Abscisic acid and ethylene are integrated in the phytoglobin (Pgb) regulation of maize somatic embryogenesis

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTERS OF SCIENCE

Department of Plant Science

University of Manitoba
Winnipeg

ABSTRACT

Kapoor,Karuna, M.Sc., University of Manitoba, March 2017

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Suppression of *Zea Mays phytoglobins* (*ZmPbg1.1* or *ZmPgb1.2*) during somatic embryogenesis induces programmed cell death (PCD) by elevating nitric oxide (NO). While *ZmPgb.1.1* is expressed in many embryonic domains and its suppression results in embryo abortion, *ZmPgb1.2* is expressed in the basal cells anchoring the embryos to the embryogenic tissue. Removal of these "anchor cells" by PCD allows the embryos to develop further. The effects of *ZmPgb* suppression on embryogenesis were abolished by exogenous applications of ABA. A depletion of ABA, ascribed to a down-regulation of biosynthetic genes, was observed in those embryonic domains where the respective *ZmPgbs* were repressed. These effects were mediated by NO. Depletion in ABA content induced the transcription of genes participating in the synthesis and response of ethylene, as well as ethylene production, which influenced embryogenesis. Somatic embryo number was reduced by high ethylene levels and increased with pharmacological treatments suppressing ethylene synthesis. The ethylene inhibition of embryogenesis was linked to the production of ROS and the execution of programmed cell death (PCD). The integration of ABA and ethylene in the *ZmPgb* regulation of embryogenesis is proposed in a model where NO accumulates in *ZmPgb* suppressing cells decreasing the level of ABA. Abscisic acid inhibits ethylene biosynthesis and the NO-mediated depletion of ABA relieves this inhibition causing ethylene to accumulate. Elevated ethylene levels trigger production of ROS and induce PCD. Ethylene-induced PCD in the *ZmPgb1.1* suppressing line [ZmPgb1.1 (A) line] leads to embryos abortion, while PCD in the ZmPgb1.2 suppressing line [ZmPgb1.2 (A) line] results in the elimination of the anchor cells and the successful development of the embryos

ACKNOWLEDGEMENTS

I feel entitled to write this section by being comprehensive. I think it is the best opportunity to thank all of the people, in writing, who have helped, instructed and supported me selflessly throughout my two years of Masters.

First and foremost I shall thank my graduate supervisor, Dr. Claudio Stasolla. His outline system for constructing a systematic project, his commitments towards his students is unrivaled and admired greatly. He is not only a very handsome advisor but his in depth knowledge in the field of plant science is incredibly impressive. I feel blessed and lucky to have completed my research under his supervision. I hope I could be as enthusiastic and energetic as him someday!! Claudio is the touchstone for what I truely value and respect in an advisor. I would also like to special thank Dr. Hill for serving on my graduate committee and for all the 'Monday, Wednesday and Friday' coffee chats that has motivated and inspired me to thrive for better. I am very grateful to Dr. Hill for his scientific advice and knowledge and many insightful discussions and suggestions.

I would also like to thank Dr. Sylvie Renault for being in my graduate committee and for her valuable suggestions.

Very special thanks to my first ever 'Canadian' advisor and a friend Dr. David Bird. Words cannot express how privileged I am to be able to work with him as an intern/assistant at Mount Royal University, Canada. He is a consummate mentor: intelligent, honest, kind and genuinely interested in his students. I never would have entered the field of 'research' if I have not had worked in his lab.

Now, I would like to extend my gratitude towards one of the most important 'behind the scene' HERO of my project. Mr. Doug Durnin, our lab technician and my savior. He is the most wonderful, generous and kind man I have ever met. He has always lent his hand for technical-help, his ears to hear my life dramas, his eyes to review my 'scientific writing' (what a relief for him!!). Words will fall short in his appraisal. He has constantly reminded me of my father and talking to him has made me feel that I have always been close to my family. Thank you Doug (for endless things you do for all of us).

I would also, like to express my gratitude to Martha Blouw for being 'my person' here in Canada. She is one of the most beautiful and kind hearted woman. She has been always there for me to reassure and encourage at the right time. I hope one day I can invite her to my house in India.

I am very grateful to Dr. Mohammed Mira for his constant guidance. I have learnt a lot of things from him during these two years. I so hope he forgives me for being so annoying at times, I know I have pestered him a lot but he has been exceptionally patient with me and I am so thankful for that.

I would also extend my thanks to the 'girl power' of the lab. Veronica, thank you for bringing me lunches, for sharing and caring for me. I am so excited to be an 'aunt' to a lovely boy. Cassandra, thank you for taking me to the 'opportunity dinners', for practicing 'mock interviews' with me. I really appreciate all of your help. Thank you Eman for bringing us beautiful gifts from Egypt. A heartfelt thank you to Dr. Shuanglong Huang for being my reference and for all the late evening talks and discussion on variety of 'out of the blue' topics.

Outside my 'plant science lab' life, I have had the support of my two very brave and beautiful best friends 'Diksha and Arzoo' who have been my pillars of strength and entertainment for the three years I have been away from home. Thank you both for all the quick 'facetime chats' for bringing smiles and happiness and immense energy every morning in my life. I have been immensely blessed to have the support of my friends and roommates Kapil, Jaipal, Samrath, Nathalie, Joyce and Madison, who I can call my 'mini canadian family'. Thank you guys, being with you have made these two years even more memorable.

And finally, behind everything, throughout the entire journey, I want to thank my favourite people, my mom- Anu Kapoor, dad- Bhartesh kapoor, my brother- Nipun Kapoor and my grandfather- Tarsem Puri for showering their love and more love through thick and thin. My parents exposed me to all sorts of opportunities as I grew up and were incredibly motivating and positive of all the crazy decisions I have made so far (that may also include, coming to Canada). My brother has been my best friend and the bond between us two has grown stronger every day. I am the luckiest daughter and sister in this world.

Any future success that I may enjoy will be built on the educational foundation and the help and support of all the people mentioned above. The extent to which I feel indebted to them is humiliating, and as I emerge from my formal education and look to start contributing to the world in earnest, I will never forget the large debt I owe to these benefactors and to society. I'm eager to start paying everyone back.

THANK YOU THANK YOU THANK YOU!!!

DEDICATION

Lovingly dedicated to the memory of my grandmother (Urmil Puri) and my sister (Garima Syal Kapoor)

I miss you both

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ABBREVIATIONS

ABA, Abscisic Acid

ACC, 1-aminocyclopropane-1-carboxylic acid

ACO, 1-aminocyclopropane-1-carboxylic acid oxidase

AOA, Aminooxyacetic acid

BI-1, Bax Inhibitor 1

Ca^{2+}, Calcium

cGMP, Cyclic Guanosine Monophosphate

CK, Cytokinin

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

DAP, Days after pollination

DPI, diphenylene iodonium

ELS, Embryo-Like Structure

ER, Endoplasmic Reticulum

ERF, Ethylene Response Factor

ET, Ethylene

ETH, Ethephon

ETI, Effortorss-Triggered Immunity

FLD, Fluridone

GA, Gibberelic acid

GTP, Guanosine Triphosphate

Hb, Hemoglobin

HR, Hypersensitive Response

IAA, indole-3-acetic acid

JA, Jasmonic Acid

LegHb, Leg hemoglobin

MAPK, mitogen-activated protein kinase

MC, Matacaspase

MCS, Multi-Cellular Structure

MT, metallothionein

$N_{2,}$ Nitrogen

NCED, 9 cis-epoxycarotenoid dioxygenase

NO, Nitric oxide

PAMP, Pathogenesis-Associated Molecular Pattern

PBS, phosphate-buffered saline

PCD, Programmed Cell Death

PEG, polyethylene glycol

PEM, Pro-Embryogenic Mass

Pgb, phytoglobin

PM, plasma membrane

PRR, Pathogen-Recognition Receptor

PTI, PAMPs-Triggered Immunity

RAM, Root Apical Meristem

RBOH, Respiratory burst oxidase homologues

ROS, Reactive Oxygen Species

SA, Salicylic Acid

SNP, Sodium nitroprusside

TUNEL, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling

WT, Wild Type

FORWARD

This thesis follows the manuscript style outlined by Department of Plant Science and Faculty of Graduate Studies at the University of Manitoba. The manuscripts follow the style recommended by "JOURNAL OF EXPERIMENTAL BOTANY". This thesis is presented as a single manuscript, containing the following abstract, introduction, results, discussion and materials and methods section. The manuscript also has supplemental figures and tables which are placed immediately following the body of the manuscript. A general introduction and literature review precedes the manuscripts and general discussion and conclusions follows the manuscript.

GENERAL INTRODUCTION

Phytoglobins (Pgbs) are globin proteins containing iron and are present in all plants (Vázquez-Limón et al. 2012). These proteins have a distinct characteristic of having the heme -Fe^{2+} ligation to both proximal and distal histidine. Further, on the basis of the biochemical structure and oxygen affinities (Hoy and Hargrove 2008; Smagghe et al. 2009; Vázquez-Limónet al. 2012) Pgbs can be divided into three classes (1-3). While class 1 and class 2 share the structural similarity in having the typical 3 on 3 globin structure and are further categorized according to their different oxygen binding characteristics, class 3 are also known as truncated globin with a 2 on 2 configuration. Additionally, class 1 and 2 Pgbs are known to be expressed in variety of dicots whereas class 1 Pgbs are present only in monocots (Smagghe et al. 2009) and their number differs notably among species, for instance, *Zea mays* contains two copies of class 1 Pgbs, also classified as *ZmPgb1.1*and *ZmPgb1.2*, and rice has five copies of class 1 Pgbs (Hoy and Hargrove 2008; Rodríguez-Alonso and Arredondo-Peter 2013).

Comprehensive studies have been done over the past three decades highlighting the structural properties, expression pattern and functional roles of Pgbs in different plant species. Studies describing the functional roles of plant hemoglobins in plant growth and development during 'stressed' conditions are scarce. However, this versatile molecule (Pgb) has been known to ligate/scavenge nitric oxide (NO) (Hill 2012; Yu et al. 2014) and thus is most likely involved in several NO-mediated responses. NO regulates a variety of plant developmental events by participating in multi-level regulation during cell division and differentiation processes and programmed cell death (PCD) mechanisms (Otvös et al. 2005; Gabaldón et al. 2005; Shen et al.

2013; Wang et al. 2013), events that systematically shape a plant body. Along with NO, Reactive Oxygen Species (ROS) are also associated with plant growth and development involving PCD (del Rio 2015).

A recent study by Huang et al. (2014) documented that alterations in expression of *Pgb* genes was observed during 'stressed' conditions such as hypoxia which resulted in manifestation of PCD, implicating the possible role of Pgb in life/death decision (Huang et al. 2014). Alteration in *Pgb* expression followed by cell death events were observed during embryogenesis (Huang et al. 2014), aerenchyma formation during hypoxia (Dordas et al 2003; Parent et al. 2011) and during plant pathogen interaction (Maassen and Hennig 2011; Mur et al. 2012). Occurrence of PCD during embryogenesis is crucial in order to shape the embryo and to remove the rest of the organs in the seed (Mira et al. 2016 b). For instance, termination of suspensor (tissue involved in nutrient transport and structural support) during mid-embryogenesis via process that resembles autolytic PCD (Bozkhov et al. 2005a) is only one of the cell death events occurring during embryogenesis (Filonova et al. 2002). Recently, Huang et al. (2014) observed manifestation of PCD during maize somatic embryogenesis when the two *Pgb* genes, *ZmPgb1.1* and *Zmpgb1.2* were suppressed and an increase in NO level was observed in the particular cells where *Pgb* genes were expressed. While the down regulation of *ZmPgb1.1*, normally expressed in several domains of the embryo, triggered massive PCD resulting in embryo abortion, down regulation of *ZmPgb1.2*, which is expressed specifically in anchoring cells, resulted in removal of these 'anchor cells' only, thus releasing the young embryos to grow and develop further (Huang et al. 2014). This suppression of two *Pgbs* resulted in differential embryo outcome, while the suppression of *ZmPgb1.1* had reduced embryo yield and the down regulation of *ZmPgb1.2* increased the embryo yield. However, in the case of a dicot, using Arabidopsis somatic

embryogenesis as the plant model, Elhiti et al. (2013) established a link between *Arabidopsis* phytoglobin 2 (*Pgb2)* and plant hormone auxin. Down-regulation of *Pgb2* resulted in increased embryonic yield by accumulating auxin during Arabidopsis somatic embryogenesis. This accumulation was associated with increased expression the enzymes participating in tryptophan and indole-3-acetic acid (IAA) biosynthesis mediated by transcription factor MYC2 (Elhiti et al. 2013). The effect was the result of an increased level of NO due to suppression of Pgb2. To further describe the interaction between different plant hormones and Pgbs, Mira et al. (2016 b) used the same Arabidopsis somatic embryogenic model and described the effect of increase in NO resulting from Pgb2 suppression on Jasmonic Acid (JA) synthesis. Furthermore, the increase in JA level due to NO accumulation in Pgb2 suppressed embryogenic cells also favored IAA accumulation through modulation of JA-responsive genes (Mira et al. 2016 b).

The fact that Pgbs effectively scavenge nitric oxide and that NO regulates several developmental processes including PCD by interacting with various signaling molecules and phytohormones is the basis of the objective of the current research, which is to investigate the role of ABA and ethylene in the *ZmPgbs* regulation of somatic embryogenesis in maize.

1 LITERATURE REVIEW

1.1 Embryogenesis

1.1.1 Introduction

Development of embryos plays a crucial role in the plant life cycle. Formation of embryos in flowering plants requires double fertilization which eventually leads to formation of an embryo and the endosperm. During embryogenesis various molecular and cellular levels take place that results in the formation of a two-celled structure called a pro-embryo. Development of embryos in angiosperms follows basic and well described developmental stages (Yang et al. 2010).

Zygotic embryogenesis in plants occurs within ovules of a female gametophyte which is usually a small structure. The forming embryo is fully embedded in the ovule and therefore difficult to extract. Thus until the 1990s studies on early embryogeny were very limited. Reproduction in flowering plants depends on the time in which male and female gametes form from the respective gametophytes. Both gametophytes arise from single haploid spores undergoing a series of mitotic divisions following different patterns of development which is often unique to species (Yang et al. 2010)

During formation of the female (macro) gametophyte the haploid spore undergoes three incomplete mitotic divisions giving rise to an eight nucleate syncytium (Fig1.1) (Ma and Sundaresan 2010; Yadegari and Drews 2004) which further undergoes cellularization and produces two synergids, one egg cell and three anitopodal cells. The synergids and the egg cells are usually located at the micropylar end while the antipodal cells are located at the chalazal end of the syncytium (Fig 1.1) (Yang et al. 2010).

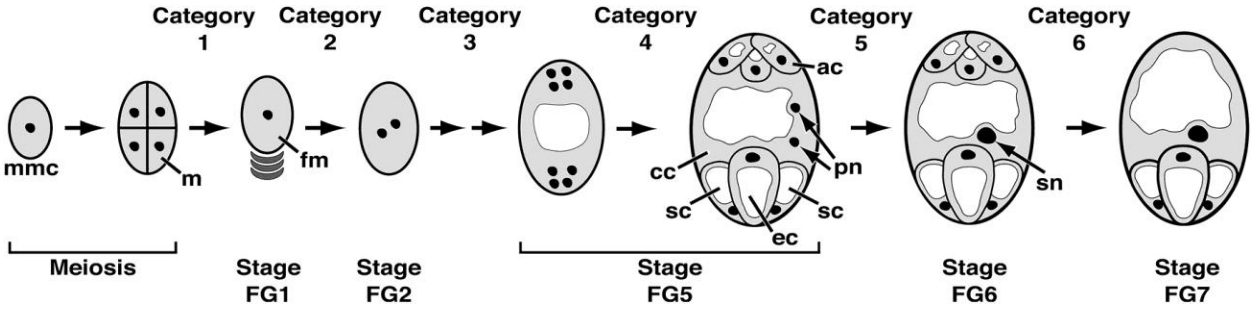

Fig.1.1: Developmental stages of female gametophyte. ac, antipodal cells; cc, central cell; ec, egg cell; fm, functional megaspore; m, megaspore; mmc, megaspore mother cell; pn, polar nuclei; sc, synergid cell; sn, secondary nucleus. Figure is from Yadegari and Drews (2004) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

The haploid spore giving rise to the male gametophyte undergoes two mitotic divisions forming a micro gametophyte containing two sperm cells. During double fertilization, one of the sperm cells fuses with the egg and forms a diploid zygote while the other sperm cell fuses with central cells generating a triploid endosperm. Formation of the diploid zygote is accompanied by mostly unknown molecular events leading to a mitotic division generating the two celled pro-embryo (Lau et al. 2012; Willemsen and Scheres, 2004). The triploid endosperm formed during double fertilization also proliferates simultaneously and undergoes cellularization around the time the embryo reaches the cotyledonary stage. The triploid endosperm surrounds as well as supports the growing embryo by providing nutrients and cues regulating the development of the embryo (Yang et al. 2010).

In monocot species, including *Zea mays* after the formation of the zygote, a two celled structure is formed which undergo multiple asymmetric cell divisions to form pro embryo. Soon after the pro embryo stage, the protoderm forms marks the beginning of "transition" phase (Fig

2) (Vernoud et al. 2005). Following the transition phase, development of root and shoot apical meristems initiate and the embryo finally reaches the coleoptilar stage (Fig 2). During these developmental stages, the suspensor disintegrates and the embryo expands in size as a result of accumulation of reserved substances in the scutellum region (Fig 1.2) (Vernoud et al. 2005).

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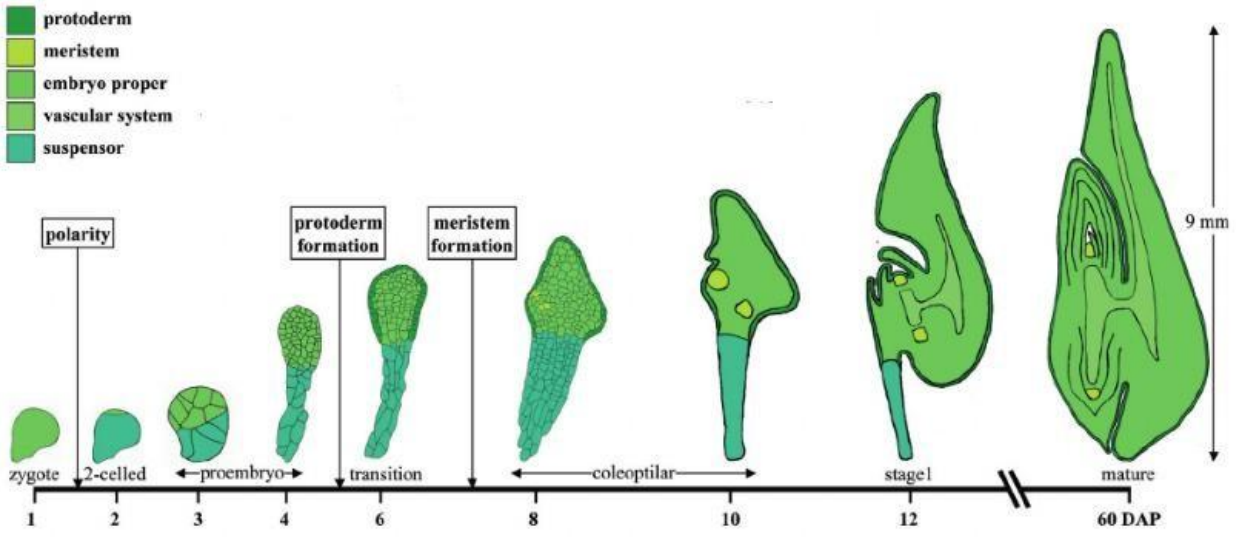

Fig. 1.2: Embryogenesis in monocot: From left to right depicting the different developmental stages according to the sizes of the embryo are shown.The time scale used is days after pollination (DAP). The stages are: zygote formation, 2 celled stage, early and late proembryo stage, transition stage, early and late coleoptilar stage, stage 1 and mature embryo. Figure is from Vernoud et al. (2005) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

Overall, embryogenesis encompasses three major events (Jurgens 1995). The first is initiated with the asymmetric division of the diploid zygote giving rise to two cells with definite cell fates; the second event involves the formation of the pro-embryo characterized by a coordinated

pattern of proliferation and differentiation; the final event produces the cotyledonary stage embryo characterized by root and shoot primordia at the opposite pole of the embryonic axis and one or more cotyledons arising from the shoot apical meristem (Dodeman et al. 1997).

Formation of embryos in higher plants can also occur without the fertilization event, such is the case of apomixis, where embryos and seeds develop directly from unfertilized egg and/or any totipotent somatic cell of the reproductive structure (Bicknell and Koltunow 2004; Koltunow et al. 1995). Furthermore, in vitro systems are also suitable to generate embryos from vegetative cells. A good example of in vitro embryogenesis is somatic embryogenesis, the formation of embryos from somatic cells, i.e. cells other than gametes (Fig 1.3) (Feher 2005). Due to the physiological and morphological similarities between somatic and zygotic embryos (Leljak-Levanic et al. 2015), some culture systems, including *Daucus sp.* and *Medicago sp.* have been used as models to study embryogeny (Dudits et al. 1991; Ozeki et al. 1990).

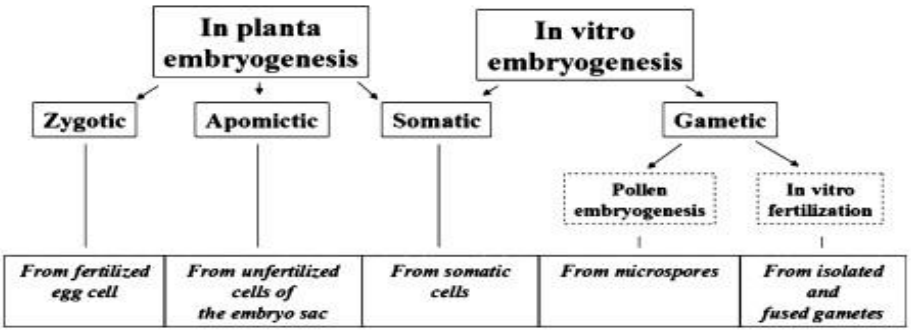

Fig. 1.3: Formation of embryo in higher plants via different pathways. This figure is from Feher (2005) and permission has been obtained from the publisher/copyright holder to incorporate it in thethesis.

To date, several strategies and protocols are available to generate somatic embryos from several genera and species (Komamine et al. 2005; Schmidt et al. 1997.) and in several systems, embryogenic cells are induced by culturing the explants under high auxin concentrations for a certain period following by a transfer on auxin-free media required for the formation of the embryos. During the somatic-embryogenic transition cells become highly cytoplasmic and congregate to form pro embryogenic masses (Halperin 1966). The percentage of somatic cells embarking in the embryogenic pathway varies from system to system and can be as low as 1-2% in carrots (de Vries et al. 1988). Therefore understanding the embryogenic inductive step is paramount for improving embryogenic output and gaining knowledge on cell fate acquisition.

Mutant analyses are a powerful tool to study plant embryogenesis. This has been the case especially for *Arabidopsis thaliana, Zea mays* where the use of mutants have expanded the knowledge on the molecular mechanisms involved in embryogenesis (Clark and Sheridan 1991, Lukowitz et al. 1996; Scanlon et al. 1994). In conjunction with the advancement of tissue culture and molecular techniques, mutant analyses have revealed the importance of several genes operating during the middle and late phases of embryogenesis (Bommert and Werr 2001). Genes controlling early embryogeny are more difficult to identify as their mutation is often lethal (Bommert and Werr 2001). The possible reason behind the arrest of embryo growth can be due to housekeeping functions of the genes, which if disrupted prevents further growth. Mutant embryo phenotype is often associated to a set of genes known as *EMBRYO DEFECTIVE*, *EMB*, genes (Meinke and Sussex 1979, Meinke 1985, Meinke et al. 2008). The mutations in these genes are also called *GERMLESS OR LETHAL EMBRYOS* (Vernoud et al. 2005).

Identification of genes related to embryogenesis has also be aided by advancements in the field of plant genetics and various molecular biology techniques such T-DNA insertion mutation (Muralla et al. 2011). Additionally, techniques like micro-dissection, next generation sequencing and microarrays have helped scientists to compare the somatic and zygotic embryogenic pathways at various stages of development (Muralla et al. 2011; Thibaud-Nissen et al. 2003). The following section examines the physiological and molecular mechanisms governing embryogenesis in maize, the plant system used in this thesis.

1.1.2 Molecular aspects of Maize embryogenesis

In the past years a lot of studies on maize have resolved many important genetic facets in relation to the regulation of gene expression and developmental aspects of embryogenesis. As presented above, sexual reproduction in higher plants is characterized by the double fertilization event, where one sperm cell of the male gametophyte fuses with the egg cell of the female gametophyte, while the second sperm cell fuses with the two nucleate central cells and develops into a triploid endosperm (Randolph 1936). According to Bommert and Werr (2001), the genetic make-up of a developing embryo and endosperm includes equal contributions from both maternal and paternal sides. However, the developmental pattern of both embryo and endosperm exhibits huge differences. For instance, the initial phases of embryo formation involve extensive cellular division while those of the primary endosperm are characterized by synctial growth with only nuclear division. The uniqueness of these developmental programs is also retained when using *invitro* techniques, in which sperms are artificially fused with egg or central cells (Kranz and Lorz 1994; Kranz et al. 1998). These developmental differences might arise from genetic

dosage differences or specific contributions from the maternal side to the egg cell or central cells (Bommert and Werr 2001).

Several mutations affecting the development of both embryo and endosperm in maize have been identified and some Mutant analyses studies showed that regulation of development in the endosperm and embryo relies on unique sets of genes (Bommert and Werr 2001)

1.1.2.1 Genes expressed during endosperm and aleurone development

Maize endosperm undergoing free nuclear division follows a relatively predictable developmental pattern. Following division, the nuclei align themselves to the periphery along the plasma membrane (Bommert and Werr 2001). Nuclear alignment is soon followed by cellularization involving distinct types of cytokinesis and resulting in the formation of three major tissue layers: the peripheral cell layer, the future aleurone layer, and the syncitial layer that will give rise to the starchy endosperm (Bommert and Werr 2001). Cellularization of the aleurone further leads to the development of four different compartments: the aleurone layer, the starchy endosperm layer, the layer surrounding embryo, and the basal transfer layer (Fig1.4) (Slocombe 1999).

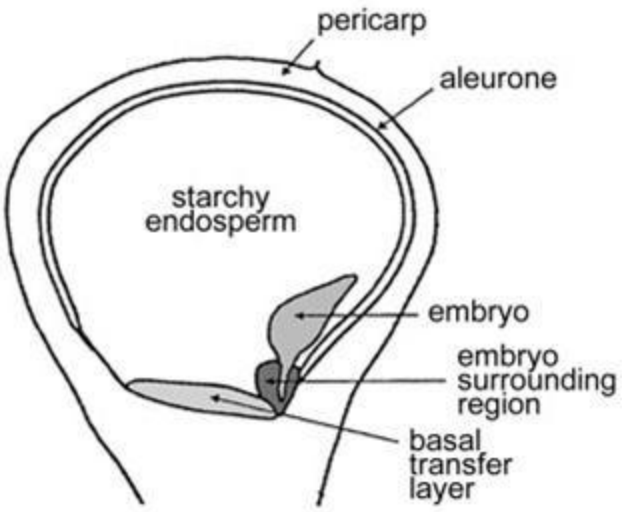

Fig. 1.4: Developing maize kernel (vertical section) showing different domains of maize embryo. This figure is from Bommert and Werr (2001) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

A well characterized marker of aleurone formation is the initiation of anthocyanin biosynthesis (Bommert and werr 2001) involving the expression of a set of genes including the transcription of *Viviparous-1* (*Vp1*) gene that encodes for viviparous protein which ultimately contributes in the regulation of anthocyanin biosynthetic pathway (Bommert and werr 2001). Expression of VP1 is then necessary for inducing *C1* on chromosome 9 which interacts with a protein encoded by the *R* gene. The heterodimer (*C1/R)* is required for the downstream activation of biosynthetic genes (Cone 1994).

Aleurone differentiation is regulated by different genes. The maize *defective kernel1 (dek1)* gene plays an important role during aleurone cell fate specification and *dek1* loss of function mutants shows absence of aleurone layer (Becraft et al. 2002; Lid et al. 2002). *Defective kernel1* encodes a membrane protein that localizes to the plasma membrane and may interact with extracellular signaling molecules (Becarft and Yi 2011; Tian et al. 2007). Another positive regulator of aleurone cell fate found in Maize is *CRINKLY4 (CR4)*, which is a receptor like-kinase (Becraft et al. 1996, 2001, Jin et al. 2000). *cr4* mutants fail to form the aleurone layer and phenocopy of *dek1* mutants (Becraft and Yi 2011).

Study by Huang et al. (2014) have highlighted the role of multiple genes in the formation of maize endosperm. These include *shrunken 2* and *brittle 2* which encode cytosolic ADP-Glucose Pyrophosphorylase subunits providing nucleotide sugars during starch biosynthesis. Another important maize gene recently reported by Sabeli et al.(2013) is *Retinoblastoma- related gene1 (RBR1)*, which plays multiple roles during endoreduplication, regulation of cell size and cell death of endodermal cells. Recessive *RBR1* mutants increase the expression of several genes including *RBR3*-type, *MINICHROMOSOME MAINTENANCE 2–7* , as well

as *PROLIFERATING CELL NUCLEAR ANTIGEN*, which encode DNA replication factors (Sabeli et al. 2013).

Molecular markers demarking the formation of the layer surrounding the embryo are the *ESR* (*Embryo Surrounding Region*) genes encoding small signal peptides (Opsahl -Ferstad et al. 1997). The transcription of these genes, restricted to the area around the embryo is limited to the "celularization period" of the endosperm. The observation that the *ESR* genes are not transcribed in embryo-less kernels implies that their expression is induced by signals produced by the embryo (Opsahl-Ferstad et al. 1997). Overall, studies on early endosperm development have provided insights into developmental cues governing endosperm regionalization (Bommart and Wer 2001).

Different types of storage proteins, including albumins, globulins, and prolamins (Landry and Moureaux 1970), are present in maize kernels. Two of the most predominant ones are 7s globulin in the embryo (Kriz 1999) and the zein fraction in the endosperm (Landry and Moureaux 1970; Wilson 1983). Genomic analysis conducted by Woo et al. (2001) suggested that the zein genes have the highest expression level in the maize endosperm. Three new zein proteins: 50kD γ- zein, 18-kDα –globulin and alegumin related protein have been identified (Woo et al. 2001).

1.1.2.2 Genes expressed during early embryogeny

During the formation of the maize kernel the development of the embryo is much slower than that of the primary endosperm (Bommert and werr 2001; Randoph 1936). Unlike dicots, division of the apical cell of the two-celled maize embryo is highly asymmetrical, resulting in the

formation of a cluster of small, highly cytoplasmic cells. This cluster of cells will give rise to all the organs of the embryo proper. Irregular cell division pattern also occurs in the basal cell, the progenitor of the suspensor characterized by several layers of cells of different size (Bommert and Werr 2001). A molecular marker specific to the early development of the embryo proper is *Zea mays homoebox (ZmHOX*). The expression of this gene, first detected 6 days after pollination (dap), is restricted to the derivatives of the apical cells, but not in the suspensor (Bommert and Werr 2001).

One of the tissues first detected in the embryo proper is the epidermal layer. Epidermal cell fate appears to be regulated by the *lipid transfer protein 2(LTP 2)* gene. This gene is expressed in the peripheral cells of the embryo proper which participates in the polar transport of lipids from the inner cells to the surface cells of the embryo (Sossountzov et al. 1991). Of note, *LTP2* is not expressed at the same levels in all epidermal cells, but rather confined especially in the abaxial side of the embryo (Bommert and Werr 2001). The absence of this gene in the adaxial cells, progenitor of the future shoot apical meristem, suggests *LTP2* is not required for the development of the apical pole.

Another set of markers identified and expressed at the pro-embryo stage are the *Zea mays outer cell layer (ZmOCL)* genes, the expression of which is also confined to the epidermis. So far five distinct transcripts of *ZmOCL* have been identified with differential expression patterns. For instance, the expression of *ZmOCL4* is weaker in the abaxial site and stronger in the adaxial site of the outer cell layer (Bommert and Werr 2001).

Recent studies have shown that plastids play an important role during embryogenesis (Li et al. 2015; Magnard et al. 2004; Shen et al. 2013). For instance, studies by Bryant et al. (2011) and

Romani et al. (2012) suggest that at least 30% of embryo defective mutants found in Arabidopsis are linked to genes that encode plastid proteins. Mutants of *ZmPRPL35a (Zea mays plastid ribosomal protein*- large subunit 35a), encoding a ribosomal protein (Magnard et al. 2004) and *maize seeds of lethal embryo* (*lem1),* encoding the ribosomal protein RPS9 (Ma and Dooner 2004) exhibit embryo lethality at very early stages. Overall, these studies suggest that plastid development plays an important role during early embryogenesis and seed development (Li et al. 2015).

1.1.2.3 Genes expressed at middle- later stages of embryogenesis

On the basis of phenotypic expression, maize seed mutants are broadly categorized into several classes, such as, *empty pericarp (emp), defective kernel (dek) embryo and defective/ specific (emb)* and *small kernel(smk)* (Becraft et al. 2002; Liu et al. 2013; Li et al. 2014; Shen et al. 2013; McCarty et al. 2005). All the genes mentioned above contribute to the development of the middle-stage embryos. For example, mutation of *Zea mays empty pericarp5 (Zmemp5)* interferes with post-transcriptional mechanisms resulting in delayed embryonic growth and formation of wrinkled pericarps (Liu et al. 2013). A similar interference with post-translational mechanisms was also observed in *smk* mutants characterized by endosperm and embryo lethality (Li et al. 2014). Malformation of the embryonic axis and distortion in the developmental pattern of leaf epidermis are apparent when *dek1* is mutated (Becraft et al. 2002). McCarty et al. (2005) suggested that ~17% of maize seed mutants follow in the category of *emb* mutants.

Unlike Arabidopsis, embryogenesis in maize follows more complex division and proliferation patters and therefore prediction of cell and tissue fate cannot rely on structural characteristics but rather on molecular markers (Nardmann and Werr 2009). A key event during embryogenesis is

the formation of the shoot apical meristem which is demarked by the expression of the homoebox transcription factor *KNOTTED 1(KN1)* in the apical domains (Jackson et al. 1994; Smith et al. 1995; Takacs et al. 2012). Expression of this gene is soon followed by the induction of *NO APICAL MERISTEM (ZmNAM)* and *Zea Mays CUP SHAPED COTYLEDONS3 (ZmCUC3).* While the former is expressed during the middle-late stages of embryogenesis and contributes together with *KN1* to the specification of meristem identity (Zimmermann and Werr 2005), *CUC3* is required during late embryogeny to demark cells at the periphery of the shoot apical meristem which will become committed to form lateral organs (Zimmermann and Werr 2005).

Formation of shoot apical meristem is preceded by the bulging of the apical domain of coleoptilar stage embryos, whereas, the root apical meristem forms in the central region between the embryo and the suspensor (Bommert and Werr 2001). Lim et al. (2000) localized the expression of a specific marker for the single endodermis layer of the maize root, *Zea Mays SCARECROW (ZmSCR).* This gene has been shown to play an important role in cell fate specification during formation of the Arabidopsis root the apical meristem (Bommert and Werr 2001).

Another important gene which encodes a transcription factor contributing to the establishment of the late maize embryo is *YABBY* (Takacs et al. 2012). Expression of YABBI is localized in the leaf and floral primordia of maize (Juarez et al. 2004). The same authors documented an induction of several YABBY members, including *Zea maysYABBY14 (ZYB14), ZYM9* and *Mays ZYB10* in meristematic tissue of the embryos (Juarez et al. 2004).

1.1.3 Somatic Embryogenesis in Maize

Maize is one of the major commercial crops that have applications worldwide; it is used not only for human and animal feed but also as a stock for producing bio-combustibles like ethanol and biodiesel (Blanco Fonseca 2010). With the advancements in plant transformation techniques, increase in commercial crop production has become more achievable. Maize has been one of the most targeted crops by scientists to generate transgenic cultivars for both basic and applied research (Salvo et al. 2014). Among the different types of *in vitro* propagation methods somatic embryogenesis is one of the most successful in maize. The technique, based on the "totipotent" nature of plant cells (West and Harada 1993) allows the formation of embryos from somatic cells and has become a model system also to study physiological and molecular events occurring during *in vivo* embryogenesis (Ikeda et al. 2006; West and Harada 1993; Zimmerman 1993;). This is because somatic embryos resemble their zygotic counterpart morphologically and to some extent physiologically. Generation of embryogenic cultures depend on various factors, such as the genetic background and type of explants (Garrocho-Villegas et al. 2012). For instance, in maize production of highly embryogenic tissue is only achieved by culturing immature zygotic embryos harvested between 12-18 days after pollination (Armstrong and Green 1985; Sanchez et al.1988).

Embryogenic cultures of maize are generally composed by two types of calli: type I and type II (Armstrong and Green 1985; Garrocho-Villegas et al. 2012), with the latter able to retain embryogenic competence for more than 2 years and is thus mostly used to generate somatic embryos during in vitro culture (Garrocho-Villegas et al. 2012). A lot of research is currently available on the physiological and molecular mechanisms characterizing embryogenic tissue

formation and regulating the progression of somatic embryo development and germination (Che et al. 2006; Duncan et al. 2003; Fontanet and Vicient 2008).

According to Garrocho-Villegas et al. (2012), plant regeneration from maize somatic embyros is achieved by following three specific culture steps: propagation of embryogenic tissue from zygotic embryos (a,b), maturation of somatic embryos from embryogenic cells (c,d). (Fig 1.5)

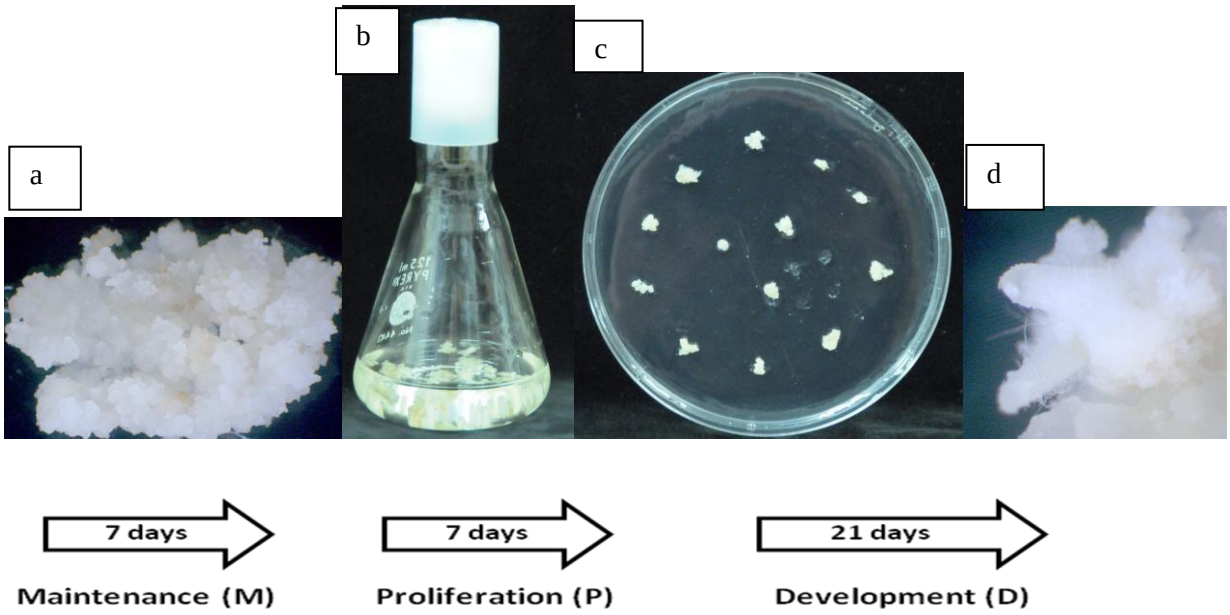

Fig.1.5: Maize somatic embryogenesis: From left to right depicting stages of maize somatic embryogenesis: (a) friable callus maintained for 7 days in maintainence media (b) followed by 7 days in liquid proliferation media and (c) 21 days in developmental media for embryo development (d) developing somatic embryo

1.3.1 Genes involved in maize somatic embryogenesis

1.1.3.1.1 Early Embryogenesis

Embryogenesis is initiated when somatic cell express totipotency (Nolan et al. 2006) and reprogram their developmental fate by becoming embryogenic cells that are able to proliferate and produce embryogenic tissue (Zavattieri et al. 2010). One gene considered a marker for the somatic-embryogenic transition is *SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK)* (Schmidt et al. 1997). Independent studies have identified *SERK* genes in a variety of plant species such as *Daucus sp.* (Schmidt et al 1997), *Arabidopsis* (Hecht et al. 2001), maize (Zhang et al. 2011), and *Musa sp.* (Huang et al. 2010). Studies conducted by Hecht et al. (2001) on *Arabidospsis* suggest a strong expression pattern of *SERK* during the early phases of embryo formation, and similar results were reported by Baudino et al. (2001) in maize. Three members of *Zea Mays SERK (ZmSERK1, ZmSERK2, ZmSERK3)* were characterized and their differential expression pattern was observed. *ZmSERK1* was expressed in the male and female reproductive tissues while *ZmSERK2* was highly expressed in microspores (Baudino et al. 2001). Both genes are also required for the proper initiation of embryo development (Baudino et al. 2001; Zhang et al. 2011). *ZmSERK3* is also important for embryogenesis with high low levels stimulating embryo initiation and high levels inhibiting the formation of the embryos (Zhang et al. 2011).

Initiation of embryogenic pathway is also regulated by *LEAFY COTYLEDON (LEC)* genes. These genes were first identified during zygotic embryogenesis and further studied by altering their expression in somatic cells (Lotan et al. 1998; Gaj et al. 2005; Yang et al. 2010). Their function is related to the establishment of embryonic cell fate, as well as cotyledon fate (Meinke

et al. 1994; West et al. 1994). Using a hybrid maize line Zhang et al. (2002) documented the expression of *Zea Mays LEAFY COTYLEDON1 (ZmLEC1)* during initial stages somatic embryo development.

Another important gene inducing somatic embryogenesis and enhancing the regeneration ability of many plants species such as *Arabidopsis*, *Brassica napus*, is *BABYBOOM (BBM)* (Boutilier et al. 2002; Yang and Zhang 2010). Global transcriptome profiling by Salvo et al. (2014) identified three maize genes with sequence similarity to the *Brassica napus BABYBOOM1 (BnBBM1)* gene. The expression of these genes increased during the initial stages of somatic embryogenic, suggesting their participation in the somatic-embryogenic transition.

Phytohormones such as auxins are known to play a significant role during the apical basal patterning of zygotic embryos (Weijers et al. 2005), cellular differentiation during embryo and endosperm development (Forestan et al. 2010), and formation of somatic embryos (Su et al. 2009). The *Zea mays PIN FORMED1 (ZmPIN1)*, encoding an auxin efflux carrier is expressed during the initial phases of zygotic embryogenesis (Forestan et al. 2010). Transcriptome analyses further revealed a high expression of *ZmPIN1a* gene during early phases of somatic embryogenesis (Salvo et al. 2014).

1.1.3.1.2 Late Embryogenesis

Independent studies have provided evidences that the genes involved during meristem formation may play an important role during the process of somatic embryogenesis (Laux et al. 1996; Mayer et al. 1998). For instance in *Arabidopsis*, genes like *WUSCHEL (WUS)* and *CLAVATA (CLV)* are important regulators of shoot and floral meristem (Laux et al. 1996; Mayer et al. 1998) and may also participate during somatic embryo development when highly expressed (Zuo et al. 2002; Gallois et al. 2004). Genetic studies have revealed that *WUS* interacts with *CLV* in a pathway in which *CLV* is upstream of *WUS* (Clarks et al. 1997). This interaction is essential for stem cell identity (Brand et al. 2000). Interestingly, in *Arabidopsis* the ectopic expression of the homeodomain transcription factor *WUS* results in formation of enlarged meristems (Schoof et al. 2000). WUSCHEL, however, is not expressed in meristem cells itself but is restricted to the cells underneath the stem cells, also known as organizing centre (Mayer et al. 1998; Schoof et al. 2000). Detailed analysis of Arabidopsis *WUS* orthologs in maize and rice by Nerdmann and Werr (2006) highlighted the expression pattern of the two maize *WUS* genes during the formation of the spikelet pair meristem, the spikelet meristem and the floral meristem at later stages of embryogenesis. These studies suggest the participation of these genes in meristematic cell formation during late embryogeny, an observation also supported by the study of Salvo et al. (2014). Besides WUS, CLV genes are also involved in Arabidopsis meristem formation and maintenance (Clark et al. 1997). Although identified in maize (Salvo et al. 2014).

, the function of this gene during maize embryogenesis is not understood

Regulation of cell division and differentiation in the shoot apical meristem is controlled by members of the *KNOTTED LIKEHOMEOBOX (KNOX)* transcription factors (Hay and Tsantis

2010; Smertenko and Bozhkov 2014; Vollbrecht et al. 1991). The absence of a functional apical pole in maize plants where *Zea Mays KNOTTED1 (KN1)* was mutated (Vollbrecht et al. 1991) suggests that this gene might also be involved in the regulation of the maize shoot apical meristem during embryogenesis and post-embryonic development. The function of this gene during maize somatic embryogenesis has not been investigated. From the studies described above, it is apparent that relative to the model plant Arabidopsis, the molecular mechanisms regulating maize somatic embryogenesis are poorly understood.

1.2 Abscisic Acid (ABA)

1.2.1 Biosynthesis, metabolism and transport

Abscisic acid (ABA) is a stress related plant hormone which participates in a variety of plant growth and developmental processes ranging from the regulation of seed dormancy (Bewley 1997) to acclimation during stress conditions (Milborrow 2001). These physiological responses depend on the amount of ABA generated endogenously and on the rate of ABA synthesis and catabolism (Endo et al. 2014). ABA is known to be synthesized in bacteria associated with plants, plant pathogenic fungi, blue green bacteria, green algae, symbiotic algae like lichens and also in human granulocytes (Bruzzone et al. 2007; Hartung 2010; Nagamune et al. 2008). Extensive studies conducted on higher plants and on various pathogenic fungi suggest two routes of ABA biosynthesis (Lichtenthaler 1999; Newman and Chapell1999). These studies have been aided by the characterization of many ABA defective mutants (Bauer et al. 2013; Ikeda-Iwai et al. 2006).

ABA is a fifteen carbon compound that is known to originate from with isopentenyl pyrophosphate (Endo et al. 2014). However, the generation of ABA can happen either directly involving the mevalonate pathway as in case of many prokaryotes and phytopathogenic fungi (Newman and Chapell 1999) or indirectly using the methylerithritol phosphate pathway as observed in cyanobacteria and all photosynthetic plants (Lichtenthaler 1999).

In the direct route ABA biosynthesis begins with farnesyldiphosphate (Hartung 2010) and a variety of ionylideneethanol isomers as precursors (Oritani and Kiyota 2003). This direct biosynthetic pathway can be broadly divided into two stages; the first stage ionylideneacetate is produced from farnesyldiphosphate , while the second stage involves several oxidation steps generating ABA (Nambara and Marion-Poll 2005).

The indirect route of ABA biosynthesis occurs in higher plants where production of ABA occurs in chloroplasts and in the cytosol (Schwartz et al. 2003; Xiong and Xu 2003). In this route the precursor, isopentenyl diphosphate, is utilized to produce C_{40} xanthophyll, an oxygenated carotenoid that breaks down to form ABA (Schwartz et al. 2003; Xiong and Xu 2003) (Fig 1.6). The first few conversions involving the generation of neoxanthin (oxidized carotenoid) from isopentenyl diphosphate take place in the plastids while the later steps involving the oxidation of xanthoxin to ABA occurs in the cytoplasm. A schematic summary of the indirect pathway is shown in Fig (1.6).

Conversion of zeaxanthin to trans –violoxanthin (C_{40} xanthophyll) can be catalysed by the Zeaxanthin epoxidase (Fig 1.6). These series of reaction, referred as “the xanthophyll cycle “ marks the beginning of the indirect pathway (Endo et al. 2014).

The next step involves the conversion of trans-violoxanthin to 9'-cis to 9'cis- violoxanthin via a poorly characterized isomerase (Endo et al. 2014). Subsequently, the 9 cis isomers of violoxanthin are converted to C15 xanthoxin by the enzyme 9 cis-epoxycarotenoid dioxygenase (NCED) or VP14. This reaction appears to be a limiting step as overexpression of NCED increases greatly ABA synthesis (Endo et al. 2014).

C15 xanthoxin is subsequently transported from the chloroplast into the cytosol where it undergoes oxidation to form abscisic aldehyde (Endo et al. 2014). Abscisic aldehyde is then converted to ABA by the activity of oxidase (AAO) and MoCo sulfurase (Endo et al. 2014).

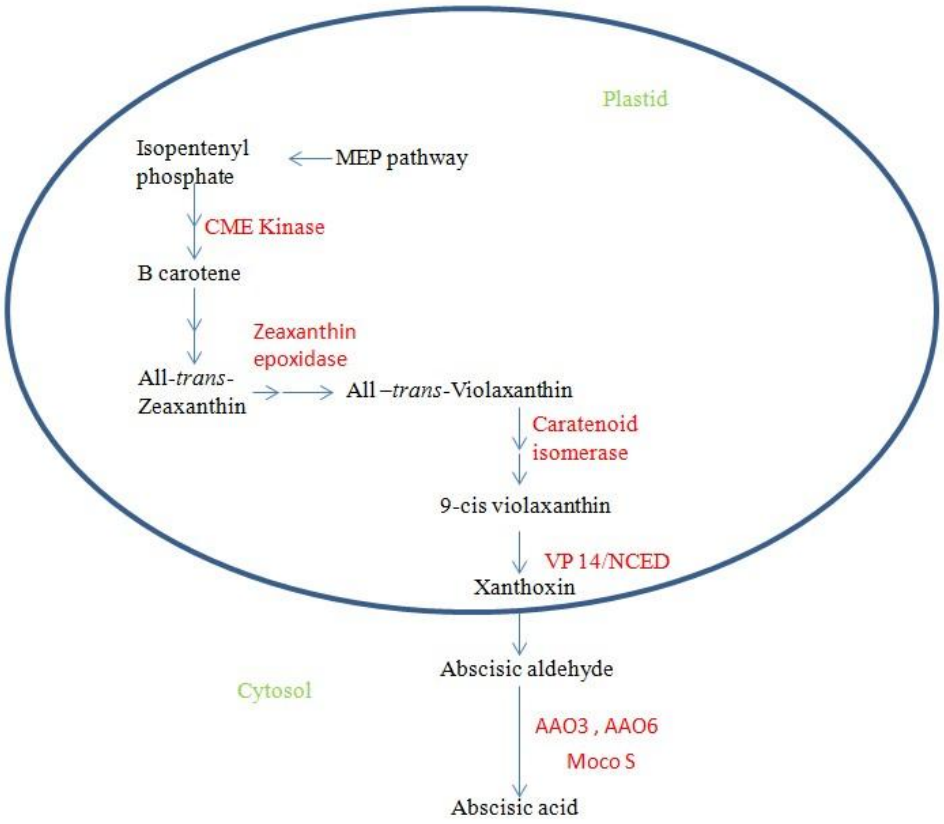

Fig. 1.6: Biosynthetic pathway of Abscisic Acid.The ABA biosynthetic pathway is initiated by a C40 carotenoid, is generated from IPP via MEP pathway. Solid arrows symbolize one-step conversion of an intermediate and dashed arrows symbolize multistep conversions of an intermediate. Enzyme names are highlighted in bold. Abbreviations used: NCED 9-cisepoxycarotenoid dioxygenase, AAO abscisic aldehyde oxidase, MoCo S MolybdenumCo sulfurase.

1.2.2 Inhibitors of ABA

In order to identify the possible physiological and morphological effects of ABA on plant growth and development, several inhibitors have been identified and used. Biosynthetic inhibitors include fluridone, norflurazon, nordihydroguaiaretic acid , and abamine, while commonly used catabolic inhibitors are uniconazole, diniconazole, and (+)-9'vinyl –ABA (Endo et al. 2014).

Biosynthetic inhibitors target the activity of NCED activity or that of phytoene desaturase, the enzyme participating in carotenoid biosynthesis (Gamble and Mullet 1986). Catabolic inhibitors alter the activity of CYP707A , the enzyme that breaks down ABA to phaseic acid (Kitahata et al. 2005; Saito et al. 2006).

1.2.3 Role of ABA during in vitro embryogenesis

Abscisic acid (ABA) has long been recognized as a key factor regulating cell proliferation, differentiation and morphogenesis both in vivo and in vitro (Feher et al. 2003). Applications of ABA during in vitro plant embryogenesis usually lead to impaired growth of the cultured tissue (Engelmann 1991; Rai et al. 2009). However, when supplied with other plant hormones or media components it might have positive effects depending on the plant system utilized (Ficcadenti and Rotino 1995; Sen et al. 1989). For instance when added in the culture medium in conjunction with auxins, ABA with maximum concentration of 1×10^{-4} M increases the number of carrot somatic embryos (Nishiwaki et al. 2000). Applications of ABA is also a requirement for promoting somatic embryogenesis in conifers, especially in combination with polyethylene glycol (PEG), a non-penetrating osmoticum which lowers the water potential thus favoring the

initiation of somatic embryos (Stasolla et al. 2002). Besides PEG, glutamine has also been often co-applied with ABA to induce plant embryogenesis (Baskaran and Jayabalan 2009).

The function of ABA during in vitro embryogenesis is often associated to the ability of this growth regulator to promote the maturation of the embryos with a lot of studies focusing on the accumulation of storage products. For example, lipid accumulation in somatic embryos of many seed oil producing plants is enhanced by the exogenous addition of ABA (Kharenko et al. 2011). Similarly, several studies showed an increased synthesis of fatty acids, sugars and late embryogenesis abundant (LEA) proteins in ABA-treated somatic embryos of both angiosperms and gymnosperms (Chugh and Khurana 2002; Dodeman et al. 1997; von Arnold et al. 2002). The induction of stress-related factors such as raffinose oligosacharides following ABA applications further confirms the requirement of ABA during the maturation stages of the somatic embryos (Chugh and Khurana 2002; Dodeman et al. 1997; von Arnold et al. 2002; Blochl et al. 2005). A similar requirement is also observed during in vivo embryogenesis (Nakagawa et al. 2001; Rajasekaran et al. 1987).

It cannot be excluded, however, that ABA is also needed during the early-middle phases of embryogenesis by regulating tissue patterning (Vahdati et al. 2006, 2008). Tissue formation and establishment of the embryo body is the result of cell division, differentiation and programmed cell death (Pennell and Lam 1997; Carimi et al. 2003). Several studies have shown that regulation of PCD, which shapes the embryo body, influences the number of somatic embryos produced in culture and ABA has been shown to alter the death fate in several embryogenic systems (Wang et al. 1999; Carimi et al. 2003).

1.3 Ethylene

Ethylene is gaseous phytohormone which plays an important role in regulating growth, development and a variety of stress responses in plants (Xu and Zhang 2015). Often referred as a "stress" hormone, ethylene production is triggered by several stress conditions, especially during senescence, fruit ripening, and pathogen attack (Kende 1993; Wang et al. 2002; Yang and Hoffman 1984; Xu and Zhang 2015). Contrary to primitive algae and ferns where some of the mechanisms of ethylene synthesis are poorly understood, the biosynthetic pathway in higher plant is well defined (Xu and Zhang 2015)

1.3.1 Ethylene biosynthetic pathway

Extensive studies on ethylene have started from the 1960s. Ethylene has a simple chemical structure consisting of two double bonded carbon molecules with hydrogens bonded to each carbon. Due to this simple chemical structure, a variety of compounds were suggested as precursors (Adams and Yang 1979; Liebermanet al. 1966; Yang 1974).

The biosynthesis of ethylene begins with S-adenosylmethionine (Lieberman et al. 1966), an activated form of methionine . S-adenosylmethionine is then converted to 1- aminocyclopropane-1-carboxylic acid (ACC) by the enzyme 1-aminocyclopropane-1-carboxylic acid synthase (ACS) (Fig 1.7). An oxidation step catalyzed by 1-aminocyclopropane-1- carboxylic acid oxidase (ACO) is responsible for the production of ethylene from ACC (Kende 1993; Yu et al. 1979; Yang and Hoffman 1984).

1-Aminocyclopropane-1-carboxylic acid synthase (ACS) is encoded by a large multigene family of which nine isoforms have been identified in Arabidopsis (Tsuchisaka and Theologies

2004b ; Yamagami et al. 2003) and *Lycopersicon* sp. (Alexander and Grierson 2002; Jiang and Fu 2000). The genes are regulated by endogenous and exogenous stimuli at both transcription and post-transcription levels (Xu and Zhang 2015). Unlike ACS, ACO is encoded by a small gene family comprised of 3-5 members exhibiting diverse response to different environmental and developmental cues (Barry et al. 1996; Blume and Grierson 1997).

Production of ethylene relies on the availability of methionine which also participates in other cellular processes, including protein synthesis and methylation of proteins and nucleic acids (Xu and Zhang 2015). Methionine is generally synthesized at low levels, an observation suggesting the presence of alternative regeneration mechanism maintaining elevated levels of methionine in the cell. In the Yang cycle, the most known methionine- regenerating cycle (Fig 1.7) (Baur and Yang 1972), 5'-methylthioadenosine is generated as a byproduct during conversion of S-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid . Through subsequent reactions 5'-methylthioadenosine continuously regenerates methionine (Miyazaki and Yang 1987)

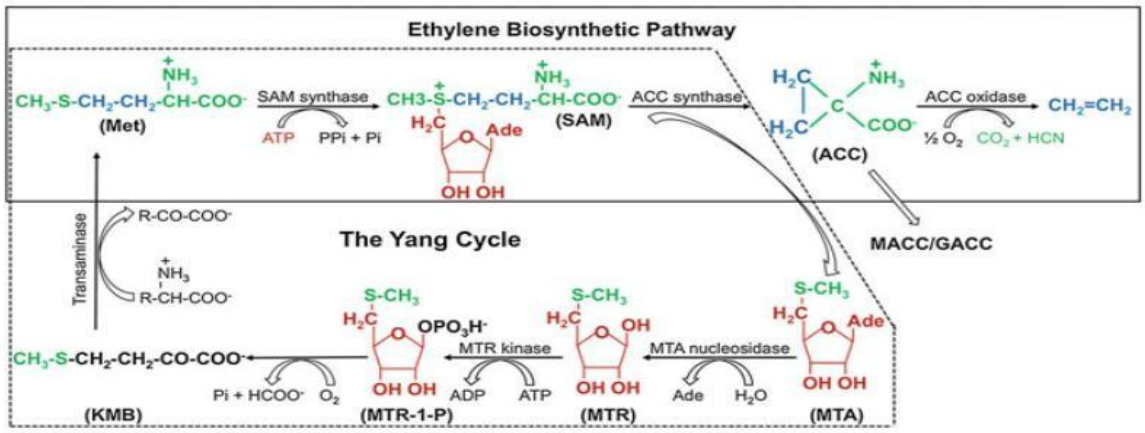

Fig. 1.7: Ethylene biosynthesis pathway and Yang cycle. Met is the precursor in ethylene biosynthesis and converts into SAM and ACC. SAM conversion to ACC by ACS produces a by-product, MTA is subsequently recycled to Met via Yang cycle. ACC undergoes oxidative cleavage to form ethylene, catalyzed by enzyme ACO. Met: methionine; SAM: S-adenosyl-L-methionine; ACC: 1-aminocyclopropane-1-carboxylate; MTA: 5′-methylthioadenosine; MTR: 5′-methylthioribose; MTR-1-P: 5′-methylthioribose-1-phosphate; KMB: 2-keto-4-methylthiobutyric acid; MACC: malonyl-ACC; GACC: 1-(γ-L-glutamylamino) ACC. Figure is from Xu and Zhang (2015) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

1.3.2 Ethylene and plant embryogenesis

Intensive studies have been done in order to explore the possible roles of ethylene on plant embryogenesis. Studies done so far highlighted the differential effects of ethylene on various developmental stages of plant embryo and also the interaction of ethylene with other phytohormones that contribute to the regulation of embryogenesis (Karami and Saidi 2010).

Independent studies have reported positive as well as negative effects of ethylene in different plant species. Research done by Thibaud et al. (2003) suggested an increase in expression level of the two ethylene biosynthetic genes in *Glycine max* somatic embryo development, implicating the possible association of ethylene during soybean somatic embryogenesis. These findings were supported by Mantiri et al. (2008) where application of ethylene induced embryogenesis in *Medicago* possibly by upregulating *SOMATIC RELATED EMBRYO FACTOR 1* (*MtSERF1*), a gene related to embryogenic competence. Similar stimulatory effects were also demonstrated on other species when disruption in ethylene biosynthetic pathway resulted in reduced embryo regeneration ability (Kepczynska et al. 2009; Kepczynska et al. 2011). Negative effects of ethylene on somatic embryogenesis were observed in *Coffea*, where inhibition of ethylene perception by $CoCl_2$ or $AgNO_3$ increased the number of embryos (Kumar et al. 2007)

The effects of ethylene on embryogenesis also appear to be dose-dependent. For example, in Arabidopsis (Zheng et al. 2013) and soybean (Zheng et al. 2013), somatic embryos were induced following small increases in ethylene levels by ACC (Chen and Chang 2003), while higher concentrations of ethylene (20 and 50uM) were required to stimulate embryogenesis in Oncidium (Chen and Chang 2003). The effect of ethylene on embryogenesis might be mediated by auxin, the inductive signal triggering embryogenic competence. Bai et al. (2013) observed a

repression of the auxin biosynthetic YUCCA genes when ethylene was applied to the Arabidopsis embryogenic tissue.

Ethylene also influences early embryogeny and seed development in both monocots and dicots. In Arabidopsis, the death of the synergid cells occurring after fertilization has been associated with the activation of the ethylene signaling pathway (Völz et al 2013). In cereals, ethylene plays a very important role during seed endosperm development by controlling events associated to PCD (Young et al. 1997; Young and Gallie 1999).

1.4 Phytoglobins (Pgb)

Hemoglobin are ubiquitous globular proteins having a secondary structure, comprising of a myoglobin – fold with A-H helices along with a heme prosthetic group that is settled in a hydrophobic cavity configuring a 3/3 sandwich of helices over one another (Vázquez-Limón et al. 2012). The two heme-Fe axial sites are coordinated either by side chains or ligands like O_2, NO, CO and few other lipid membranes. O_2 binding hemoglobins in the vertebrates were among the well- researched proteins whose structure and function have been extensively studied for more than 50 years (Hill 2012) (Fig 1.8). Phytoglobins are hemoglobins present in plants; they have a variety of functions and are expressed in many parts of a plant body (Hill 2012).

Comprehensive studies including genome sequencing ranging from bacteria to higher plants have been done as these proteins are known to manifest a variety of biological functions (Hill 2012). Leghemoglobin or symbiotic Phytoglobins were first identified in root nodules (Kubo 1939) of plants like soybeans, where they are involved in nitrogen (N_2) fixation by facilitating oxygen transport to symbiotic nitrogen fixing bacteria. One of the breakthrough discovery that

suggested the presence of distinct phytoglobins was the identification of a non-symbiotic phytoglobin in *Hordeum sp.* which had ~40% nucleotide sequence identity with leghemoglobin (Hill 2012). Characterization of additional phytoglobins in non-nodulating plants such as *Parasponia, Trema tomentosa, Casuarina* and others (Bogusz et al. 1988; Christensen et al. 1991; Hill 1998; Landsmann et al. 1986) suggested phytoglobins are functionally different than leghemoglobins (Hill 2012). To date there are more than 50 plant species known to express distinct phytoglobins (Huang et al. 2014).

1.4.1 Characteristics and classification of Pgbs

Hemoglobins are often associated to erythrocytes that facilitate oxygen transport in the animal circulatory system. The concentration of erythrocyte in animals may range from millimolar to submillimolar, which imparts a red color to the blood (Kundu et al. 2003). The concentration of phytoglobins in plants is quite low (5-20uM) (Hill 1998).

Biochemically, phytoglobins share structural similarity with animal hemoglobins as they contain a globular structure that is further attached to prosthetic groups facilitating the binding of ligands such as O_2, NO, CO H_2S and certain membrane lipids (Kundu et al. 2003) (Fig 1.9). On the basis of their chemical structure Pgbs can be hexa-coordinated and penta-coordinated (Fig 1.9) (Hill 2012).

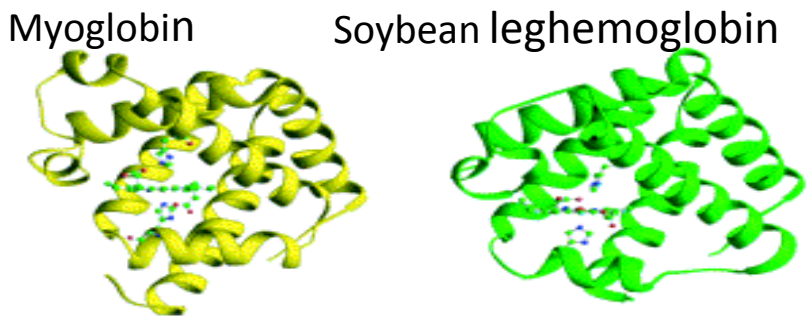

Fig 1.8: Myoglobin and LegHb structure. This figure is from Kundu et al. (2003) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

In the penta-coordinated structure, only the proximal histidine coordinates with the fifth site of the heme iron, leaving the sixth site open for reversible binding of ligands such as O_2 and NO (Fig 1.9). However, in the hexa-coordinated structure, both the proximal and distal histidine coordinate with the heme iron, facilitating tight binding of O_2 that can further accept an electron from iron and oxygenate NO to form nitrate (NO_3^-) (Fig 1.9) (Gupta et al. 2011).

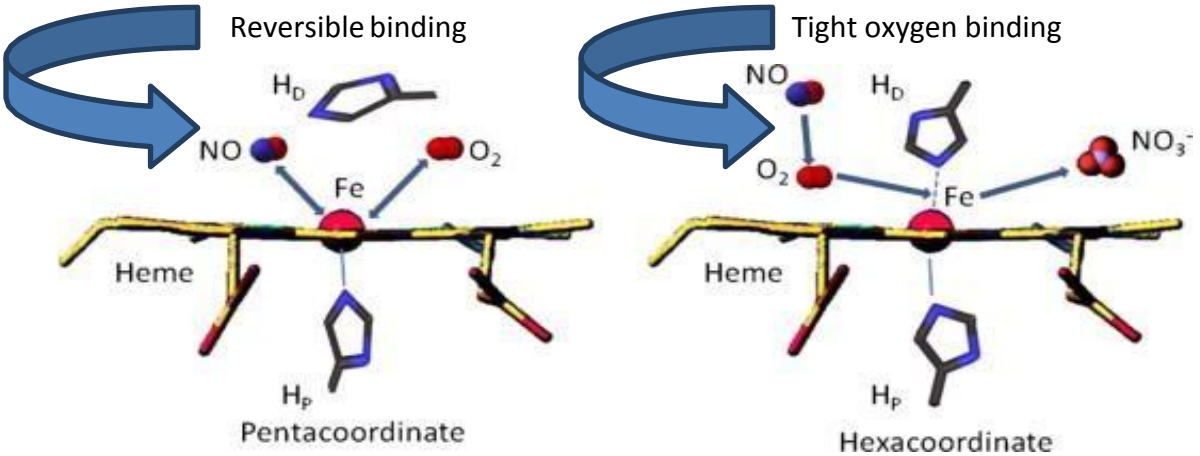

Fig. 1.9: Chemical structures of Pgb showing Penta coordination and Hexa coordination. This figure is from Gupta et al. (2011) and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

Phytoglobins are broadly divided into three main classes (class1-3) according to the phylogenetic analysis conducted by Gupta et al. (2011). Class 1 phytoglobins possess weak hexa-coordination characteristic and are expressed in cells under low oxygen tension (Hargrove et al. 2000). Upon binding of a ligand, such as oxygen, the distal histidine moves away from the iron atom and the protein attains in a more stable conformation (Hoy et al. 2007) which allows a very tight but slow oxygen binding during the scavenging of NO under near anaerobic conditions (Fig 1.9) (Perazzolli et al. 2004). During this interaction, Pgb and oxygen interacts to form oxyPgb that participates in oxygen dependent NO binding and/or scavenging under oxygen deficit conditions and produces non toxic nitrate (NO_3^-) and metPgb (Igamberdiev and Hill 2004; Nienhaus et al. 2010) (Fig 1.10). In the same model reductase activity is needed to convert the ferric state in metPgb to the ferrous state (Igamberdiev et al. 2006) (Fig 1.10)

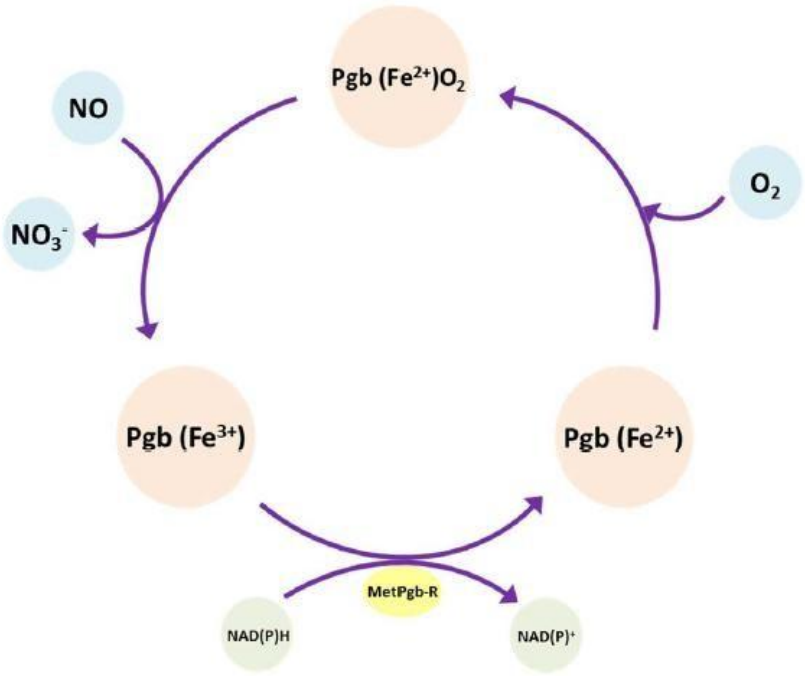

Fig 1.10: Formation of NO and MetPgb under hypoxic conditions. This is adapted from Igamberdiev and Hill, 2004 and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

Unlike class 1 phytoglobins, class 2 phytoglobins are usually not induced under hypoxia (Garrocho-Villegas et al. 2008) but their overexpression may promote the survival under low oxygen conditions (Kakar et al. 2010). Class 2 phytoglobins display tighter hexa-coordination than class1 phytoglobins, which makes them more efficient in sensing low oxygen level and less sensitive to scavenge NO (Kakar et al. 2010). A function of class 2 phytoglobin in oxygen storage and diffusion was also reported by Vigeolas et al. (2011). The authors further demonstrated that through this characteristic phytoglobins facilitate oxygen supply in developing seeds, thus promoting accumulation of polyunsaturated fatty acids (Vigeolas et al. 2011).

Class 3 Pgbs, also known as truncated Pgbs, were originally described by Watts et al. (2001) in *Arabidopsis*. Truncated Pgbs are generally expressed throughout the plant where they function as oxygen transporters with moderate oxygen affinity (Watts et al. 2001). Structurally these protein are different from other phytoglobins as their deoxygenated state displays temporary hexa-coordination, which involves ligand binding to the sixth bond of the heme iron molecule. The protein reverts back to its original and stable oxygenated penta-coordinate state in about 20 min (Gupta et al. 2011).

1.4.2 Expression pattern and functionality of phytoglobin

Independent studies have revealed that the timing of expression of phytoglobins is associated to the initiation of several embryogenic and post-embryogenic events modulated by NO (Igamberdiev et al. 2016).

1.4.2.1 Pgb expression and function during embryogenesis

Phytoglobins are highly expressed during *in vitro embryogenesis* and their expression might be involved in the formation of embryos. Smagghe et al. (2007) reported that both Pgb1 and Pgb2 are expressed in non-embryogenic chicory cells; however, Pgb2 was also expressed abundantly in embryogenic cells with potential to produce somatic embryos. A more comprehensive study was performed by Elhiti et al. (2013) who reported that suppression of the Arabidopsis Pgb2 enhanced somatic embryo yield by increasing the expression of key enzymes regulating the tryptophan and indoleacetic acid pathways. These changes led to increasing levels of auxin, the inductive signal required for the formation of embryogenic tissue. The same authors also demonstrated that these effects were initiated by the accumulation of NO, as a result of Pgb2 suppression, resulting in the repression of MYC2, an inhibitor of auxin synthesis.

This model has been expanded further by Mira et al. (2016 b) who suggested that the increase in NO levels by suppressing *Pgb2* stimulated the production of jasmonic acid which in turn elevated the levels of auxin by up-regulating *JAZ1(JASMONATE-ZIM-PROTEIN*) (JA-inducible nuclear-localized protein). These effects were also mediated by the suppression of MYC2. Using the maize somatic embryogenic system, Huang et al. (2014) reported that suppression of *ZmPgb1.1* inhibited the formation of somatic embryos while suppression of *ZmPgb1.2* enhanced embryogenesis. These divergent effects were due to the different expression patterns of the two phytoglobins: ZmPgb1.1 was expressed throughout the embryo body, while ZmPgb1.2 in those cells anchoring the immature embryos to the embryogenic tissue (anchor cells). Suppression of either ZmPgbs induced PCD in the cells where the respective ZmPgbs were expressed. Suppression of ZmPgb1.1 induced PCD in many embryogenic cells leading to embryo abortion,

while suppression of ZmPgb1.2 induced death in the anchor cells, thus allowing the immature embryos to separate from the embryogenic tissue and develop further. The induction of the death program as a result of ZmPgb suppression was mediated by NO and Zn^{2+} and executed by metacaspases and ROS (Huang et al. 2014; Mira et al 2016 b).

Besides regulating embryogenic tissue formation, Pgbs also influence the organogenesis. Wang et al. (2011) reported the induction of Arabidopsis shoot organogenesis following the upregulation of both Pgb1 and Pgb2. These effects were associated to changes in expression of genes involved in cytokinin synthesis and signaling

1.4.2.2 Pgb expression and function during post embryonic development

Phytoglobins are involved at the onset of germination in several species including barley (Guy et al. 2002), tobacco (Seregelyes et al. 2003) and wheat (Sen 2010). Through the modulation of cellular NO phytoglobins induce glucose catabolic processes (Arc et al. 2013; Igamberdiev and Hill 2004) regulating dormancy and germination. Phytoglobins have also been identified in the aleurone layer, especially under condition of low oxygen levels, (Taylor et al. 1994) and their expression in cereals grains during imbibition has been suggested to be a marker for seed viability and quality (Guy et al. 2002). Phytoglobins are also involved during post-embryonic development. In Arabidopsis Pgb1 is expressed abundantly in leaf hydathodes, and shoot and root apical meristems, and its suppression causes major developmental defects (Hebelstrup et al. 2006). In the same study the authors documented seedling lethality in double mutant plants in which both Pgb1 and Pgb2 were suppressed. This observation suggests that the expression of the two phytoglobins is essential for normal development.

Up-regulated during hypoxic conditions, phytoglobins support growth by reducing NO levels and maintaining a high energy status. This has been demonstrated by experimentally altering the levels of Pgbs in *Medicago sp.* cultured roots (Dordas et al. 2003). The role of phytoglobins in supporting growth of hypoxic tissue has also been demonstrated in several systems (Vinogradov et al. 2006; Perazzolli et al. 2004; Yang et al. 2005). Recent studies also showed that the expression of Pgbs is essential for plant and cell survival under flooding conditions. Youssef et al. (2016) found a positive correlation in corn between flooding tolerance and expression of phytoglobins. Plants over-expressing phytoglobins exhibited improved growth and photosynthetic rate, possibly due to a more effective antioxidant system able to limit the levels of ROS responsible for damage to photosynthetic tissue. Phytoglobins also participate in acclimation of root cells to hypoxia. In corn phytoglobins are mainly present in the root tip where they exercise a protective role by preventing hypoxia-induced death through the suppression of ethylene synthesis and response (Mira et al. 2016 b).

Pgb also plays a very important role during plant pathogen interaction. Over expression of the *Gossypium sp.*class 1 nsPgb in *Arabidopsis* increased pathogen resistance as well as enhanced tolerance to NO (Qu et al. 2005, 2006). A contrasting result was reported by Mur et al. (2013) while examining the role Pgb in the interaction between *Arabidopsis* and several hemibiotrophic and necrotrophic fungi strains. They observed increased disease resistance towards the pathogenic strains when the level of Pgb1 was reduced, and these effects were ascribed to elevated levels of NO (Mur et al. 2013).

1.5 Nitric Oxide (NO)

Nitric oxide is a bioactive signaling molecule participating in a variety of physiological events occurring during the plant life cycle (Rőszer 2012) and ranging from growth and developmental processes to abiotic and biotic responses occurring (Freschi 2013; Wang et al. 2013; Yu et al. 2014). The following sections will describe the involvement of NO as a major signaling molecule, and its regulation by phytoglobins.

1.5.1 Biochemistry of NO

In plants, NO production via reductive pathways is due to elevated redox levels which usually prevail during various stress conditions such as hypoxic stress. Furthermore, deoxygenated heme proteins, molybdocofactors, mitochondria, cytosol and the plasma membranes are also involved in NO generation (Gupta et al. 2011; Stőhr et al. 2001; Yamasaki and Sakihama 2000). NO production as a result of elevated redox levels is controlled by feedback loop mechanisms which in turns decrease cellular redox levels (Igamberdieve and Hill 2004). However, NO production via the oxidative pathway involving NO synthase (NOS) is still debatable and unclear (Crawford 2006).

The increase in redox level also results in the formation of reactive oxygen species (ROS) both in oxygen rich (Igamberdieve et al. 2016) and oxygen deprived (Blokhina et al. 2001) conditions, as opposed to NO productions which mainly occurs under low oxygen levels. The association of ROS generation and NO production at different oxygen concentrations leads to formation of physiologically important compounds known as reactive nitrogen species (RNS), such as nitrogen dioxide (NO_2), and peroxynytrite ($ONOO^-$) (Igamberdieve et al. 2016; Gupta et

al. 2011, 2014). Another important chemical reaction involving NO which results in the formation of an essential RNS species, S-nitrosoglutathione reductase (GSNO), occurs via nitrosylation of the SH groups of glutathione. S-nitrosoglutathione influences the cell redox levels, the glutathione pool size and many signaling pathways regulating cell division and differentiation (Leterrier et al. 2011). NO thus, is a very crucial signaling molecule which regulates a variety of developmental processes including those associated to the death/survival fate. (Igamberdieve et al. 2016). Plant responses mediated by NO are extremely crucial especially during hypoxic stress (Igamberdieve et al. 2016)

1.5.2 PGB regulates NO turnover and exhibit differential cell response

Interaction between non symbiotic Pgb class 1 and nitric oxide is known to maintain the energy levels of the cells under hypoxia (Dordas et al. 2003b, 2015). Studies in the past have shown that over expressing Pgb1 lines in different plant species such as maize, alfa alfa and *Arabidopsis* have resulted in higher production of ATP due to Pgb1-NO interaction and the plant cells and root cultures of the overexpressing Pgb lines displayed better resistance towards hypoxic stress (Dordas et al. 2003a,b; Igamberdieve et al. 2004; Perazzolli et al. 2004; Sowa et al. 1998). Additionally, underexpressing Pgb lines were also studied and an inverse co relation was found between NO production and Pgb expression, where higher levels of NO was produced in underexpressing Pgb lines (Dordas et al. 2003,2004; Perazolli et al. 2004). Thus it was suggested that Pgb regulates NO levels which may further influence the NO dependent physiological events occurring in the plants (Dordas et al. 2003, 2004).

An example of NO regulation by Pgbs occurs at onset of germination in several species including barley (Guy et al. 2002), tobacco (Seregelyes et al. 2003) and wheat (Sen 2010).

Through the modulation of cellular NO, Pgbs induce glucose catabolic processes (Igamberdiev and Hill 2004; Arc et al. 2013) regulating dormancy and germination. Additionally, NO emission spectra studies of germinating barley and sorghum seeds revealed sources of NO generation during the initial stage of seed germination (Bethke et al. 2004b; Simontacchi et al. 2004). Phytoglobins have also been identified in the aleurone layer, especially under condition of low oxygen levels,(Taylor et al. 1994) and their expression in cereals grains during imbibition has been suggested to be a marker for seed viability and quality (Guy et al. 2002).

Meristematic cells are fast dividing, compact cells that often experience hypoxic stress and NO generation in these cells is very crucial (Mancuso and Boselli 2002). Perturbation of NO content in meristematic affects cell viability (Igamberdieve et al. 2016). Studies have also reported the expression of hypoxically induced class 1 Pgb in root and shoot meristem and in root branches domain (Heckmann et al. 2006). The function of class 1 Pgb during meristem development was also documented by Hebelstrup and Jensen (2008), who observed altered meristem function in Arabidopsis plants suppressing Pgb1. These alterations were related to changes in auxin's acropetal transport (Fernandez-Marcos et al. 2011). Recently, Mira et al. (2016 a) investigated the role of Pgb/NO interaction in corn lines over-expressing or down-regulating Pgb and showed that Pgbs prevent hypoxia induced cell death in root apical meristems through scavenging NO and regulation of ethylene synthesis and response and ROS production (Mira et al. 2016 a). The role of Pgbs as a determinant factor in cell death/survival might also be involved in other cell events, including aerenchyma formation (Igamberdiev et al. 2016) and embryo production (Huang et al. 2014). Using maize somatic embryogenesis as a model system, Huang et al. (2014) observed that downregulation of ZmPgb1.2 enhanced embryo yield whereas

downregulation of ZmPgb1.1 repressed embryo formation. The differential embryo outcome was associated with the differential expression pattern of PGB, with ZmPgb1.2 expressed in the "anchor" cells anchoring the immature embryos to the embryogenic tissue, while ZmPgb1.1 is expressed in many embryogenic cells. Thus, suppression of ZmPgb1.2 eliminated the anchor cells (by PCD) and released the young embryos allowing their further development. Suppression of ZmPgb1.1 resulted in the death of many embryonic cells causing embryo abortion (Huang et al. 2014). The mechanisms of PCD induced by repression of ZmPgb1.1 and ZmPgb1.2 were mediated by NO and Zn^{2+} levels (Huang et al. 2014).

1.5.3 Role of NO in hormone regulated processes

Plant hormones play a predominant role in various growth and development events occurring during the plant life cycle under "unstressed" and stressed conditions (Davies 2010). Their effects are often modulated by signal molecules such as NO (Vanstraelen and Benková 2012), which influences a wide ranges of hormonal responses such as those experienced during abiotic and biotic stresses like cold, drought, salinity, light, and pathogen attack (Durner and Klessig 1999; Gracía-Mata and Lamattina 2002; Procházková et al. 2012; Zhang et al. 2006; Zhao et al. 2007). Some of these responses are described in the sections.

1.5.4 Role of NO during biotic stress

NO plays a crucial role as a signaling molecule to different plant responses under biotic stresses (Mur et al. 2013a). Plants adapt different strategies to overcome biotic stresses which predominantly depend on the nature of the pathogenic interaction. For instance, to counteract the advance of biotrophic and hemi biotrophic pathogens, requiring living cells, plants triggers

programmed cell death (PCD) at the site of infection. This hypersensitive response (HR) required NO (Igamberdieve et al. 2016) and involves the participation of several growth regulators including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Mur et al. 2013b). SA and JA/ET pathways have been suggested to trigger antagonistic pathways (Glazebrook 2005; Mur et al. 2013) with NO influencing both (Mur et al. 2013). For instance, in the case of the ET signaling pathway during HR, NO regulates the response by both inducing the expression of the ET biosynthetic genes, and inhibiting the synthesis of S-adenosylmethionine transferase through nitrosylation mechanisms (Mur et al. 2013b). In *Nicotiana sp.* bacterial infection causes a rapid increase in NO and ET levels with NO acting upstream of ethylene (Mur et al. 2008, 2012). Treatments with the NO donor SNP elevate the expression of ACC oxidase and ethylene responsive genes (Chun et al. 2012).

In addition to the traditional SA- and JA/ET-mediated responses (Glazebrook 2005), NO influences plant defense against pathogens through ABA (Bellin et al. 2013), which triggers "alternative" pathways linked to HR (Robert-Seilaniantz et al. 2011). For instance, NO and ABA have antagonistic roles in triggering effector triggered immunity (ETI), a plant immunity which is activated when R proteins in plants recognize pathogen effectors (Delledonne et al. 1998). Specifically, ETI is induced by NO and repressed by ABA. Delledonne et al. (1998) examined the positive regulatory effect of NO by treating wild type *Arabidopsis* with NO biosynthesis inhibitors and demonstrated enhanced susceptibility to *P.syringae avrRpm1 and avrRps4*. Similar results were demonstrated by using NO-deficient *Arabidopsis* mutants (Mandal et al. 2012). Application of ABA also resulted in enhanced susceptibility of *Arabidopsis* plants

to avirulent pathogen *P.syringae* suggesting that this hormone suppresses ETI (Mohr and Cahill 2003).

1.6 Programmed Cell Death (PCD)

PCD is a genetically defined process that leads to the programmed suicide of cells in both plants and animals (Rantong and Gunawardena 2015). The term PCD is defined as an explicit mechanism manifested by eukaryotes that involves disintegration of cells, tissues and organs. This unique pathway requires various intrinsic factors and energy driven events (Conradt 2009; Lockshin and Zakeri 2004; Olvera-Carrillo et al. 2012). Broadly, PCD is divided into two classes: environment related PCD and development related PCD. Environment related PCD events are induced by abiotic and biotic stress and examples include the death of cells surrounding the infection site during plant pathogen interaction (Drew et al. 2000; Greenberg 1996), or the formation of aerenchyma by the dismantling of cortical cells under conditions of low oxygen (Takahashi et al. 2014). Development related PCD generally occurs under "un-stress' conditions and examples include the formation of treachery elements that form the vascular tissues and removal of senescent old tissues and organs (Bozhkov et al. 2005a; Greenberg et al. 1996; Pennel and Lamb 1997; Olvera Carrillo et al. 2012; Smertenko and Bozhkova 2014).

PCD mechanism in animal system has been investigated deeply. However, research on PCD in plant systems is scarce (Reape et al.2008; van Doorne 2011). Broadly, the morphological characteristics of PCD can be categorized in two forms; necrosis and vacuolar cell death (Gunawardena 2008; van Doorn et al. 2011). Vacuolar cell death, commonly observed during

organ formation or development of tracheary elements (Filonova et al. 2000), involves the gradual decrease in cytoplasmic components due to the rupture of the tonoplast and the release of vacuolar hydrolytic enzymes triggering the rapid destruction of cell protoplast and wall. On the other hand, necrosis is characterized by the bursting of the plasma membrane followed by the immediate removal of cytoplasmic components. The process is triggered in hypersensitive responses during plant-pathogen interaction (van Doorn et al. 2011).

As reviewed by Rantong and Gunawardena (2015), abiotic and biotic stimuli such as stress conditions (drought and heat), UV light, and pathogen infection are able to trigger plant cell death mechanism via PCD through production of reactive oxygen species (ROS). Reactive oxygen species are important signaling molecules conserved across kingdoms (Overmyer et al. 2003). Although the molecular signal pathways induced by ROS are not completely understood, it is suggested that production of ROS is encouraged by stress and their effects are mediated by their interaction with hormones or other signal molecules (Apel and Hirt 2004). It is well established that during plant pathogen interaction ROS are generated via plasma-membrane located NADPH oxidase (Bolwell and Wojtaszek 1997) and cell-wall bound peroxidases (Kawano 2003). Production of ROS in the infected area triggers NO and salicylic acid responses culminating with the selective elimination of cells via PCD, a process delaying the advance of the pathogen (Gadjev et al. 2008). The complexity of stimuli elevating ROS production and the down-stream events leading to PCD are summarized in Fig1.11. Plant hormones such as salicylic acid, jasmonic acid, ethylene and ABA are also known to regulate ROS production (Berrocal-Lobo et al. 2002; Cao et al. 1994; Mur et al. 2008;Ton et al. 2009), while MAPK and calcium-

dependent calmodulin cascades and Ca^{2+} act as downstream components of PCD responses (Fig1.11). Death of plant cells by PCD generally requires metacaspace activity and the transcriptional activation of master regulator genes including Retinoblastoma related genes (RBR) and the Bax inhibitor (BI 1) gene which will be discussed later in this section.

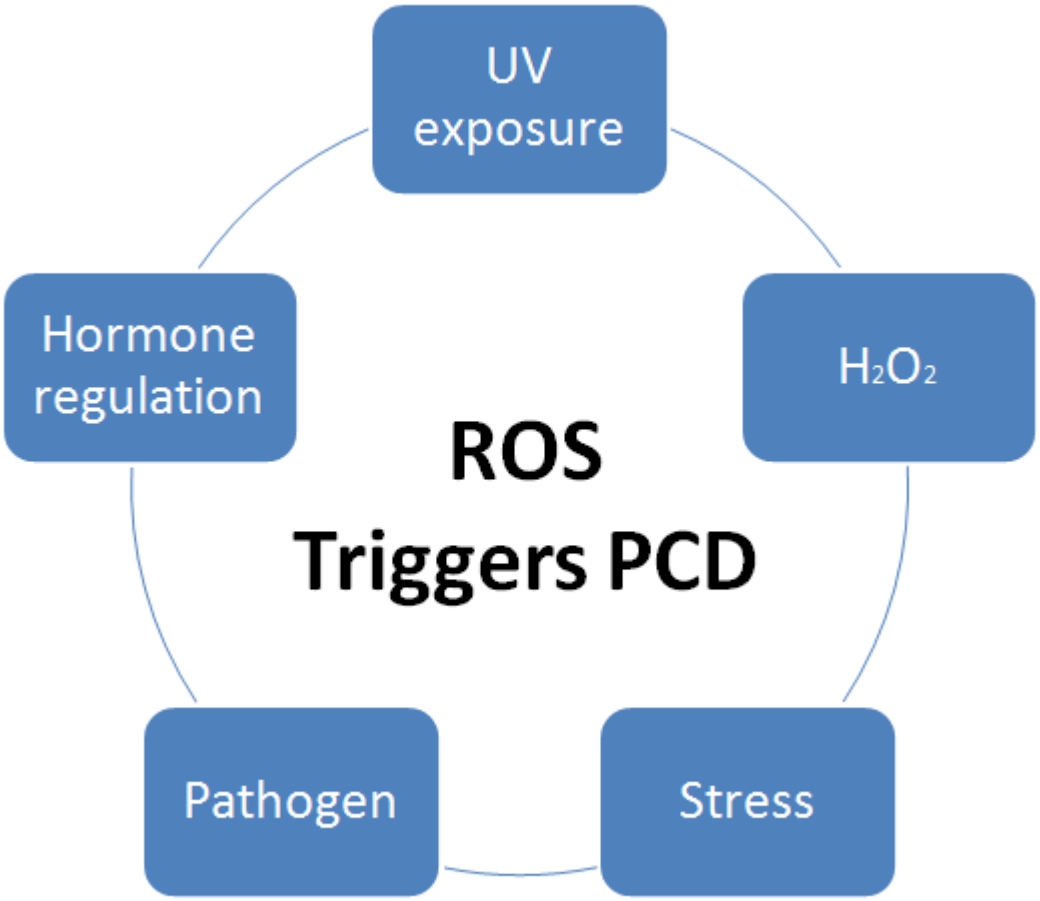

Fig. 1.11: Regulators of plant Programmed Cell Death (PCD)The diagram highlights variety of stimuli such as UV light, hydrogen peroxide (H_2O_2), pathogen attack, developmental cues, and stress conditions such as heat and drought. The model is based around reactive oxygen species (ROS) triggering PCD as a result of various environmental cues.

1.6.1 PCD during embryogenesis

During morphogenesis the shaping of organs require the selective elimination of specific cells. Examples of activation of the death program during morphogenesis have been described post-embryonically in leaf (Schippers et al. 2007), flowers (Rogers 2013), seeds (Domínguez et al. 2015) and during the differentiation of the vascular tissue characterized by the development of dead tracheary elements (Escamez and Tuominen 2014). In embryogenesis execution of PCD is essential for the elimination of the suspensor and the removal of subordinate embryos.

1.6.1.2 PCD in suspensor cells

Fertilization leads to the formation of a zygote which in several plant species divided asymmetrically forming an apical cell and a basal cell (Raghavan 2006). The apical cell undergoes a series of cell divisions forming the 'embryo proper' whereas, the basal cell divides to form the suspensor (Raghavan 2006). While not important during post-embryonic development, the suspensor is crucial for embryogenesis as it provides positional cues for the formation of the embryo proper and supplies nutrients and growth regulators such as auxin and gibberellins (Ceccarelli et al. 1981;Picciarelli et al. 2001).

Independent research has shown that terminally differentiated suspensors undergo vacuolar PCD in a pattern which is species dependent. For instance, Lombardi et al. (2007) demonstrated that elimination of the suspensor in *Phaseolus coccineus* occurs through waves of PCD starting from the uppermost suspensor layer, adjacent to the embryo proper, and progressing downwards to the bottom of the suspensor.

Based on this pattern, Bozkhov et al.(2005a) proposed two theories accounting for the progression of the death program. While the first theory argues for the presence of a ‘pro- death signal’ produced by the embryo proper and released in a basipetal pattern towards the suspensor, the second theory is based on an ‘anti- death factor’ first produced by the base of suspensor and moving acropetally towards the embryo proper.

Variation in the timing of suspensor elimination is also apparent. While in some species PCD only occurs after the suspensor has reached morphological maturity (Bozkhov et al. 2005a), in others, including spruce the death program is activated soon after the first divisions of the basal cell originating from the asymmetric division of the zygote (Bozhkov et al 2005b).

1.6.1.3 Elimination of subordinate embryos and survival of a single dominant embryo

In gymnosperms division of the zygote produces multiple embryos, a process referred as monozygotic polyembryony (Bouquet 1980). Of the multiple embryos one become the dominant embryo and continues growing while the others “subordinate” embryos abort (Filonova et al (2002). Elimination of the subordinate embryos occurs by PCD (Singh 1978).the execution of the cell death mechanism during elimination of subordinare embryos is a very well regulated event and has been documented by Filonova et al. (2002) as a three step process. The first step involves the formation and growth of multiple embryo from a single zygote followed by the second step, where one of the embryo acquires ‘dominant’ characterstics and overgrows the subordinate embryos. Lastly, the elimination of subordinate embryos take place while the dominant embryo further develops and reach the maturation phase. In addition the researchers also performed terminal deoxynucleotydil transferase mediated dUTP nick end labeling (TUNEL) assay

in order to detect DNA degradation in the subordinate embryos undergoing cell death. The researchers further suggested that initiation of PCD behins from the basal part of the embryo and proceeds in an acropetal fashion reaching the uppermost layer of the embryo in about four weeks (Filonova et al. 2002).

1.6.1.4 Somatic embryogenesis undergoes PCD

Besides cell division and differentiation, PCD plays a crucial role during the formation of embryo in culture (Thorpe and Stasolla 2001; Smertenko and Bozkhov 2014). Filonova et al. (2000b) highlighted the relevance of PCD in spruce somatic embryogenesis, where the pro embryogenic masses (PEMs) derived from cultured zygotic embryos form cellular aggregates (PEM I-III) in the presence of auxin and cytokinin (Filonova et al. 2002).

The PEM I aggregates consist of cluster of cytoplasmic-rich cells linked with a suspensor cell. The formation of additional suspensor cells produce PEM II which will differentiate further into PEM III characterized by a larger size and more suspension cells encircling the central cytoplasmic-rich cluster of cells. Removal of both auxin and cytokinins induces PCD within cells of PEM III leading to the formation of immature somatic embryos (Filonova et al. 2000b). Execution of PCD is an obligatory event and when the death program is abrogated no embryos form (Filonova et al. 2000b). Once the somatic embryos develop, a second wave of PCD contributes to the dismantling of the suspensor. Termination of the suspensor occurs in a basipetal fashion, starting from the uppermost cells of the suspensor and progressing to the bottom cells (Smertenko et al. 2003).

Recently using the maize somatic embryogenic system, Huang et al. (2014) reported that

suppression of *Zea mays phytoglobin 1.1* (*ZmPgb1.1*) inhibited the formation of somatic embryos while suppression of *Zea mays phytoglobin 1.2 (ZmPgb1.2)* enhanced embryogenesis. These divergent effects were due to the different expression patterns of the two phytoglobins: *ZmPgb1.1* was expressed throughout the embryo body, while *ZmPgb1.2* in those cells anchoring the immature embryos to the embryogenic tissue (anchor cells). Suppression of either *ZmPgbs* induced PCD in the cells where the respective *ZmPgbs* were expressed. Suppression of *ZmPgb1.1* induced PCD in many embryogenic cells leading to embryo abortion, while suppression of *ZmPgb1.2* induced death in the anchor cells, thus allowing the immature embryos to separate from the embryogenic tissue and develop further. The induction of the death program as a result of ZmPgb suppression was mediated by NO and Zn^{2+} and executed by metacaspases and ROS (Huang et al. 2014; Mira et al. 2016 a).

1.6.2 Modulators of Plant PCD

Regulation of PCD in plants is somewhat similar to the regulation of apoptosis in animals although much more research has been conducted in animals relative to plants (Rantong and Gunawardena 2015). As indicated in the previous section, execution of the death program can occur via necrosis or vacuolar cell death, and both processes require the activation of nucleases, proteases, catalases and retinoblastoma related proteins, many of which have been identified in several systems (Hackenberg et al. 2013; Kermoda 2003; Tsiatsiani et al. 2011; Sabelli et al. 2013).

Reactive oxygen species (ROS) can act both as modulators or executors of PCD as their involvement in the death program has been documented in many plant species (Apel et al. 2004;

Chamnogpol et al. 1998). Independ\ent studies have proposed ROS as key factors in the regulation of both developmentally and environmentally induced PCD (Gross et al. 2013; Petrov et al. 2015). Biochemically, ROS consist of an heterogeneous array of molecules such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical and singlet oxygen, all generated in plants as a result of oxygen reduction during various metabolic events (Baxter et al. 2014). The participation of ROS during PCD is especially apparent during plant pathogen interaction where their over-production following infection triggers PCD, a component of the hypersenitive response which delays the spreading of the pathogen. (Mittler et al. 2004; Mittler et al. 2011). As over-production of ROS causes severe damages to cellular components (Moller et al. 2007), it is not surprising that plant cells have developed systems to attenuate ROS accumulation. An efficient system is the antioxidant machinery consisting of an array of enzymes, such as catalases (CAT), superoxide dismustase (SOD) and ascorbate peroxidase (APX) which operate together to scavenge ROS and protect cellular components under conditions of stress. Several reviews are available in literature describing the function of the antioxidant system in attenuating ROS-induced death (Hackenberg et al. 2013; Mhamdi et al. 2010).

Other molecules, executors of the death program in both animals and plants, are proteases with caspases-like activity (Minina et al. 2014). In plants these molecules are referred to as metacaspases. The main function of caspases in animals and metacaspases in plantsis to trigger the dismantling of cellular components by attacking and degrading amino acid residues in proteins (López-Fernández and Maldonado 2015). In plants metacaspases detected preferentially arginine and lysine residues (Watanabe and Lam 2005). Metacaspase activity during plant PCD was demonstrated by Bozhkov et al. (2005b) which identified the spruce metacaspase (mcII-Pa)

as required for the execution of PCD during somatic embryogenesis. The metacaspase gene is expressed in all those tissues and organs characterized by death events, such as procambial and suspensor cells, and suppression of *mcII-Pa* during spruce somatic embryogenesis was sufficient to reduced PCD in PEM III and reduce the formation of spruce somatic embryos. (Bozkhov et al. 2005b).

Retinoblastoma related genes (RBR) are a family of protein contributing to the regulation of the cell cycle, transcription, and cell proliferation and differentiation (Gutzat et al. 2012). Grafi et al. (1996) reported a regulatory role of RBR 1 in DNA endo-reduplication processes during endosperm development of *Zea mays*. RNAi-mediated suppression of RBR1 was sufficient to stimulate DNA endo-reduplication cell cycles and the death program during endosperm development (Lee et al. 2009). This down regulation of *RBR1* by RNAi resulted in enhanced cell death mechanism as well as stimulated both the mitotic and DNA endo-reduplication cell cycles.

Another regulator of PCD is the Bax inhibitor 1, a ubiquitous protein highly expressed under stress conditions, some of which leading to death (Coupe et al. 2004; Lam 2004).While the over-expression of BI-1 represses the cell death mechanisms in Arabidopsis cells exposed to extreme temperature, and pathogen or fungal attack (Yamada et al. 2004), its suppression as a result of carbon starvation induces PCD (Bolduc and Brisson 2002). During maize somatic embryogenesis Huang et al. (2014) observed extensive PCD in those domains where a maize homolog of the Arabidopsis BI-1 was experimentally expressed.

Taken together, these studies demonstrate the existence of a variety of signal molecules triggering and executing PCD.

CHAPTER ONE: Abscisic acid and ethylene are integrated in the phytoglobin (Pgb) regulation of maize somatic embryogenesis

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Paper would be submitted shortly and the text has been edited by some of the co-authors**

K.K. performed the majority of the experiments and contributed to data analysis and writing the manuscript. M.M. contributed in optimizing some of the techniques used in the research B.T.A. conducted the ABA quantification experiment. R.D.H contributed in the interpretation of the data. C.S. assisted in the design of experiments and interpretation of data.

2.1 Abstract

Suppression of *Zea Mays phytoglobins* (*ZmPbg1.1* or *ZmPgb1.2*) during somatic embryogenesis induces programmed cell death (PCD) by elevating nitric oxide (NO). While *ZmPgb.1.1* is expressed in many embryonic domains and its suppression results in embryo abortion, *ZmPgb1.2* is expressed in the basal cells anchoring the embryos to the embryogenic tissue. Removal of these "anchor cells" by PCD allows the embryos to develop further. The effects of *ZmPgb* suppression on embryogenesis were abolished by exogenous applications of abscisic acid (ABA). A depletion of ABA, ascribed to a down-regulation of biosynthetic genes, was observed in those embryonic domains where the respective *ZmPgbs* were repressed. These effects were mediated by NO. Depletion in ABA content induced the transcription of genes participating in the synthesis and response of ethylene, as well as the accumulation of ethylene, which influenced embryogenesis. Somatic embryo number was reduced by high ethylene levels and increased with pharmacological treatments suppressing ethylene synthesis. The ethylene inhibition of embryogenesis was linked to the production of ROS and the execution of programmed cell death (PCD). Integration of ABA and ethylene in the *ZmPgb* regulation of embryogenesis is proposed in a model where NO accumulates in *ZmPgb*-suppressing cells, decreasing the level of ABA. Abscisic acid inhibits ethylene biosynthesis and the NO-mediated depletion of ABA relieves this inhibition causing ethylene to accumulate. Elevated ethylene levels trigger production of ROS and induce PCD. Ethylene-induced PCD in the *ZmPgb1.1*-suppressing line [ZmPgb1.1 (A) line] leads to embryo abortion, while PCD in the *ZmPgb1.2*-suppressing line [ZmPgb1.2 (A) line] results in the elimination of the anchor cells and the successful development of the embryos.

Keywords: Abscisic acid, embryogenesis, ethylene, maize, phytoglobin, programmed cell death.

2.2 Introduction

Hemoglobins are ubiquitous iron-containing proteins which participate in a variety of cellular functions. In vertebrates, where they were originally identified, hemoglobins are mainly involved in the storage and transport of oxygen and other molecules, such as CO_2 and NO (reviewed by Hill 2012). In plants, proteins with hemoglobin-like properties were found to be expressed in the nodules of leguminous species and are associated with the activity of nitrogen fixing bacteria (Smagghe et al. 2009). Other plant hemoglobin-like proteins, not associated with nodules, were subsequently identified and referred to as non-symbiotic hemoglobins (reviewed Hill 2012), and more recently as phytoglobins (Pgbs) (Hill et al. 2016).

Studies on Pgbs have been mainly focussed on their oxygen binding properties, expression patterns and phylogenetic relationships (Hunt et al. 2001) and, based on these parameters, three distinct classes of Pgbs have been identified. While class 3 Pgbs are similar to truncated globins and are the least characterized, class 1 and 2 have a unique 3-on-3 α-helical loop surrounding the heme (Watts et al. 2001). Both class 1 and 2 Pgbs have a very high oxygen binding affinity (Hoy and Hargrove 2008; Dordas 2009) and their initially proposed function as oxygen sensors has now been discounted (Hill 1998). Independent studies have confirmed that the major role of Pgbs is to scavenge nitric oxide (NO), thereby modulating many responses where NO acts as a signal molecule (reviewed by Hill 2012).

A large body of information is available on the function of Pgbs during abiotic stress, especially conditions of low oxygen availability. Over-expression of class 1 *Pgb* enhances growth of hypoxic alfalfa roots that develop fewer aerenchyma (Dordas et al. 2003). An increased survival rate, as a result of NO depletion, was also observed in *Arabidopsis* roots over-expressing class 1 Pgb (Hunt et al. 2002). The protective role of class 1 Pgbs is not necessarily restricted to the root system as corn plants over-expressing the class 1 *ZmPgb1.1* and *ZmPgb1.2* retain a higher photosynthetic rate when waterlogged (Youssef et al. 2016). Like class 1 Pgbs, constitutive expression of class 2 Pgbs confers enhanced performance under restricted oxygen conditions (Hebelstrup et al. 2006) through their NO-scavenging characteristics (Hebelstrup and Jensen 2008). The ability to modulate NO is also relevant in biotic stress responses where alterations of Pgbs have been used to alter plant resistance to diverse pathogens (Mur et al. 2012; 2013). Collectively, these studies place Pgbs as prominent regulators of NO-mediated stress responses.

The function of NO is not restricted to stress responses, as this molecule is also engaged in many physiological events occurring during plant development, including embryogenesis and seed development (Sanz et al. 2015). The ability of NO to stimulate embryogenic cell formation from alfalfa leaf-protoplasts (Otvös et al. 2005) has contributed to extensive studies of Pgbs during somatic embryogenesis, the generation of somatic embryos from somatic cells. During Arabidopsis somatic embryogenesis, suppression of class 2 Pgb enhances the embryogenic competence by increasing NO levels necessary to repress the transcription factor MYC2 (Elhiti et al. 2013), an inhibitor of auxin biosynthesis. The increased auxin synthesis following Pgb suppression encourages the formation of embryogenic tissue and generation of

somatic embryos at high frequency. Extension of this study reveals a more complex mode of action of Pgbs, possibly through the regulation of hormone signalling. Using the same Arabidopsis embryogenic system, Mira et al. (2016b) showed that jasmonic acid signalling is an integral component of Pgb action which can further modulate a specific biological outcome such as PCD during embryogenesis and that factors modulating plant-pathogen interaction also influence somatic embryo formation. The effect of *Pgbs* on embryogenesis is not restricted to dicots. Repression of two class 1 maize Pgbs, *ZmPgb1.1* and *ZmPgb1.2* influences somatic embryogenesis by regulating the cell survival/death decision as opposed to the effect on hormonal responses in dicots. Suppression of either *ZmPgb* is sufficient to induce programmed cell death (PCD) through the activation of a pathway initiated by an elevation of NO and leading to over-production of reactive oxygen species (ROS) (Huang et al. 2014). These distinctive effects of the two *ZmPgbs* on embryogenic response are due to their unique cell-specific expression. *ZmPgb1.1* is expressed in many embryonic domains and its suppression results in extensive accumulation of NO and ROS, as well as massive PCD causing embryo abortion. *ZmPgb1.2*, on the other hand, is transcribed in the basal cells anchoring the immature embryos to the embryogenic tissue. Suppression of *ZmPgb1.2* results in a localized accumulation of NO and ROS in the "anchor cells" leading to their removal by PCD, releasing the embryos and allowing them to develop further (Huang et al. 2014). This work, in conjunction with the role played by ABA and ethylene during PCD-related processes (Young and Gallie 2000; Steffen and Sauter 2005), is the basis of the present study assessing the participation of ABA and ethylene in the *ZmPgb* regulation of maize somatic embryogenesis.

Data presented here reveal that suppression of *ZmPgbs* results in a NO-mediated depression of ABA levels causing the transcriptional activation of ethylene biosynthetic and responsive genes inducing production of ROS and PCD. Combined manipulations of ABA and ethylene levels suggest that both hormones are integrated in the ZmPgb regulation of embryogenesis in a proposed model where NO acts up-stream of ABA with ethylene as a downstream executor of PCD through ROS.

2.3 MATERIALS AND METHODS

2.3.1 Plant material and treatments

The corn embryogenic lines suppressing *ZmPgb1.1* [line ZmPgb1.1(A)] or *ZmPgb1.2* [line ZmPgb1.2(A)] were those characterized in previous studies (Huang et al. 2014). Production of *in vitro* embryos through somatic embryogenesis was achieved through three distinct culture steps, exactly as reported by Garrocho-Villegas et al. (2012). Briefly, embryogenic tissue grown on solid maintenance medium (M medium), was transferred in a liquid auxin-containing proliferation (P) medium of similar composition. Cells in the P medium proliferated rapidly and immature somatic embryos attached to the subtending embryogenic tissue became visible. After 7 days, the tissue was transferred onto a solid auxin-free development (D) medium which allowed the immature embryos to grow into fully functional mature embryos able to germinate and regenerate viable plants (Supplemental Fig.1). Pharmacological treatments were applied during the 7 days in P medium. 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 100μM and 10μM respectively (Elhiti et al. 2013). The ABA biosynthetic inhibitor, fluridone (FLD), and exogenous ABA were used at concentrations of 2μM

and 5μM, respectively. The ethylene donor ethephon (ETH) and the ethylene biosynthetic inhibitor aminooxyacetic acid(AOA) were both used at a concentration of 10 μM. The NADPH oxidase inhibitor diphenylene iodonium (DPI) was applied at a concentration of 5 μM (Drummond et al. 2011; Huang et al. 2014).

2.3.2 Gene expression studies

Total RNA, extracted from the embryogenic tissue using the TRI Reagent method (Huang et al. 2014), was first treated with DNaseI RNase-free (Promega), and further utilized for cDNA synthesis using the cDNA Reverse Transcription Kit (Applied Biosystems). All primers used for gene expression studies are listed in Supplementary Table 1. The relative gene expression level was analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) using actin as the reference gene (Huang et al. 2014).

2.3.3 ABA, ROS, and PCD localization

Localization of ABA was conducted as reported in Baron *et al*. (2012). Tissue was fixed in FAA (formaldehyde:acetic acid:ethanol:H_2O) at a ratio (v/v) of [10:5:50:35] and kept overnight at 4°C. Samples were then dehydrated in an ethanol series (50% - 100%) followed by an ethanol:xylene series (3:1, 1:1, 1:3) and embedded in wax. Tissue was sectioned with a microtome and placed on L-polylysine-coated slides, dewaxed, and incubated for 15 minutes in FAA buffered with 10% PBS, following three washes in PBS (10 min each). Post-fixed slides were then incubated in a blocking solution [10 mM PBS (pH 7.0), 0.1% Tween-20, 1.5% glycine and 5% (w/v) bovine serum albumin (BSA)] for 45 min at room temperature, and washed with a low salt buffer 1 (10 mM PBS, 0.8% NaCl, 0.1% Tween-20, 0.8% BSA). Primary

ABA monoclonal antibodies raised in mouse (Phytodetek ABA; Agdia, Elkhart, IN, USA) were diluted (1:100) in PBS and added to the slides. Incubation was performed for 24h at 4°C. The slides were washed twice in a high salt buffer (10 mM PBS, 0.1% Tween-20 and 0.8% BSA), once in buffer 1, and twice more in PBS to remove excessive Tween-20. The slides were then incubated overnight with secondary antibodies [anti-mouse IgG alkaline phosphatase conjugate (1 mg/ml), Promega, USA] and washed twice in a solution composed of 1× PBS containing 0.88 g/L NaCl, 0.1% Tween-20, and 0.8% BSA. Color detection was performed with Western Blue stabilized substrate for Alkaline Phosphatase (Promega, Madison, WI).

Reactive oxygen species (specific for superoxide detection) were localized using dihydroethidium, exactly as described by Mira et al. (2016 a) .

In Situ Cell Death Detection Kit-Fluorescein (Roche) was used to detect cells undergoing PCD, exactly as reported in Huang et al. (2014). Briefly, tissue was fixed in 4% paraformaldehyde, dehydrated in ethanol series and embedded in wax. Sections (10 µm) were de-waxed in xylene and labeled with the TUNEL kit (Roche) according to the manufacture's protocol, with the exclusion of the permeabilization step by proteinase K. Omission of TdT was used for negative controls.

2.3.4 ABA quantification

Freeze dried embryogenic tissues were ground into fine powder in liquid nitrogen with mortar and pestle and then extracted with 80% (v/v) acetonitrile containing 1% (v/v) acetic acid and the internal standard. ABA was extracted and purified from the homogenate as described in Son et al. (2016). Quantification of the ABA level in the tissues was performed with liquid

chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS, Agilent 1260-6430) using the conditions described in Yoshimoto et al. (2009). ABA extraction and analysis were performed from three independent replicates.

2.3.5 RNA in situ hybridization

RNA in situ hybridization analysis was performed as reported in Elhiti et al. (2010). Maize cells were first fixed in 4% (w/v) freshly prepared paraformaldehyde dissolved in phosphate-buffered saline (pH 7.4), and then dehydrated in an ethanol series (30%, 50%, 70%, 95%, 100%, and 100%). The samples were then treated with increasing concentrations of xylene, embedded gradually in paraffin, and sectioned (10μm). For hybridization, cDNAs encoding 1-aminocyclopropane-1-carboxylic acid synthase (*ZmAcs*), aminocyclopropane-1-carboxylic acid oxydase (*ZmAco),* and the ethylene responsive genes EIN3-binding F-box protein 1 (*ZmEbf1*) and ethylene responsive factor 2 (*ZmErf2)* were amplified and used for the preparation of digoxigenin (DIG)-labelled sense and antisense riboprobes, following the procedure described in the DIG Application Manual (Roche Diagnostics). Tissue treatments and pre-hybridization steps were performed as described by Cantón et al. (1999).

Sections were hybridized with sense or antisense probe in 1X Denhardt's, 1 mg/ml-1 tRNA, 10% dextran sulfate, 50% formamide, and 1X salts (Regan et al. 1999). Hybridization was conducted at 50^{o}C for 16 h. Post-hybridization washes and antibody treatments were performed as described by Regan et al. (1999). Detection of DIG-labeled probes was carried out using a Western Blue stabilized substrate for Alkaline Phosphatase (Promega, Madison, WI).

2.3.6 Ethylene measurements

Ethylene measurements were conducted according to Geisler-Lee et al. (2010). Embryogenic tissue (about 1 g) was incubated in the dark in a sealed 5-mL syringe for 2 h at 22°C. The gas accumulated in the headspace (1 ml) was analyzed with a Bruker 450-GC gas chromatograph. Data analysis was conducted using the Bruker Compass Data analysis 3.0 software.

2.3.7 Statistical analysis

Analysis of data was conducted by one way ANOVA using the SPSS program (IBM Corp. Released 2010. 466 IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.). Treatments means were compared by Tukey test ($\alpha = 0.05$) to compare the significance differences.

2.4 RESULTS

2.4 1 Applications of ABA abolish the effects of *ZmPgbs* on maize somatic embryogenesis

Somatic embryogenesis in maize is achieved through two steps (Garrocho-Villegas et al. 2012). Embryogenic tissue, initially cultured on solid maintenance medium (M), is transferred to a liquid auxin-containing proliferation (P) medium of identical composition (Supplemental Fig 1). After 7 days of proliferation, the embryogenic tissue, composed of immature embryos connected to the subtending tissue, is plated onto solid development (D) medium devoid of auxin. Removal of auxin encourages the development of fully functional mature embryos that can be harvested after 21 days (Supplemental Fig. 1).

Ectopic expression of *ZmPgbs* [(ZmPg.1.1(S) and ZmPgb1.2(S) lines] did not influence somatic embryogenesis, while embryo number was severely reduced by suppressing *ZmPgb1.1* [ZmPgb1.1(A) line] and increased when *ZmPgb1.2* was repressed [ZmPgb1.2 (A) line] (Fig. 2.1). These opposite trends are ascribed to the cell-specific localization of *ZmPgbs* and their regulation of PCD (Huang et al. 2014). *ZmPgb1.1* is expressed in several domains of the immature embryos and its suppression induces massive PCD resulting in embryo abortion. This is in contrast to *ZmPgb1.2* which is expressed in a few cells anchoring the embryos to the embryogenic tissue and its suppression induces PCD in these "anchor cells" releasing the embryos and allowing them to develop further (Huang *et al*. 2014).

Applications of 5μM ABA , the highest concentration not affecting embryo production in the WT line (Supplemental Fig. 2), abolished the effects of suppression of *ZmPgbs* by reverting embryo formation to WT values in both the ZmPgb1.1(A) and ZmPgb1.2(A) lines (Fig. 2.1). The effects of ABA were further investigated in the two ZmPgb(A) lines.

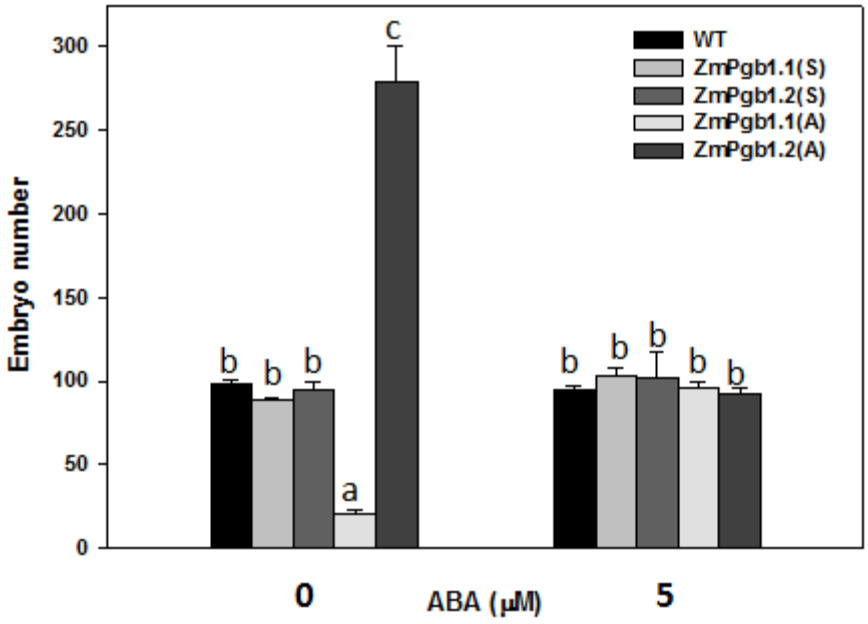


Fig. 2.1**:** Effects of ABA treatments on the number of fully developed maize somatic embryos produced by the WT line and lines down-regulating (A) or up-regulating (S) *ZmPgb1.1* or *ZmPgb1.2*. Values (means of at least three biological replicates) ± SE are expressed as percentages of the WT value (0 uM ABA) set at 100%. Letters on bars indicates statistically significant differences ($p < 0.05$).

2.4.2 Suppression of *ZmPgbs* reduces ABA levels through NO

The opposite embryogenic behaviour of the ZmPgb1.1(A) and ZmPgb1.2(A) lines, and the ability of ABA to revert embryo production to WT levels (Fig. 2.1), were examined on the basis of the following premise: *ZmPgbs* are efficient NO scavengers (Hebelstrup et al. 2008, Huang et al. 2014), and NO is intimately linked to ABA (Hancock et al. 2011).

Gene expression analyses were conducted at the end of the proliferation (P) period for three early ABA biosynthetic genes (*vp14/NCED, CME kinase*, and *carotenoid-isomerase*) and three participating in later steps (*aldehyde oxidase AAO3, AAO6*, and *molybdenum cofactor sulfurase*) (Endo et al. 2008; Ernst et al. 2010). Expression of many ABA biosynthetic genes were repressed in the ZmPgb1.1(A) and ZmPgb1.2 (A) lines (Fig. 2.2A).

The effect of NO on the ABA biosynthetic genes was examined pharmacologically using the NO donor SNP and the NO scavenger cPTIO. The specificity of both compounds was tested in previous studies (Huang et al. 2014), showing that during maize embryogenesis NO levels are increased by SNP and these effects can be reversed by cPTIO. In the present study, SNP was used to raise NO in the WT line accumulating low levels of NO, while cPTIO to scavenge NO in the NO over-producing ZmPgbs(A) lines (Huang et al. 2014). Elevation of NO by SNP significantly reduced the expression of *vp14/NCED* and *molybdenum cofactor sulfurase* in the WT line, while applications of cPTIO induced the expression of many ABA biosynthetic genes, especially in the ZmPgb1.1(A) line (Fig. 2.2A).

These results prompted the quantitation of ABA in the different lines. Compared to WT, the level of ABA was significantly reduced when NO was lowered either pharmacologically (by

SNP) or by repressing *ZmPgbs* (Fig. 2.2B). Addition of cPTIO in the ZmPgb1.1(A) line, characterized by the most pronounced accumulation of NO (Huang et al. 2014), raised the ABA content (Fig. 2.2B).

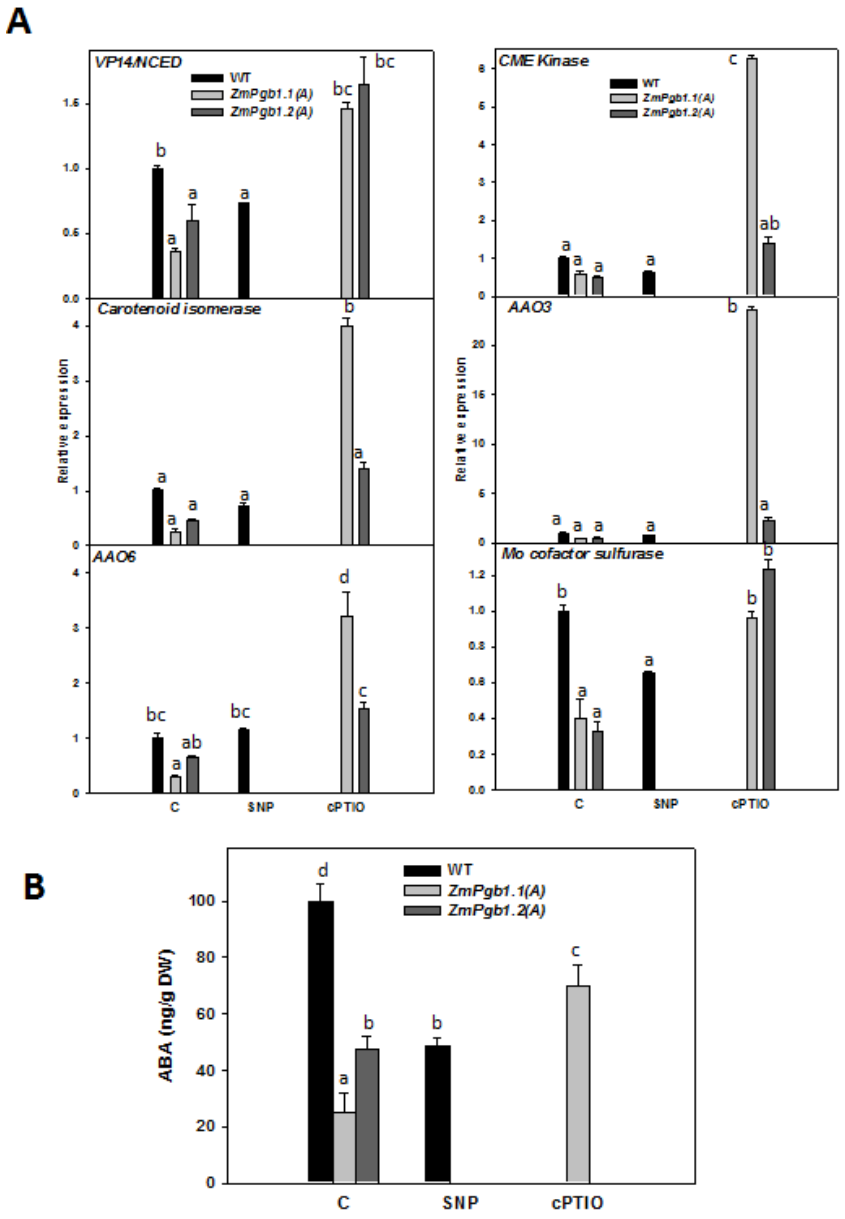


Fig. 2.2: Effects of suppression of *ZmPgbs* and nitric oxide (NO) manipulations on ABA biosynthetic genes and ABA level in maize somatic embryos. **(A)** Expression level of *vp14/NCED, CME kinase, carotenoid-isomerase, aldehyde oxidase AAO3, AAO6,* and *molybdenum (Mo) cofactor sulfurase* measured at the end of the proliferation period in the WT line and lines down-regulating (A) *ZmPgb1.1* or *ZmPgb1.2*. Values (means of at least three

biological replicates) ± SE are normalized to the WT (control, C) value set at 1. Letters on bars indicate statistically significant differences ($p < 0.05$). Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). **(B)** Endogenous ABA level measured at the end of the proliferation medium in the same lines utilized in (A). Values (means of three biological replicates) ± SE. Letters on bars indicate statistically significant differences ($p < 0.05$).

To further establish the spatial relationship between ABA and the previously documented localization of *ZmPgbs* transcripts (Huang et al. 2014), immunolocalization of ABA was conducted in immature embryos of the different lines. Heavy ABA staining was observed throughout the WT embryos (Fig. 2.3) which under similar conditions also do not stain for NO (Fig. 1E in Huang et al. 2014). In the transgenic lines, ABA staining was very faint in all the embryonic domains of the ZmPgb1.1(A) embryos and in the basal cells (anchor cells) of the ZmPgb1.2(A) embryos (arrow, Fig. 2.3). These ABA-depleted areas coincide very closely with those accumulating NO (Fig. 1E in Huang et al. 2014) and expressing the respective *ZmPgbs* under normal conditions (Fig. 1C in Huang et al. 2014).

Taken together these results confirm that suppression of *ZmPgbs* has opposite effects on maize embryogenic competence, and suggest that the NO-dependent depletion of ABA in the *ZmPgbs* down-regulating line occurs in those domains [throughout the embryos of the ZmPgb1.1(A) line, and in the anchor cells of the ZmPgb1.2(A)] where the respective *ZmPgbs* are expressed under normal conditions.

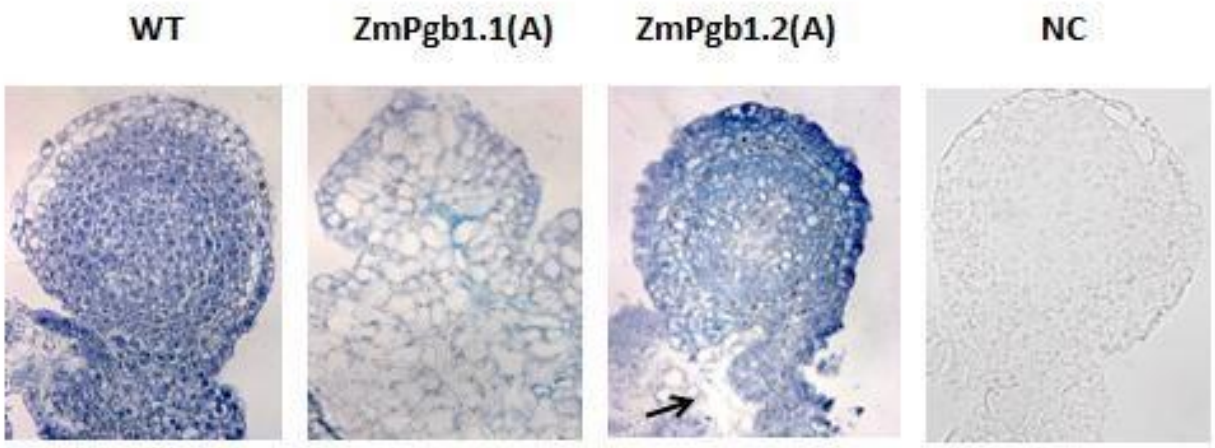


Fig. 2.3: Immunolocalization of ABA in the immature embryos of the WT line and lines down-regulating (A) *ZmPgb1.1* or *ZmPgb1.2* collected at the end of the proliferation medium treatment. Arrow indicates anchor cells connecting the developing embryos to the subtending embryogenic tissue. NC, negative control (WT embryo where the primary antibody was omitted).

2.4.3 Depletion of ABA induces ethylene synthesis and response

The established connection between ABA and ethylene in stress responses, many of which lead to PCD (Overmyer et al. 2003), prompted the analysis of the ethylene biosynthetic (*ZmACOs* and *ZmACSs*) and responsive (*ZmEBF1* and *ZmERF2*) genes, known mediators of ethylene signalling in maize (Geisler-Lee et al. 2010; Takahashi et al. 2015). Relative to WT, suppression of *ZmPgbs* increased the transcript levels of all genes measured (Fig. 2.4, 2.5A). In WT embryos, characterized by low levels of NO (Huang *et al*. 2014) and high levels of ABA (Fig. 2.2B and 2.3), a pharmacological increase of NO (SNP) or decrease of ABA (FLD) induced the expression of several ethylene genes. In the same embryos the addition of ABA reversed the effect of SNP (SNP+ABA). In the transgenic embryos displaying high levels of NO (Huang et al. 2014) and reduced levels of ABA (Fig. 2.2B and 2.3), removal of NO (cPTIO) or addition of ABA reduced the expression of several genes (Fig. 2.4,2.5A). The observation that suppression of ABA (FLD) reversed the effects of cPTIO (cPTIO+FLD) suggests NO acts upstream of ABA in the transcriptional regulation of ethylene synthesis and response.

Measurements of ethylene levels were also conducted on the treatments used for transcriptional analyses. In WT, elevated ethylene levels were evident in those situations where NO was increased (SNP) or ABA reduced (FLD) (Fig. 2.5B). Applications of ABA reversed the effects of SNP (SNP+ABA). The removal of NO (cPTIO) or exogenous applications of ABA in the ZmPgb down-regulating lines repressed ethylene production while the combined application of cPTIO + FLD induced the accumulation of ethylene relative to cPTIO treatments (Fig. 2.5B).

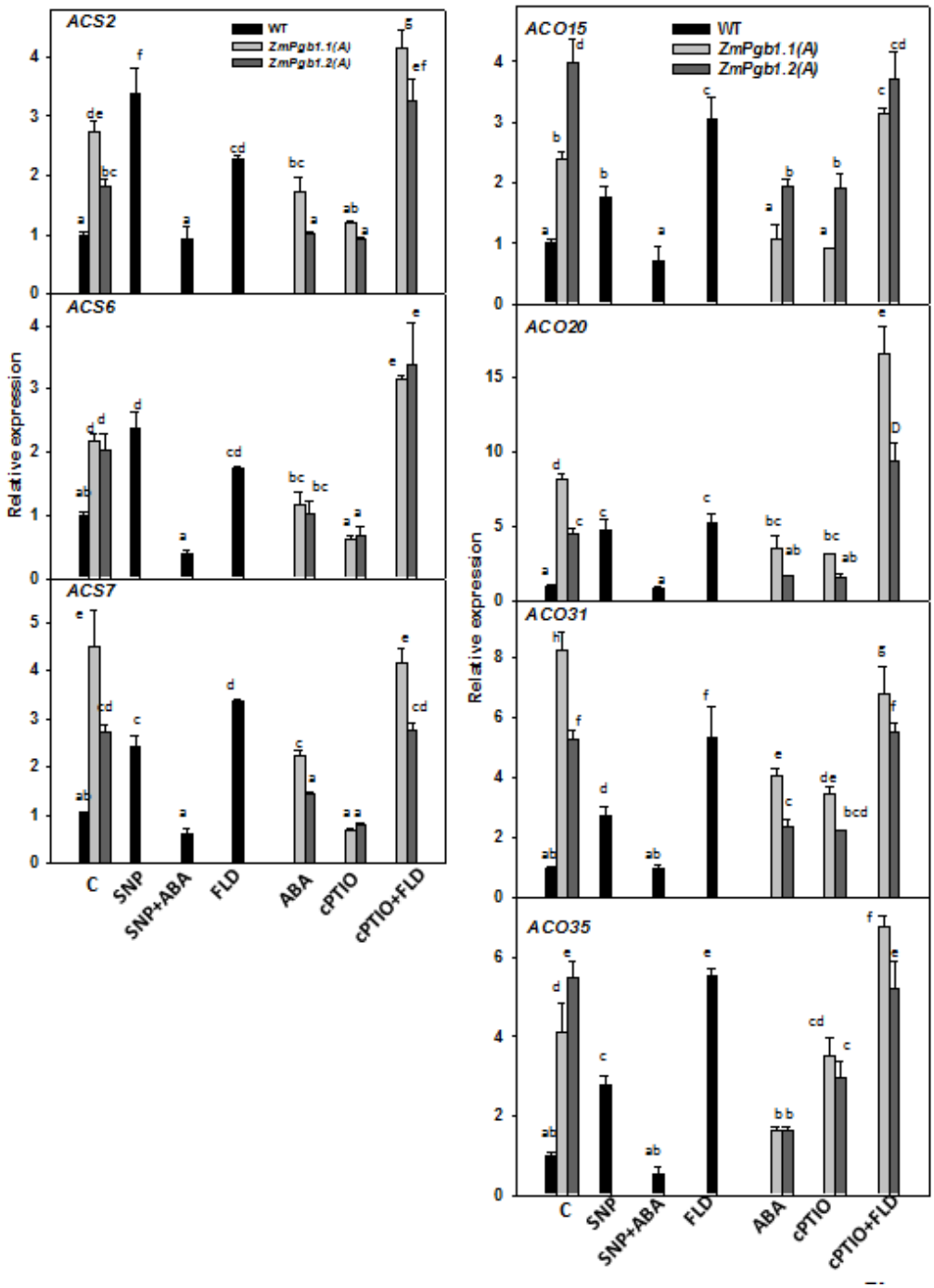


Fig. 2.4: Effects of suppression of *ZmPgbs* and nitric oxide (NO) and ABA manipulations on ethylene biosynthetic genes. Expression level of *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS2, 6, and 7*) and *oxidase* (*ACO 15, 20, 31,* and *35*) measured at the end of the

proliferation period in the WT line and lines down-regulating (A) *ZmPgb1.1* or *ZmPgb1.2*. Values (means of at least three biological replicates) ± SE are normalized to the WT (control, C) value set at 1. Letters on bars indicate statistically significant differences ($p < 0.05$). Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), fluridone (FLD).

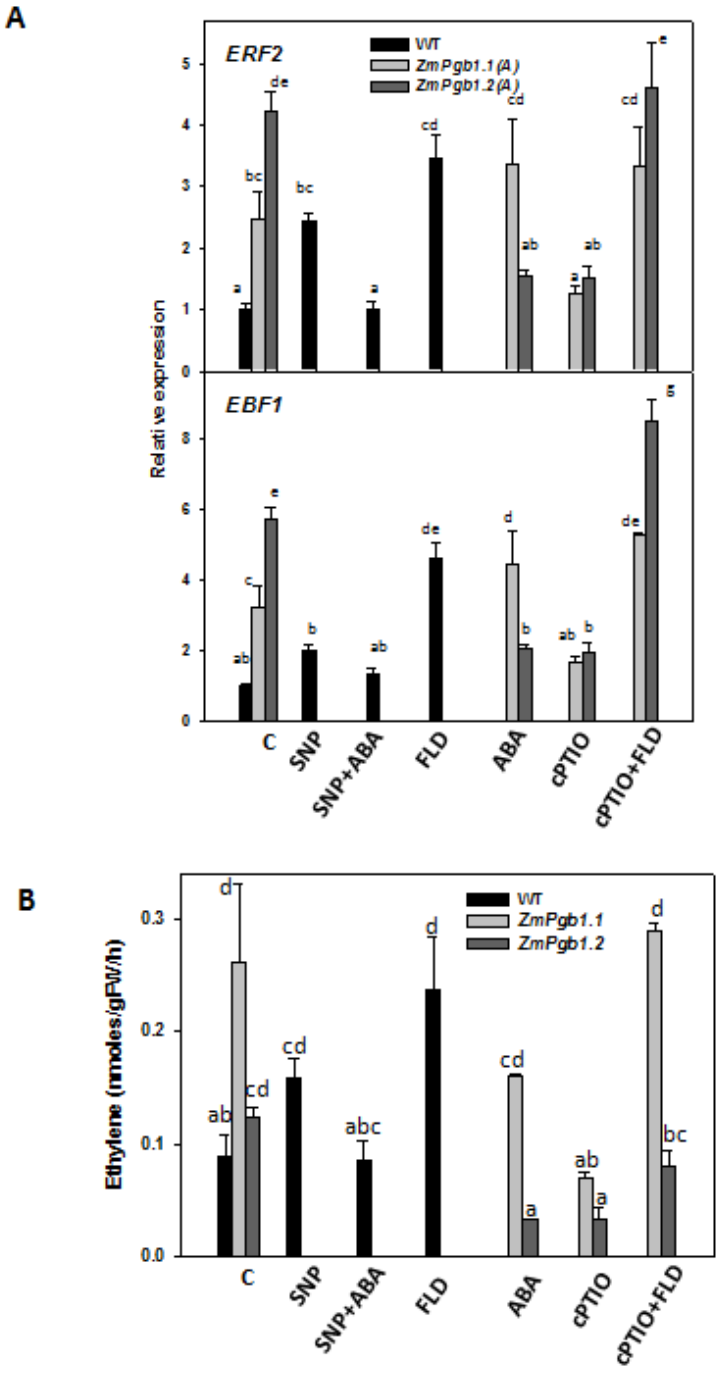


Fig. 2.5: Effects of suppression of *ZmPgbs* and nitric oxide (NO), and ABA manipulations on ethylene responsive genes and ethylene levels. **(A)** Expression level of *EIN3-binding F-box protein 1* (*ZmEBF1*), and *ethylene responsive factor 2* (*ZmERF2*) measured at the end of the

proliferation period in the WT line and lines down-regulating (A) *ZmPgb1.1* or *ZmPgb1.2*. Values (means of at least three biological replicates) ± SE are normalized to the WT (control, C) value set at 1. Letters on bars indicate statistically significant differences ($p < 0.05$). **(B)** Ethylene levels in embryogenic tissue of the different lines. Values are means of at least three biological replicates ± SE. Letters on bars indicate statistically significant differences ($p < 0.05$). Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), floridone (FLD).

The cause-effect relationship between ABA and ethylene was further substantiated using pharmacological approaches. In the WT embryos, a decline in ABA (FLD) reduced the number of fully mature somatic embryos and this inhibitory effect was abolished by co-applications with the ethylene biosynthetic inhibitor aminooxyacetic acid (AOA) (FLD+AOA) (Fig. 2.6). Applications of ethephon (ETH), increasing ethylene levels, reduced the number of embryos, and this effect could not be fully restored by ABA (ABA+ETH). Suppression of ethylene synthesis (AOA) abolished the effects of both ZmPgb1.1 and ZmPgb1.2 down-regulation by reverting embryo production to the WT values (Fig. 2.6).

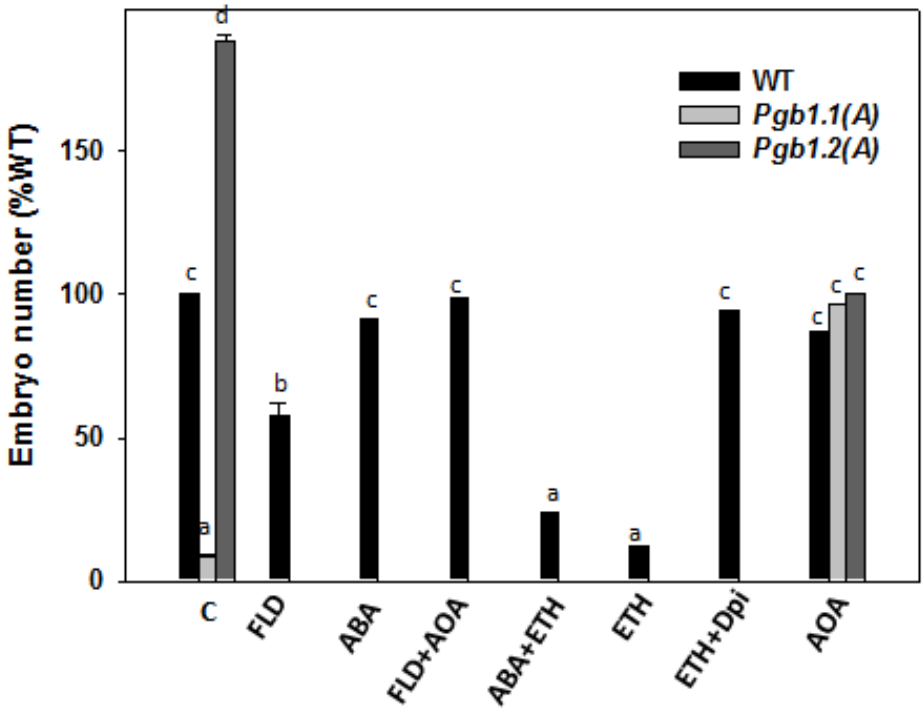


Fig. 2.6: Effects of ABA, ethylene, and ROS manipulations on the number of fully developed maize somatic embryos produced by the WT line and lines down-regulating (A) *ZmPgb1.1* or *ZmPgb1.2*. Values (means of at least three biological replicates) ± SE are expressed as percentages of the WT (control, C) set at 100%. Letters on bars indicate statistically significant differences ($p < 0.05$). Fluridone (FLD), ethephon (ETH), aminooxyacetic acid (AOA), diphenylene iodonium (Dpi).

To assess the domains expressing the ethylene genes, RNA in situ hybridization was performed on immature embryos collected from the WT line and lines down-regulating *ZmPgbs* [line ZmPgb1.1(A) and ZmPgb1.2(A)]. As a result of the elevated levels of nucleotide similarity among some of the genes, the localization studies show the combined expression of *ZmACS2* and *ZmACS7* (probe ZmACS2/7), *ZmACO15* and *ZmACO31* (probe ZmACO15/31), and *ZmACO20* and *ZmACO35* (probe ZmACO20/35). While the transcripts of many genes analysed accumulated heavily in many cells of the ZmPgb1.1(A) embryos, those of *ZmACO15/31*, *ZmACO20/35*, and *ZmERF2* were limited to the basal cells (anchor cells) of the ZmPgb1.2(A) embryos (Fig.2. 7). The spatial location of these domains is similar to the location of domains depleted in ABA (Fig. 2.3), accumulating NO (Fig.1E in Huang et al. 2014), and expressing the respective *ZmPgbs* under normal conditions (Fig. 1C in Huang et al. 2014).

Collectively these data suggest that 1) an elevation of NO or a depletion of ABA, either pharmacologically (by giving treatments with SNP and FLD respectively) or by suppression of *ZmPgbs,* induces ethylene synthesis and response, and 2) ethylene inhibits embryogenesis with NO and ABA acting as upstream components.

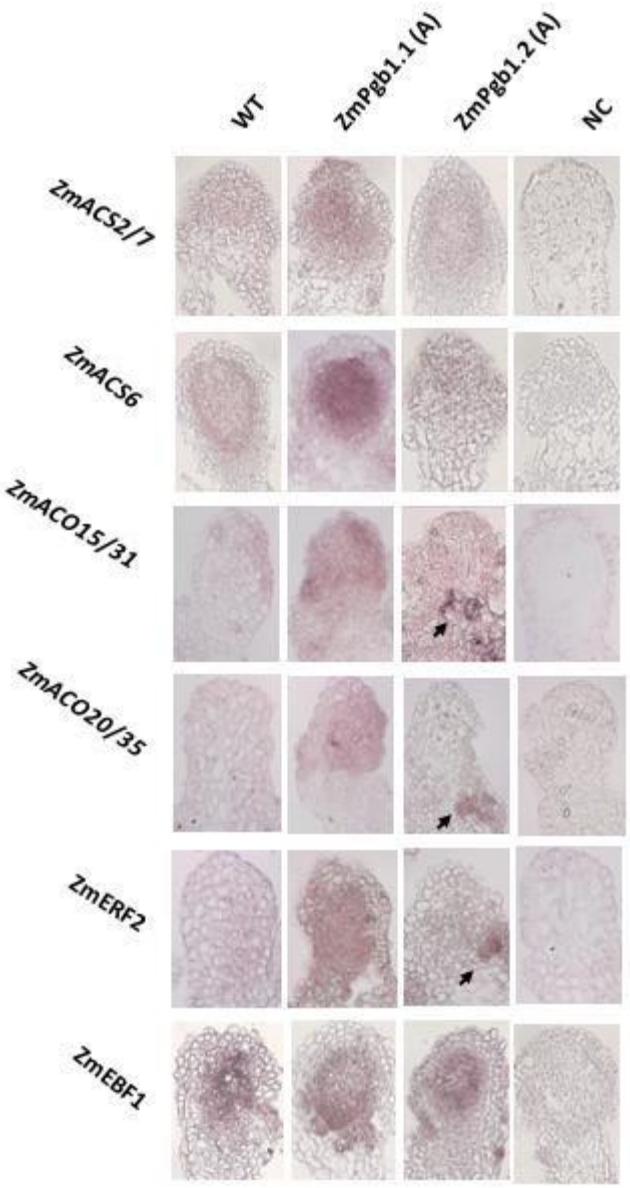


Fig. 2.7**:** RNA in situ hybridization of the ethylene biosynthetic genes 1-aminocyclopropane-1-carboxylic acid synthase (ACS2, 6, and 7) and oxidase (ACO 15, 20, 31, and 35), as well as response genes EIN3-binding F-box protein 1 (ZmEBF1), and ethylene responsive factor 2 (ZmERF2). Due to the elevated levels of nucleotide similarity among some of the genes, the localization studies show the combined expression of ZmACS2 and ZmACS7 (probe

ZmACS2/7), ZmACO15 and ZmACO31 (probe ZmACO15/31), and ZmACO20 and ZmACO35 (probe ZmACO20/35). Arrow indicates anchor cells. NC, negative controls hybridized with sense riboprobes

2.4.4 Production of reactive oxygen species (ROS) are induced by ethylene

Suppression of *ZmPgb1.1* or *ZmPgb1.2* during somatic embryogenesis leads to ROS-activated PCD in those cells where the respective *ZmPgbs* are expressed under normal conditions (Huang et al. 2014). Production of ROS in plants occurs through oxidative bursts induced by several mechanisms, the majority of which rely on membrane-bound NADPH oxidases (Sagi and Fluhr 2006). Maize respiratory burst oxidase homologues [ZmRBOH (A-D)], homologs to gp91 phox, are reliable indicators of NADPH oxidase activity (Lin et al. 2009). Relative to WT, suppression of *ZmPgbs* induced the expression of many *ZmRBOHs* (Fig.2. 8). In WT embryos an induction of several *ZmRBOHs* was observed when the levels of ABA were decreased (FLD), or those of ethylene (ETH) or NO (SNP) increased. A raise in ABA or a reduction in NO (cPTIO) or ethylene (AOA) reduced the expression of several *ZmRBOHs* in the ZmPgb-suppressing lines. The observation that ABA reverses the effect of SNP [compare SNP with ABA+SNP in *ZmRBOH(C)*] in WT embryos, and FLD reverses that of cPTIO (compare cPTIO and FLD+cPTIO) in the ZmPgb lines, places NO upstream of ABA (Fig. 2.8).

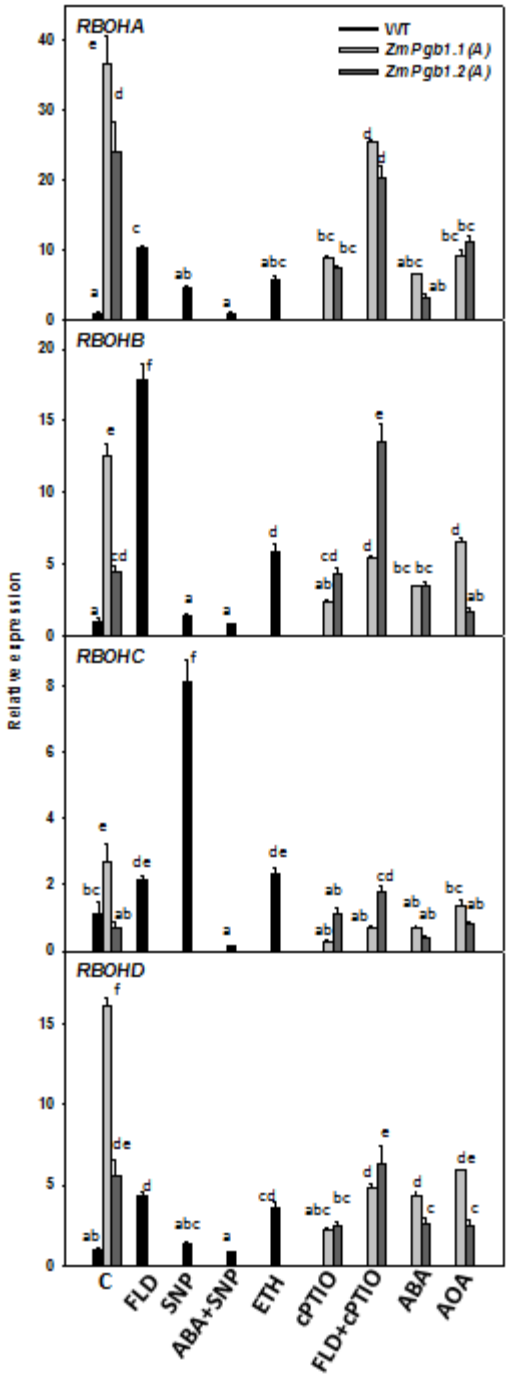


Fig. 2.8**:** Effects of suppression of *ZmPgbs* and nitric oxide (NO), ABA, and ethylene manipulations on the *Respiratory Burst Oxidase Homologs* (Zm*RBOH*) genes measured at the end of the proliferation period in the WT line and lines down-regulating (A) *ZmPgb1.1* or

ZmPgb1.2. Values (means of at least three biological replicates) ± SE are normalized to the WT (control, C) value set at 1. Letters on bars indicate statistically significant differences ($p < 0.05$). Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), fluridone (FLD), ethephon (ETH), aminooxyacetic acid (AOA).

In situ localization of ROS with dihydroethidium, previously used in maize cells (Huang et al. 2014), was also conducted to establish the function of ethylene in the accumulation of ROS. In WT embryos, over-production of ethylene (ETH) increased ROS staining (Fig. 2.9). Inhibition of ABA synthesis (FLD) also resulted in elevated ROS staining and this effect was partially abolished by the co-application of the ethylene biosynthetic inhibitor AOA (FLD+AOA). In the transgenic lines [ZmPgb1.1(A) and ZmPgb1.2(A)] ROS accumulated in those domains expressing the respective *ZmPgb* under normal conditions (Huang et al. 2014), and inclusion of AOA was sufficient to decrease ROS staining in both lines (Fig. 2.9).

Production of ROS in maize cells can be reduced by diphenylene iodonium (Dpi) (Huang et al. 2016), an inhibitor of NADPH oxidase in many systems (Bindschedler et al. 2006; Davies et al. 2006). During maize somatic embryogenesis Dpi is sufficient to reverse the effects of suppression of *ZmPgb1.1* and *ZmPgb1.2* by attenuating PCD (Huang et al. 2014). To further determine if ROS participate in ethylene responses, as suggested by the ROS localization studies (Fig. 2.9), Dpi was applied in WT embryos under conditions of elevated ethylene levels (Fig.2. 6). Reduction of ROS by Dpi abolished the ethylene inhibition on embryogenesis (compared ETH with ETH+Dpi in Fig. 2.6).

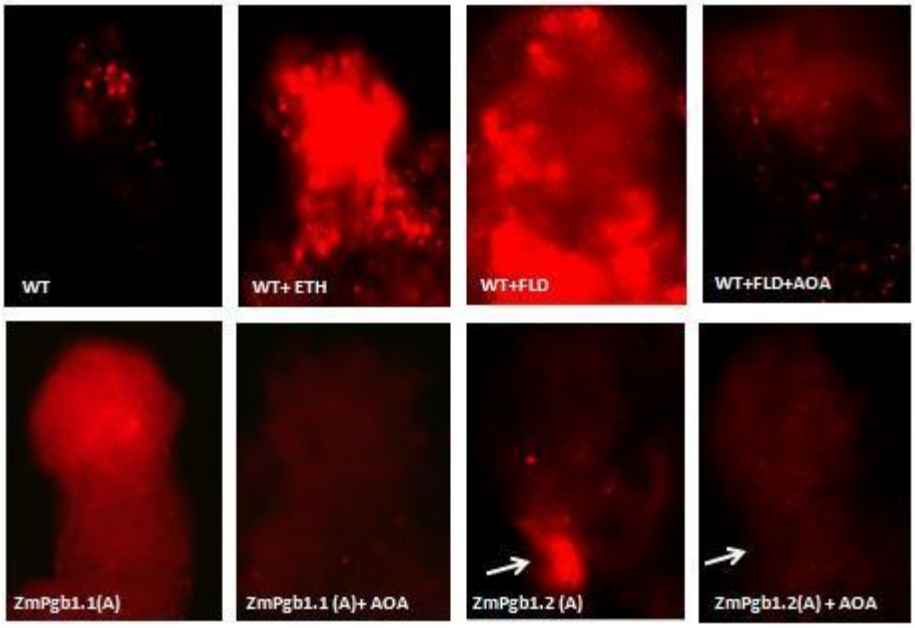


Fig. 2.9**:** Localization of reactive oxygen species (ROS) with dihydroethidium in embryos of the WT line and lines down-regulating (A) *ZmPgb1.1* or *ZmPgb1.2* harvested at the proliferation period. Arrows indicate anchor cells. Fluridone (FLD), ethephon (ETH), aminooxyacetic acid (AOA).

2.4.5 NO, ABA, and ethylene influence the pattern of PCD

Programmed cell death was monitored by TUNEL assays in immature maize somatic embryos. In WT embryos, a raise in NO (SNP) and ethylene (ETH), or a decrease in ABA (FLD) increased the number of TUNEL positive nuclei and this effect was attenuated in SNP+ABA and FLD+AOA treated embryos (Fig. 2.10). In ZmPgb1.1(A) embryos, massive PCD program was alleviated by reducing NO (cPTIO) and ethylene (AOA), or increasing ABA (ABA). Applications of ETH and FLD reversed the respective effects of ABA and cPTIO (Fig. 2.10). In ZmPgb1.2(A) embryos PCD, restricted to the anchor cells (arrow in Fig. 2.9), was abolished by reducing NO (cPTIO) or increasing ABA. In these embryos co-application of cPTIO and FLD (cPTIO+FLD) triggered PCD in many cells (Fig. 2.10).

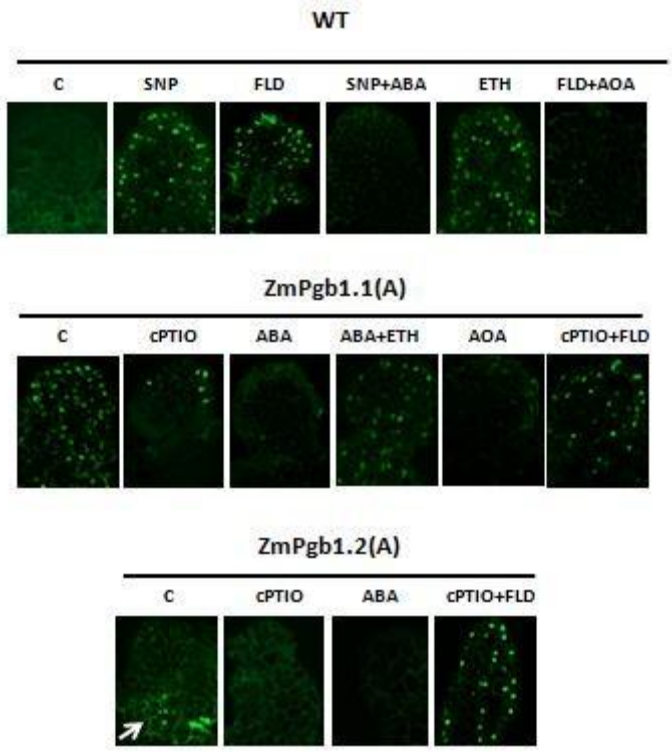


Fig. 2.10: Programmed cell death pattern measured by TUNEL in embryos of the WT line and lines down-regulating (A) *ZmPgb1.1* or *ZmPgb1.2* harvested at the end of the proliferation period. Arrows indicate anchor cells. Sodium nitroprusside (SNP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), fluridone (FLD), ethephon (ETH), aminooxyacetic acid (AOA).

2.5 DISCUSSION

Phytoglobins are effective NO scavengers (Hebelstrup et al. 2006) and their suppression influences maize somatic embryogenesis by triggering PCD through accumulation of NO and ROS (Huang et al. 2014). While *ZmPgb1.1* is expressed in many embryonic cells and its repression causes extensive PCD leading to embryo abortion, *ZmPgb1.2* is mainly transcribed within the basal cells, anchoring the young embryos to the embryogenic tissue. Suppression of *ZmPgb1.2* raises the content of NO and ROS in the "anchor cells" leading to their removal by PCD, and allowing the embryos to develop at high frequency (Huang et al. 2014, Fig. 1). Based on this evidence, this work examines the participation of the plant hormones ABA and ethylene, in the ZmPgb control of embryogenic competence.

The regulation of *ZmPgb* suppression on embryogenesis is mediated by a depletion of ABA. Besides the observation that ABA supplementations fully reverse the effects of *ZmPgb* suppression on the number of somatic embryos (Fig. 2.1), ABA content is significantly lower in ZmPgb1.1(A) and ZmPgb1.2(A) tissues (Fig. 2.2B). This is possibly due to the down-regulation of several ABA biosynthetic genes (Fig. 2.2A). Furthermore, a depletion of ABA in the transformed lines occurs in those domains where the respective *ZmPgbs* are expressed under normal conditions (throughout the embryo body for *ZmPgb1.1* and in the anchor cells for *ZmPgb1.2*) (compare Fig. 2.3 with Fig. 1C in Huang *et al*. 2014).

A possible intermediate linking ZmPgbs to ABA content is NO. Initially considered a downstream component of ABA responses, NO has now been shown to functionally interact with ABA through more complex mechanisms (Leon et al. 2013). In some instances NO can act as an upstream regulator of ABA responses (Liu et al. 2009; Wang et al. 2015). From our study

it emerges that depletion of ABA occurs as a result of elevated levels of NO, accumulating in *ZmPgb* suppressing cells (Huang *et al*. 2014). The inhibitory role of NO on ABA synthesis might occur at a transcriptional level. While enrichment of NO (by SNP), a condition inhibiting maize somatic embryogenesis (Huang et al. 2014), downregulates the expression of the ABA biosynthetic genes *vp14/NCED* and *Molybdenum cofactor sulfurase*, depletion of NO (by cPTIO) in the NO-over-producing ZmPgb(A) lines induces the transcripts of many ABA biosynthetic genes (Fig. 2A). The most pronounced transcriptional induction was observed when cPTIO was applied to the ZmPgb1.1(A) embryos, accumulating under control conditions the greatest amount of NO among all the lines utilized (Huang et al. 2014). The NO repression on ABA was further confirmed by the ability of SNP to reduce the endogenous ABA content in WT embryos, and cPTIO to elevate ABA levels in the ZmPgb1.1(A) line (Fig. 2B).

Abscisic acid is known to regulate several plant responses by influencing the synthesis of other hormones, including ethylene. The ABA regulation of ethylene is dichotomous, with ABA promoting ethylene production in some systems (Luo et al. 2014) but decreasing it in others (Trivellini et al. 2011). During maize embryogenesis, conditions lowering ABA levels (by FLD, or suppression of *ZmPgbs*) increase ethylene production (Fig. 5B) and the genes participating in ethylene synthesis (*ACS* and *ACO*) and response (*EBF1* and *ERF2*) (Fig. 4,5A). While the transcripts of many of these genes localized throughout the ZmPgb1.1(A) embryos, which stained more intensely than their WT counterparts, probes for ZmACO15/31, ZmAO20/35 and ZmERF2 were mainly detected in the basal domains of the embryos (Fig. 7). These localization domains correspond closely with those depleted in ABA (Fig. 3), enriched in NO (see Fig. 1E in Huang et al. 2014), and expressing the respective *ZmPgbs* under normal

conditions (see Fig. 1C in Huang et al. 2014). The transcriptional up-regulation of ethylene biosynthetic genes in tissues suppressing *ZmPgbs* agrees with the induction of ACO activity, corresponding to an increase in ethylene synthesis, in maize cells down-regulating the class 1 barley *Pgb* (Manac'h-Little et al. 2005). If a depletion of ABA induces ethylene accumulation and the expression of ethylene biosynthetic and responsive genes in the WT line, exogenous ABA applications in the *ZmPgb* down-regulating lines (characterized by low levels of endogenous ABA, Fig 2.2B), have opposite effects (Fig. 2.4, 2.5).

Like ABA, accumulation of ethylene and transcription of ethylene biosynthetic and responsive genes was also influenced by NO [with SNP increasing ethylene accumulation and gene expression in the WT line, and cPTIO decreasing ethylene accumulation and gene expression in the ZmPgb(A) lines, Fig. 2.4,2.5]. Nitric oxide is a signal molecule known to stimulate ethylene production in some systems (Lindermayr et al. 2006) while repressing it in others (Hebelstrup et al. 2012). The ability of exogenous ABA to revert the effect of SNP in the WT line, and FLD that of cPTIO in the ZmPgb(A) lines suggests that NO acts up-stream in the ABA regulation of ethylene synthesis and response.

Ethylene regulates embryogenesis and, consistent with work on conifer and angiosperm embryogenic systems (Kong and Yeung 1994; Leroux et al. 2009), maize embryogenesis is inhibited when ethylene is experimentally increased by ETH (Fig. 2.6). One of the possible consequences of ethylene over-production, previously implicated in morphological defects such as cellular separation of meristematic cells (Kong and Yung 1994), is to induce production of ROS. In plants, the ROS-generating oxidative burst is regulated by NADPH oxidases (Sagi and Fluhr 2006), large protein complexes with some cytoplasmic domains which include the

p47phox and a NADPH-binding cytochrome comprising the glycosylated transmembrane protein gp91phox and the non-glycosylated p22phox (Torres and Dangl, 2005). The transcript levels of the four *ZmRBOH* (*A-D*), homologs to gp91 phox, good indicators of NADPH oxidase activity (Lin et al. 2009), are induced in the WT embryos when ethylene is overproduced (ETH) and repressed in the ZmPgbs(A) lines when ethylene level is reduced with AOA (Fig. 2.8). This regulation, verified by *in situ* localizations studies (Fig. 2.9) is consistent with a model where NO and ABA act upstream of ethylene.

Among the diverse functions as signal molecules during development and in response to stress conditions, ROS have been implicated with PCD (van Breusegem and Dat 2006). Targeted accumulation of ROS often precedes the initiation of the death program leading to the elimination of cells, tissues and organs (Hauser 2006). During both *in vivo* and *in vitro* embryogenesis execution of PCD is an obligatory event shaping the embryo body, and Pgbs have been identified as cellular switches of the death fate (Hill et al. 2013; Huang et al. 2014). Besides confirming the characteristic death pattern in the *ZmPgb*-suppressing embryos [many cells in the ZmPgb1.1(A) embryos and anchor cells of the ZmPgb1.2(A) embryos], the TUNEL assay reveals an activation of the death program under conditions where the level of NO or ethylene are increased and that of ABA decreased (Fig. 2.10). The pattern of PCD also confirms the upstream participation of NO and ABA in the ethylene modulation of the death program.

Based on the results, a model is proposed integrating NO, ABA and ethylene in the ZmPgb regulation of embryogenesis (Fig. 2.11). The model builds on our previous findings (Huang et al. 2014), documenting a cell-specific localization of the *ZmPgbs*. *ZmPgb.1.1* expression extends throughout the immature somatic embryo while expression of *ZmPgb.1.2* is restricted to

a few basal cells, i.e. “anchor cells”, anchoring the embryos to the subtending embryogenic tissue. An elevation in NO content is observed in these domains following suppression of the respective *ZmPgb* (Huang et al. 2014). Nitric oxide down-regulates several ABA biosynthetic genes resulting in a depletion of ABA in those domains suppressing *ZmPgbs* and accumulating NO. Abscisic acid inhibits ethylene biosynthesis and the NO-mediated depletion of ABA relieves this inhibition causing ethylene to accumulate. Elevated ethylene levels trigger production of ROS and induce PCD in *ZmPgb*-suppressing cells. Ethylene-induced PCD in the *ZmPgb1.1* suppressing cells, scattered throughout the embryo, causes abortion while PCD in the *Zmpgb1.2.* suppressing cells results in the elimination of the anchor cells and the successful development of the embryos.

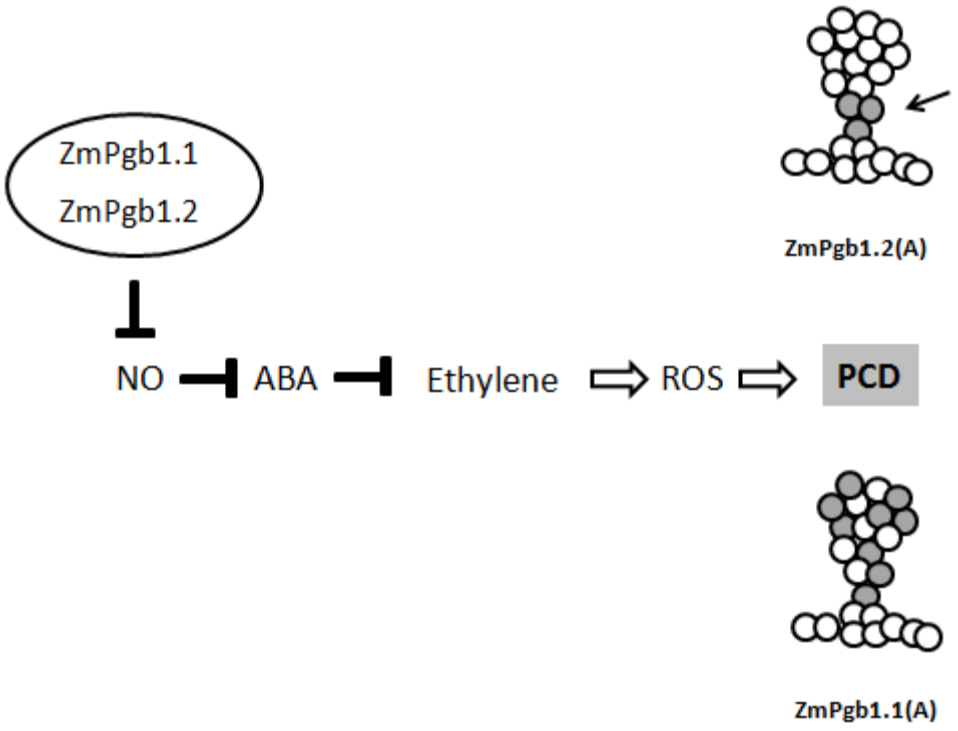


Fig. 2.11: Proposed model integrating ABA and ethylene in the ZmPgb regulation of somatic embryogenesis. Suppression of *ZmPgb1.1* or *ZmPgb1.2* releases the inhibitory effects on NO. Accumulation of NO blocks the synthesis of ABA thus allowing production of ethylene. Ethylene induces the formation of ROS, which trigger PCD in the *ZmPgb* suppressing cells. The cell specific localization of the respective *ZmPgb* determines which cells are eliminated by PCD (grey cells). Suppression of *ZmPgb1.1* results in the death of many embryonic cells leading to the abortion of the embryos, while suppression of *ZmPgb1.2* induces PCD in the basal cells (anchor cells) anchoring the embryos to the subtending embryogenic tissue. Elimination of the anchor cells (arrow) allows the further development of the embryos.

2.6 GENERAL DISCUSSION AND CONCLUSION

Results presented above thus fit in a model that integrates ABA and ethylene in *ZmPgb* regulation of cell death mechanism by modulating NO levels during *Zea mays* somatic embryogenesis. Previous research has well established a pathway that links *Pgbs* to cell death/survival decisions during *in vitro* embryogenesis by modulating NO that further interferes with biosynthesis and signaling of several phytohormones such as auxin, JA, ABA and ethylene along with ROS homeostasis (Huang et al. 2014, Mira et al. 2016 b). Plant regeneration is a process regulated by fine tuning of genetic and epigenetic events that involve various developmental programs including cell division, differentiation and programmed cell death (PCD) (Huang et al. 2014). Somatic embryogenesis is thus used to characterize the totipotency of plants as it displays a zygote like embryogenic process, in which somatic cells grow, differentiate and develop into fully mature embryos when cultured under appropriate culture conditions. Cell division and differentiation were considered as two important steps during somatic embryogenesis but recent evidence has suggested PCD as another important event that controls the initial stages of *in vitro* embryogenesis (Smertenko and Bozkhov 2014; Thorpe and Stasolla 2001; Yang and Zhang 2010).

Plant hormones such as auxin, ABA and ethylene, along with NO play critical roles during various stages of the embryogenic development (Freschi 2013; Rodríguez-Serrano et al. 2012; Otvös et al. 2005; Jiménez 2005). Therefore, regulation of NO homeostasis, along with biosynthesis and signaling of plant hormones, is critical for proper development of the embryos. Reduction in cellular NO may occur through various mechanisms and recent studies have documented a plausible role of Pgbs as efficient NO scavengers (Hill 2012). Furthermore, the

suppression of *ZmPgb* triggered PCD via NO and ROS over-production during maize somatic embryogenesis (Huang et al. 2014). These studies have thus prompted us to investigate the participation of ABA and ethylene in the ZmPgb regulation of somatic embryogenesis.

The results in chapter 2 investigate the role of ABA during the *ZmPgb* regulation of embryogenic competence by modulating NO levels during *Zea mays* somatic embryogenesis. Inclusion of ABA into the culture media nullified the effects of *ZmPgb* repression on the somatic embryo count number (Fig 2.1). Furthermore, the level of ABA was significantly reduced in lines down-regulating Pgb [ZmPgb1.1(A) and ZmPgb1.2(A)] (Fig 2.2B). These results were supported by observing a down regulation in the expression pattern of key ABA biosynthetic genes such as *vp14/NCED, CME kinase,* and *molybdenum cofactor sulfurase* (Fig 2.2 A). Additionally, localization of cellular ABA conducted in the down regulating ZmPgb lines showed a depletion of ABA in those domains where the respective *ZmPgb* genes are expressed during normal conditions - for the *ZmPgb1.1(A)* line throughout the embryogenic cells, and for the *ZmPgb1.2(A)* line in the anchoring cells of the immature embryo (Fig 2.3 and Huang et al. 2014). The results further prompted us to investigate the role of the signaling molecule NO as a possible link between *ZmPgb* and ABA. Besides confirming the work of Huang et al. (2014), this thesis shows that a reduction in ABA content occurs as a result of elevated NO levels due to *ZmPgb* repression. This inhibitory effect of NO on ABA levels can be observed at transcriptional levels as elevation in NO (by SNP), compromising somatic embryo production (Huang et al. 2014), represses the expression of ABA biosynthetic genes such as *vp14/NCED* and *Molybdenum cofactor sulfurase.* Depletion of NO by cPTIO in the ZmPgb1.1 (A) line increased the expression levels of ABA biosynthetic genes when compared to other lines (Fig 2.2 A).

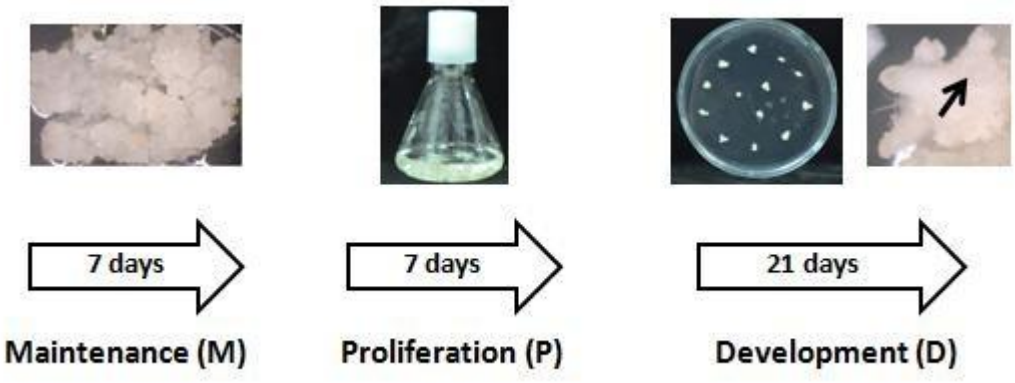
ABA is also known to interact with other hormones such as ethylene in many plant systems (Luo et al. 2014; Trivelleni et al. 2011). In the present study, the results observed suggest that ABA regulates ethylene synthesis and signaling during maize somatic embryogenesis. Depletion of ABA (by FLD or using down-regulating ZmPgb lines) increases ethylene content in the embryogenic lines (Fig 2.5 B), and elevates the expression of key ethylene biosynthetic and responsive genes (*ACS, ACO,EBF1* and *ERF2*) (Fig 2.4, 2.5A). The RNA *in-situ* localization results further supported the link between ABA and ethylene interaction, with NO as a down-stream component. The transcripts of several ethylene biosynthetic and responsive genes were localized in many cells of the ZmPgb1.1(A) line, while ZmACO15/31, ZmAO20/35 and ZmERF2 were detected only in the anchoring cells of the ZmPgb1.2(A) embryos (Supplemental Fig 2.7). These localization domains are possibly those devoid of ABA content (Fig 2.3), enriched in NO (Huang et al. 2014), and expressing the respective *ZmPgbs* under normal conditions (Huang et al. 2014). These results were further supported by gene expression data showing a transcriptional regulation of ethylene by ABA. For example, the addition of exogenous ABA in the ZmPgb down-regulated lines (having reduced ABA content, Fig 2.2 B) results in reduced ethylene biosynthetic and responsive gene expression (Fig 2.4and 2.5 A). Thus NO along with ABA, also regulates the transcription of ethylene biosynthesis and responsive genes. Additionally the fact that inclusion of exogenous ABA reverts the effects of SNP in the WT, while applications of FLD that of cPTIO in ZmPgb(A) lines, suggest that NO is positioned upstream in the ABA regulation of ethylene biosynthesis.

Accumulation of ethylene in culture is associated with several morphological defects such as the separation of meristematic cells and generation of reactive oxygen species (ROS) (Kong and

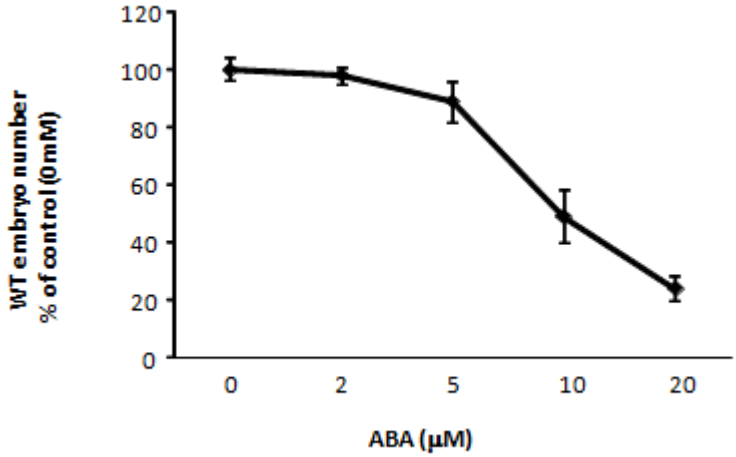
Yeung 1994). The present study provides evidence that ROS production is encouraged when the levels of ethylene are increased (by Ethephon), and repressed following a reduction in ethylene (by AOA). These results were also confirmed by analyzing the transcription of the four *ZmRBOH(A-D)* genes (Fig 2.8). Reactive oxygen species have also been closely associated with PCD during various developmental stages and stress responses (Breusegem and Dat 2006). Over-production of ROS often initiates the cell death mechanisms (Huaser et al. 2006). A recent study by Huang et al. (2014) implicated PCD as a requiring step to shape the embryo body, a process influenced by Pgbs.

Together, results of this thesis not only confirm the 'specific' death pattern in the *ZmPgb* suppressed lines [many cells throughout the ZmPgb1.1(A) embryos and anchoring cells of the ZmPgb1.2(A) embryos], but also propose a model ((Fig. 2.11) integrating ABA and ethylene in the Pgb regulation of embryogenesis. It is plausible that this model might operate not only during embryogenesis, but also in those developmental and stress-related responses modulated by Pgbs; an hypothesis that could be tested in future studies.

2.7: SUPPLEMENTAL DATA



Supplementa Figure1: Somatic embryogenesis in maize. Embryogenic tissue, maintained on solid auxin-containing medium (M) can be induced to proliferate on liquid proliferation (P) medium for 7 days. Transfer of the tissue on the auxin-free development (D) medium induces the formation of developing somatic embryos (arrow) which will become fully mature after 21 days. All pharmacological treatments were performed on P.



Supplemental Figure 2: Effects of increasing concentrations of ABA on the number of fully developed somatic embryos. Values (means of at least three biological replicates) ± SE are expressed as percentages of the WT (control, C) set at 100%.

Supplementa Table 1: List of primers for gene expression

ABA	
Name	Sequences
Actin-F	GATGGTCAGGTCATCACCATTG
Actin-R	AACAAGGGATGGTTGGAACAAC
ZmCME Kinase-F	GCAAGCCTCGGTTGCTGACTG
ZmCME Kinase-R	TTG TCT TGT GAA CAA TCC CCG CC
ZmCar iso-F	GATGGCTGAGAACACTTGCATAGGACG
ZmCar iso-R	GTTTGCCCTGGCATCCCTAATTACAGAG
Zmvp14/NCED-F	GGCTTCCACGGCACCTTCATCACGGGC
Zmvp14/NCED-R	CGGGGAACTGATCTGGGCTCCCTCTGG
ZmAAO3-F	GCCCAAACACAGCGAAAGCATAGATCCAGC
ZmAAO3-R	CGCTCCTCGCAGTTCCTGAGTTCCCC
ZmAAO6-F	CACCCACCCTCATGCCCATGTA
ZmAAO6-R	GTGCTTCGTCTCCCATCATCGG
ZmMoco S-F	CGGCAGGTGTACTTTGGGCAAA
ZmMoco S-R	CGGGGTCCTGATTCGGTCACTCAG

ETHYLENE	
ZmACS2-F	ATCGCGTACAGCCTCTCCAAGGA
ZmACS2-R	GGCCATGAACTCCGCGTCC
Zm-ACS6-F	CGCGCCGCCACGGACGACG
Zm-ACS6-R	ATCTTGGTGGCCGCGGAGAC
Zm-ACS7-F	ATCGCGTACAGCCTCTCCAAGGA
Zm-ACS7-R	TGCCATGAACTCCGCGTCGG
Zm-ACO15-F	AGCGGCGGCGACGCATACC
Zm-ACO15-R	GGAGATGACTTGGGCGCTGCAA
Zm-ACO20-F	CGTTCGGCACCAAGGTGAGC
Zm-ACO20-R	ACGTCCACCCACTCCCCGC
Zm-ACO31-F	AGCGGCGGCGACGCATACC
Zm-ACO31-R	GGAGATGACTTTGGCGCCCC
Zm-ACO35-F	CGTTCGGCACCAAGGTGAGC
Zm-ACO35-R	CACGTCCACCCACTCCCCG
ZmEBF1-F	CTGTCCGGCTGTATGAAGGT
ZmERF2-R	AATTGCTCCCGAGCTTATCG

ZmERF2-F	AGACAATGAGGCGTGCAAGT
ZmEBF1-R	TGGTTGCCAATGAAGTTGAA
Zm-ACO35-F	CGTTCGGCACCAAGGTGAGC
RESPIRATORY BURST OXIDASE HOMOLOGUES	
ZmrbohA-F	CACACGTGACCTGCGACTTC
ZmrbohA-R	CCCCAAGGTGGCCATGA
ZmrbohB-F	GGCCAGTACTTCGGTGAAACA
ZmrbohB-R	ATTACACCAGTGATGCCTTCCA
ZmrbohC-F	TTCTCTTGCCTGTATGCCGC
ZmrbohC-R	CTTTCGTATTCCGCAGCCA
ZmrbohD-F	CCGGCTGCAGACGTTCTT
ZmrbohD-R	CCTGATCCGTGATCTTCGAAA

Supplemental Table 2: Primers for Ethylene *Insitu* Localization

Name	Sequences
ZmACS2-F	TGACTGTTGCTGGAGGTCAG
ZmACS2-R	CGTTGAGCTTCACCTTGTGT
ZmACS6-F	CTCATCACCAACCCTTCCAA
ZmACS6-R	CTTCTTCCACAGCTCCATCTC
ZmACO15-F	GGACTGGGAGGACATCTTCTA
ZmACO15-R	GTTGCTGAGCACCTCAATCT
ZmACO20-F	ACTGGGAGAGCACCTTCTT
ZmACO20-R	TACGTGGTGGCCTTCTTCT
ZmEBF1-F	CAAGGCTGTAGGTCGTTTCT
ZmEBF1-R	GCCCACTGTTTGAACTCTTTATC
ZmERF2-F	AGCTTCGGGATCCTGGT
ZmERF2-R	ACGAGTCCGAGGAGGTG

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