 QCs. Letters indicate statistically significant differences ($P<0.05$).

SUPPLEMENTARY TABLES

Supplemental table 1. List of primers used for (q)RT-PCR analyses

Gene		Forward	Reverse
Actin1	*ZmActin1*	GATGGTCAGGTCATCACCATTG	AACAAGGGATGGTTGGAACAAC
Phytoglobin1	*ZmPgb1.1*	TCCGCTTCTTTCTCAAGGTCTTCG	AGGTCATGACGAAGACGGACAT
Plethora	*ZmPLT*	GAGCAACCACTACATCGGCA	TGCAGACATCCTTCCGCCAC
Wuschel homeobox protein	*ZmWOX5*	TCCTGACGGAGCTCTTCC	GTAGAAGACGTTCTTGCTCTCC
Amidase1	*ZmAMI1*	TTTGCCAGCTGAGCATTCCACTTG	ATGAACTCCAGCTCCAGGCTTTCT
Nitrilase2	*ZmNIT2*	AGCTGCCAAGAGTGATATCGATACTAAG	CACAAGGAACATAACTGCGGCC
Aldehyde oxidase1	*ZmAAO1*	GGGAGGCTGTGTACGTTGAT	TCTCCACCGCTTGGAATATC
Yucca 1	*ZmYUC1*	ATCTCCTGACCACCAACAT	CACCTTCTCCGTCATCTCT
Auxin response factor 2	*ZmARF2*	CACTCCACCATGCCCTATATC	TTCCTGTCGTCAGATCTCTCT
Auxin response factor 5	*ZmARF5*	CTGGGATGATTCCTCTCCATTATC	GCAGATCCTCGCTCCATTT
Auxin response factor 6	*ZmARF6*	CAACGGGCTCGAGAGTTATTT	GTATGCCTTGCTGACCAGTAG
Auxin response factor 7	*ZmARF7*	CAGTGGAAGGAGTCGTATCAAG	TCAAACATCTCTCCAACCTCAC
Auxin response factor 8	*ZmARF8*	GCCTTATCCGAGGTAATGTGAG	AGGGTAGTACACCACCTCAA
Auxin response factor 10	*ZmARF10*	GCCCTTCAGAGTTCATCATACC	CCTCTTCTCCTTCAAACCTCATC
Lipoxygenase7	*ZmLOX7*	GCGAATGCGAGCTGTTTTGA	CGAACGATCTCCCCAGTAGC
Lipoxygenase8	*ZmLOX8*	CAGTACCGACAGACAGCCAT	GTTTCGGACCACCAAATCAA
Allene oxide synthase 1	*ZmAOS1a*	ACAAGGTGGAGAAGAAGGAC	GTCGTTGAGCTTGTTGAACT
Allene Oxide Synthase 3	*ZmAOS3*	GTACTGACACGCTCGCCTTA	ACAGCGAGTGACGTGTGCA
Allene Cylcase	*ZmAOC1*	ACGCATCCCTCGACAACTAC	AGCCAAACTACGGCTACGAG
Oxo-Phytodienoic Acid Reductase7	*ZmOPR7*	CCCATGGCTACCTCATCGAT	CGTCAGTCCGGTCGTTGAT
MYC2	*ZmMYC2*	TATATGGATCAGCCGTCGTCCT	TCCATTGCAAAGCGAAATCCTG