Mechanisms of Programmed Cell Death Modulated by Phytoglobins in Maize Somatic Embryogenesis

by

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ABSTRACT

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Mechanisms of programmed cell death modulated by phytoglobins in maize somatic embryogenesis

Supervisor: Dr. Claudio Stasolla and Dr. Belay T. Ayele

Hemoglobins (Hbs) are heme-containing proteins belonging to the globin superfamily that are ubiquitous in most living organisms including prokaryotes and eukaryotes. In addition to the first legHbs found in leguminous plants, there are another three classes of phytoglobins (Pgbs) identified in various plant species including dicots and monocots. The ability of heme groups to bind gaseous ligands such as oxygen, carbon monoxide and nitric oxide (NO) places Pgbs as multifunctional players in various processes during plant growth and development under normal or stress conditions. The objective of this project is to investigate how transcriptional manipulation of *ZmPgb1.1* and *ZmPgb1.2* influences somatic embryogenesis in maize (*Zea mays*). Suppression of either of the two genes is sufficient to induce programmed cell death (PCD) through a pathway initiated by accumulation of nitric oxide (NO) and zinc (Zn^{2+}), and mediated by production of reactive oxygen species (ROS). The effect of the death program on the fate of the developing embryos is dependent upon the localization patterns of the two *Pgbs*. During somatic embryogenesis, *ZmPgb1.2* transcripts are restricted to a few cells anchoring the embryos to the subtending embryogenic tissue, while *ZmPgb1.1* transcripts extend to several embryonic domains. Suppression of *ZmPgb1.2* induces PCD in the anchoring cells allowing the embryos to develop further, while suppression of *ZmPgb1.1* results in massive PCD leading to embryo abortion. Cells suppressing the *Pgb* genes are also depleted of endogenous auxin (indole-3-acetic acid, IAA) localization established by polar auxin transport (PAT), thus suggesting a possible involvement of this plant hormone in the observed processes. Collectively, it appears that the cell specific expression of *Pgbs* has the capability to determine the developmental fate of embryogenic tissue during maize somatic embryogenesis through their effect on PCD. This novel regulation has implications for development and differentiation in other species.

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DEDICATION

Lovingly dedicated to the memory of my mother

Xiu’e You (尤秀娥)

TABLE OF CONTENTS

ABSTRACT i
ACKNOWLEDGEMENTS ii
DEDICATION iii
TABLE OF CONTENTS iv
LIST OF TABLES viii
LIST OF FIGURES ix
ABBREVIATIONS xii
FORWARD xvi
1 GENERAL INTRODUCTION 1
2 LITERATURE REVIEW 4
2.1 Biological functions of phytoglobins 4
2.1.1 Introduction 4
2.1.2 Characteristics and expression patterns of Pgbs 5
2.1.2.1 Characteristics of Pgbs 5
2.1.2.2 Pgb location and timing of expression 8
2.1.2.2.1 Embryo development, seed germination and dormancy 8
2.1.2.2.2 Somatic embryogenesis 10
2.1.2.2.3 Post-embryonic development 11
2.1.3 Nitric oxide (NO) signaling in plants 14
2.1.3.1 The role of NO in hormone signal transduction 14
2.1.3.2 NO and abiotic stress 15
2.1.3.2.1 Hypoxic stress 16
2.1.3.2.2 Salt stress 18
2.1.3.2.3 Cold stress 19
2.1.3.2.4 Nutrient deficiency 20
2.1.3.3 NO and plant-pathogen interactions 21
2.1.3.4 NO and plant-rhizobium symbiosis 23
2.1.4 Conclusions 25
2.2 Programmed cell death in plant embryogenesis 26
2.2.1 Introduction 26

2.2.2 Basic characteristics of PCD in plants 27
2.2.3 Functional roles of PCD in plant embryogenesis 29
2.2.3.1 Role of PCD during *in vivo* plant embryogenesis 30
2.2.3.1.1 Elimination of the suspensor 30
2.2.3.1.2 Survival of a single dominant embryo 33
2.2.3.2 Role of PCD during *in vitro* plant embryogenesis 34
2.2.3.2.1 PCD during somatic embryogenesis 35
2.2.3.2.2 PCD during microspore-derived embryogenesis 36
2.2.4 Key regulators of PCD in plant embryogenesis 38
2.2.4.1 Bax inhibitor 1 39
2.2.4.2 Metacaspases 40
2.2.4.3 Nitric oxide 41
2.2.5 Conclusions 43
3 CHAPTER ONE: PHYTOGLOBIN CONTROL OF CELL SURVIVAL/DEATH DECISION REGULATES IN VITRO PLANT EMBRYOGENESIS* 44
3.1 Abstract 45
3.2 Introduction 45
3.3 Results 48
3.3.1 Suppression of ZmPgbs affects somatic embryogenesis 48
3.3.2 ZmPgbs regulate intracellular Zn^{2+} through the NO-dependent metal release from metallothioneins 52
3.3.3 ZmPgb modulation of embryogenesis involves PCD and production of ROS 59
3.3.4 ROS accumulation in maize cells is required for the execution of PCD 61
3.3.5 The oxidative burst contributes to ROS production 65
3.3.6 The production of ROS necessitates an active MAPK cascade 68
3.3.7 Targeted PCD in ZmPgb-expressing cells phenocopies suppression of Pgbs 71
3.4 Discussion 73
3.4.1 ZmPgbs modulate somatic embryogenesis through PCD 73
3.4.2 ZmPgb regulation of PCD is mediated by cellular NO and Zn^{2+} and requires ROS 75
3.4.3 Inhibition of the oxidative burst and MAPK cascade abolishes ROS formation 78
3.4.4 A unique model linking ZmPgbs to cell death/survival decision during *in vitro* embryogenesis 79

3.5 Materials and methods 83
3.5.1 Plant materials and treatments 83
3.5.2 Plant transformation 84
3.5.3 Isolation of the maize embryo-specific ZmMT4 85
3.5.4 ZmMT4 expression and purification 85
3.5.5 Release of Zn^{2+} from the purified ZmMT4 86
3.5.6 In situ localization of NO, Zn^{2+}, and PCD 87
3.5.7 Localization of superoxide, hydrogen peroxide, and reduced ASC 87
3.5.8 Expression studies 88
3.5.9 Caspase-like activity 88
3.5.10 RNA in situ hybridization 88
3.5.11 Statistical analysis 89
3.6 Supplemental data 89
4 CHAPTER TWO: SUPPRESSION OF PHYTOGLOBINS INFLUENCES CELLULAR AUXIN LOCALIZATION PATTERN IN MAIZE SOMATIC EMBRYOGENESIS 101
4.1 Abstract 102
4.2 Introduction 103
4.3 Results 106
4.3.1 Endogenous IAA localization is affected by suppression of phytoglobins 106
4.3.2 Nitric oxide (NO) and zinc (Zn^{2+}) mediate the phytoglobin-regulation of IAA localization 109
4.3.3 Alteration of Pgbs does not affect the total level of IAA in the embryogenic tissue 111
4.3.4 Pgbs affect the transcription of genes involved in auxin biosynthesis 112
4.3.5 Localization patterns of VT2 and ZmAO1 119
4.3.6 Pgbs affects the expression pattern of IAA transporters 122
4.3.7 Pgbs affect the expression pattern of auxin-responsive genes 125
4.3.8 Auxin and polar auxin transport are required for somatic embryogenesis 126
4.4 Discussion 128
4.5 Materials and methods 134
4.5.1 Plant material and treatments 134
4.5.2 Expression studies 134
4.5.3 RNA in situ hybridization 134
4.5.4 Endogenous IAA localization 135

4.5.5 Free IAA quantification 135
4.5.6 Immunolocalization of ZmPIN1 proteins 135
4.5.7 Statistical analysis 136
4.6 Supplemental data 136
5 GENERAL DISCUSSION AND CONCLUSIONS 138
6 LITERATURE CITED 142

LIST OF TABLES

Table 3.1 Effects of the NO donor DEA/NO on the release of Zn^{2+} from the recombinant MBP5 and the MBP5-ZmMT4 fusion protein……..54

Table S3.1 List of primers used in PCD studies……98

Table 4.1 Key genes involved in auxin biosynthesis pathways in maize……113

Table S4.1 List of primers used in auxin studies…….135

LIST OF FIGURES

Fig. 2.1 The Pgbs/NO cycle during hypoxia……...…….7

Fig. 3.1 Pgb regulation of maize somatic embryogenesis…….50

Fig. 3.2 Effects of NO and Zn^{2+} on the production of mature somatic embryos (d 21, P medium)…….57

Fig. 3.3 PCD during maize somatic embryogenesis……..63

Fig. 3.4 Requirement of NADPH oxidase in the ZmPgb response…….....67

Fig. 3.5 An active MAPKK cascade is required in the ZmPgb response……..69

Fig. 3.6 Induced cell death in ZmPgb-expressing domains regulates embryogenesis……...72

Fig. 3.7 Proposed model of ZmPgb action during somatic embryogenesis……...81

Fig. S3.1 Amino acid sequence alignment of ZmPgb1.1 and ZmPgb1.2 with Clustal W using BLOSUM protein weight matrix ……..89

Fig. S3.2 Regulation of ZmPgb1.1 and ZmPgb1.2 by auxin…….90

Fig. S3.3 Detection of Zn^{2+} in maize embryos by Zinquin……92

Fig. S3.4 Effects of increasing concentrations of the NO donor (DEA/NO) on the release of Zn^{2+} from a constant concentration of recombinant ZmMT4 protein ….……..93

Fig. S3.5 Percentage of TUNEL-positive cells in immature (d 7, P medium) embryos cultured in environments with altered levels of NO and Zn^{2+}…….94

Fig. S3.6 Cytological and molecular hallmarks of PCD in ZmPgb-suppressing embryos……95

Fig. S3.7 Localization of reduced ASC in immature (d 7, P medium) maize embryos produced by the wild-type line and ZmPgb1.1(A) and ZmPgb1.2(A) lines……...96

Fig. S3.8 Expression level of four respiratory burst oxidase homologs (ZmrbohA-D) in immature embryos (d 7, P medium) produced from the wild-type lines and ZmPgb1.1(A) and ZmPgb1.2(A) lines……97

Fig. 4.1 Suppression of phytoglobins affects somatic embryogenesis in maize……..107

Fig. 4.2 Localization of endogenous IAA in the WT, ZmPgb1.1 (A) and ZmPgb1.2 (A) lines at day 7 on proliferation medium…….109

Fig. 4.3 Measurement of free IAA levels in WT, ZmPgb1.1(A) and ZmPgb1.2(A) embryogenic tissue at day 3 and 7 on proliferation (P) medium……...110

Fig. 4.4 Auxin biosynthesis pathways in maize……...112

Fig. 4.5 The expression levels of TSB1 and TSB2 in the WT, ZmPgb1.1 (A) and ZmPgb1.2 (A) lines…….....114

Fig. 4.6 The expression levels of ZmAMI1, SPI1, and ZmNIT1,2 in the WT, ZmPgb1.1 (A) and ZmPgb1.2 (A) lines…….....116

Fig. 4.7 The expression levels of VT2 and ZmAO1 in the WT, ZmPgb1.1 (A) and ZmPgb1.2 (A) lines……..117

Fig. 4.8 RNA in situ hybridization of VT2 in the WT, ZmPgb1.1(A) and ZmPgb1.2(A) lines at day 7 on proliferation medium…….119

Fig. 4.9 RNA in situ hybridization of ZmAO1 in the WT, ZmPgb1.1(A) and ZmPgb1.2(A) lines at day 7 on proliferation medium…….120

Fig. 4.10 Relative expression levels of ZmPIN1s in the WT, ZmPgb1.1(A) and ZmPgb1.2(A) lines at day 3 and 7 on proliferation medium……..122

Fig. 4.11 Immunolocalization of the ZmPIN1 protein in the WT, ZmPgb1.1(A) and ZmPgb1.2(A) lines at day 7 on proliferation medium……122

Fig. 4.12 Relative expression levels of auxin transport genes in the WT, ZmPgb1.1(A) and ZmPgb1.2(A) lines at day 3 and 7 on proliferation medium……...123

Fig. 4.13 Relative expression levels of two early auxin-responsive genes in the WT, ZmPgb1.1(A) and ZmPgb1.2(A) lines at day 3 and 7 on proliferation medium…….124

Fig. 4.14 Effects of 2,4-D (Top) and TIBA (Bottom) on mature embryo production in the WT, ZmPgb1.1(A) and ZmPgb1.2(A) lines…….....126

ABBREVIATIONS

ABA, Abscisic Acid

AEBSF, 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride

ASC, Ascorbic acid

ARR, Arabidopsis Response Regulator

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

2,4-D, 2,4-Dichlorophenoxyacetic Acid

DAF-2DA, 4,5-diaminofluorescein diacetate

DEA/NO, Diethylamine NONOate

DPI, diphenylene iodonium

DTZ, diphenyl thiocarbazone

DW, dry weight

ELS, Embryo-Like Structure

ER, Endoplasmic Reticulum

ERF, Ethylene Response Factor

ET, Ethylene

ETI, Effortorss-Triggered Immunity

GA, Gibberellin

GTP, Guanosine Triphosphate

Hb, Hemoglobin

HR, Hypersensitive Response

IAA, indole-3-acetic acid

JA, Jasmonic Acid

KOD, Kiss Of Death

MAPK, mitogen-activated protein kinase

MAPKK, mitogen-activated protein kinase kinase

MBP, maltose binding protein

MC, Matercaspase

MCS, Multi-Cellular Structure

MT, metallothionein

PAMP, Pathogenesis-Associated Molecular Pattern

PAT, Polar Auxin Transport

PBS, phosphate-buffered saline

PCD, Programmed Cell Death

PD098059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one

PEM, Pro-Embryogenic Mass

Pgb, phytoglobin

PM, plasma membrane

PRR, Pathogen-Recognition Receptor

PTI, PAMPs-Triggered Immunity

PTM, Post Translational Modification

RAM, Root Apical Meristem

ROS, Reactive Oxygen Species

SA, Salicylic Acid

SL, Sphingolipid

SNP, sodium nitroprusside

TIBA, 2,3,5-triiodobenzoic acid

TPEN, N,N,N’,N’-tetrakis (2 pyridylmethyl) ethylenediamine

TUNEL, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling

WT, Wild Type

Zinquin, 2-methyl-8-(toluene-p-sulfonamido)-6-quinolyloxyacetic acid

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 Plant Physiology. This thesis is presented as two manuscripts, each containing an abstract, introduction, results, discussion and materials and methods section. For each manuscript, supplemental figures and tables 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 manuscripts.

1 GENERAL INTRODUCTION

Hemoglobins (Hbs), iron containing proteins first described in vertebrates for their ability to transport and deliver oxygen, are ubiquitously distributed among living organisms including bacteria and plants (Kundu et al., 2003; Vázquez-Limón et al., 2012). Other than the symbiotic Hbs or leghemoglobins (legHbs) discovered in plants, there are three other classes (class 1-3) of phytoglobins (Pgbs) identified across the plant kingdom, based on their oxygen affinities and sequence/structural properties (Hoy and Hargrove, 2008; Smagghe et al., 2009; Vázquez-Limón et al., 2012). While legHbs were the first to be identified in plants due to their involvement in nitrogen (N_2) fixation processes, Pgbs were discovered in later years and identified in a variety of plant species (Hill, 2012). The first two classes, class 1 and 2, of Pgbs are present in dicots while only class 1 Pgbs have been identified in monocots (Smagghe et al., 2009). Evolved within monocots, their number varies significantly among species, with two copies of class 1 Pgbs, designated as *ZmPgb1.1* and *ZmPgb1.2*, characterized in maize (*Zea mays*), and five copies of class 1 Pgbs present in the rice (*Oryza sativa*) genome (Hoy and Hargrove, 2008; Rodríguez-Alonso and Arredondo-Peter, 2013). Different from the first two classes which have a three-on-three α-helical loop structure surrounding the heme moiety, the third class of Pgbs possess a two-on-two α-helical structure, similar to truncated Hbs identified in bacterial species, also designated as truncated Pgbs (Vázquez-Limón et al., 2012; Hemschemeier et al., 2013).

Over the past three decades, studies on Pgbs have been advanced significantly and the structural properties and functional roles of Pgbs have been determined in several species. Compared to legHbs, the function of Pgbs in plant growth and development is less understood in spite of their induced expressions under different abiotic and biotic stress conditions. However,

due to their ability to bind/scavenge nitric oxide (NO) (Hill, 2012) and being one of the most versatile signaling molecules in plant development (Yu et al., 2014), Pgbs are most likely involved in many, if not all NO-mediated responses. The regulation of NO in plant development is fulfilled through its multi-level regulation of cell division, cell differentiation, and programmed cell death (PCD), processes that coordinately shape the plant body (Otvös et al., 2005; Gabaldón et al., 2005; Shen et al., 2013; Wang et al., 2013). Furthermore, NO interacts in a synergistic or antagonistic fashion with several other signaling molecules and with almost all plant hormones (Freschi, 2013). Fine-tuning of these interactions ensure the needed developmental flexibility which characterizes the plant kingdom (Santner et al., 2009; Vanstraelen and Benková, 2012). Characterized as one of the first plant hormones, auxin has been shown to exert a dominant role in a number of developmental processes, such as the early ontogeny events of embryogenesis *in vivo* (Möller and Weijers, 2009; Lau et al., 2012) and somatic embryogenesis, that is the ability of somatic cells to generate zygotic-like embryos (Thorpe and Stasolla, 2001; Raghavan, 2004; Karami et al., 2009). The role of auxin during somatic embryogenesis is well known in that, by playing as the inductive signal in many embryogenic systems, it favours the de-differentiation of somatic cells which subsequently re-embark into an embryogenic pathway culminating in the production of somatic embryos (Thorpe and Stasolla, 2001). In conjunction with auxin, another required early event for the proper execution of the embryogenic process is PCD which results in the selective elimination of specific cells that shapes the immature embryos. In addition, the requirement of PCD during embryogenesis has been documented both *in vivo* and *in vitro* (Pennell and Lamb, 1997; Smertenko and Bozhkov, 2014).

On the premises that Pgbs are effective NO scavengers, and that NO regulates several processes including PCD through its interaction with other signaling molecules and plant hormones, the objective of this research program is to investigate how the altered expression of monocot class 1 *Pgbs* regulates maize embryogenesis in culture. The hypothesis tested is that manipulations in *Pgbs* levels alter cellular NO homeostasis and, perturbations in NO levels interfere with down-stream cellular events affecting the death program and ultimately the production of somatic embryos.

2 LITERATURE REVIEW

2.1 Biological functions of phytoglobins

2.1.1 Introduction

Hemoglobins (Hbs) belong to the superfamily of globin proteins that is defined by a characteristic α-helical globin polypeptide fold with a bound heme prosthetic group (Hargrove et al., 2000; Smagghe et al., 2009). Hbs were originally identified in mammals where they evolved for the distinctive functions in oxygen transport and storage, and now are widely recognized for their ubiquitous presence in almost all living organisms including archaeobacteria, eubacteria, and eukaryotes (Vinogradov et al., 2006; Hoy and Hargrove, 2008). In plant systems, phytoglobins (Pgbs) were first isolated from the root nodules of legumes and designated as leghemoglobins (legHbs) or symbiotic Hbs (Kubo, 1939), which are structurally similar to mammalian globins and act to facilitate oxygen transport in plant-rhizobium symbiosis (Gupta et al., 2011). Later, Pgbs were also identified in non-leguminous plants (Bogusz et al., 1988), a discovery suggesting that Pgbs may have important functions beyond their involvement in symbiotic nitrogen fixation. Since then, as discussed later in this review, identification of new family members such as the three classes (class 1-3) of Pgbs in different species has increased remarkably. These studies have mainly focussed on structural properties, expression patterns and biological functions in a wide range of organisms (Taylor et al., 1994; Sowa et al., 1998; Hoy and Hargrove, 2008; Hill, 2012; Vázquez-Limón et al., 2012; Mukhi et al., 2013; Shah et al., 2013).

2.1.2 Characteristics and expression patterns of Pgbs

2.1.2.1 Characteristics of Pgbs

The globin family are proteins with a characteristic α-helical secondary structure comprised of helices A-H, and a heme group enclosed within a hydrophobic pocket formed by the typical 3/3-folding or the truncated 2/2-folding helices (Vinogradov et al., 2005; 2006). The heme group contains an iron atom and four of the six coordination sites, and it is further attached to histidines of the globin moiety (Gupta et al., 2011). This unique sequence/structure composition is highly conserved within the family members including Pgbs, and determines their gene classifications and ligand binding properties.

In addition to the first symbiotic Hbs or legHbs identified from leguminous plants, three distinct classes (class 1-3) of Pgbs have been identified in numerous plant species (Smagghe et al., 2009; Vázquez-Limón et al., 2012). Of these three classes, class 1 and 2 Pgbs are found in non-leguminous plants, while class 3 or truncated Pgbs are present in both legumes and non-leguminous plants (Bustos-Sanmamed et al., 2011; Vázquez-Limón et al., 2012). From the perspective of sequence/structure similarity and oxygen affinity (Smagghe et al., 2009), the first two classes of Pgbs are highly divergent in that (1) both classes of Pgbs are found in dicots, while only class 1 occurs in monocots, (2) evolved within monocots, class 1 Pgbs can be further diversified into clade I and II Pgbs, corresponding to the respective class 1 and 2 Pgbs in dicots, and (3) the number of monocot-specific class 1 Pgb genes varies among species, for example, maize has two (*ZmPgb1.1* and *ZmPgb1.2*) while rice has five (*OsPgb1.1*-*OsPgb1.5*) (Smagghe et al., 2009; Rodríguez-Alonso and Arredondo-Peter, 2013). Unlike the typical 3/3-folding helices in legHbs and the first two classes of Pgbs, truncated or class 3 Pgbs in plants are structurally truncated into 2/2-folding helices and are highly homologous to the truncated bacterial Hbs

(Vinogradov et al., 2006; Reeder and Hough, 2014). Furthermore, the number of truncated Pgbs is also species-specific particularly in monocots (Rodríguez-Alonso and Arredondo-Peter, 2013).

The divergent complexity of these Pgbs has also attracted interest in studying their biochemistry. Based on the coordination site of the heme iron, symbiotic legHbs are penta-coordinated, while the other three classes (class 1-3) of Pgbs are hexa-coordinated (Smagghe et al., 2009; Gupta et al., 2011). However, within monocots, clade I of Pgbs is hexa-coordinated, while clade II of Pgbs can be either hexa-coordinated or penta-coordinated (Rodríguez-Alonso and Arredondo-Peter, 2012). For example in maize, both *ZmPgb1.1* (clade I) and *ZmPgb1.2* (clade II) are hexa-coordinated, while the rice *OsPgb1.1-OsPgb1.4* (clade I) are hexa-coordinated and *OsPgb1.5* (clade II) is penta-coordinated (Rodríguez-Alonso and Arredondo-Peter, 2012). In the penta-coordinated structure, only the proximal histidine coordinates with the fifth site of the heme iron, leaving the sixth open for reversible binding of ligands such as O_2 and NO. However, in the hexa-coordinated structure, both the proximal and distal histidine coordinate with the heme iron, facilitating the tight binding of O_2 that can further accept an electron from iron and oxygenate NO to form nitrate (NO_3^-) (Gupta et al., 2011). Compared to class 1 Pgbs, a stronger extent of hexa-coordination has been observed in class 2 Pgbs, resulting in a relatively lower O_2 affinity (Smagghe et al., 2009). These interactions between O_2 and Pgbs to form oxyPgbs have been further identified being responsible for the O_2 dependent NO binding/scavenging under near-anaerobic environment, resulting in the formation of NO_3^- and metPgbs (Fig. 2.1; Igamberdiev and Hill, 2004; Nienhaus et al., 2010). In the same model, reductase activity is required to convert the ferric in the metPgbs back to a ferrous state (Igamberdiev et al., 2006a).

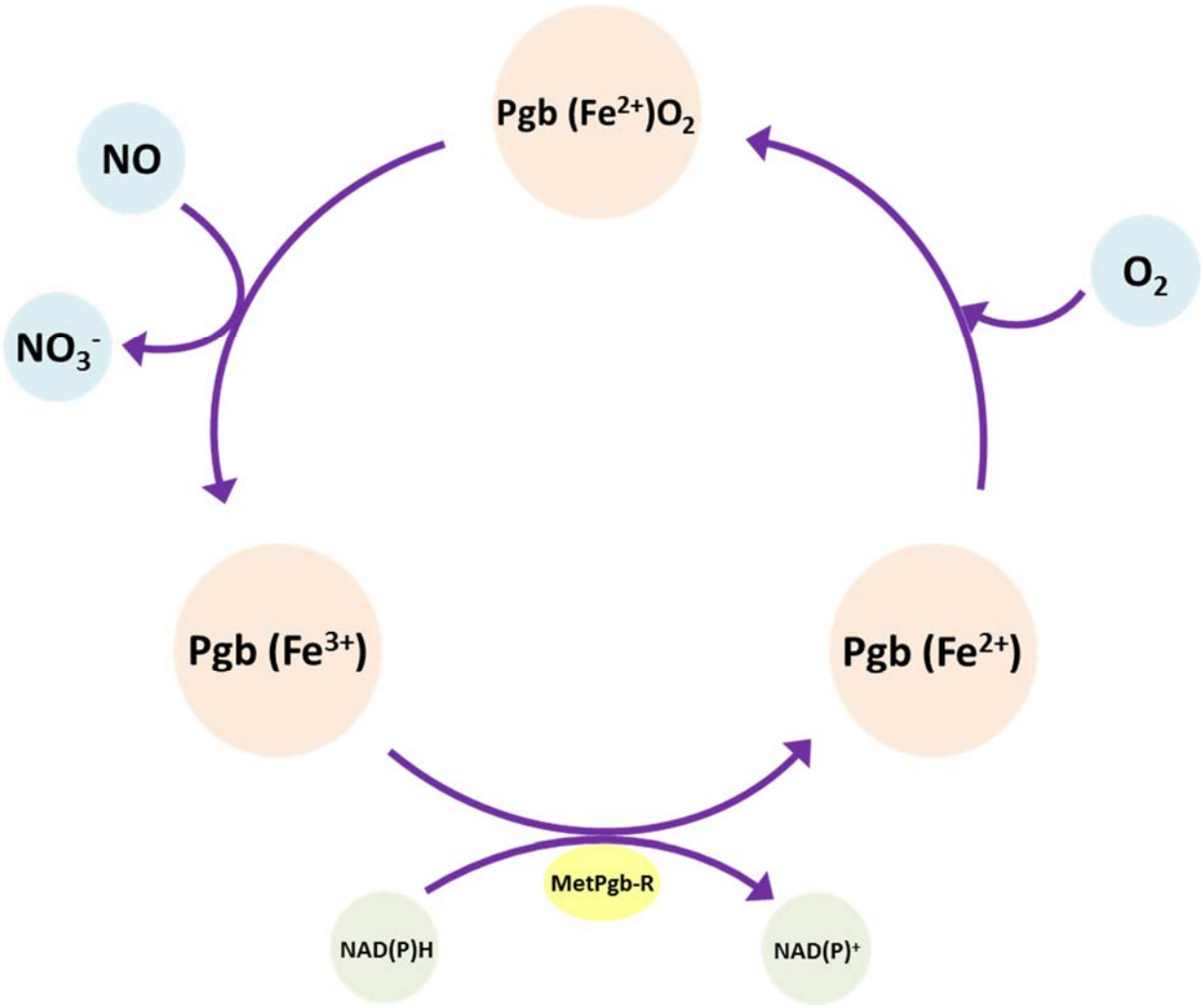

Fig. 2.1 The Pgbs/NO cycle during hypoxia. This is adapted from Igamberdiev and Hill, 2004, and permission has been obtained from the publisher/copyright holder to incorporate it in the thesis.

It must be mentioned, however, that the distinction in O_2 affinity facilitates the genetic origin/classification of Pgbs, the biological roles of a given *Pgb* gene in different plant developmental processes are not the same due to overlapping functions (Hill 2012). To some extent, these unique functions largely rely on their involvement in NO binding/scavenging, and this places Pgbs as crucial regulators of fundamental processes (Hill 2012; Hebelstrup et al., 2013; Matilla and Rodríguez-Gacio Mdel, 2013), as NO is a highly versatile signal molecule (Yu et al., 2014). This notion can be further supported by the expression pattern of distinctive *Pgbs*, which primarily refers to their regulatory roles in NO homeostasis at the cellular level.

2.1.2.2 Pgb location and timing of expression

To complete their life cycles, higher plants undergo a series of developmental events which include gametogenesis, fertilization, embryogenesis, and seed germination, growth and maturation. All these biological processes are controlled by complicated genetic and epigenetic networks (He et al., 2011). Through the modulation of cellular NO levels, Pgbs acts as key regulators in several, and possibly many, of these processes and information on their functional role has emerged over the past few years (Hill, 2012). The following sections outline the involvements of Pgbs during different stages of the plant life cycle, as well as *in vivo* and *in vitro* morphogenesis.

2.1.2.2.1 Embryo development, seed germination and dormancy

In several monocots and dicot species, including Arabidopsis (Vigeolas et al., 2011; Thiel et al., 2011), sweet gale (Heckmann et al., 2006), barley (Taylor et al., 1994; Nie and Hill, 1997; Duff et al., 1998), maize (Aréchaga-Ocampo et al., 2001), and rice (Ross et al., 2001; Hoy and Hargrove, 2008; Lira-Ruan et al., 2011), *Pgbs* are expressed during embryo/seed development and germination, although with often distinct and specific patterns. In rice seeds, for example,

transcripts of both *OsPgb1.1* and *OsPgb1.2* are detected in the embryos and seminal roots, but only that of *OsPgb1.2* was found in the coleoptiles (Lira-Ruan et al., 2011). The increased expression of *Pgbs* during seed germination could be partially explained by their de novo synthesis, as revealed by experiments using cycloheximide (Ross et al., 2001). In barley (*Hordeum vulgare*) grains, *HvPgb1* transcripts accumulated quickly after imbibition and their expression continued to increase until radicle emergence (Guy et al., 2002; Hebelstrup et al., 2007). *HvPgb1* was identified as a germination marker to evaluate seed viability and quality in barley, possibly through maintenance of the energy status within the grain (Guy et al., 2002). The fact that Pgbs can facilitate seed germination is apparent in tobacco plants over-expressing the alfalfa *Pgb1* (Seregélyes et al., 2003), and this is due to the promotion of dormancy release. According to two independent studies, the Pgb-mediated regulation of NO influences glucose catabolism via the pentose phosphate pathway which controls the dormancy-germination transition (Igamberdiev and Hill, 2004; Arc et al., 2013). Besides contributing to the induction of seed germination in wheat, over-expression of *Pgb1* also affects seed production (Sen, 2010). Arabidopsis plants with elevated levels of *Pgb1* have increased seed weight, a consequence of the NO accumulation due to the tight binding reaction with *Pgb1* or a transcriptional regulation of *NIA2* (*nitrate assimilation 2*) and *NiR1* (*nitrite reductase 1*), a major enzymatic source of NO synthesis in plants (Desikan et al., 2002; Thiel et al., 2011). Seed storage product accumulation is also under the control of Pgbs, as documented by increases in polysaturated fatty acids and total oil content in Arabidopsis plants over-expressing *Pgb2* (Vigeolas et al., 2001). These results were ascribed to enhanced oxygen availability in the developing seeds. It is therefore apparent from all these studies that seed development and germination are processes influenced by Pgbs, although information related to their precise functional role is scarce.

2.1.2.2.2 Somatic embryogenesis

Recapitulation of embryogenesis can also occur *in vitro* where cultured cells subjected to specific conditions can produce embryos sharing physiological and morphological characteristics to their *in vivo* counterpart (Thorpe and Stasolla, 2001). The detailed mechanisms regulating the formation of *in vitro* embryos will be discussed later, this section documents studies underlying the involvement of Pgbs in the process.

Independent studies have shown that *Pgbs* are expressed during *in vitro* embryogenesis. In the development of chicory somatic embryos *Pgb1* was specifically expressed during the auxin-mediated induction phase, coinciding with the de-differentiation of the somatic cells and the acquisition of embryogenic potential (Hendriks et al., 1998). This expression pattern was inherent to the development of the embryos, as it was unrelated to wounding, commonly occurring during the excision of the explant, and to exogenous auxin applications. At the ultracellular level, Pgbs were localized near the chloroplasts and in the vicinity of the vascular tissue of the original explant (Smagghe et al., 2007). The authors speculated that the presence of Pgbs is necessary for the initial mitotic divisions preceding the formation of embryogenic cells.

An important question arising from these studies is whether the different types of Pgbs fulfill the same function during embryo formation. Using embryogenic and non-embryogenic chicory lines it was determined that while *Pgb1* and *Pgb 2* are expressed in the non-embryogenic line, only *Pgb2* was present in the line competent to produce embryos (Smagghe et al., 2007). The participation of Pgbs during somatic embryogenesis was also investigated in Arabidopsis. In this system suppression of *Pgb2* increased the number of embryos while the down-regulation of *Pgb1* showed a significantly negative effect on embryo yield (Elhiti et al., 2013). The authors further demonstrated that the increased embryo formation in the *Pgb2*-suppressing lines was due

to the elevation of NO in the embryogenic cells resulting in the down-regulation of the transcription factor MYC2, a repressor of auxin biosynthesis (Dombrecht et al., 2007). The increased auxin synthesis in *Pgb2* suppressing cells was sufficient to promote embryogenesis (Elhiti et al., 2013). This study shows that through the regulation of NO, Pgbs play a central role in regulating the development of embryos *in vitro*.

2.1.2.2.3 Post-embryonic development

Information about the role of Pgbs during post-embryonic development is extremely scarce, although indirect evidence can be derived from expression and localization studies. Expression of *Pgbs* during post-embryonic development has been observed in several plant species, including Arabidopsis (Hunt et al., 2001; Hunt et al., 2002; Hebelstrup et al., 2006; Hebelstrup and Jensen, 2008; Wang et al., 2011), tobacco (Holmberg et al., 1997), tomato (Wang et al., 2003) and rice (Lira-Ruan et al., 2011). In rice, *OsPgb1.1* was expressed in leaves and roots, while *OsPgb1.2* in coleoptiles, embryos, leaves and seminal roots (Lira-Ruan et al., 2011). A differential expression pattern for *Pgbs* was also observed in tomato, where *Pgb1* transcripts were mainly found in the stem, with low abundance in immature fruit, leaves, and flowers, while *Pgb2* was expressed at high levels in young leaves (Wang et al., 2003). Evidence also suggests that Pgbs might control development and affect growth. Transgenic tobacco plants over-expressing the bacterial-specific hemoglobin from *Vitreoscilla* (*VHb*), exhibited enhanced growth, and on average 80-100% more dry weight after 35 days of growth compared to wild-type (WT) controls (Holmberg et al., 1997). These *VHb* overexpressing plants contained, on average, 30-40% more chlorophyll and 34% more nicotine than controls. The expression of *VHb* also resulted in an altered distribution of secondary metabolites with a reduction of anabasine (Holmberg et al., 1997).

The majority of information relating Pgbs to post-embryonic plant development has been derived from Arabidopsis studies. Arabidopsis *Pgb2* was expressed in roots, leaves and inflorescence and could be induced by cytokinin (CK) in young plants (Hunt et al., 2001). This was in contrast to *Pgb1* which was expressed in germinating seeds and was induced by hypoxia and increased sucrose supply, but was not affected by CK (Hunt et al., 2002). To be more precise, strong expression of the Arabidopsis *Pgb1* was observed in root tips and in the cells surrounding lateral root branches. In the aerial parts of the plant, expression of *Medicago gale Pgb1* was also localized at the end points of vascular tissues in leaves, corresponding to hydathodes, and within axilliary meristems (Heckmann et al., 2006). Activation of the auxillary meristems leading to the production of new tissues resulted in reduced *Pgb1* expression. As compared to *Pgb1*, *Pgb2* expression is generally weaker within the meristematic tissues and totally absent from the hydathodes.

The expression of both *Pgb1* and *Pgb2* in post-embryonic meristems is intriguing and suggestive of a regulation of meristematic cell activity with repercussions on morphogenesis and development. This notion was in part demonstrated by transgenic studies suppressing or elevating the two *Pgbs*. An elevation of *Pgb1* expression was sufficient to reduce flowering time while a suppression of the same gene induced several developmental deficiencies consistent with the expression patterns of *Pgb1* (Hebelstrup and Jensen, 2008). In addition to forming abnormal hydathodes, a reduction in *Pgb1* level affected meristem function with a loss of apical dominance, and a delay in bolting and in the vegetative-reproductive transition of the apical meristems (Hebelstrup and Jensen, 2008). The plants remained in the vegetative stage for longer time and produced more rosette leaves. Interestingly, ectopic axilliary meristems are formed along the inflorescence producing aerial rosette of leaves (Hebelstrup and Jensen, 2008). These

phenotypic abnormalities were not observed when the expression of *Pgb2* was altered. The *pgb2* knockout mutant lines showed a normal developmental phenotype, although over-expression of *Pgb2* enhanced survival under hypoxic stress conditions. Co-suppression of both *Pgb1* and *Pgb2* induced death in the early developmental stages (Hebelstrup et al., 2006).

The phenotypes observed following alteration in *Pgb1* or *Pgb2* were associated with a differential accumulation of NO in the affected organs. In line with their role of NO scavengers, a suppression of the two *Pgbs* resulted in elevated cellular NO signals (Hebelstrup et al., 2006; Hebelstrup and Jensen, 2008). Although information linking NO to meristematic activity is very scarce, it is evident that the high respiratory rate of active meristematic tissue depletes oxygen with a concomitant rise in NO (Bidel et al., 2000).

The regulation of *Pgbs* on meristem function *in vivo* was also verified during shoot organogenesis *in vitro*. In contrast to the repression of *Pgb2*-inhibited organogenesis, the over-expression of either *Pgb1* or *Pgb2* enhanced the number of shoots produced in culture, and altered the transcription levels of genes participating in CK perception and signaling (Wang et al., 2011). The up-regulation of either *Pgb1* or *Pgb2* activated CKI1 (CYTOKININ-INDEPENDENT 1) and AHK3 (ARABIDOPSIS HISTIDINE KINASE 3), genes encoding CK receptors, and affected the transcript levels of ARABIDOPSIS RESPONSE REGULATOR (ARR). The expression of Type-A ARRs (ARR4, 5, 7, 15, and 16), feed-back repressors of the cytokinin pathway, was repressed in both Hb over-expression lines whereas that of several Type-B ARRs (ARR2, 12, and 13), transcription activators of cytokinin responsive genes, was induced. Based on these results, the authors suggested that the two *Pgbs* might regulate meristematic function and formation of shoots by alterations in CK signaling, whereas CK is a key hormone controlling the formation and maintenance of shoot meristems (Bartrina et al., 2011).

2.1.3 Nitric oxide (NO) signaling in plants

Nitric oxide (NO), a free radical first identified in mammalian systems and soon after in plants, is considered an important molecule in plant signaling networks. In spite of this, the precise source for NO and the machinery converting NO signals into physiological responses is not fully known (Mur et al., 2012a). In addition, through dynamic interactions with other signal molecules such as hormones, NO is actively involved in the regulation of developmental processes that account for growth, development, abiotic and biotic responses in plants (Freschi, 2013; Wang et al., 2013; Yu et al., 2014). To achieve this NO likely affects transcription of target genes and/or causes structural modifications of target proteins via the NO-dependent post translational modifications (PTMs) such as S-nitrosylation of cysteine residues, transducing the NO message into different responses (Freschi, 2013). Furthermore, the homeostasis of endogenous NO levels can be effectively modulated by the expression of plant *Pgbs*, which indirectly influence and/or modify NO signaling, action sites, and NO interactions with hormones (Hill, 2012).

2.1.3.1 The role of NO in hormone signal transduction

Proper plant growth, development and defense require the coordination of complex signaling networks including a variety of molecular components (Vanstraelen and Benková, 2012). Among these components, NO has been shown to interact dynamically with different hormones, acting either upstream or downstream of the hormonal signaling route (Freschi, 2013; Simontacchi et al., 2013). In this context, the NO-triggered transcriptional modifications and/or PTMs might influence hormonal responses and/or change plant hormone levels, distribution, and signaling (Freschi, 2013).

Of the NO-interacting hormones, auxin is one of the most versatile players that are also important for proper plant growth and development (Zhao, 2010). By acting as a downstream component of auxin signaling, NO mediates auxin response both embryonically and post-embryonically. Examples include the formation and maintenance of root apical meristem (RAM) and the development of adventitious roots (Lanteri et al., 2008; Fernández-Marcos et al., 2011; Terrile et al., 2012). In addition, during the initial phases of *in vitro* embryogenesis, NO regulates cell division and de-differentiation resulting in embryogenic callus formation (Otvös et al., 2005; Rodríguez-Serrano et al., 2012). The level of NO has also been shown to influence somatic embryogenesis, where an increase of NO levels by suppression of class 2 *Pgb2* in Arabidopsis facilitates the acquisition of embryogenic competence and enhances the production of somatic embryos through alterations in auxin synthesis (Elhiti et al., 2013).

The crosstalk between NO and different plant hormones is not restricted to the primary (classical) hormones such as auxin, CK, gibberellin (GA) abscisic acid (ABA), and ethylene (ET), but also includes jasmonic acid (JA) and salicylic acid (SA) (Freschi, 2013; Yu et al., 2014). Most of these interacting modules will be further integrated in the following sections, particularly in plant responses to abiotic/biotic stresses, plant-pathogen interactions and plant-rhizobium symbiosis.

2.1.3.2 NO and abiotic stress

Being sessile, plants have evolved extreme levels of physiological flexibility, which allow them to survive in changing environments under severe abiotic/biotic stress conditions (Atkinson and Urwin, 2012). The involvement of Pgbs in coping with unfavourable environmental challenges is not only justified by their induced expression under various stress conditions but also, and above all, by their tight link with NO (Hill, 2012). As presented in the next sections,

NO is an important factor in plant response mechanisms and regulation of NO homeostasis is crucial for plant survival.

2.1.3.2.1 Hypoxic stress

Being aerobic organisms, plants need oxygen for energy production and may experience oxygen deficiency, such as hypoxia or anoxia, when oxygen diffusion from the environment cannot satisfy the demand set by metabolic rates (Bailey-Serres et al., 2012). The N-end rule pathway of targeted proteolysis has been identified as a specific mechanism in plants to cope with low oxygen levels (Gibbs et al., 2011; Licausi et al., 2011a), which uses hypoxia-associated ethylene response group VII (ERF-VII) transcription factors as substrates. Under normoxic conditions, ERF-VII transcription factors are degraded via the N-end rule pathway, while under hypoxic conditions, the oxidation of cysteine is mitigated and the N-end rule pathway is inhibited (Kosmacz and Weits, 2014). For example, during the anaerobic response, constitutive expression of ERF-VII members, such as RAP2.12, has been shown to activate responsive genes for hypoxia acclimation (Licausi et al., 2011a; Bailey-Serres et al., 2012). In this context, *Pgb1* has been shown to be one of the direct targets of RAP2.12, and belongs to the core genes that are remarkably up-regulated in response to hypoxia (Licausi et al., 2011b). On the other hand, the fact that nitric oxide (NO) has been shown to be strongly associated with low oxygen levels (Dordas et al., 2003; Dordas, 2009; Igamberdiev et al., 2004), and that NO levels can be modulated by Pgbs (Hill, 2012), suggest that Pgbs might protect RAP2.12 from degradation and maintain hypoxic responses for increased tolerance (Igamberdiev et al., 2014).

In this scenario, however, two basic elements must be considered when interpreting the possible roles of Pgbs/NO in relation to hypoxic responses. As discussed in above sections, the first one is that the expression pattern of Pgbs and/or the action pattern of NO are

cell/tissue/organ specific within different plant species. The second is that plants may experience hypoxic conditions at different levels and/or durations. For instance, overexpression of *Pgb1* in Arabidopsis shows a more moderate induction of hypoxic response genes as compared to the WT seeds exposed to mild hypoxia (Thiel et al., 2011), while a similar overexpression of *Pgb1* favours seedling survival challenged by hypoxic conditions (Hunt et al., 2002).

In addition to their direct effects in hypoxic responses, Pgbs/NO in plants may also participate indirectly in tuning survival in response to oxygen deficiency through other regulatory factors. One of the most typical examples is aerenchyma formation in environments with excess water (Drew et al., 2000; Takahashi et al., 2014). Under low oxygen conditions, the formation of aerenchyma facilitates the rapid transport of gases, such as oxygen and carbon dioxide, between and within shoots and roots (Evans, 2003). Two types of primary aerenchyma occur in plants, schizogenous aerenchyma and lysigenous aerenchyma (Takahashi et al., 2014). The former is formed by the physical separation of adjacent files of cortical cells and by the enlargement of existing intercellular spaces via differential cell division/expansion. The latter is formed by the selective collapse and lysis of cortical cell files through programmed cell death (PCD), a process involving several important signaling molecules such as ethylene (ET), calcium ion (Ca^{2+}), reactive oxygen species (ROS) and NO (Drew et al., 2000; Evans, 2003; Igamberdiev et al., 2004; Parent et al., 2011; Wang et al., 2013), which are indirectly affected by Pgb levels.

Suppression of Pgbs increases NO and/or ethylene levels in several plant systems under hypoxic stress conditions (Manac'h-Little et al., 2005; Ederli et al., 2006; Sánchez et al., 2010; Hebelstrup et al., 2012). Furthermore, Pgbs also actively participate in the regulation of ROS homeostasis, including hydrogen peroxide (H_2O_2), a signal molecule in PCD (Gadjev et al., 2008; Fukao et al., 2011; Petrov and Van Breusegem, 2012). For example, over-expression of *Pgb1* in

Arabidopsis reduces the levels of H_2O_2 under severe hypoxia (Yang et al., 2005), and this effect might be exercised through the modulation of the cellular antioxidant system, which is also mediated by NO (Yang et al., 2005). This model links Pgbs and NO to the ascorbate-glutathione cycle (Yang et al., 2005; Igamberdiev et al., 2006a; Zou et al., 2010; Sairam et al., 2012). Collectively, these studies suggest that Pgbs are key regulators of hypoxic responses and their roles might be exercised through different routes.

2.1.3.2.2 Salt stress

By altering the activities of cytosolic enzymes, salinity imposes severe osmotic stresses on plants including nutritional disorders and oxidative damage due to the overproduction of reactive oxygen species (ROS) (Farooq et al., 2013). In many systems, NO accumulates under salinity conditions (Valderrama et al., 2007; Zhao et al., 2007; Qiao et al., 2009; Tanou et al., 2009a,b; David et al., 2010) resulting in deleterious or beneficial effects. While compromising the ROS-scavenging antioxidant defense system by interfering with the electron transport process during respiration (Zottini et al., 2002; Kopyra and Gwozdz, 2003; Molassiotis et al., 2010), NO maintains a high K^+/Na^+ ratio in the cytoplasm by elevating the H^+-ATPase and H^+-PPase activities in the plasma membrane (PM) (Ruan et al., 2004; Zhao et al., 2004; Zhang et al., 2006; Shi et al., 2007; Siddiqui et al., 2011). Furthermore, the accumulation of organic osmolytes and other solutes important for the maintenance of cell turgor and water acquisition are influenced by NO (Guo et al., 2009). Under high salinity conditions NO alters gene expression and enzymatic activity. On one hand, genes involved in enhanced salinity resistance, such as NtGRAS1 in tobacco, a stress-induced member of the GRAS family chacracterized by the functions of GAI (GIBBERELLIN-INSENSITIVE)/RGA (REPRESSOR GAI) and SCR (SCARECROW) genes (Pysh et al., 1999), were induced by NO (Czikkel and Maxwell, 2007). Under salinity stress, the

PM HA1 (H^+-ATPase 1) activity, and the salt tolerance locus SOS1 (salt overly sensitive 1) encoding the PM Na^+/H^+ antiporter (Shi et al., 2000; 2002), were also induced by NO in mangrove plants (Chen et al., 2010). In the same line, the enzymatic activities of stress-activated protein kinases and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), involved in glycolysis and energy supply, are also increased under high salinity conditions. The unequivocal participation of NO in salt stress responses was demonstrated by Zhao et al. (2007), who showed that exogenous NO applications in the NO deficient *noa1* (*nitric oxide associated 1*) knockout Arabidopsis mutant rescued the NO-associated phenotype, and improved salinity resistance by mitigating salinity-induced oxidative damages (Zhao et al., 2007). It is therefore plausible that by regulating NO homeostasis, Pgbs might play an indirect role in salt stress responses.

2.1.3.2.3 Cold stress

Temperature is another important factor influencing plant growth and development, and NO triggers responses to both high and low temperatures. In conjunction with key stress responsive players such as ABA, Ca^{2+} and H_2O_2, NO is associated with signal transduction events linked to cold stress (Gupta et al., 2011). For instance, applications of exogenous NO confer tolerance to cold conditions in several plant systems including wheat, maize and tomato (Neill et al., 2003). This NO-response might be mediated by its antioxidant properties, which reduce peroxidative metabolism (Beligni et al., 2002; Neill et al., 2002). In addition to the well established relationships between NO and calcium or phosphatidic acid signaling, other interactions of NO with sphingolipids (SLs) have been suggested to be important for enhancing tolerance to cold stress (Ruelland et al., 2002; Berkey et al., 2012; Markham et al., 2013). It has been shown that NO negatively regulates the phosphorylation of SLs triggered by cold temperature, and that this event might mitigate the stress (Cantrel et al., 2011). However, how NO regulates phospho-SL

synthesis during cold stress response remains unclear. The generic term SLs designate both membrane-located complex SLs and their metabolic precursors, such as long chain bases (LCB) and ceramides (Cer) (Pata et al., 2010). In relation to this, low temperature reduces the total LCB amount significantly in WT Arabidopsis plants (Guillas et al., 2013), but not in plants over-expressing *Pgb1* which had increased root growth (Perazzoli et al., 2004). These observations suggest that Pgbs could be potential regulators of cold stresses by modulating the amount of NO, and therefore interfering with the effect of NO on the signal molecules SLs, which trigger downstream responses. This model is partially supported by the observation that *Pgb* over-expression has been shown to selectively suppress the expression of defensive genes induced by cold stresses (Novillo et al., 2004; Novillo et al., 2007; Gery et al., 2011).

2.1.3.2.4 Nutrient deficiency

The availability of essential nutrients, including both macro-nutrients such as nitrogen, phosphorous, potassium, calcium, and magnesium, and micro-nutrients such as iron and zinc, is critically important for plant growth and development (Maathuis and Diatloff, 2013). Moreover, plant growth and development, under normal conditions or abiotic stresses such as nutrient deficiency, is finely tuned by endogenous signaling molecules such as nitric oxide (NO) and hormones (Graziano and Lamattina, 2007; Wang et al., 2010a; Kiba et al., 2011; Krouk et al., 2011; Meng et al., 2012). Current studies on the roles of NO in nutrient deficiency are mostly related to phosphorous and iron deficiency. In the case of phosphorous deficiency, exogenous supplementations of NO promote the growth of phosphorous deficiency-induced lateral roots and cluster roots in *Lupinus albus* (Wang et al., 2010a) via the activation of the SCARECROW (SCR) transcription factors SCR1 and SCR2, both required for the asymmetric cell division and radial root patterning (Meng et al., 2012). In the case of iron deficiency, addition of NO donors induce

the expression of the FER (Fe-Efficient Regulator) transcription factor which increases iron uptake in roots, and activate genes such as *FRO1* (*Ferric Reduction Oxidase 1*) and *IRT1* (*Iron Regulated Transporter 1*), which stimulate growth in iron-deficient tomato roots (Graziano and Lamattina, 2007). This regulation is possibly mediated by auxin and ethylene, which also participate in the activation of FRO1 and IRT1 under iron-deficient conditions (Schmidt et al., 2001; Lucena et al., 2006).

Through the regulation of NO, Pgbs might be implicated in the responses described above, and in other processes related to nutrient uptake and possibly metabolism (Hill, 2012). For instance, *Pgb1* is up-regulated in aleurone cells of barley exposed to high nitrate levels, under aerobic or anaerobic conditions (Nie and Hill, 1997). This nitrate-inducible feature of *Pgbs* was also revealed by microarray studies in other systems. For example, Arabidopsis *Pgb1*, but not *Pgb2*, was induced by high concentrations of nitrate in liquid cultures (Wang et al., 2000). A similar behavior was also apparent in tomato roots where the induction of *Pgb1* was highly increased during the early (1-6 h) and middle (12 h) stages after nitrate exposure, before declining afterward (24-48 h) (Wang et al., 2001; 2003). In the same tomato system (Wang et al., 2003), *Pgb1* was up-regulated under potassium, phosphorus or iron deprivation conditions. These observations suggest a possible involvement of Pgbs in nutrient deficiency responses although more studies are needed to elucidate their mode of action.

2.1.3.3 NO and plant-pathogen interactions

In parallel to abiotic stress defense, plants have evolved to deploy effective physiological and molecular recognition and response strategies against potential pathogens (Mysore and Ryu, 2004). For example, different pathogens are recognized by the plant cell surface-localized pathogen-recognition receptors (PRRs), which interact with pathogenesis-associated molecular

patterns (PAMPs) and pathogenesis effectors (Boller and Felix, 2009). Plants are equipped with the innate PAMPs-triggered immunity (PTI) or the inducible effectors-triggered immunity (ETI) which are the first layers of defense against pathogen infection (Ausubel, 2005; Dodds and Rathjen, 2010). Both the PTI and the ETI responses modulate hormonal homeostasis resulting in the transcriptional reprograming of defensive genes (Denancé et al., 2013a). Plant hormones participating in these responses include not only the classical "stress" hormones, such as SA, JA, ET, and ABA, but also auxin, CK and GA (Robert-Seilaniantz et al., 2011; Denancé et al., 2013b). Many of these hormones have also been implicated in the systemic acquired resistance (SAR), which leads to the expression of antimicrobial pathogenesis-related (PR) genes in un-inoculated cells/tissues/organs (Fu and Dong, 2013), and in the hypersensitive response (HR) culminating in the localized programmed cell death (PCD) at the infection site (Caplan et al., 2008).

The well characterized involvement of SA and JA/ET during plant-pathogen interactions is often effected by NO which plays the dual role of a pathogen virulence factor and a host defence component (Bellin et al., 2013; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2014). Within the SA and JA/ET pathways of the host system, NO can act either as an inducer or a suppressor in plant defense signal transductions (Pieterse et al., 2012; Mur et al., 2013). While in some systems, NO promotes localized PCD in combination with other ROS (Mur et al., 2006; Tan et al., 2013), in others NO behaves as a pro-survival factor by scavenging ROS (Beligni et al., 2002; Crawford and Guo, 2005). These diverse responses make studies on NO during plant-pathogen interactions often difficult to interpret.

By modulating NO homeostasis, Pgbs might indirectly participate in the NO-regulations described above (Hill, 2012). For example, the best known involvement of NO in PCD is

mediated by the S-nitrosylation of NADPH oxidases (Yun et al., 2011), a process which is possiblely affected by Pgbs (Igamberdiev et al., 2014). *Pgbs* are generally induced during the early phases of infection. In cotton, *Pgb1* expression increased rapidly upon challenge with the necrotrophic fungus *Verticillum dhaliae*, or in un-challenged plants treated with JA and ET (Qu et al., 2005; Zhang et al., 2012). Further investigations into this interaction (Qu et al., 2006) showed that introduction of cotton *Pgb1* into Arabidopsis plants facilitated both JA/ET and SA defense responses by increasing the expression levels of the defense genes *PDF1.2* (plant defensin 1.2) and *PR1* (pathogenesis-related 1). The latter gene was also induced in tobacco plants over-expressing alfalfa *Pgb1* (Seregélyes et al., 2004). Several pathogen inducible genes related to JA and ET responses were also up-regulated in Arabidopsis plants ectopically expressing alfalfa *Pgb1* (Maassen and Hennig, 2011). The authors speculated that the observed transcriptional regulation might have been mediated by NO.

Between the first two classes of Pgbs, class 1 seems to be more directly involved in plant-pathogen interactions. In Arabidopsis manipulations of *Pgb1* (but not *Pgb2*) levels alter the resistance to both *Pseudomonas syringae* and *Botrytis cinerea*, and these responses occur through the modulation of NO, H_2O_2, SA, and JA/ET (Mur et al., 2012b). However, other studies revealed that *Pgb1* has no effect on the NO-mediated HR triggered by *Pseudomonas syringae* (Perazzolli et al., 2004). This discrepancy reflects the complexity of the participation of Pgbs during plant-pathogen interactions.

2.1.3.4 NO and plant-rhizobium symbiosis

The interaction between legume plants and rhizobia results in the formation of root nodules, newly differentiated organs where bacteroids fix atmospheric nitrogen (N_2) by nitrogenase activity (Oldroyd and Downie, 2008). The presence of NO has been observed at different stages

of the symbiotic process, ranging from early formation steps in the developing nodules to the senescence steps in the mature nodules (Baudouin et al., 2006; Shimoda ta el., 2009; Meilhoc et al., 2011). However, like in many other processes, the role played by NO in the symbiotic N_2 fixation can be either deleterious or beneficial (Sasakura et al., 2006; Shimoda et al., 2009; Kato et al., 2010; Melo et al., 2011). This discrepancy is due to variations in the patterns of NO action, slight changes in concentration, location and timing of action (Boscari et al., 2013). On one hand, for example, over-production of NO was reported to repress the symbiotic N_2 fixation efficiency (Sasakura et al., 2006; Shimoda et al., 2009; Kato et al., 2010), either through transcriptional regulations or post translational modification (PTM) events of the nitrogenase subunits leading to a reduction in the nitrogenase activity (Sanchez et al., 2010; Xue et al., 2010; Puppo et al., 2013), or by activating early nodule senescence (Cam et al., 2012). Conversely, the energy status in the functioning nodules under normoxic or hypoxic conditions is largely dependent on the activity of nitrite reductase, which converts nitrite to NO and in turn, the latter is involved in the maintenance of cellular energy status (Horchani et al., 2011).

In this context, cellular NO homeostasis during the establishment and maintenance of the symbiosis can be modulated by Pgbs (Smagghe et al., 2009; Hill, 2012). As mentioned in previous sections, there are legHbs or symbiotic Hbs, and three other classes of Pgbs. These distinct types of Pgbs, especially legHbs, are all involved in plant-rhizobium symbiosis. For example, in spite of their variable copies existing in different plant species (Smagghe et al., 2009), legHbs are specifically expressed in the root nodules where they facilitate oxygen diffusion, and maintain a very low oxygen level to prevent the inactivation of the rhizobial nitrogenase complex that is responsible for N_2 fixation (Herold and Puppo, 2005; Smagghe et al., 2009; Gupta et al., 2011). In addition, legHbs protect the nodules from senescence during the

early stage of symbiosis, and provide defense against stress conditions such as low temperature (Sasakura et al., 2006; Cam et al., 2012). Besides legHbs, other Pgbs are also distributed in legumes where they contribute to the establishment of the symbiotic relationship (Nagata et al., 2008; Bustos-Sanmamed et al., 2011). This redundant role is possibly due to the common shared functions of all Pgbs in controlling cellular nitric oxide (NO) homeostasis (Hill, 2012; Boscari et al., 2013). For instance, gene expression studies showed that over-expression of *Pgb1* facilitates N_2 fixation in the nodules of *Lotus japonicus*, possibly by mitigating the inhibitory effect of cellular NO at a high concentration (Shimada et al., 2009; Kato et al., 2010).

2.1.4 Conclusions

As thoroughly discussed above, with their conserved sequence/structure and ligand binding properties *Pgbs* are broadly expressed in a number of cells/tissues/organs, where they play important roles in various biological processes including embryo development, seed germination and dormancy, somatic embryogenesis, and post-embryo development. Their stress-inducible expression patterns and ability to bind/scavenge NO clearly suggest that Pgbs are also key regulators in defense responses against a number of abiotic and biotic stresses.

Data available in the literature clearly suggest that Pgbs might be indirectly involved in all those processes requiring changes in NO homeostasis. Among these processes is PCD, which together with cell division and differentiation regulates plant development. As shown in the next section, PCD is crucial during embryogenesis where it shapes the body of the developing embryo.

2.2 Programmed cell death in plant embryogenesis

2.2.1 Introduction

Similarly to cell division and differentiation, PCD is a genetically controlled process required for normal growth and development in both animal and plant systems (Conradt, 2009; Olvera-Carrillo et al., 2012). The term PCD encompasses several distinct pathways unique in eukaryote systems (Kroemer et al., 2008) that lead to the selective dismantling and elimination of unwanted cells, tissues and/or organs. This finely tuned process, which "shapes" the body of organism, is controlled by endogenous factors and relies on energy-dependent events (Lockshin and Zakeri, 2004; Conradt, 2009; Olvera-Carrillo et al., 2012). In the case of plant systems, manifestation of PCD is developmentally and environmentally regulated and observable throughout the lifecycle. The best characterized examples of development-regulated PCD are apparent during xylogenesis, the maturation and death of xylem cells required for the formation of the vascular system, during reproduction, in the specific elimination of embryogenic cells or the selective killing of female primordia, and during senescence, where PCD ensures the removal of old tissue and the turnover of macromolecules (Greenberg, 1996; Pennel and Lamb, 1997; Bozhkov et al., 2005a; Olvera-Carrillo et al., 2012; Smertenko and Bozhkov, 2014).

In addition to the described developmental processes (Kuriyama and Fukuda, 2002), activation of PCD is a common component of responses to biotic and abiotic stress conditions. For instance, during flooding selective removal of cortical cells maintains a continuous supply of oxygen to the under-water organs through the formation of aerenchyma (Takahashi et al., 2014). Similarly, during plant-pathogen interactions the programmed elimination of cells limits pathogen growth and reduces the infection sites (Greenberg, 1996; Drew et al., 2000).

In animal systems the mechanisms regulating PCD have been well investigated and, based on morphological, biochemical, and molecular characteristics, three types of PCD are distinguished: apoptosis, autophagy, and necrosis (Kroemer et al., 2008; van Doorn, 2011). In spite of the early recognition of PCD that might be initiated by the autophagy mechanism (Minina et al., 2014), knowledge on the biochemical and molecular events underlying PCD in plants is scarce and classification of the death pathways is solely based on morphological criteria (Reape et al., 2008; van Doorn, 2011). The morphological classification suggests that PCD in plant cells can be categorized as necrosis or vacuolar cell death (van Doorn et al., 2011). While necrosis is generally caused by the rupture of the plasma membrane and the shrinkage of the cytoplasmic components, as often observed under abiotic stresses, vacuolar cell death is characterized by the clearance of the cytoplasmic components caused by the rupture of the tonoplast and the release of vacuolar hydrolytic enzymes. The execution of vacuolar cell death is very common during development where it is involved in organ formation (Gunawardena, 2008). However, it must be kept in mind that the categorization of cell death into these two morphological pathways, either necrosis or vacuolar cell death, is simplistic to some extent, as atypical examples of cell death may not fall into either category (van Doorn et al., 2011).

2.2.2 Basic characteristics of PCD in plants

Initially considered as an "unprogrammed" cellular event, necrosis has been recently included as an integral pathway of PCD (van Doorn, 2011). Necrosis is typically characterized by two early structural hallmarks: the increase in cellular volume and the rupture of the cytoplasm leading to the release of the intracellular content (Kroemer et al., 2008). Although relatively less understood in plant cells, necrosis in animal systems is accompanied by increases in cytosolic Ca^{2+} level and changes in mitochondrial and lysosomal function, ultimately leading

to the accumulation of ROS that act as important signaling components in PCD pathways upon different stimuli (Overmyer et al., 2003; Christofferson and Yuan, 2010). As summarized in previous studies differentiating morphological classes of cell death in plants, necrosis is typical of the hypersensitive response and cells challenged with necrotrophic pathogens (van Doorn et al., 2011).

Unlike necrosis, vacuolar cell death is better characterized and manifested by the rupture of the tonoplast and the release of the hydrolytic enzymes. Plant cells are equipped with two major types of vacuoles, storage vacuoles which preferentially accumulate proteins, and lytic vacuoles enriched with several hydrolytic enzymes including aspartate and cysteine proteases and nucleases (van Doorn, 2011). Manifestation of vacuolar cell death can be either non-disruptive, if the tonoplast fuses with the plasma membrane and releases the hydrolytic enzymes in the apoplast, or disruptive, if the collapse of the tonoplast discharges the hydrolytic enzymes within the cytoplasm (Hara-Nishimura and Hatsugai, 2010). Of these two contrasting events, disruptive vacuolar PCD has been shown to occur during lysogenous aerenchyma formation through three temporally distinct steps. The first step is characterized by the swelling of the lytic vacuole which occupies most of the symplast. The second step is initiated by the invagination of the tonoplast which engulphs and degrades cytoplasmic regions through processes analogous to autophagy of animal cells (Kundu and Thompson, 2005). The third and final step is characterized by the lysis of the tonoplast and the release of the hydrolytic enzymes, which clear cytoplasmic components starting from the endoplasmic reticulum (ER) and terminating with the nucleus and mitochondria (van Doorn, 2011). However, there is evidence deviating from this sequence, such as the early disruption of the cell wall preceding the rupture of the vacuole (Webb and Jackson, 1986).

The most characteristic cytological events occurring during PCD are DNA degradation, chromatin condensation and nuclear fragmentation (Arends et al., 1990), which compromise the ability of the DNA to transcribe and replicate. The degradation of DNA is executed by nucleases and occurs through two distinct sequential phases, including the initial cleavage of the DNA at the interloop sites of chromatin DNA generating fragments of about 50-300 kbp, and the cleavage at the internucleosomal sites of chromatin DNA producing 200 bp-sized DNA fragments (Peitsch et al., 1993). These events occur in conjunction with the condensation of chromatin, which requires de-polymerization of F-actin (Widłak and Garrard, 2005), and the fragmentation of the nucleus which is very typical of apoptotic PCD in animals (Eleftheriou, 1986). While nuclear fragmentation is generally one of the last events of PCD, it was identified as the first sign of aerenchyma formation (Drew et al., 2000; Schussler and Longstreth, 2000). This discrepancy shows that PCD can occur through different and often unpredictable sequences of events.

2.2.3 Functional roles of PCD in plant embryogenesis

Embryogenesis is one of the most important developmental processes during the lifecycle of plants (Lau et al., 2012). The zygote, originating from a single fertilization event in gymnosperms and a double fertilization event in angiosperms undergoes a precise pattern of cell divisions culminating in the formation of a fully developed embryo. The subsequent imposition of a maturation period, in which the seed experiences water stress, is required for the termination of the developmental program and the initiation of germination (Kermode, 1990). On the other hand, recapitulation of embryogenesis can also be achieved in culture through judicious manipulations of culture media and environment. In this context, two methods routinely used to generate *in vitro* embryos are somatic and gametophytic embryogenesis (Thorpe and Stasolla,

2001). The former method is employed to generate *in vitro* embryos from cells other than gametes, such as somatic cells/tissues/organs, while the latter uses male or female gamethophytes as explants to produce *in vitro* embryos. For instance, androgenesis consists in the utilization of male gametophytes to produce embryos, by exploiting the ability to re-route the developmental fate of immature pollens such as microspores from a gametophytic to an embryogenic pathway (Touraev et al., 2001). Both somatic and gametophytic embryogenic systems are widely used as model systems to investigate biochemical and molecular events accounting for the initiation and development of plant embryos. Similar to cell division and cell differentiation, expression of PCD is an integral component of embryonic development both *in vivo* and *in vitro* as it is essential for the establishment of the embryo body through elimination of specific cells, tissues and/or organs (Bozhkov et al., 2002).

2.2.3.1 Role of PCD during *in vivo* plant embryogenesis

Manifestation of programmed cell death (PCD) is important as it participates in different phases of *in vivo* embryogenesis in various plant systems. For example, PCD can be observed in several phases of embryogenesis, such as during the dismantling of the suspensor, removal of supernumerary embryos produced by polyembryonic seeds, and degradation of nucellus, endosperm and aleurone cell layer (Sreenivasulu and Wobus, 2013). It must be mentioned that, dismantling of the suspensor and removal of supernumerary embryos produced by polyembryonic seeds are the most typical examples of PCD intimately related to the formation and maturation of plant embryos. These two events will be discussed in the following sections.

2.2.3.1.1 Elimination of the suspensor

The suspensor consists of a population of terminally differentiated cells connecting the embryo proper to the surrounding tissues during early seed development (Kawashima and

Goldberg, 2010). Formation and specification of the suspensor is concomitant to that of the embryo proper. In angiosperms, the first asymmetric division of the zygote originates an apical cell and a sub-apical cell. Later, the apical cell gives rise to the embryo proper, which will go through a series of developmental stages defined as globular, heart, cotyledon, and torpedo stages. Meanwhile, through transverse cell divisions the sub-apical cell gives rise to the suspensor (Raghavan, 2001). In addition to the most primitive function of anchoring the embryo to the seed, the suspensor transfers nutrients to the embryo proper and it participates in the establishment of the polar-basal embryonic axis by modulating the flow of auxin (Friml et al., 2003). The suspensor is short-lived and after serving its functions it is dismantled through massive PCD. Elimination of the suspensor usually occurs at, or right after the cotyledon stage of embryo development (Bozhkov et al., 2005a; Kawashima and Goldberg, 2010). In all cases examined, PCD is required for the elimination of the suspensor regardless of the shape and morphology which may differ remarkably among plant species. For example, the suspensor in orchids is single-celled, the suspensor in Arabidopsis is composed of about seven cells and the suspensor in runner bean has over two hundred cells (Kawashima and Goldberg, 2010). Such variations in the number of suspensor cells are also observed within the same family (Lersten, 1983). In addition, profound morphological differences of the suspensor are also apparent between angiosperms and gymnosperms. The suspensor in angiosperms is generally composed of a file of single cells characterized by two regions, the neck including suspensor cells adjacent to the embryo proper and the knob comprising suspensor cells in close proximity to the seed integuments (Lombardi et al., 2007). More complex morphological arrangements are observed in gymnosperms, such as *Picea abies*, where the suspensor consists of defined tiers of cells with the upper tier “embryonal tube cells” produced by the asymmetric division within the embryo proper

(Bozhkov et al., 2005b). Independent evidence suggests that elimination of the suspensor by PCD progresses basipetally, starting from the top suspensor cells adjacent to the embryo proper and terminating to the bottom portion of the suspensor. For instance, in *Phaseolus coccineus* the execution of PCD occurs in a basipetal fashion and DNA fragmentation is first visible in the neck region of the suspensor before proceeding to the knob region (Lombardi et al., 2007). This unique execution of PCD is also evident in other species, including maize and spruce (Giuliani et al., 2002; Bozhkov et al., 2005b) and two scenarios have been proposed to account for this conserved pattern (Bozhkov et al., 2005b). The first involves the presence of a "pro-death signal" produced by the embryo proper which is released basipetally towards the suspensor cells, while the second may require the depletion of an "anti-death factor" in the late stage of suspensor termination which is first produced by cells occupying the base of the suspensor. However, the generation and analysis of mutants is necessary to resolve the nature of PCD progression in the suspensor.

An intriguing question arising from the progressive spreading of PCD is whether the suspensor cells are universally committed to die only after they are fully differentiated. Arabidopsis suspensor cells are targeted by PCD only after the suspensor is fully formed, thus suggesting that death occurs in terminally differentiated cells. This notion is also substantiated by analyses of the *tween* mutant embryos. In these mutants, the suspensor cells can re-differentiate into embryogenic cells and this capability is retained only up to the globular stage, after which the suspensor cells become fully differentiated and committed to die (Vernon and Meinke, 1994). In spruce, however, the death program is initiated in newly formed suspensor cells which are not terminally differentiated (Bozhkov et al., 2005b). These suspensor cells are added from asymmetric divisions of the embryo proper and the death program is activated soon after they are

formed. Also, elimination of the suspensor is a slow process as suspensor cells are not subjected to rapid disruption. This is possibly required for the proper differentiation of the embryo, given the function of the suspensor in transporting nutrients (Bozhkov et al., 2005a). Time-course analysis of zygotic and somatic embryogenesis in gymnosperms (Hakansson, 1956; Filonova et al., 2000a) suggests that the death and removal of a suspensor cell occurs in a period of at least five days (Bozhkov et al., 2005b). The information above argues strongly for the involvement of PCD in the elimination of the suspensor and it provides a picture on the timing and progression of PCD. However, variations in the execution of PCD are not uncommon. Different patterns of PCD timing and progression in suspensor cells of different species exist and, in the case of the *Leguminosae* family, have been categorized (Edo, 2012).

2.2.3.1.2 Survival of a single dominant embryo

In embryology, the phenomenon in which multiple embryos are derived from only one zygote is referred to as monozygotic polyembryony, a common developmental event observed in animals (Craig et al., 1997). This process, the genetic bases of which are unknown, requires the physical splitting of embryonic cells after a few rounds of mitotic divisions leading to the formation of two or more genetically identical embryos. In plants, however, the process of embryogenesis is more flexible. For example, the formation of a supernumerary embryo from double fertilization can be found in angiosperms (Vernon and Meinke, 1994), while monozygotic polyembryony is particularly common in gymnosperms where several embryos are produced in one seed, but only a “dominant” one continues the completion of the developmental process. The remaining “subordinate” embryos are eliminated by PCD (Singh, 1978). This intrinsic regulation is well documented in spruce where it is executed in three distinct phases (Filonova et al., 2002). The first phase is characterized by the formation of multiple embryos from the same zygote.

These embryos share the same growth rate without any dominance. Acquisition of the "dominant" characteristics in one of the embryos, which overgrows the subordinate embryos, demarks the second phase. In the third phase, the subordinate embryos are eliminated by PCD, while the dominant embryo completes the maturation process. Manifestation of PCD in the subordinate embryos follows a specific pattern (Bozhkov et al., 2005b). By using terminal deoxynucleotydil transferase mediated dUTP nick end labeling (TUNEL) to detect DNA degradation in the subordinate embryos, it was shown that the initiation of PCD starts from the basal part of the embryo proper and progresses acropetally reaching the apical cells in approximately four weeks (Filonova et al., 2002). This pattern might be established by the presence of a death signal expanding acropetally, or a survival signal which accumulates preferentially in the apical region of the embryo (Bozhkov et al., 2005b). The nature and origin of the signal are unknown, but the signal might be produced by the megagametophyte, where PCD is observed prior to the autodestruction of the embryonic cells (Filonova et al., 2002).

Regardless of the initial stimulus propagating the death signal, orchestration of the death program in megagametophytic and embryonic cells is required for normal seed development (Bozhkov et al., 2005a). As in any other developmental process governed by PCD, the response to the death program can be quite flexible and environmentally influenced, as demonstrated by the presence of more than one dominant embryo capable of completing the entire developmental processes (Krutovskii and Politov, 1995).

2.2.3.2 Role of PCD during *in vitro* plant embryogenesis

In addition to embryo development *in vivo*, recapitulation of the embryogenic process can also occur *in vitro* through somatic embryogenesis and androgenesis (also referred to as

microspore-derived embryogenesis). In both processes, removal of cells through PCD is a crucial event required for the proper development of the embryos (Smertenko and Bozhkov, 2014).

2.2.3.2.1 PCD during somatic embryogenesis

The process of somatic embryogenesis relies on a number of nutritional or environmental factors (Thorpe and Stasolla, 2001), with PCD having a central role (Smertenko and Bozhkov, 2014). Manipulations in hormone supplementations and cell density have been shown to induce the death program in several somatic embryogenic systems (McCabe et al., 1997; Yan et al., 1999; Zhou et al., 1999). The relevance of PCD for the successful formation of somatic embryos was first experimentally proven in spruce (Filonova et al., 2000b; Filonova et al., 2002), where the embryogenic pathway has been well characterized (Filonova et al., 2002). Generated from cultured zygotic embryos, the pro-embryogenic masses (PEMs) in this system are maintained in a media supplemented with the plant hormones auxin and cytokinin. The pro-embryogenic masses (PEMs) consist of three defined cellular aggregates (PEM I-III). The PEM I stage is composed of clusters of highly cytoplasmic cells subtended by a single suspensor-like cell. Addition of other suspensor cells to PEM I forms PEM II. As more cells are added to the PEM II the cluster grows in size and further differentiates into PEM III. In the presence of plant hormones, the three PEMs co-exist without forming embryos. Removal of plant hormones from the culture medium stimulates the trans-differentiation of PEM III into somatic embryos, and this transition requires PCD (Filonova et al., 2000b). The massive execution of the death program in the PEM III re-shapes the cell cluster and allows the formation/development of somatic embryos. Independent evidence suggests that PCD is obligatory for proper and successful embryogenesis. In addition to the positive correlation observed between the extent of PCD in the PEM III and the number of somatic embryos produced (Filonova et al., 2000b), inhibition of embryogenic PCD

through manipulations of the culture medium compromises the normal differentiation of the PEM III cell clusters into somatic embryos (Bozhkov et al., 2002). Consistent with the requirement of the death program, lines composed by PCD-deficient PEMs are unable to produce embryos (van Zyl et al., 2003), possibly because of their inability to reprogram their transcriptional machinery (Stasolla et al., 2004). These results are analogous to developmental studies in Drosophila showing that blockage of PCD by mutagenesis results in prenatal death (White et al., 1994). Furthermore, the ablation of PEM III cells by the death program is followed by a second wave of PCD which ultimately removes the suspensor of the somatic embryos during the late phases of embryogenesis (Filonova et al., 2000b). During this second wave, PCD follows a basipetal pattern, as described in the previous section, starting from the suspensor cells adjacent to the embryo proper and proceeding towards the basal region of the suspensor (Smertenko et al., 2003).

2.2.3.2.2 PCD during microspore-derived embryogenesis

Unlike somatic embryogenesis, microspore-derived embryogenesis relies on the ability of immature microspores to redirect their normal gametophytic developmental pathway toward a sporophytic route. This cell reprogramming can be triggered in culture by imposition of diverse stress treatments including heat shock, starvation, low temperature conditions, ethanol and gamma irradiation (Touraev et al., 1997; Seguí-Simarro and Nuez, 2008). The fulfillment of this embryogenic process requires two developmental phases, the formation of multicellular structures (MCSs) within the exine wall of the isolated microspores upon inductive stimuli, and the differentiation of MCSs into embryo-like structures (ELSs) (Touraev et al., 2001). The formation of MCSs can be achieved through three different pathways. In the first pathway, the

microspore nucleus divides asymmetrically forming a generative and a vegetative cell, and further divisions of the vegetative cells give rise to MCSs (Sunderland, 1974). In the second pathway, as commonly observed in rapeseed, potato and tobacco, MCSs are formed directly from symmetric divisions of the microspore nucleus. The last pathway is characterized by the simultaneous divisions of the vegetative and generative cells, both contributing to the formation of MCSs (Raghavan, 1986).

Other than these cell division and differentiation events, manifestation of PCD is an integral component of microspore-derived embryogenesis, especially in the early phases as most of the anther tissue enclising microspores undergo massive death. For example, the degeneration of cells by PCD is first apparent in the tapetum of the anthers at the pre-meiosis stage (Varnier et al., 2005). This first wave of the death program, which contributes to the overall elimination of the tapetal cell layers, has an effect on the early microspores soon after meiosis (Varnier et al., 2009). It has been suggested that the death message might migrate from the dying tapetum to the microspores and trigger their degradation (Caredda et al., 2000), thus compromising their redirection towards the embryogenic pathway. Therefore, the ability to control and manipulate the execution of PCD in the early microspores is crucial for ensuring a high frequency of embryo recovery.

One of the key factors inducing microspore-derived embryogenesis is the imposition of a stress pre-treatment to the microspores, which is required for redirecting their fate from a gametophytic to an embryogenic program. The observation that in some systems this stress treatment does not cause PCD, but rather induces PCD inhibitors, such as the bax inhibitor BI-1, suggests that the arrest of the death pathway in the microspores is a necessary prerequisite for redirecting their fate towards embryogenesis has been made (Varnier et al., 2009). What is

contrasting is the role of PCD after this developmental redirection has occurred. While in some systems microspores undergo a symmetric division without the appearance of PCD (Joosen et al., 2007; Malik et al., 2007), in others, including barley, microspores previously subjected to a stress treatment exhibited an increased level of mortality during the subsequent stages (Rodriguez-Serrano et al., 2012). These discrepancies in PCD occurrence are possibly due to different plant systems and stress conditions utilized.

In relation to the developmental phase, the second wave of PCD during microspore-derived embryogenesis occurs during the differentiation of MCSs into ELSs. In barley, the development of mannitol-stressed microspores into haploid embryos is characterized by the formation of MCSs composed by two distinct cell domains derived from proliferation of the vegetative cell and the generative cell, respectively. These two domains have distinct developmental fates, that is, the generative domain is eliminated by PCD, while the vegetative domain develops into ELSs (Maraschin et al., 2005). With regard to the developmental role of PCD, the elimination of the generative domain marks the site of exine rupture from where the globular embryos will emerge.

The above studies suggest that PCD plays an integral and important role not only during the redirection of the microspores from a gametophytic to an embryogenic pathway, but also during the early morphogenetic events required for proper embryo development. Thus, the capacity to experimentally manipulate the death program during both processes would provide valuable insights into the requirement of PCD for microspore-derived embryogenesis.

2.2.4 Key regulators of PCD in plant embryogenesis

The large number of components involved in the death program makes PCD a difficult event to study. However, the fact that common PCD hallmarks are shared across species is a

premise that initial investigations on plant PCD should be focusing on analogous components triggering PCD in animals. The following section will discuss the role of some specific proteins and signaling molecules during plant embryogenesis.

2.2.4.1 Bax inhibitor 1

Apoptosis in animal systems is mainly orchestrated by the Bcl-2 protein family, which includes pro-survival and pro-apoptotic members (Czabotar et al., 2014). Of these, the pro-apoptotic members, such as those of the Bax subfamily, trigger cell death events through the release of cytochrome C from the mitochondria. On the other hand, the pro-survival components such as Bax inhibitor 1 (BI-1) abrogate these events (Watanabe and Lam, 2004). Initially characterized in humans for its ability to repress the yeast death pathway activated by the over-expression of the mouse Bax gene (Xu and Reed, 1998), BI-1 has been isolated in many species of yeasts, plants and animals where it is expressed under stress conditions and in senescent tissues (Coupe et al., 2004; Lam, 2004). The pro-survival nature of this protein was also defined in plants through genetic transformation studies. Cell death induced by pathogens, fungal elicitors, temperature stress and hydrogen peroxide (H_2O_2) was repressed in Arabidopsis plants ectopically expressing BI-1 (Kawai-Yamada et al., 2004). On the contrary, a down-regulation of BI-1 accelerated the death program in carbon starved tobacco cells (Bolduc and Brisson, 2002).

In addition to the regulation of PCD induced by these conditions, the role of BI-1 had not been investigated during embryogenesis until independent studies showed a transcriptional activation of the barley *BI-1* following stress treatments that induce embryogenesis possibly through the suppression of the PCD pathway (Maraschin et al., 2006). Localization studies together with analysis of structural and/or functional domains suggest that BI-1 proteins reside in the endoplasmic reticulum (ER) membranes where they play a protective role against the ER-

stress induced PCD, a condition where basic ER functions are compromised (Csala et al., 2006). This cytoprotective role of BI-1 is mediated by its ability to modulate Ca^{2+} homeostasis and response in the ER by interacting with several calcium-binding proteins (Ihara-Ohori et al., 2007).

As indicated above, the pro-survival role of BI-1 proteins is to counteract the pro-death effect of other factors, including kiss of death (KOD), a small amino acid peptide which triggers PCD in Arabidopsis (Blanvillain et al., 2011). Using two mutant *kod* alleles and *KOD* over-expressing lines, the authors demonstrated the involvement of KOD in the elimination of the suspensor cells during embryogenesis and its participatory executions in early PCD events including the depolarization of mitochondrial membrane (Blanvillain et al., 2011). The ability to trigger the suicide program by the sole expression of *KOD* makes this gene a suitable tool to target and dismantle cells by PCD in other species.

2.2.4.2 Metacaspases

The activation of apoptosis in animal cells is triggered by caspases, a family of endoproteases that are able to cleave proteins at specific amino acid residues (McIlwain et al., 2013). Expressed as inactive pro-enzymes, caspases are activated at the onset of the death program where they initiate an irreversible proteolytic cascade of events involving the induction of other caspases and culminating in rapid cell death (Elmore, 2007). These caspases are broadly divided into three distinct types, acting as initiators (caspase 2, 8, 9, and 10), executioners (caspase 3, 6, and 7), and inflammatory caspases (caspase 1, 4, and 5), based on their expression and functional roles along this cascade (Cohen, 1997; Rai et al., 2005).

In plants, however, direct homologues of caspases are not found. Instead, proteins with similar functions have been identified as metacaspases (MCs), characterized by caspase-like secondary structures and catalytic domains (Uren et al., 2000; Tsiatsiani et al., 2011). Involvement of metacaspases in PCD has been demonstrated in yeast, where the survival/death fate is dependent upon metacaspase expression (Madeo et al., 2002), and more recently in plant embryos (Suarez et al., 2004). This latter study showed that a spruce (*Picea abies*) metacaspase (mcII-Pa) was expressed during spruce somatic embryogenesis in PCD-committed tissues such as the suspensor cells of immature embryos and procambium of late embryos, and that RNAi-mediated suppression of *mcII-Pa* prevented the differentiation of somatic embryos from PEM III clusters by repressing the death program. Besides establishing metacaspases and mcII-Pa specifically, as executioners of PCD in plant embryogenesis, this work emphasized the critical link between the death program and the formation of embryos in culture. Moreover, the cellular function exercised by mcII-Pa requires its cysteine-dependent arginine-specific proteolytic activity and its ability to migrate from the cytoplasm into the nucleus to induce the fragmentation of DNA and the disassembly of the nuclear envelope in cells committed to die (Bozhkov et al., 2005b).

2.2.4.3 Nitric oxide

Nitric oxide (NO) is a signal molecule fundamental for a broad range of plant developmental and environmental responses, including hormone signaling, cell cycle mechanisms, and biotic and abiotic stress responses (Mur et al., 2012a; Freschi, 2013; Yu et al., 2014). Over the past years the role of NO acting as modulator of PCD has emerged and its participation in the embryogenic process has received increasing attention from researchers. In animal systems, the pro-apoptotic role of NO occurs through several mechanisms. For example,

being able to induce p53 and CD95, two important caspase activators in animals (Brune, 2003), NO influences the death program by post translational modifications (PTMs) such as protein nitrosation/nitrosylation, and by modulating the level of cellular cGMP (cyclic guanosine monophosphate) (Hill et al., 2013). This role is retained in plant-pathogen interactions, where protein nitrosylation via reaction with NO regulates the activity of many stress response enzymes, including metacaspase 9 (Belenghi et al., 2007). In addition, NO also influences the pool of cGMP by binding to the ferrous heme group of the guanylate cyclase-coupled receptor converting GTP (guanosine-5'-triphosphate) to cGMP, an effector of apoptosis (Blaise et al., 2005). In plants, administration of NO increases PCD through an elevation of cGMP which opens Ca^{2+} channels through intermediates including cADPR (cyclic adenosine diphosphate-ribose) (Besson-Bard et al., 2008). A spike in cellular Ca^{2+} increases mitochondria permeability and triggers the death program (Wang et al., 2010b). Of note, applications of 8-Br-cGMP, an analogue of cGMP, suppresses caspase-like activity and PCD in Arabidopsis (Clarke et al., 2000). Although these regulatory mechanisms not having been demonstrated in embryogenesis, the NO-mediated activation of caspase-like activity and PCD has been recently shown to occur during the early phases of microspore-derived embryogenesis in barley (Rodriguez-Serrano et al., 2012). Independent studies showed that the involvement of NO signaling in PCD regulation during embryogenesis is also mediated by intracellular Zn^{2+} levels (Helmersson et al., 2008) that are released from metallothionins through the destruction of the zinc-sulphur clusters (Aravindakumar et al., 1999).

In addition to these versatile regulatory roles played in PCD, cellular NO homeostasis is critical for plant growth and development (Wang et al., 2013). The uniqueness of Pgbs in binding and scavenging NO places them at the center of developmental processes (Hill, 2012; Hill et al.,

2013). In parallel to studies in animal systems showing the ability of Pgbs to influence the developmental programs by modulating NO, the involvement of Pgbs in development/defense processes is largely expanded (Hill, 2012; Hebelstrup et al., 2012; Mur et al., 2012a), including the participatory regulation of somatic embryogenesis in Arabidopsis (Elhiti et al., 2013).

2.2.5 Conclusions

Removal of damaged/unwanted/non-functional plant cells by PCD is an important factor for embryonic and post-embryonic development. During *in vivo* embryogenesis, activation of the death program is required for the elimination of the suspensor, once the function of this organ is not needed, and for the selective termination of supernumerary embryos in early polyembryonic seeds. Recent advances on the role of PCD during *in vitro* embryogenesis have identified the death program as an obligatory event in the early phases of embryo formation. Untill now, the ability to alter the embryonic death program with molecular and pharmacological approaches has been pivotal in the identification of important regulators of the death pathway, while more information is required to identify the early inductive signals triggering death. Specific attention should be given to the initial steps of the death commitment, the reversibility of the commitment process, and most importantly the identification of potential cues such as positional and/or timing patterns responsible for selective death programs executed within the cultured tissue. Answers to these questions may open new avenues for targeted applications and manipulations of PCD to enhance embryo quality and yield.

3 CHAPTER ONE: PHYTOGLOBIN CONTROL OF CELL SURVIVAL/DEATH DECISION REGULATES IN VITRO PLANT EMBRYOGENESIS*

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S.H. performed the majority of the experiments and contributed to data analysis and writing the manuscript. S.K.J. isolated the maize Pgbs and prepared the constructs for the antisense lines. G.D. performed the nitric oxide-metallothionein experiment. O.S.W. prepared the KOD constructs, assisted in the preparation of the PZmPgb1.1:GUS and PZmPgb1.2:GUS constructs, and contributed to the design of experiments related to PCD. B.T.A. contributed to the design of experiments related to PCD and interpretation of results. C.S. and R.D.H. assisted in the design of experiments, interpretation of data and writing of the manuscript.

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3.1 Abstract

Programmed cell death (PCD) in multicellular organisms is a vital process in growth, development, and stress responses that contributes to the formation of tissues and organs. Although numerous studies have defined the molecular participants in apoptotic and PCD cascades, successful identification of early master regulators that target specific cells to live or die is limited. Using *Zea mays* somatic embryogenesis as a model system, we report that the expressions of two phytoglobin genes (*ZmPgb1.1* and *ZmPgb1.2*) regulate the cell survival/death decision that influences somatic embryogenesis through their cell-specific localization patterns. Suppression of either of the two *ZmPgbs* is sufficient to induce PCD through a pathway initiated by elevated NO and Zn^{2+} levels and mediated by production of reactive oxygen species. The effect of the death program on the fate of the developing embryos is dependent on the localization patterns of the two *ZmPgbs*. During somatic embryogenesis, *ZmPgb1.2* transcripts are restricted to a few cells anchoring the embryos to the subtending embryogenic tissue, whereas *ZmPgb1.1* transcripts extend to several embryonic domains. Suppression of *ZmPgb1.2* induces PCD in the anchoring cells, allowing the embryos to develop further, whereas suppression of *ZmPgb1.1* results in massive PCD, leading to abortion. We conclude that regulation of the expression of these *ZmPgbs* has the capability to determine the developmental fate of the embryogenic tissue during somatic embryogenesis through their effect on PCD. This unique regulation might have implications for development and differentiation in other species.

3.2 Introduction

Selective elimination of overproduced, superfluous, or damaged cells by programmed cell death (PCD) is a common feature of cellular homeostasis. In conjunction with cell division and differentiation, spatial and temporal execution of the death program helps develop the

organism's shape and sculpts its final form. Necrosis, autophagy, and apoptosis are the recognized forms of PCD in vertebrates (for review, see Portt et al., 2011), whereas necrosis and autophagy are the identifiable forms of PCD in plants (for review, see van Doorn et al., 2011). Common features of PCD are loss of osmoregulation in the cell, activation of caspases, nuclear and DNA fragmentation, generation of lysosomal enzymes, and mitochondrial swelling with the release of cytochrome C (for review, see van Doorn et al., 2011). Autophagy differs between vertebrates and plants in that autophagosomes are produced in the former, whereas lytic vacuoles are a common feature in plant cells undergoing PCD. Variations and flexibility in the execution of these events and presence of crosstalk among the different death pathways (Whelan et al., 2010) attest to the complexity of the death program. This complexity is further aggravated by the ambiguous involvement of several signal molecules, including Zn^{2+}, in the cell death/survival decision. The participation of Zn^{2+} in cell death is dichotomous because proapoptotic and antiapoptotic effects have been shown in different systems. Although Zn^{2+} deficiencies are associated with apoptosis in some systems, including human Burkitt lymphoma B cells (Truong-Tran et al., 2000), elevated Zn^{2+} levels induce caspase activity in others (Schrantz et al., 2001; Helmersson et al., 2008).

As with vertebrates, plant PCD is an integral component of growth and development, which shapes organs through selective and controlled elimination of cells. Execution of PCD has been reported during different phases of the plant life cycle. It establishes polarity in immature embryos, it eliminates suspensor cells in mature embryos, it contributes to the formation of vascular tissue throughout development, and it is involved in senescence-related processes (Reape et al., 2008). Execution of the death pathway is also a key component of abiotic (aerenchyma formation during hypoxia) (Evans, 2003) and incompatible pathogen infection

(Pennell and Lamb, 1997). Although a great deal is now known about the molecular and biochemical events regulating the death pathways in vertebrates and plants, information on the mechanisms by which specific cells are targeted to either live or die is scarce.

Hemoglobins (Hbs) are ubiquitous heme-containing proteins found in all nucleated organisms (for review, see Weber and Vinogradov, 2001). Depending on organisms and tissues, they may act as oxygen carriers or stores or bind other small ligands, such as CO_2, CO, or NO. In addition to the first found legHbs or symbiotic Hbs, there are other distinct types of phytoglobins (Pgbs) identified in plants. LegHbs are generally restricted to root nodules of nitrogen-fixing plants, whereas the other Pgbs are found in shoots and roots of both monocots and dicots (Hill, 2012). From a phylogenic perspective, these Pgbs are categorized into three classes (classes 1–3; Hunt et al., 2001). Classes 1 and 2 Pgbs share a three-on-three α-helical loop structure surrounding the heme moiety, whereas class 3 Pgbs possess a two-on-two α-helical structure, similar to truncated bacterial globins. NO scavenging is one of the major functions of Pgbs, and evidence of their action in this fashion has been shown in plant development and in response to both biotic and abiotic stress (for review, see Hill, 2012).

Although expressed during embryo development (Smagghe et al., 2007), the majority of information related to the function of Pgbs has been obtained during postembryonic growth. Embedded in the maternal tissue and difficult to dissect, plant embryos are not readily amenable to physiological and molecular studies. An alternative model to *in vivo* embryogenesis is somatic embryogenesis, the production of embryos from somatic cells. Other than elucidating key molecular mechanisms governing embryogenesis (Thorpe and Stasolla, 2001), this system has been used to show the requirement of PCD for proper embryo development. In spruce (*Picea abies*), the activation of PCD is an obligatory step for the formation of somatic embryos, and

experimental suppression of the death program compromises embryogenesis (for review, see Bozhkov et al., 2005a).

Using maize (*Zea mays*) somatic embryogenesis as a model system, the relationship between the expression of two maize Pgbs (*ZmPgb1.1* and *ZmPgb1.2*), NO, and PCD has been examined during the formation of somatic embryos. The two ZmPgbs are shown to regulate the death program through similar mechanisms that interfere with the cascade of events mediated by NO and Zn^{2+}, the mitogen-activated protein kinase (MAPK) cascade, and accumulation of reactive oxygen species (ROS) that lead to PCD. This regulation, which influences the embryogenic pathway in a fashion consistent with the localization patterns of *ZmPgb1.1* and *ZmPgb1.2*, identifies Pgbs as unique key regulators of the survival/death decision.

3.3 Results

3.3.1 Suppression of ZmPgbs affects somatic embryogenesis

In maize, production of *in vitro* embryos through somatic embryogenesis is achieved through three distinct culture steps (Garrocho-Villegas et al., 2012; Fig. 3.1A). Embryogenic tissue grown on solid maintenance medium was transferred into 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 d, 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. Pharmacological treatments were applied during 7 d in P medium (Fig. 3.1A). For the purpose of this study, immature embryos were harvested from P medium at d 7, whereas mature embryos were collected from the D medium at d 21 (Fig. 3.1A).

Expression and localization of two maize Pgb genes *ZmPgb1.1* (Accession number: AF236080) and *ZmPgb1.2* (Accession number: DQ171946) (Smagghe et al., 2009; Fig. S3.1), were examined during different phases of the embryogenic process. The expression of both genes gradually increased in the P medium, with a sharp increase occurring between d 1 and 7 in the D medium (Fig. 3.1B). To verify whether this increase was because of the removal of auxin in the D medium or other differences in chemical composition between the P and D media, the expression of the *ZmPgbs* genes was also measured in a D medium devoid of (−) or enriched with (+) auxin. The transcript levels of both *ZmPgb1.1* and *ZmPgb1.2* increased in the D medium devoid of auxin (Fig. S3.2).

In immature embryos (d 7 in P medium; Fig. 1A), a different localization pattern was observed for the two *ZmPgbs* (Fig. 3.1C). Unlike *ZmPgb1.2*, with expression that was restricted to a cluster of basal cells anchoring the embryos to the subtending embryogenic tissue (Fig. 3.1C, 2 and 5) or other immature embryos (Fig. 1C, 3), *ZmPgb1.1* transcripts were detected in extended domains of the embryos (Fig. 1C, 1 and 4).

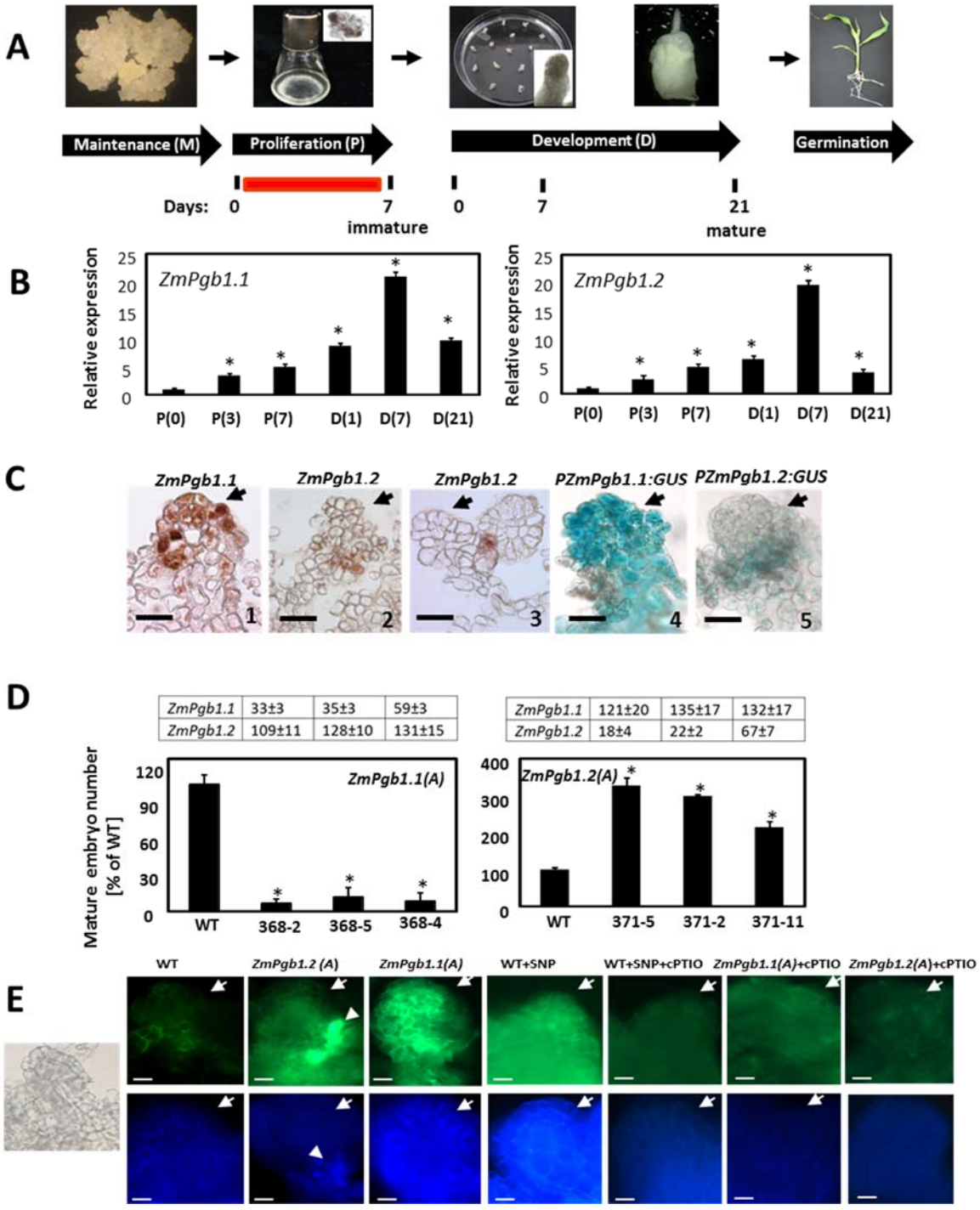

A
Maintenance (M)
Proliferation (P)
Development (D)
Germination
Days: 0 7 0 7 21
immature
mature
B
ZmPgb1.1
ZmPgb1.2
Relative expression
P(0) P(3) P(7) D(1) D(7) D(21)
C
ZmPgb1.1
ZmPgb1.2
ZmPgb1.2
PZmPgb1.1:GUS
PZmPgb1.2:GUS
1 2 3 4 5
D
ZmPgb1.1 33±3 35±3 59±3
ZmPgb1.2 109±11 128±10 131±15
ZmPgb1.1 121±20 135±17 132±17
ZmPgb1.2 18±4 22±2 67±7
Mature embryo number [% of WT]
ZmPgb1.1(A)
ZmPgb1.2(A)
WT 368-2 368-5 368-4
WT 371-5 371-2 371-11
E
WT
ZmPgb1.2 (A)
ZmPgb1.1(A)
WT+SNP
WT+SNP+cPTIO
ZmPgb1.1(A)+cPTIO
ZmPgb1.2(A)+cPTIO

Fig. 3.1 Pgb regulation of maize somatic embryogenesis. A, Schematic representation of the maize somatic embryogenic system comprising maintenance (M), proliferation (P), development (D), and germination (G) phases. Representative stages of embryo development and days in culture are also depicted. The red line indicates the timing of pharmacological treatments, which were all applied between d 0 and 7 in P medium. B, Expression level by quantitative RT-PCR of *ZmPgb1.1* and *ZmPgb1.2* during the P and D phases of embryogenesis. Values are means ± SE of three independent biological replicates and normalized to the value of P(0) set at 1. Numbers in parentheses represent days in the respective phase. *, Statistically significant difference ($P \leqslant 0.05$) from P(0). C, Localization of *ZmPgb1.1* (1 and 4) and *ZmPgb1.2* (2, 3, and 5) in immature embryos (d 7, P medium) using RNA in situ hybridization (1–3) and *PZmPgb1:GUS* and *PZmPgb2:GUS* reporter lines (4 and 5). Arrows indicate the apical domain of the developing embryos. Bars = 20 μm. D, Percentage of mature (d 21, D medium) somatic embryos obtained from antisense lines suppressing *ZmPgb1.1* [*ZmPgb1.1(A)*] or *ZmPgb1.2* [*ZmPgb1.2(A)*]. Values (wild type set at 100%) are means ± SE of four independent biological replicates. *, Statistically significant difference ($P \leqslant 0.05$) from wild type. Numbers in charts indicate the expression levels of *ZmPgb1.1* or *ZmPgb1.2* in the transgenic lines compared with the wild-type value set at 100%. E, Localization of NO (top) and Zn^{2+} (bottom) in immature embryos (d 7, P medium). Arrows indicate the apical domain of the embryos, whereas arrowheads indicate the preferential accumulation of NO and Zn^{2+} in a few cells located at the base of the embryos down-regulating *ZmPgb1.2*. Left, The micrograph shows the representative development of embryos used for the localization studies. Endogenous NO level was modulated by applications of SNP and/or cPTIO. WT, Wild type. Bars = 20 μm.

The functional role of *ZmPgb1.1* and *ZmPgb1.2* in the embryogenic process was further analyzed through antisense-mediated suppression studies. Competence to produce mature somatic embryos (d 21 on D medium; Fig. 3.1A) was highly repressed in lines down-regulating *ZmHb1.1* [*ZmPgb1.1(A)* lines] and induced in lines down-regulating *ZmPgb1.2* [*ZmPgb1.2(A)* lines; Fig. 3.1D]. These embryogenic differences were solely ascribed to a repression of the respective *ZmPgb* gene, because the antisense suppression of one *ZmPgb* gene did not significantly alter the expression of the other (charts in Fig. 3.1D). Additional studies were performed using the 368-2 *ZmPgb1.1(A)* line and the 371-5 *ZmPgb1.2(A)* line, because they showed the greatest differences in embryo number and the most pronounced repression of the respective *ZmHb*.

3.3.2 ZmPgbs regulate intracellular Zn^{2+} through the NO-dependent metal release from metallothioneins

The documented ability of Pgbs to scavenge NO (Weber and Vinogradov, 2001) was retained by *ZmPgb1.1* and *ZmPgb1.2* as revealed by the fluorescent signal of 4,5-diaminofluorescein diacetate (DAF-2DA), which is routinely used to detect cellular NO (Hebelstrup and Jensen, 2008; Elhiti et al., 2013). In the antisense lines, NO accumulation was elevated in areas where the specific *ZmPgb* was detected in wild-type tissue (i.e. small clusters of basal cells in the *ZmPgb1.2(A)* line; arrowhead in Fig. 3.1E, top) and a larger group of embryonic cells in the *ZmPgb1.1(A)* line (compare Fig. 3.1, C with E). Specificity of the signal was verified in the wild-type line with the commonly used NO donor, sodium nitroprusside (SNP), and/or an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Hebelstrup and Jensen, 2008; Elhiti et al., 2013). Applications of cPTIO reduced NO and Zn^{2+} signals in the transformed lines.

In vertebrates, Zn^{2+} homeostasis regulates several cell responses, some of which are modulated by NO (Lee and Koh, 2010). Intracellular Zn^{2+} was detected using the membrane-permeable Zinquin-ethyl-ester (Zalewski et al., 1993), which is highly sensitive to variations in Zn^{2+} in maize embryos (Fig. S3.3). Zinquin is often used to image free Zn^{2+} for its strong specificity because it does not react with other divalent cations (Helmersson et al., 2008). An increased intracellular Zn^{2+} pool was observed in cells accumulating NO, whereas cells with reduced NO content had less intense Zn^{2+} signal (Fig. 3.1E, bottom). The relationship between Zn^{2+} levels and NO presence was substantiated by pharmacological manipulations of NO content using SNP or cPTIO. Free Zn^{2+} level increased after applications of the NO donor SNP, whereas it decreased in cells treated with the NO scavenger cPTIO (Fig. 3.1E).

Despite the lack of information relative to the regulation of intracellular Zn^{2+} pool by NO in plants, the role of metallothioneins (MTs) in the storage and homeostasis of Zn^{2+} and other heavy metals is becoming more evident. Based on the arrangements of Cys residues, four types of plant MTs (MT1–MT4) have been identified (Cobbett and Goldsbrough, 2002). The expression and localization of MTs vary between tissues and organs. In both Arabidopsis (*Arabidopsis thaliana*) and barley (*Hordeum vulgare*), MT4 isoforms contribute to the storage and release of Zn^{2+} during embryogenesis (Hegelund et al., 2012; Ren et al., 2012). To investigate whether the maize ZmMT4 is capable of storing Zn^{2+} and releasing it in a high-NO environment, a recombinant maltose binding protein (MBP) fusion with ZmMT4 was constructed and expressed in *Escherichia coli*. *In vivo E. coli* feeding with $ZnSO_4$ yielded a recombinant ZmMT4 protein loaded with Zn^{2+} (Table 3.1), which was assessed by acid release and dithizone (DTZ) measurement. Treatments with the NO donor diethylamine NONOate (DEA/NO) released a substantial percentage of the Zn^{2+} contained in the recombinant ZmMT4 during the 45-min

incubation time (Table 3.1). No Zn^{2+} was detected on DEA/NO treatment of MBP alone. The amount of Zn^{2+} released was dependent on the amount of NO provided (Fig. S3.4). This unloading of Zn^{2+} clearly shows that NO can regulate intracellular Zn^{2+} level through its release from MTs, a process that has not been described in plants.

Table 3.1 Effects of the NO donor DEA/NO on the release of Zn^{2+} from the recombinant maltose binding protein (MBP5) and an MBP5-ZmMT4 fusion protein

Medium was supplemented with 0.4 mM $ZnSO_4$. Values are means ± SE of three biological replicates. Zinc measurements were performed by the dithizone method (Paradkar and Williams, 1994). The percentage of Zn^{2+} recovered after the DEA/NO treatment (Zn^{2+} released by NO) compared to the acidification assay (Zn^{2+} stored in MT4).

Protein	Concentration (μM)	Zinc released (μM)		Percentage	
		Acidification	DEA/NO	Acidification	DEA/NO
MBP5	30.5 ±0.13	0.01±0.01	0.01±0.01		
MBP5-ZmMT4	31.12 ±0.21	0.76±0.02	0.63±0.05	100%	83%

To further investigate the requirement of NO in modulating intracellular Zn^{2+} during the *ZmPgb* response, we examined embryo production in the transformed lines cultured in a medium enriched with or depleted of NO and/or Zn^{2+} (Fig. 3.2). In the wild-type line, increased NO levels by SNP repressed the production of mature somatic embryos, whereas removal of NO by cPTIO had no effects (Fig. 3.2A). The possibility that SNP had toxic effects on embryogenesis was excluded by the combined applications of SNP and cPTIO. A similar embryogenic inhibition by SNP was also observed in the *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines, whereas cPTIO had opposite effects, promoting the production of mature somatic embryos in the former and repressing it in the latter (Fig. 3.2A).

Alterations in Zn^{2+} using a pharmacological procedure described by Helmersson et al. (2008) also affected embryogenesis. Supplementation of Zn^{2+} was detrimental for the production of mature somatic embryos in all lines (Fig. 3.2B). Sequestration of Zn^{2+} by the Zn^{2+} chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) promoted embryogenesis in the *ZmPgb1.1(A)* line and repressed embryo yield in the *ZmPgb1.2(A)* line (Fig. 3.2B).

To assess the effects of combined alterations of NO and Zn^{2+} levels on embryogenesis, the cell lines were subjected to different treatments. The wild-type line, characterized by the lowest accumulation of NO (Fig. 3.1E), was used to test the effects of an experimental increase of NO in a low Zn^{2+} environment, whereas the NO-accumulating *ZmPgb1.1(A)* line (Fig. 3.1E) was used to investigate the effects of a depletion of NO levels in a Zn^{2+}-enriched environment. The repressive effect of NO (SNP treatment) on wild-type embryo number was reversed by decreasing Zn^{2+} levels (SNP and TPEN treatment; Fig. 3.2C). Consistent with this observation, the increased production of mature embryos in the *ZmPgb1.1(A)* line with low NO levels (cPTIO

treatment) was abolished by elevating intracellular Zn^{2+} levels (cPTIO and Zn^{2+} treatment; Fig. 3.2C). These observations place Zn^{2+} as a downstream component of NO in the *ZmPgb* response.

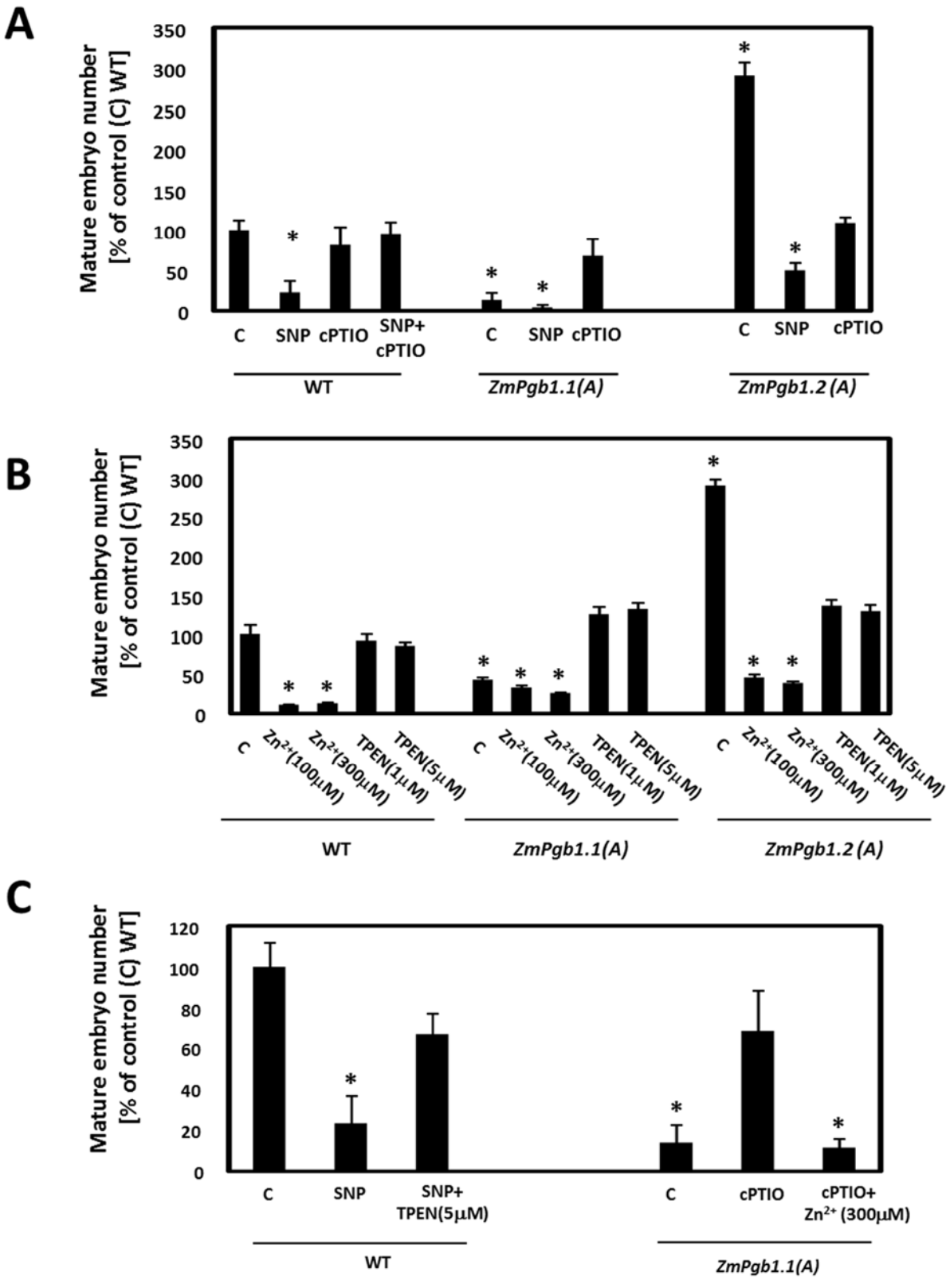

A
Mature embryo number
[% of control (C) WT]
350
300
250
200
150
100
50
0
C
SNP
cPTIO
SNP+
cPTIO
C
SNP
cPTIO
C
SNP
cPTIO
WT
ZmPgb1.1(A)
ZmPgb1.2 (A)
B
Mature embryo number
[% of control (C) WT]
C
Zn2+(100μM)
Zn2+(300μM)
TPEN(1μM)
TPEN(5μM)
WT
ZmPgb1.1(A)
ZmPgb1.2 (A)
C
Mature embryo number
[% of control (C) WT]
120
100
80
60
40
20
0
C
SNP
SNP+
TPEN(5μM)
C
cPTIO
cPTIO+
Zn2+ (300μM)
WT
ZmPgb1.1(A)

Fig. 3.2 Effects of NO and Zn^{2+} on the production of mature somatic embryos (d 21, D medium). A, Effects of the NO donor SNP and NO scavenger cPTIO on the number of somatic embryos. B, Effects of the Zn^{2+} chelator TPEN and Zn^{2+} supplementations (using $ZnSO_4$) on the number of somatic embryos. C, Effects of combined manipulations of NO and Zn^{2+} on the number of somatic embryos. Values are means ± SE of at least four biological replicates. *, Statistically significant differences ($P \leqslant 0.05$) from control (C); wild type (WT) set at 100%.

3.3.3 ZmPgb modulation of embryogenesis involves PCD and production of ROS

In vertebrates, apoptosis and autophagy are influenced by essential trace elements. The role of Zn^{2+} homeostasis in both processes is ambiguous. Although Zn^{2+} deficiencies are associated with apoptosis in some systems (Truong-Tran et al., 2000; Helmersson et al., 2008), elevated Zn^{2+} levels induce caspase activity in others (Schrantz et al., 2001). Controlled cell death is essential in both vertebrates and plants because it shapes the body of the organism by eliminating specific cells and tissues. To verify whether the *ZmPgb* regulation of somatic embryogenesis is executed through PCD, we assayed nuclear DNA fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Fig. 3.3A). Although no or little PCD was observed in immature wild-type embryos, the distribution of the TUNEL signals in the *ZmPgb1.2(A)* line was restricted to small clusters of basal cells, which was in contrast to the *ZmPgb1.1(A)* line, where PCD targeted a large number of embryonic cells (Fig. 3.3A). Cells undergoing PCD are most likely those suppressing *ZmPgbs* and with elevated levels of NO and Zn^{2+}, which are shown by the similar distribution patterns of the TUNEL-positive nuclei, *ZmPgb* expression, and NO and Zn^{2+} signals (compare Fig. 3.1, C and E with Fig. 3.3A). The requirement of NO and Zn^{2+} for the ZmPgb regulation of PCD was confirmed pharmacologically in the wild-type line. Although increasing levels of either NO- (by SNP) or Zn^{2+}-induced PCD, depletion of NO (by cPTIO) or Zn^{2+} (by TPEN) reduced the frequency of TUNEL-positive cells (Fig. 3.3A). Using the *ZmPgb1.1(A)* line characterized by the highest levels of NO and Zn^{2+} (Fig. 3.1E), it is shown that a reduction of NO (by cPTIO) or Zn^{2+} (by TPEN) was sufficient to reduce PCD (Fig. 3.3A). These results were also verified by calculating the percentage of TUNEL-positive cells (Fig. S3.5).

Manifestation of PCD in plant cells is characterized by biochemical and molecular hallmarks, such as fragmentation of nuclei, increased expression of metacaspases, and caspase-like activities (Reape et al., 2008). Both antisense lines showed signs of fragmentations in the nuclei undergoing PCD and increasing caspase 3-like activity (Fig. S3.6). The expression of the metacaspase *ZmMCII-3* increased significantly in the *ZmPgb1.1(A)* line but only slightly in the *ZmPgb1.2(A)* line. In the transgenic lines, the *ZmMCII-3* transcripts localized in similar areas, where the respective *ZmPgb* was detected in wild-type tissue (compare Fig. 3.1C with Fig. S3.6). These results suggest that expression of *ZmMCII-3* and caspase 3-like activity might be involved in the *ZmPgb* mediation of PCD.

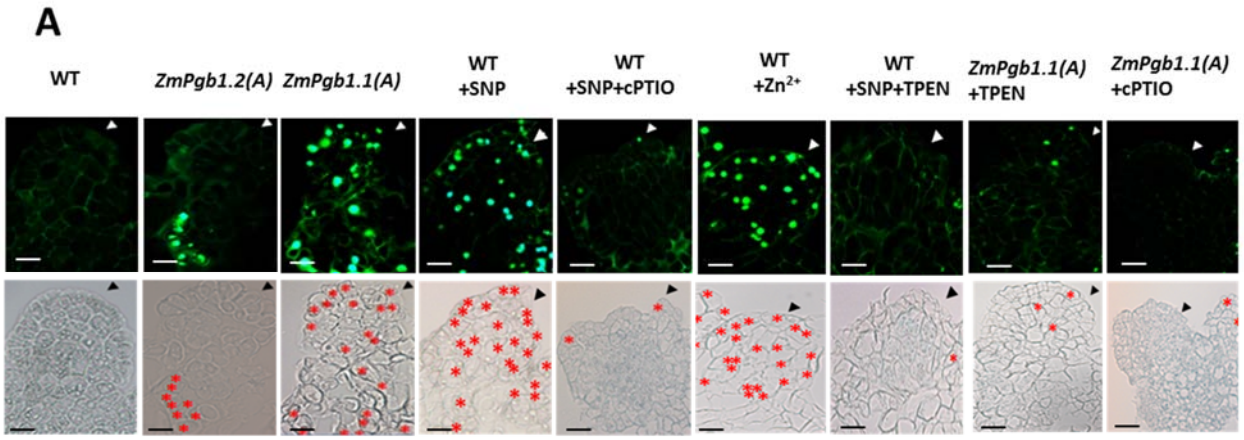
Additionally, cells programmed to die accumulate ROS (Van Breusegem and Dat, 2006). To examine whether maize cells targeted by PCD accumulated ROS, we stained with nitroblue tetrazolium and 3,3'-diaminobenzidine, which detect superoxide and hydrogen peroxide, respectively (Thordal-Christensen et al., 1997; Lin et al., 2009b). Although the specificity of each individual dye should be interpreted with due caution, the use of both ensures an accurate localization of ROS in plant cells (Lin et al., 2009b). The staining signal of superoxide and hydrogen peroxide coincided with the distribution pattern of TUNEL-positive nuclei (compare Fig. 3.3A with Fig. 3.3B), thus suggesting that ROS accumulate in cells targeted to die by the suppression of *ZmPgbs*. As observed for PCD, accumulation of ROS required NO and Zn^{2+}, whereas a chelation of Zn^{2+} by TPEN decreased ROS levels in an environment enriched with NO (by SNP). These results suggest that Zn^{2+} is a necessary downstream component in the NO production of ROS (Fig. 3.3B).

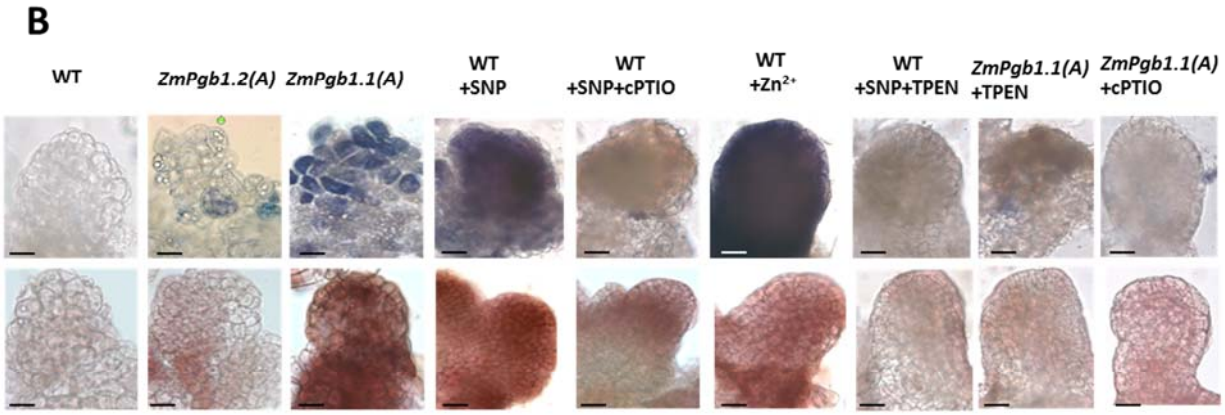
3.3.4 ROS accumulation in maize cells is required for the execution of PCD

In plant cells, ROS homeostasis is regulated by antioxidant molecules, including reduced ascorbic acid (ASC). Through the ascorbate-glutathione redox system, ROS are scavenged in a series of sequential reactions converting ASC to dehydroascorbate (Potters et al., 2002). Depletion of cellular ASC occurs in cells overproducing ROS (Potters et al., 2002), a characteristic also observed in our system (Fig. S3.7). The antioxidant properties of ASC were used to further investigate the requirement of ROS for the activation of the death program (Fig. 3.3C). Using the *ZmPgb1.1(A)* line, which accumulated higher levels of NO and ROS and displayed extensive PCD (Figs. 3.1E and 3.3, A and B), it is shown that ASC reduced both NO and ROS signals as well as decreased the number of TUNEL-positive nuclei (Fig. 3.3C). Furthermore, exogenous ASC applications modulated embryogenesis by increasing the production of mature somatic embryos in the *ZmPgb1.1(A)* line and decreasing it in the *ZmPgb1.2(A)* line to wild-type values (Fig. 3.3C). This finding was consistent with the opposite effects of the *ZmPgb*-induced PCD on embryogenesis.

Collectively, these data suggest that suppression of the two *ZmPgbs* triggers identical molecular responses, but their different localization pattern results in opposite embryogenic behavior. Repression of *ZmPgb1.2*, which is normally expressed in the basal cells anchoring the immature embryos to the embryogenic mass (Fig. 3.1C), elevates NO levels, which, in turn, increase free intracellular Zn^{2+} (Fig. 3.1E). A rise in the Zn^{2+} pool triggers ROS-mediated PCD (Fig. 3.3), eliminating these anchor cells and allowing the embryos to develop further at a higher frequency (Fig. 3.1D). Induction of PCD through similar events also occurs in *ZmPgb1.1* down-regulating cells. However, the enlarged localization pattern of *ZmPgb1.1*, encompassing most of

the embryo proper (Fig. 3.1C), results in the elimination of numerous cells (Fig. 3.3A), leading to embryo lethality and suppression of embryogenesis (Fig. 3.1D).


A
WT
ZmPgb1.2(A)
ZmPgb1.1(A)
WT +SNP
WT +SNP+cPTIO
WT +Zn2+
WT +SNP+TPEN
ZmPgb1.1(A) +TPEN
ZmPgb1.1(A) +cPTIO


B
WT
ZmPgb1.2(A)
ZmPgb1.1(A)
WT +SNP
WT +SNP+cPTIO
WT +Zn2+
WT +SNP+TPEN
ZmPgb1.1(A) +TPEN
ZmPgb1.1(A) +cPTIO

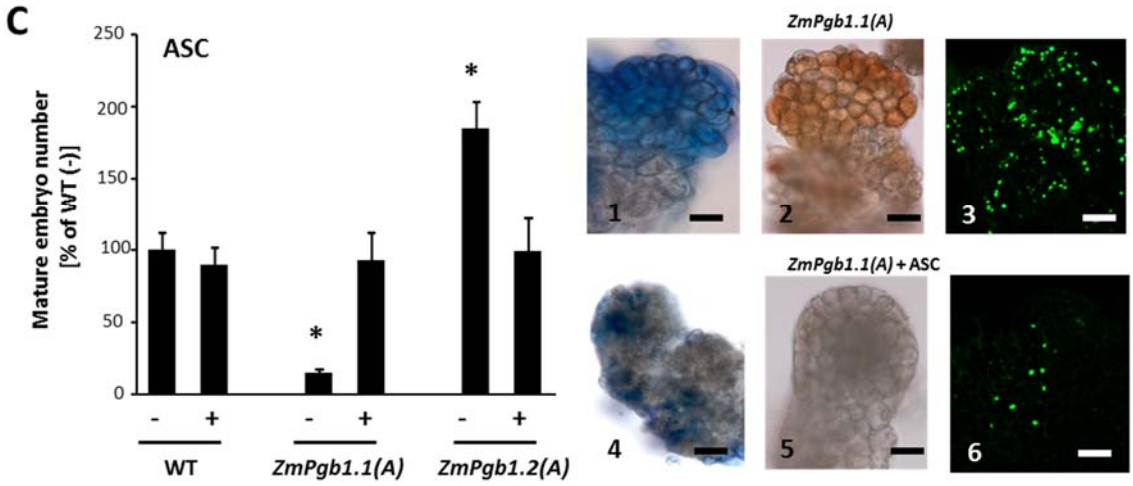

C
ASC
Mature embryo number [% of WT (-)]
250
200
150
100
50
0
*
*
- + - + - +
WT
ZmPgb1.1(A)
ZmPgb1.2(A)
ZmPgb1.1(A)
1
2
3
ZmPgb1.1(A) + ASC
4
5
6

Fig. 3.3 PCD during maize somatic embryogenesis. A, Identification of TUNEL-positive cells in immature embryos (d 7, P medium) of the *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines as well as embryos subjected to alterations in NO and Zn^{2+} levels using compounds from Fig. 3.2 *, Enhanced visualization of TUNEL-positive cells in the same embryos (bottom). Arrowheads mark the apical domains of the embryos. Bars = 20 μm. B, Localization of superoxide (top) and hydrogen peroxide (bottom) in immature embryos (d 7, P medium) subjected to alterations in NO and/or Zn^{2+} level. Bars = 20 μm. C, Effects of applications of ASC on mature somatic embryo number (d 21, D medium), accumulation of superoxide (1 and 4) and hydrogen peroxide (2 and 5), and distribution of TUNEL-positive cells (3 and 6) in immature embryos (d 7, P medium). Values of embryo numbers are means ± SE of at least four biological replicates. *, Statistically significant differences ($P \leqslant 0.05$) from control; wild type (WT) set at 100%. Bars = 20 μm.

3.3.5 The oxidative burst contributes to ROS production

In plants, the ROS-generating oxidative burst is mainly controlled by NADPH oxidases, a family of membrane-bound enzymes activated in a variety of stress and developmental responses (Sagi and Fluhr, 2006). In mammalian systems, NADPH oxidase is a large protein complex comprising cytosolic regulatory proteins, which include the $p47^{phox}$ and a membrane-bound NADPH-binding cytochrome consisting of the glycosylated transmembrane protein $gp91^{phox}$ and the nonglycosylated $p22^{phox}$ (Torres and Dangl, 2005). Investigations on the function of NADPH oxidases have been limited by the reduced specificity of pharmacological inhibitors, such as 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF; Bayraktutan, 2005) and diphenylene iodonium (DPI; Bindschedler et al., 2006). Although effective in preventing the activation of the ROS-producing NADPH oxidase in cell-free systems and intact stimulated macrophages by interfering with the translocation of $p47^{phox}$ to the plasma membrane (Diatchuk et al., 1997), AEBSF is a Ser protease inhibitor (Wind et al., 2010). Undesirable side effects have also been reported for DPI, which other than inhibiting NADPH oxidase in many systems (Bindschedler et al., 2006; Davies et al., 2006), also inhibits other flavoenzymes (Wind et al., 2010). In light of these side effects, although the efficacy of each individual inhibitor must be interpreted with due caution, the use of both should provide a solid indication on the participation of NADPH oxidase in the production of ROS.

Applications of both AEBSF and DPI reduced the intensity of superoxide and hydrogen peroxide signal as well as the number of TUNEL-positive nuclei in the *ZmPgb1.1(A)* line (Fig. 3.4) used for its elevated levels of ROS and extensive PCD (Fig. 3.3, A and B). Furthermore, both inhibitors modulated embryogenesis in a pattern mimicking that resulting from depletion of ROS (compare Fig. 3.3C with Fig. 3.4) and consistent with the regulation of PCD by the two

ZmPgbs (compare Fig. 3.3A with Fig. 3.4). The identical responses obtained with both inhibitors suggest that NADPH oxidase is involved in the accumulation of ROS during maize somatic embryogenesis.

To further validate this notion, the expression pattern of four maize respiratory burst oxidase (*ZmrbohA-D*) homologs to $gp91^{phox}$, a component of the mammalian NADPH oxidase complex, was measured in immature embryos. All genes, with expression patterns that correlate to the activation of NADPH oxidase (Lin et al., 2009a), were induced in lines suppressing *ZmPgb1.1(A)* and *ZmPgb1.2(A)* in a fashion consistent with a regulation mediated by NO and Zn^{2+} (Fig. S3.8).

Collectively, these data confirm the requirement of the oxidative burst in the *ZmPgb* regulation of PCD and strongly suggest the participation of NADPH oxidases in the accumulation of ROS.

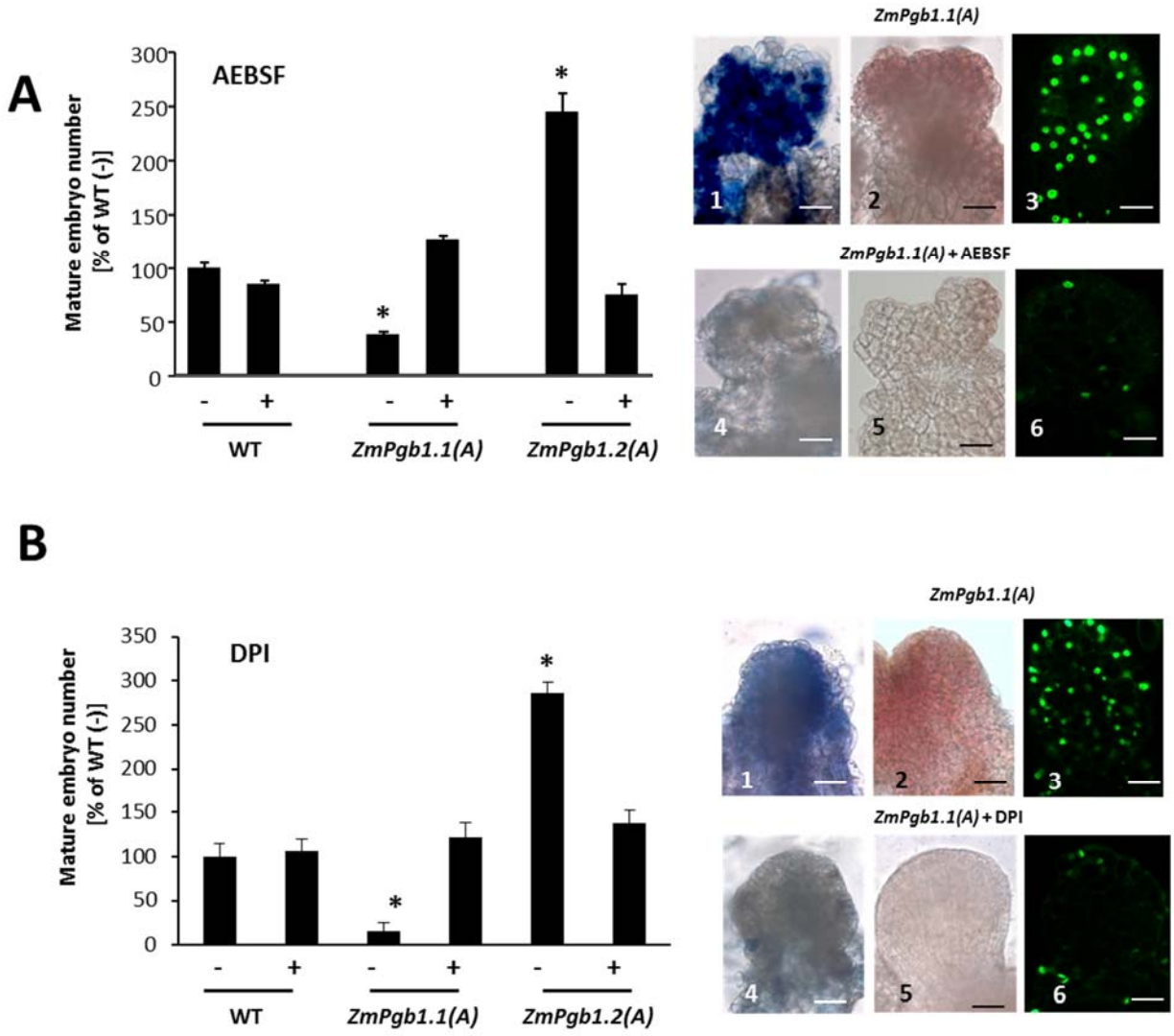
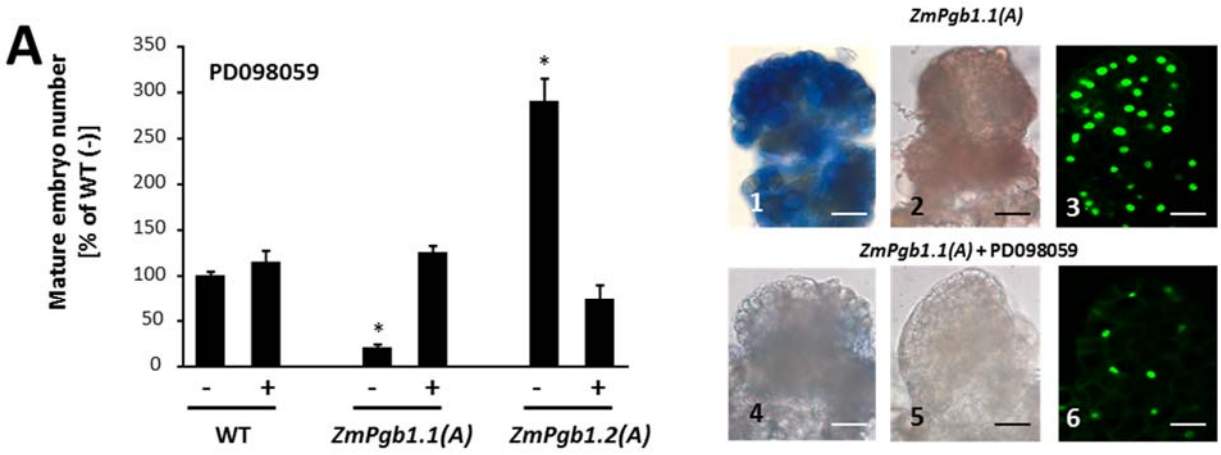


Fig. 3.4 Requirement of NADPH oxidase in the *ZmPgb* response. Effects of applications of the NADPH oxidase inhibitors AEBSF (A) and DPI (B) on mature (d 21, D medium) somatic embryo number, accumulation of superoxide (1 and 4) and hydrogen peroxide (2 and 5), and distribution of TUNEL-positive cells (3 and 6) in immature embryos (d 7, P medium). Values of embryo numbers are means ± SE of at least four biological replicates. *, Statistically significant differences ($P \leqslant 0.05$) from wild type (WT; −) set at 100%. Bars = 20 μm.

3.3.6 The production of ROS necessitates an active MAPK cascade

The MAPK cascade transduces many stimuli, some of which are mediated by NO (Keshet and Seger, 2010), into cellular responses. This cascade, triggered by the sequential activation of the mitogen-activated protein kinase kinase (MAPKK) and the MAPKK kinase by MAPK, culminates in downstream events, including protein phosphorylation and gene expression (Keshet and Seger, 2010). The participation of the MAPK cascade in the production of ROS was investigated pharmacologically using 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD098059), an effective MAPK cascade inhibitor that prevents the phosphorylation of MAPKK in animals and plants (Favata et al., 1998; Samuel and Ellis, 2002; Wang et al., 2010c). Inactivation of the MAPK cascade by PD098059 abolished the accumulation of ROS and reduced PCD in the *ZmPgb1.1(A)* line as well as modulated embryogenesis by increasing mature embryo yield in the *ZmPgb1.1(A)* line and decreasing it in the *ZmPgb1.2(A)* line (Fig. 3.5A). These results, which phenocopy closely those obtained by depleting ROS with ASC or inhibiting NADPH oxidase with AEBSF or DPI (compare Fig. 3.3C with Fig. 3.4), identify the MAPK cascade as a requirement for the production of ROS and activation of PCD.

Suppression of the MAPK cascade by PD098059 also repressed the expression of the four maize respiratory burst oxidase homologs (*ZmrbohA-D*; Fig. 3.5B), a result consistent with the requirement of an active MAPK cascade for the function of NADPH oxidase (Lin et al., 2009a).


A
Mature embryo number
[% of WT (-)]
350
300
250
200
150
100
50
0
PD098059
*
*
*
- + - + - +
WT
ZmPgb1.1(A)
ZmPgb1.2(A)
ZmPgb1.1(A)
1
2
3
ZmPgb1.1(A) + PD098059
4
5
6

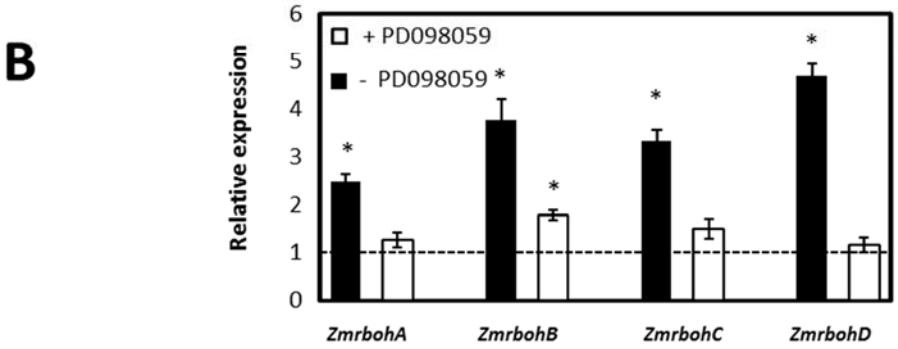

B
Relative expression
6
5
4
3
2
1
0
+ PD098059
- PD098059 *
*
*
*
*
ZmrbohA
ZmrbohB
ZmrbohC
ZmrbohD

Fig. 3.5 An active MAPKK cascade is required in the *ZmPgb* response. A, Effects of applications of the MAPKK inhibitor PD098059 on mature somatic embryo (d 21, D medium) number, accumulation of superoxide (1 and 4) and hydrogen peroxide (2 and 5), and distribution of TUNEL-positive cells (3 and 6) in immature embryos (d 7, P medium). Values of embryo numbers are means ± SE of at least four biological replicates. *, Statistically significant differences ($P \leqslant 0.05$) from control wild type (−) set at 100%. Bars = 20 μm. B, Expression level of the four NADPH respiratory burst oxidase homologs (*ZmrbohA-D*) in the *ZmHb1(A)* tissue (d 7, P medium) cultured in the presence (+) or absence (−) of PD098059. Values are means ± SE of three independent biological replicates and normalized to the expression level of each gene in the wild-type line (set at 1). WT, Wild type. *, Statistically significant difference ($P \leqslant 0.05$) from wild type.

3.3.7 Targeted PCD in ZmPgb-expressing cells phenocopies suppression of Pgbs

Evidence presented so far suggests that the opposite embryogenic response of the *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines is caused by the execution of PCD in different domains encompassing a large number of cells in the *ZmPgb1.1(A)* embryos and a few cells anchoring the embryos to the embryogenic tissue in the *ZmPgb1.2(A)* line. To show more precisely that cell death in these domains is a sufficient factor responsible for the observed embryogenic behavior, we induced death in *ZmPgb*-expressing cells by driving the Arabidopsis gene *KISS OF DEATH* (*KOD*), a known executor of PCD (Blanvillain et al., 2011), under the control of *ZmPgb1.1* or *ZmPgb1.2* promoters. Expression of *AtKOD* was sufficient to induce death in *ZmPgb*-expressing domains (compare Fig. 3.1C with Fig. 3.6A). Furthermore, the embryogenic response in the *P ZmPgb1.1:AtKOD* and *P ZmPgb1.2:AtKOD* lines mimicked that of the *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines, respectively (compare Fig. 3.1D with Fig. 3.6B), thereby showing the sufficient role of PCD in the *ZmPgb* regulation of embryogenesis.

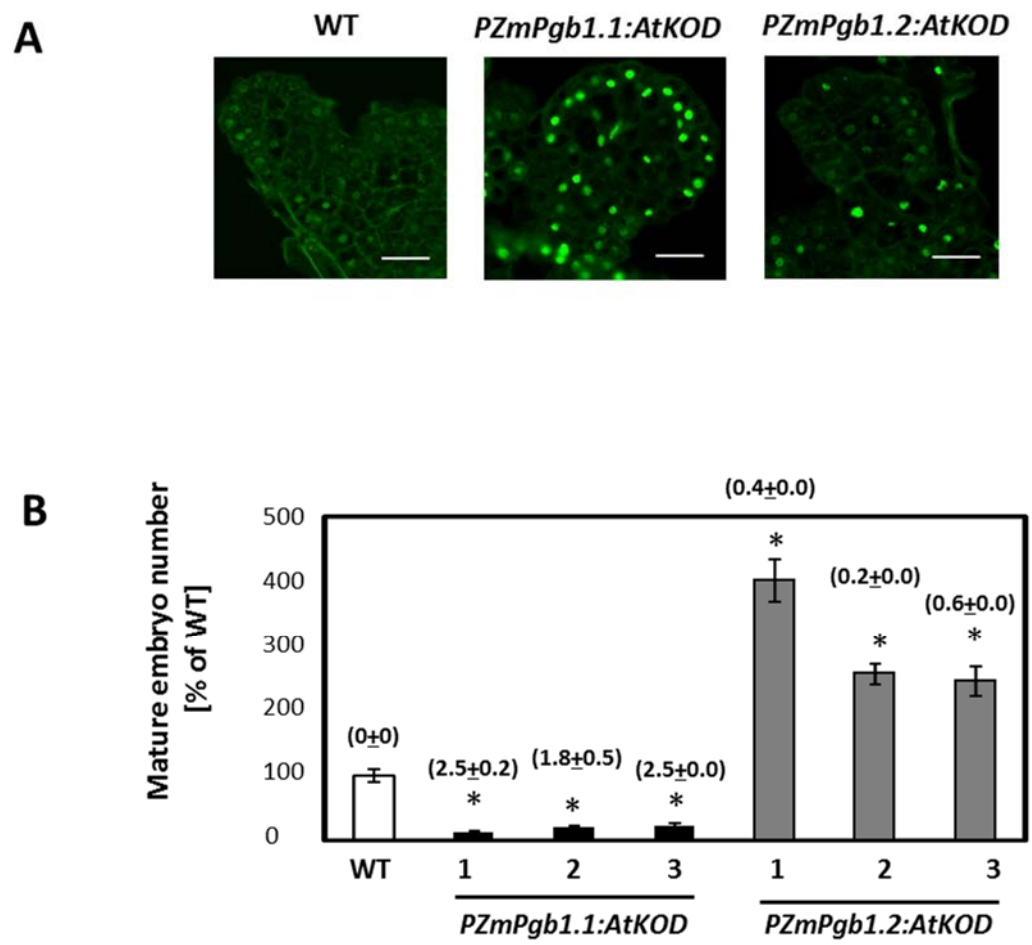


Fig. 3.6 Induced cell death in ZmPgb-expressing domains regulates embryogenesis. A, Identification of TUNEL-positive cells in immature embryos (d 7, P medium) obtained from the *PZmPgb1.1:AtKOD* lines, the *PZmPgb1.2:AtKOD* lines, and the wild-type line. Bars = 20 μm. B, Number of mature embryos (d 21, D medium) produced by three *PZmPgb1.1:AtKOD* lines and three *PZmPgb1.2:AtKOD* lines. Numbers in parentheses indicate the KOD-actin ratio in the respective lines. Values are means ± SE of four biological replicates and expressed as a percentage of wild type (set at 100%). WT, Wild type. *, Statistically significant difference ($P \leqslant 0.05$) from wild type.

3.4 Discussion

3.4.1 ZmPgbs modulate somatic embryogenesis through PCD

Somatic embryogenesis, the ability to generate embryos from somatic cells, has been often used as a model system to investigate basic aspects of plant growth and development because the embryogenic pathway can be easily controlled, manipulated, and/or redirected (Thorpe and Stasolla, 2001). In many plant species, including maize, an obligatory event of this process is the differentiation of immature somatic embryos from the surface of the embryogenic tissue consisting of a heterogeneous cell population. This differentiation step is facilitated by removal of plant hormones (Fig. 3.1A), and in spruce, it requires the selective elimination of embryogenic cells through PCD (Filonova et al., 2000b). Abrogation of the death program using either a pharmacological approach or PCD-deficient cell lines decreases the number of somatic embryos (Bozhkov et al., 2005a). The programmed nature of cell death during spruce embryogenesis was confirmed by the requirement of metacaspases, proteases with similar functions to animal caspases that are components of the late phases of the apoptotic pathway (Bozhkov et al., 2005a). However, identification and characterization of molecular components participating in the early phases of the developmental PCD process, not only in embryogenesis but also during advanced stages of animal and plant development, have been elusive (Elmore, 2007).

Here, we show that the spatial regulation of *ZmPgbs* is an early factor of the death program, which influences maize somatic embryogenesis. Suppression of both *ZmPgb1.1* and *ZmPgb1.2* triggers the death program in specific domains of the embryogenic tissue, resulting in embryogenic outcomes consistent with their respective localization patterns. Although suppression of *ZmPgb1.1* triggers death in extended domains of embryogenic tissue, leading to embryo abortion, suppression of *ZmPgb1.2* eliminates selected cells (i.e. the anchor cells)

connecting the developing embryos to the subtending tissue and increases the formation of mature somatic embryos. Although the effects of the *ZmPgb1.2*-mediated dismantling of anchor cells on embryo formation are beyond the scope of this work, they might be required for shaping the embryo body and promoting growth, which was documented in animal embryogenesis (Elmore, 2007) or, most likely, reducing embryonic density, an impeding factor during *in vitro* embryogenesis (Thorpe and Stasolla, 2001), through the physical separation of immature embryos from the embryogenic tissue.

The possibility that factors other than PCD are accountable for the embryogenic behavior of the *ZmPgbs*-suppressing lines was dismissed by triggering death in *ZmPgb1.1*- and *ZmPgb1.2*-expressing domains using KOD. Identified as an early inducer of the death pathway in Arabidopsis, where it contributes to the depolarization of the mitochondria membrane in the Bax-mediated death pathway, heterologous expression of *KOD* is sufficient to activate the suicide mode (Blanvillain et al., 2011). Expression of *KOD* in *ZmPgbs* domains closely phenocopied the death pattern and the embryogenic response of lines suppressing *ZmPgbs* (compare Figs. 3.1D, 3.3A, and 3.6), a similarity that proves unequivocally the sufficient requirement of the death program for the observed behavior of the transformed lines. In addition to regulating the cell death/survival decision during embryogenesis, as evidenced by our results, Pgbs might fulfill a similar function during postembryonic development in an analogous fashion to animal neuroglobins. Expressed at high levels in metabolically active cells, neuroglobins protect the brain from Alzheimer's disease by preventing neuron PCD (Khan et al., 2007; Greenberg et al., 2008) through mechanisms interfering with apoptotic events, such as the release of mitochondrial cytochrome C (Brittain et al., 2010). In plants, *Pgbs* are regulated by many abiotic and biotic stress responses characterized by the manifestation of PCD (Hill, 2012).

Independent studies show that overexpression of *Pgbs* reduces aerenchyma formation (Dordas et al., 2003) and represses cell death after pathogen infection (Mur et al., 2012a).

3.4.2 ZmPgb regulation of PCD is mediated by cellular NO and Zn^{2+} and requires ROS

A key role played by Pgbs during development and stress responses is to scavenge NO through an NADH-dependent dioxygenase (Igamberdiev et al., 2014). In harmony with previous studies documenting that suppression of *AtPgbs* in Arabidopsis increased cellular NO and applications of NO donors had an antagonistic effect to AtPgbs (Hebelstrup and Jensen, 2008), our results showed a rise in NO level in cells suppressing *ZmPgb1.1* or *ZmPgb1.2* (Fig. 3.1E). Elevated NO levels were apparent in many embryonic cells of the *ZmPgb1.1(A)* line and a few basal embryonic cells of the *ZmPgb1.2(A)* line. These NO-enriched domains coincided with the localization patterns of the respective *ZmPgb* in the wild-type line (compare Fig. 3.1C with Fig. 3.1E). DAF-2DA fluorescence, as an indicator of NO presence, must be interpreted with caution (Mur et al., 2011). DAF-2DA fluorescence is quenched at basic pH, whereas PCD is associated with acidification of the cytoplasm (Bosch and Franklin-Tong, 2007). Fluorescence quenching caused by cellular basic pHs should not, therefore, be an issue in this circumstance, and in any event, fluorescence quenching should reduce the observed fluorescence, not increase it. Ascorbate and dehydroascorbate can also react with DAF-2DA, resulting in fluorescence similar to that observed with NO. Previous work (Igamberdiev et al., 2006a) has shown that expression of *Pgb* increases cell ascorbate and dehydroascorbate. In this work, no fluorescence was observed in cells with suppressed levels of NO. Furthermore, lower levels of reduced ASC were observed in cells of *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines (Fig. S3.7). Increases in ascorbate/dehydroascorbate cell levels resulting in false NO detection are unlikely in this situation.

Many studies suggest that, in both animals and plants, accumulation of NO modulates several developmental and physiological responses, including PCD (Brüne, 2003). The participation of NO in the death program is dichotomous because both antiapoptotic and proapoptotic pathways have been shown to be regulated by NO (Brüne, 2003). Proapoptotic pathways are triggered by NO through a variety of mechanisms, including the inhibition of respiration and reduction in membrane potential, the activation of caspase activity (Brüne, 2003), and the covalent modification of target proteins by nitrosylation (for review, see Igamberdiev et al., 2014). During maize embryogenesis, the increased levels of NO in *ZmPgb*-suppressing cells induce PCD (Fig. 3.3A), and modulation of NO levels affects embryogenesis in a pattern consistent with the expression of *ZmPgbs*. Specifically, a reduction of NO with cPTIO increases the number of mature somatic embryos in the *ZmPgb1.1(A)* line, whereas it reduces it in the *ZmPgb1.2(A)* line (Fig. 3.2).

Accumulation of NO elevated the levels of cellular Zn^{2+}, an important regulator of cellular integrity and response to endogenous and exogenous stimuli (Roohani et al., 2013). Elevated Zn^{2+} signals were detected in domains enriched in NO and suppressing *ZmPgbs* (Fig. 3.1E) as well as in cells treated with the NO donor SNP (Fig. 3.1E). Furthermore, depletion of NO with cPTIO reduced Zn^{2+} levels. The requirement of NO for Zn^{2+} accumulation can be ascribed, at least in part, to the ability of NO to release Zn^{2+} from MTs (Table 3.1; Fig. S3.4), small heavy-metal binding proteins able to regulate cellular Zn^{2+} homeostasis (St Croix et al., 2002). The participation of MTs in NO signaling by sequestration or release of Zn^{2+} is a unique concept in plants, which other than having potential central roles in other NO-mediated responses unrelated to PCD, provide evidence of similar NO-regulatory mechanisms operating across organisms. In both hippocampus and endothelial cells, NO increases the amount of labile Zn^{2+} (Berendji et al.,

1997; Cuajungco and Lees, 1998), and elevated Zn^{2+} levels can be generated from the destruction of the Zn^{2+}-sulfur clusters of MTs by NO (Aravindakumar et al., 1999). In animal cells, the contribution of the MT-mediated Zn^{2+} release to the overall cellular Zn^{2+} pool can be significant and is detectable with the same Zn^{2+}-specific fluorophore Zinquin (St Croix et al., 2002) used in this study.

Unequivocal experimental evidence indicates that elevation of Zn^{2+} levels in *ZmPgb*-suppressing maize cells mediates the NO activation of PCD. First, chelation of Zn^{2+} abolishes death by NO, whereas increasing Zn^{2+} levels induce PCD in environments depleted of NO (Fig. 3.3). Second, manipulations of Zn^{2+} in the culture medium override the effects of NO on embryo yield (Fig. 3.2). The proapoptotic role of Zn^{2+} reported in our study agrees with work on animal systems (Wätjen et al., 2002; Land and Aizenman, 2005), which show that the death-triggering accumulation of Zn^{2+} originates solely from MTs (Lee et al., 2003), but not with others documenting PCD in Zn^{2+}-deficient cells (Rogers et al., 1995; Helmersson et al., 2008). This discrepancy is possibly caused by differences in perception of Zn^{2+} homeostasis operating across organisms. When manifested, the proapoptotic role of Zn^{2+} in animal cells is often mediated by ROS (Zhang et al., 2007a), key regulators of developmental and environmental death programs (Elmore, 2007). This finding is in agreement with our results showing the accumulation of ROS in Zn^{2+}-supplemented cells, their depletion in Zn^{2+}-deficient cells (Fig. 3.3B), and the abolishment of the death program after scavenging of ROS by ASC in the *ZmPgb1.1(A)* line characterized by high levels of Zn^{2+} (Fig. 3.3C). Furthermore, the observation that Zn^{2+} manipulations (supplementation or chelation) override the effects of NO on ROS production (Fig. 3.3B) places ROS downstream of NO and Zn^{2+} in the Pgb response.

3.4.3 Inhibition of the oxidative burst and MAPK cascade abolishes ROS formation

Generation of ROS in plants occurs through an oxidative burst triggered by diverse mechanisms, some of which involve NADPH oxidases, membrane-bound enzymes implicated in several responses, including PCD (Sagi and Fluhr, 2006). A reduction in ROS production during maize embryogenesis was achieved pharmacologically using both AEBSF and DPI (Diatchuk et al., 1997; Tiwari et al., 2002). Other than decreasing superoxide and hydrogen peroxide, a result consistent with previous studies (Bindschedler et al., 2006; Davies et al., 2006), these inhibitors reduced PCD in the *ZmPgb1.1(A)* line characterized by elevated levels of ROS and TUNEL-positive nuclei (Fig. 3.4). Furthermore, both AEBSF and DPI abolished the effects of *ZmPgb* suppression on embryogenesis, consistent with the reduction of ROS and PCD (compare Fig. 3.3C with Fig. 3.4).

Additional evidence supporting the involvement of NADPH oxidase in the accumulation of ROS during the ZmPgbs regulation of embryogenesis is apparent by the behavior of the four *ZmrbohA-D* genes, homologs of the human neutrophil pathogen-related $gp91^{phox}$ (a component of the NADPH oxidase) that are expressed at high levels during active production of ROS by NADPH oxidases (Lin et al., 2009a). Suppression of *ZmPgb1.1* or *ZmPgb1.2* induces the expression of the four genes through the mediation of NO by Zn^{2+} (Fig. S3.8).

Activation of the oxidative burst is linked to several transduction mechanisms, including the MAPK cascade (Lin et al., 2009a), which transduces many stimuli, some of which are mediated by NO (Browning et al., 2000). Inhibition of the MAPK cascade by the cell-permeable PD098059, which prevents the phosphorylation of MAPKK (Favata et al., 1998; Samuel and Ellis, 2002; Wang et al., 2010c), reduces ROS production, abolishes the death program, and modulates embryogenesis in a pattern consistent with the depletion of ROS (Fig. 3.5A). The

requirement of active MAPK for the accumulation of ROS might be exercised through the transcription of the four *ZmrbohA-D* (Fig. 3.5B), the expressions of which have been shown to correlate to the activation of NADPH oxidase (Lin et al., 2009a).

3.4.4 A unique model linking ZmPgbs to cell death/survival decision during *in vitro* embryogenesis

Results presented here fit a model in which *ZmPgb* controls PCD by regulating cellular NO during maize somatic embryogenesis (Fig. 3.7). NO is produced as an initiator of the cascade of events ending in death of the cell. Most of the PCD pathway has previously been described, but this work has shown the unique finding that Zn^{2+}, like in vertebrate systems, is a critical component in this cascade and released from MTs. Depending on characteristics within a specific maize embryonic cell, *ZmPgb1.1* or *ZmPgb1.2* may be expressed to inhibit this process. The oxygenated form of the Pgb reacts with cellular NO, producing nitrate and metphytoglobin (Pgb [Fe^{3+}]). Therefore, the cellular expression of a specific *Pgb* gene has the capability to determine the fate of that cell under conditions where mobile or transportable hormones or effectors, such as auxin, ethylene, or NO, are present in an organ. Expression of *ZmPgb1.1* during maize somatic embryogenesis protects a large number of embryonic cells from PCD by scavenging NO; therefore, when this gene's expression is suppressed [*ZmPgb1.1(A)*], significant embryo abortion occurs. Expression of *ZmPgb1.2* protects a few basal embryonic cells from PCD. Suppression of expression of this gene [*ZmPgb1.2(A)*] results in death of these anchor cells, increasing the number of mature somatic embryos produced. Programming of PCD in suspensor cells to establish embryo polarity plays a critical role in survival and development during zygotic embryogenesis (Smertenko and Bozhkov, 2014). Our results would suggest that the two maize

Pgbs may function to both protect the developing maize embryo from PCD (*ZmPgb1.1*) and orchestrate the timing of suspensor PCD (*ZmPgb1.2*) during zygotic embryogenesis.

Manifestation of PCD is also important for key events during the *in vivo* reproductive phase. The dismantling of the suspensor during the middle late phases of embryogenesis, the selective elimination of nucellus and aleurone cells during endosperm development, pollen-stigma interaction in the initial steps of fertilization, male and female organ sculpturing in developing flowers, and parthenogenesis are all examples of events modulated by PCD. Based on the premises of this model, it is possible that Pgbs are intimately linked with these events, and their manipulation can be used as a tool to enhance the reproductive outcome.

It cannot be excluded that the regulation of PCD by Pgbs can also operate across species. The majority of plants that have had Pgbs identified contain at least two *Pgb* genes, with rice (*Oryza sativa*) having as many as five genes (Vázquez-Limón et al., 2012). Cytoglobin and neuroglobin are two Pgbs that potentially could function in a similar fashion in vertebrates (Burmester and Hankeln, 2009). They are up-regulated during hypoxia, capable of scavenging NO, and prevent PCD. We conclude that *ZmPgbs* modulate somatic embryogenesis in maize through PCD and identify Pgbs as potential regulators of morphogenesis in other systems.

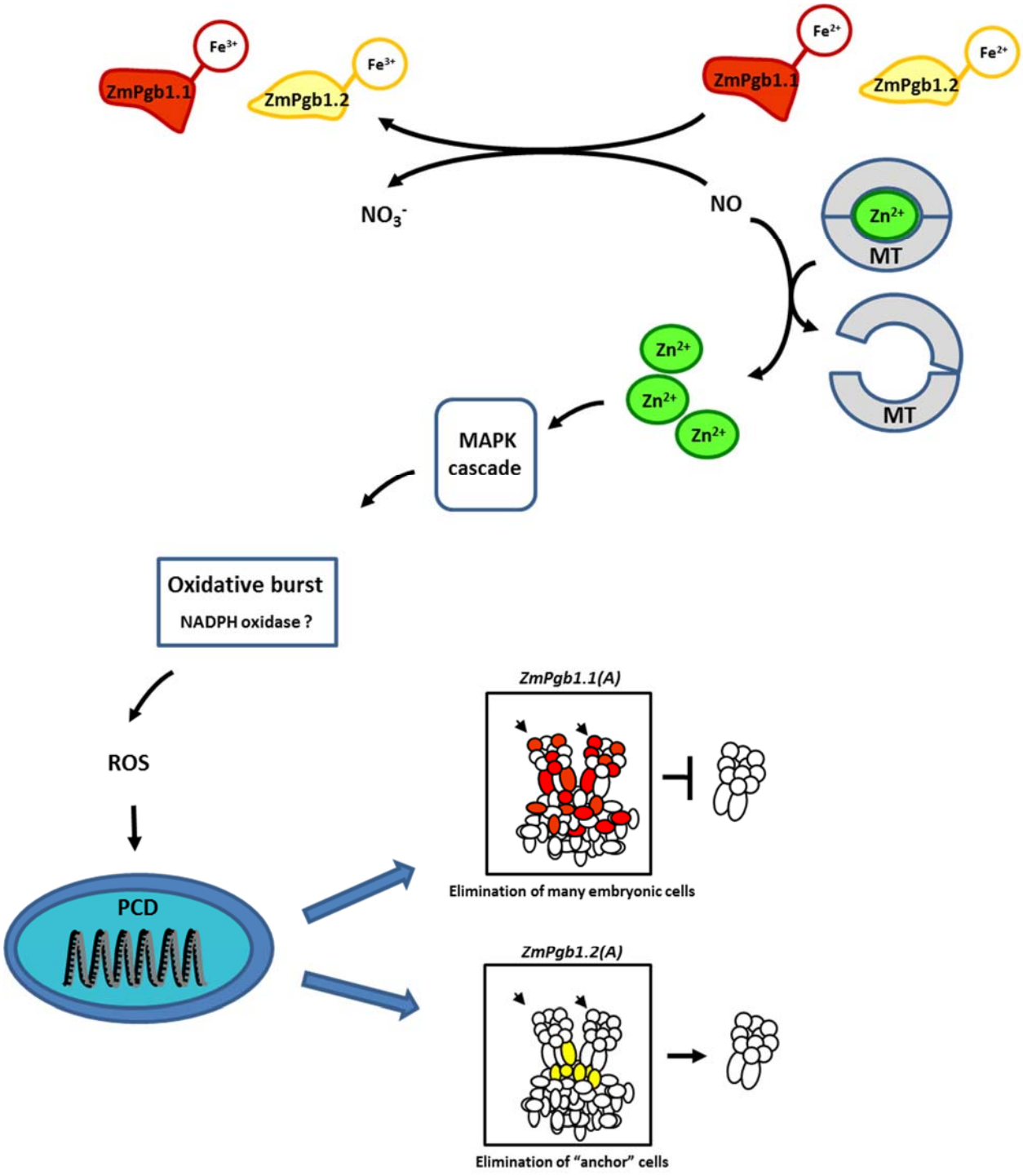

Fe3+
ZmPgb1.1
Fe3+
ZmPgb1.2
Fe2+
ZmPgb1.1
Fe2+
ZmPgb1.2
NO3-
NO
Zn2+
MT
Zn2+
Zn2+
Zn2+
MT
MAPK cascade
Oxidative burst
NADPH oxidase ?
ROS
PCD
ZmPgb1.1(A)
Elimination of many embryonic cells
ZmPgb1.2(A)
Elimination of "anchor" cells

Fig. 3.7 Proposed model of *ZmPgb* action during somatic embryogenesis. Suppression of *ZmPgbs* releases the inhibitory effects of NO on PCD through a cascade involving intracellular Zn^{2+}, the MAPK cascade, and accumulation of ROS, which are possibly mediated by NADPH oxidase. The distinct expression domains of *ZmPgb1.1* and *ZmPgb1.2* identify cells targeted by PCD and, ultimately, embryo formation. Cells undergoing PCD in the two lines [red, *ZmPgb1.1(A)*; yellow, *ZmPgb1.2(A)*] are outlined. Small arrows indicate the apical domain of developing embryos embedded in the embryogenic tissue.

3.5 Materials and methods

3.5.1 Plant materials and treatments

Maize (*Zea mays*) Hi II Type II embryogenic tissue was obtained from the Plant Transformation Facility at Iowa State University (http://agron-www.agron.iastate.edu/ptf/). Somatic embryogenesis was induced as reported by Wang and Frame (2009) by transferring embryogenic tissue maintained on a solid 2,4-dichlorophenoxyacetic acid-containing maintenance medium into a liquid P medium of similar composition. After 7 d, the tissue was plated on solid hormone-free D medium to allow embryo maturation, which lasted 21 d. Pharmacological treatments were carried out for 7 d in the P medium. The NO donor SNP was applied at a concentration of 10 µM, and the NO scavenger cPTIO was applied at a concentration of 100 µm (Elhiti et al., 2013). $ZnSO_4$ was used as a Zn^{2+} source and applied at a concentration of 100 or 300 µM (Helmersson et al., 2008). In the Zn^{2+} supplementation experiments, control cells were also grown in a medium devoid of $ZnSO_4$. The membrane-permeable Zn^{2+} chelator TPEN was applied at a concentration of 1 or 5 µM (Helmersson et al., 2008). The specific inhibitor of MAPKK PD098059 was administered at a concentration of 50 µM (Wang et al., 2010c). The NADPH oxidase inhibitors AEBSF and DPI were applied at concentrations of 1 mM and 5 µM, respectively (Diatchuk et al., 1997; Drummond et al., 2011; Ryder et al., 2013). Reduced ASC was used at a concentration of 100 µM (Stasolla and Yeung, 1999). Treatments were interrupted at d 7 in the P medium, and the tissue was either cultured on solid D medium to assess embryo yield or collected for gene expression, immunological, and histological analyses. When not specified, $ZnSO_4$ and TPEN were applied at concentrations of 300 and 5 µM, respectively.

3.5.2 Plant transformation

For construction of antisense maize lines, the two Pgbs, *ZmPgb1.1* (Accession number: AF236080) and *ZmPgb1.2* (Accession number: DQ171946), were amplified from complementary DNA (cDNA) prepared from maize embryogenic tissue by reverse transcription (RT)-PCR using gene-specific primers and subcloned into a pGEM-T Easy vector (Promega). Sequences were confirmed by DNA sequencing, and *BamHI* sites were added to both 5' and 3' ends of both genes by PCR. The maize polyubiquitin1 (*ubi-1*) promoter was isolated from pUbiSXR vector, and *KpnI* restriction sites were added to the 5' and 3' ends by PCR. The nopaline synthase terminator was isolated from pBI121 with SacI restriction sites and added to the 5' and 3' ends. The entire cassette was assembled through sequential ligation of the *ubi-1* promoter, *nos-t*, and *ZmPgb1.1* or *ZmPgb1.2* into pBluescript SK−. Confirmed antisense constructs were cobombarded into immature maize embryos and selected on glufosinate-containing media (Wang and Frame, 2009).

For the promoter constructs, putative *P ZmPgb1.1* and *P ZmPgb1.2* were isolated by PCR from maize Hi II Type II callus genomic DNA using specific primers (MaizeGDB, http://www.maizegdb.org/; BDGP, http://www.fruitfly.org/; Zhao et al., 2008). The promoters (898 and 1,251 bp, respectively) were subcloned into pGEM-T Easy vector and confirmed by DNA sequencing. Using Gateway Cloning Technology (Invitrogen), both promoters were assembled into the destination vector pKGWFS7 (Karimi et al., 2002) to generate *PZmPgb1.1:GUS* and *PZmPgb1.2:GUS* lines.

For the KOD constructs, *AtKOD* was isolated by PCR from genomic DNA of Arabidopsis (*Arabidopsis thaliana*) ecotype Colombia-0 using gene-specific primers (Blanvillain et al., 2011), subcloned into pGEM-T Easy vector, and confirmed by DNA sequencing. The cloning fragments

were amplified by *SpeI*-AtKOD-F and puc19-M13-R primers and ligated into the SpeI-digested and -dephosphorylated *pGEM-T:PZmPgb1.1* and *pGEM-T:PZmPgb1.2*, respectively. Using Gateway Cloning Technology (Invitrogen), both *PZmPgb1.1:AtKOD* and *PZmPgb1.2:AtKOD* were transferred into the destination vector pEarleygate 301 (Earley et al., 2006).

All primers used are listed in Table S3.1.

3.5.3 Isolation of the maize embryo-specific ZmMT4

Total RNA was isolated from wild-type maize tissue using the Dynabeads Kit (Life Technologies). The cDNA of ZmMT4 embryo-specific U10696 (protein accession no. CAA84233; Leszczyszyn et al., 2013) was obtained by RT-PCR. The amplified product was purified and cloned into the pCR4-blunt TOPO vector (Life Technologies) and confirmed by sequencing. An MBP fusion construct was assembled in pMAL-c5X vector (NEB) using an Infusion strategy (Clontech) by linearizing the vector with *XmnI* and *EcoRI* followed by amplification with the primers MT4_fw and MT4_rev (Table S3.1).

3.5.4 ZmMT4 expression and purification

A single colony of NEB expression *Escherichia coli* harboring the pMAL-c5X vector alone or the positive vector containing ZmMT4 fusion was used to inoculate an overnight starter culture in 4 mL of Luria-Bertani broth incubated at 37°C with vigorous shaking. Two milliliters of the starter was used to inoculate 200 mL of double-concentrated Luria-Bertani broth grown at 37°C. When the optical density$_{600}$ reached 4.5, the expression was induced by 0.5 mM isopropyl-β-d-1-thiogalactoside in a medium supplemented with or without 0.4 mM $ZnSO_4$. The cells were grown for an additional 3 h, harvested by centrifugation, and then resuspended in 8 mL of extraction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 1 mM AEBSF, 10 μM

trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane, 20 μM Bestatin, 0.1 mM Phosphoramidon, 4 mM dithiothreitol, 0.01% [v/v] Triton X-100, and 20% [v/v] glycerol). After sonication, the lysate was clarified by centrifugation and purified on 10 mL of amylose resin according to the manufacturer's specifications (NEB). Eluted control MBP and MBP-ZmMT4 fusion proteins were dialyzed and concentrated using vivaspin 20 centrifugal devices (GE Healthcare). Approximately 10 mg of recombinant MBP was recovered from 200 mL of culture. Protein quantification was performed with the method by Bradford (1976), whereas purity was confirmed by SDS-PAGE with Coomassie staining.

3.5.5 Release of Zn^{2+} from the purified ZmMT4

The NO donor DEA/NO (sodium salt hydrate) was prepared as a 0.5 M stock solution in 10 mM NaOH. To quantify the presence of Zn^{2+} in the purified MBP or MBP-ZmMT4 proteins, 0.2 to 0.26 mg of each protein, in triplicate, was incubated with 1 N HCl for 45 min at room temperature and centrifuged at 20,000g for 2 min. The release of Zn^{2+} was quantitatively evaluated by DTZ (diphenyl thiocarbazone) by following published protocols (Paradkar and Williams, 1994).

For the Zn^{2+} release by DEA/NO, one concentration of ZmMT4 and MBP (as negative control), in triplicate, was incubated at room temperature for 45 min in 0.05 M Tris-HCl (pH 7.5) with 3 mM DEA/NO or the same amount of water (negative control). The amount of Zn^{2+} released was directly assayed by the DTZ method described above.

The choice of different NO donors (SNP for embryogenesis and DEA/NO for the MTs experiment) was based on their different characteristics. SNP is the NO donor used most routinely *in vivo* (Hebelstrup and Jensen 2008; Elhiti et al., 2013). NO release from SNP is not

spontaneous but relies on chemical reactions after SNP is absorbed by the cells (Miller and Megson, 2007). Therefore, SNP was used only for the embryogenesis studies but not the MTs experiment. The effectiveness of SNP was always verified by monitoring NO levels. DEA/NO spontaneously dissociates, releasing NO at neutral or slight alkaline pH. The release of NO from DEA/NO is more homogeneous and more suitable for *in silico* studies because it can be regulated/controlled by adjusting the pH of the buffer used. A pH of 7.5 was used for the MT experiment. Because of its pH-dependent properties, the use of DEA/NO is not recommended *in vivo*, especially in culture, where changes in pH often occur over time.

3.5.6 In situ localization of NO, Zn^{2+}, and PCD

NO was assayed using DAF-2DA (Elhiti et al., 2013). Zinquin-ethyl-ester was used to localize Zn^{2+} (Helmersson et al., 2008). Nuclear DNA fragmentation was detected with the In Situ Cell Death Detection Kit-Fluorescein (Roche). Tissue was fixed in 4% (w/v) paraformaldehyde, dehydrated in ethanol series, and embedded in wax. Sections (10 μm) were dewaxed in xylene and labeled with the TUNEL kit (Roche) according to the manufacturer's protocol, with the exclusion of the permeabilization step by proteinase K. Omission of terminal deoxynucleotidyl transferase was used for negative controls.

3.5.7 Localization of superoxide, hydrogen peroxide, and reduced ASC

Localization of hydrogen peroxide in maize cells was performed as described by Thordal-Christensen et al. (1997). Suspension cells (0.2 mL) were stained with 3,3'-diaminobenzidine (pH 3.8) for 3 h in the dark. Superoxide was visualized by incubating cells in a solution containing 0.05% (w/v) nitroblue tetrazolium, 10 mM sodium azide, and 50 mM phosphate-buffered saline (pH 7.6; Lin et al., 2009b). Reduced ASC was localized following the procedure by Liso et al. (2004).

3.5.8 Expression studies

TRI Reagent Solution was used to extract RNA following the manufacturer's instruction (Invitrogen), and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis. Gene expression studies were performed by quantitative RT-PCR. All primers used for gene expression studies are listed in Table S3.1. The relative gene expression level was analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using actin as the reference gene.

Expression of *AtKOD* was detected by quantitative RT-PCR, Taqman technology using AtKOD-F/R with AtKOD-FAM probe, and Actin1-F/R with Actin1-HEX probe (Table S3.1).

3.5.9 Caspase-like activity

Caspase 3-like activity was assayed using the substrate Ac-DEVD-AFC (Coffeen and Wolpert, 2004; Ye et al., 2013). The assay was conducted using a fluorescence spectrophotometer (excitation of 400 nm and emission of 505 nm).

3.5.10 RNA in situ hybridization

RNA in situ hybridization studies were conducted as described (Elhiti et al., 2010). The two full-length *ZmPgb1.1* and *ZmPgb1.2* cDNAs were cloned into a pGEM-T Easy Vector System (Promega). The two cDNAs were subsequently amplified from the vector using T7 and SP6 primers and used for the preparation of digoxigenin-labeled sense and antisense riboprobes following the procedure outlined in the DIG Application Manual (Roche Diagnostics). Slide preparation, hybridization conditions, and color development were performed as documented previously (Elhiti et al., 2010).

3.5.11 Statistical analysis

Tukey's post hoc test (Zar, 1999) was used for comparison and analysis of significant differences.

3.6 Supplemental data

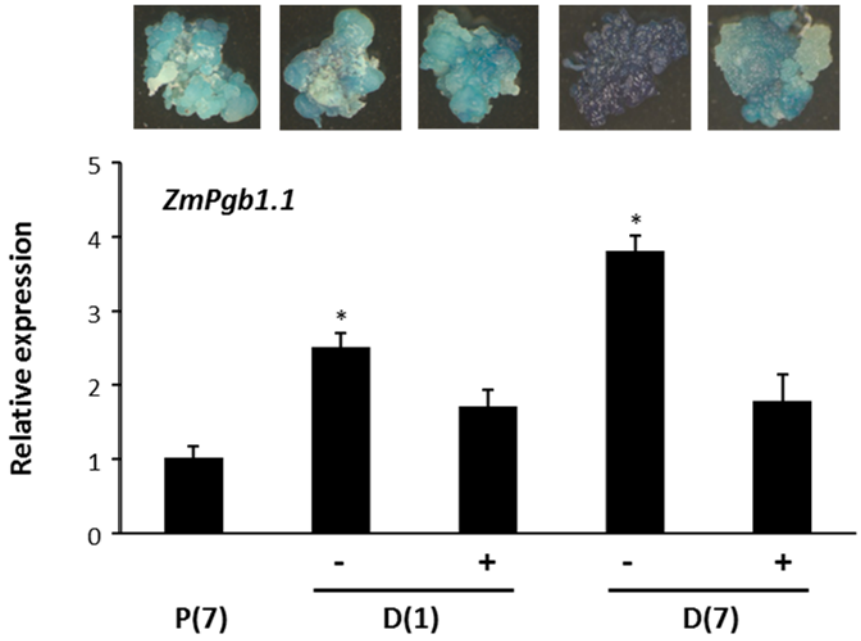
```
ZmPgb1.1          MALAEADDGAVVFGEEQEALVLKSWAVMKKDAANLGLRFFLKVFEIAPSAKQMFSFLRDS 60
ZmPgb1.2          ----------MGFSEAQEELVLRSWKAMKSDSESTALKFFLRIFEIAPGAKQMFSFLRDA 50
                            : *.* ** ***:** .**.*: . .*:***::*****.**********:

ZmPgb1.1          -DVPLEKNPKLKTHAMSVFVMTCEAAAQLRKAGKVTVRETTLKRLGATHLRYGVADGHFE 119
ZmPgb1.2          GDAPLEKHPKLKAHAVTVFVMACESATQLRSTGDVKVREATLKRLGATHARAGVADAHFE 110
                   *.****:****:**::****:**:*:***.:*.*.***:********* * ****.***

ZmPgb1.1          VTGFALLETIKEALPADMWSLEMKKAWAEAYSQLVAAIKREMKPDA-------------- 165
ZmPgb1.2          VVKTALLDTIRDAVP-DMWTPEMKAAWEEAYDQLAAVIKEEMKNAAAAEEQTKNAATAAE 169
                  *.  ***:**::*:* ***: *** ** ***.**.*.**.***  *

ZmPgb1.1          ----------------------
ZmPgb1.2          ETTNAAAAEEETTNAAAAVDAS 191
```

Fig. S3.1 Amino acid sequence alignment of ZmPgb1.1 and ZmPgb1.2 with Clustal W using BLOSUM protein weight matrix (Larkin et al., 2007).


ZmPgb1.1
Relative expression
0
1
2
3
4
5
*
*
-
+
-
+
P(7)
D(1)
D(7)

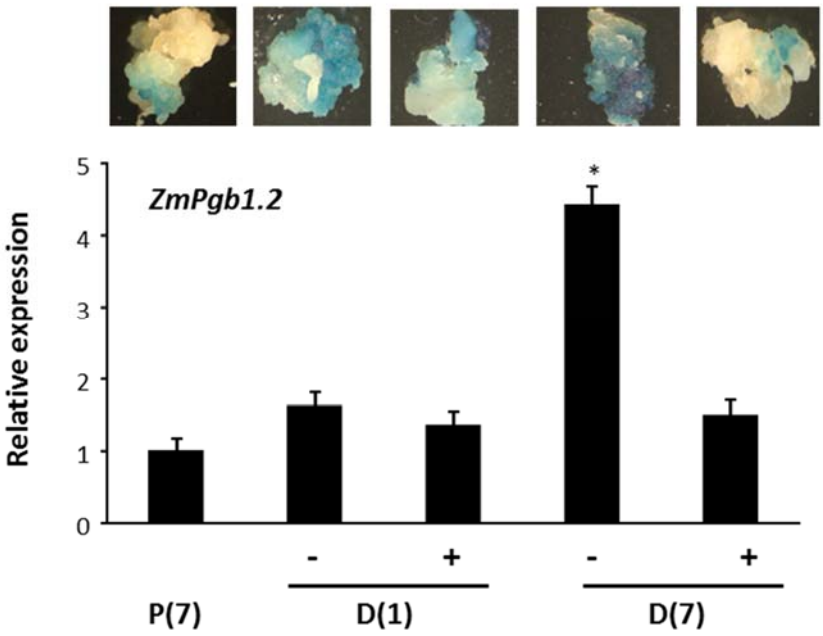

ZmPgb1.2
Relative expression
0
1
2
3
4
5
*
-
+
-
+
P(7)
D(1)
D(7)

Fig. S3.2 Regulation of *ZmPgb1.1* and *ZmPgb1.2* by auxin. Expression level of *ZmPgb1.1* and *ZmPgb1.2* in WT maize tissue cultured for 7 days in 2,4-D –containing proliferation (P) medium and then transferred on development (D) medium supplemented with (+) or devoid of (-) 2,4-D. Numbers in brackets indicate days in culture. Values ± SE are means of three biological replicates and are normalized to the P(7) value set at 1. * Statistically significant difference ($p \leqslant 0.05$) from the respective P(7) value. Micrographs show the GUS staining in the *PZmPgb1.1:GUS* and *PZmPgb1.2:GUS* lines at the respective days in culture.

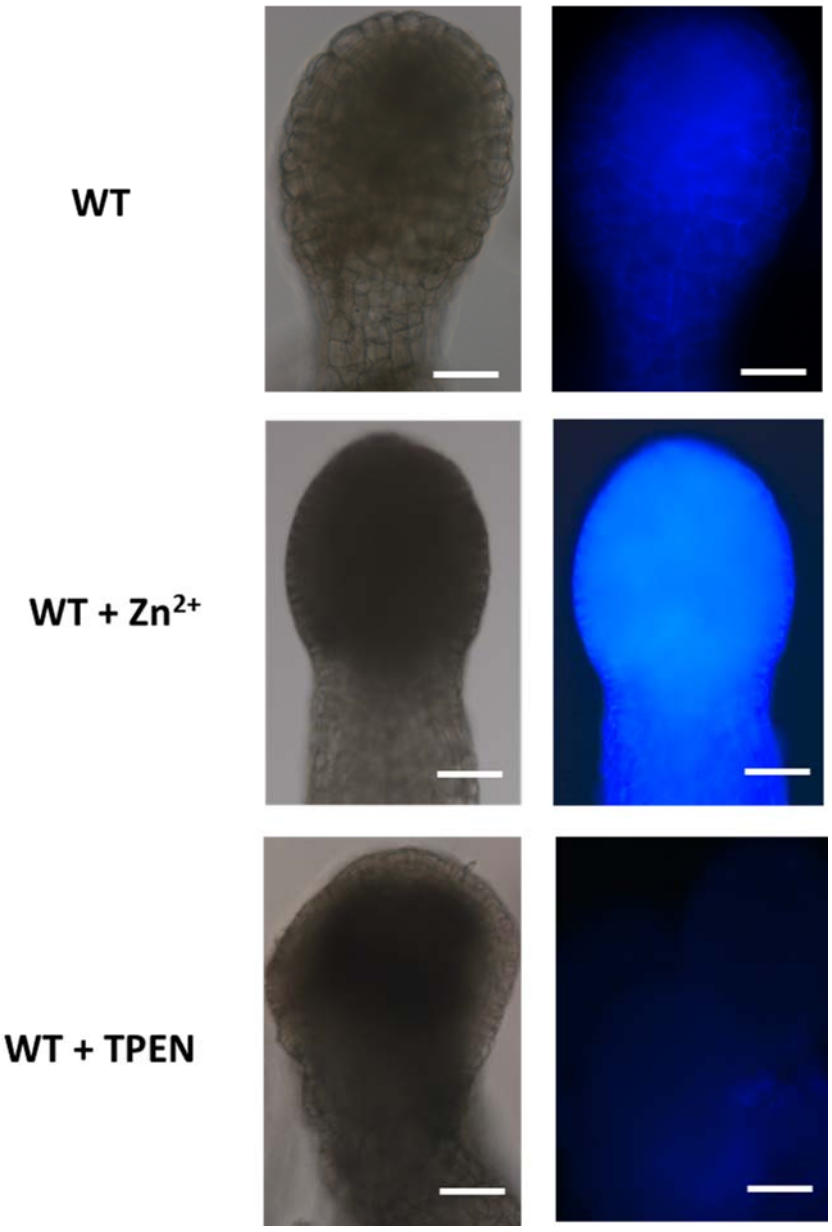


Fig. S3.3 Detection of Zn^{2+} in maize embryos by Zinquin. Localization of Zn^{2+} in WT developing embryos cultured for 7 days in the P medium supplemented with 300 µM $ZnSO_4$ (Zn^{2+}) or with 5 µM of the Zn^{2+} chelator TPEN. See material and methods for details. Scale bars = 30 µm.

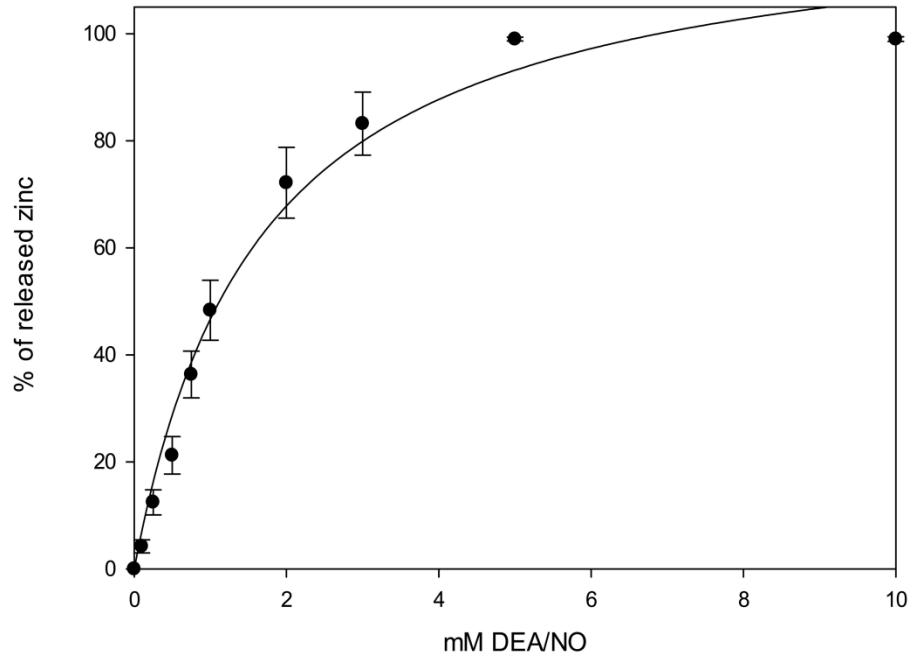


Fig. S3.4 Effects of increasing concentrations of the NO donor (DEA/NO) on the release of Zn^{2+} from a constant concentration of recombinant ZmMT4 protein (see Table 3.1). Values ± SE are means of three biological replicates.

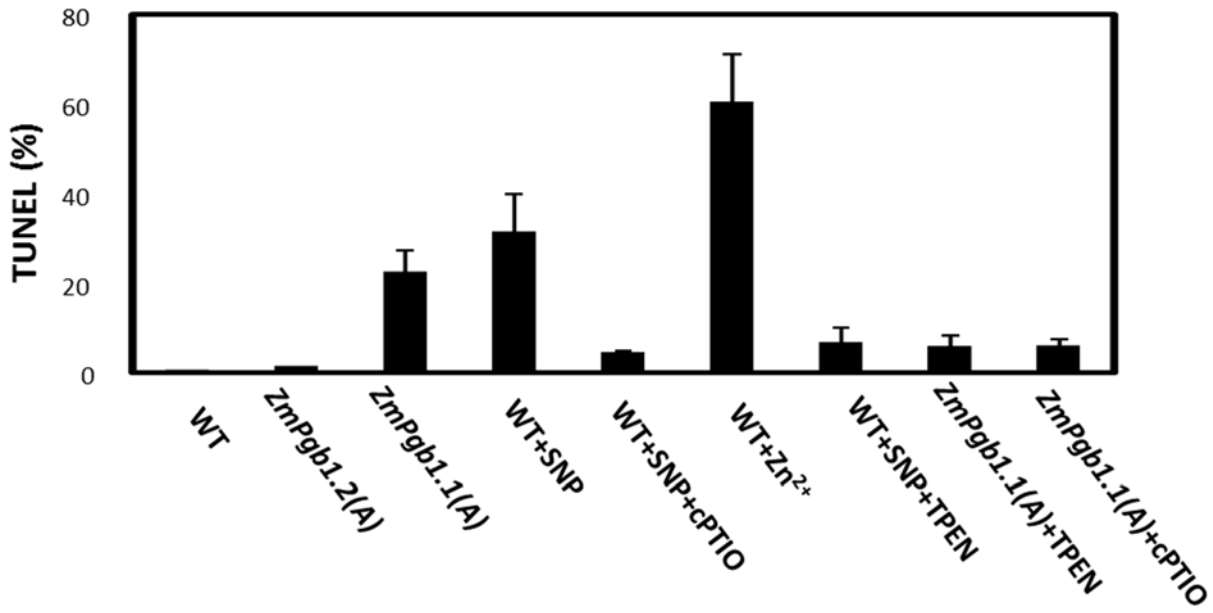


Fig. S3.5 Percentage of TUNEL-positive cells in immature (day 7, P medium) embryos cultured in environments with altered levels of NO and Zn^{2+}.

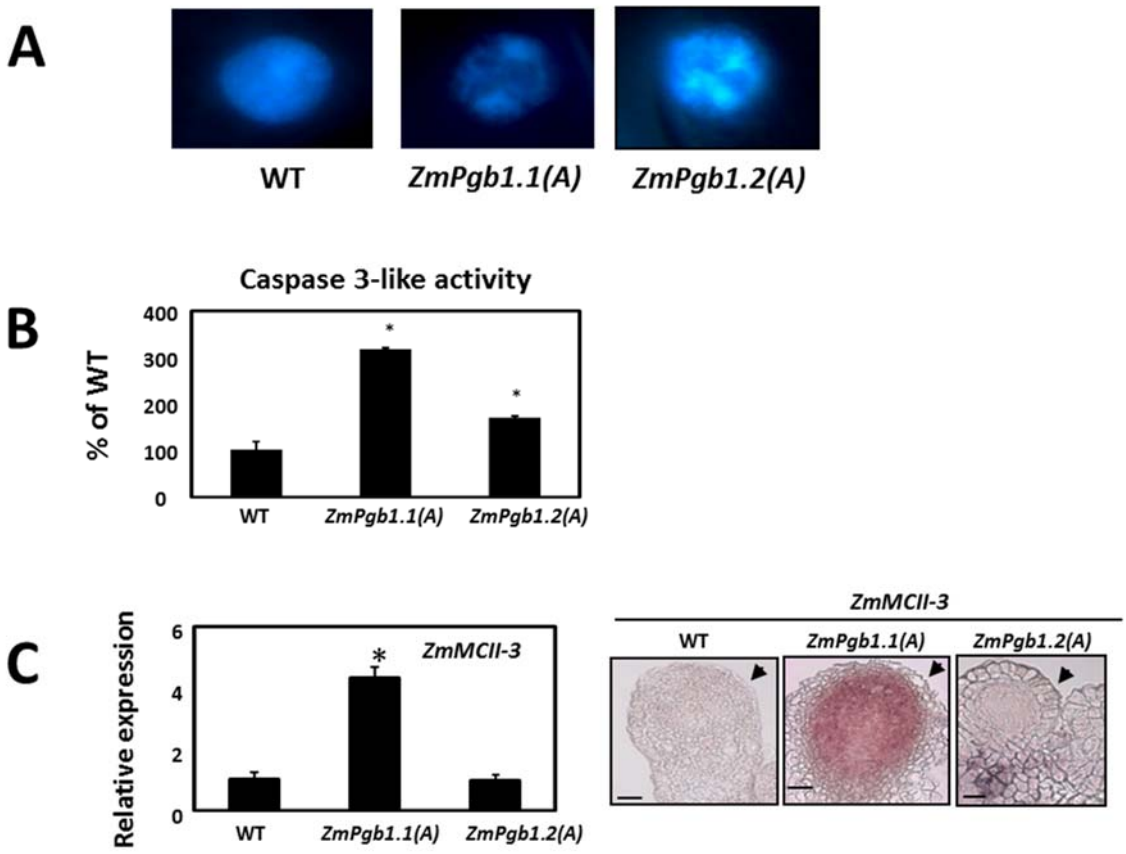


Fig. S3.6 Cytological and molecular hallmarks of PCD in *ZmPgb*-suppressing embryos. (A) Nuclear fragmentation in cells targeted by PCD in immature (day 7, P medium) embryos. (B) Caspase 3- like activity in immature (day 7, P medium) embryos. * Statistically significant difference ($p \leqslant 0.05$) from WT set at 100%. (C) Expression levels of metacaspase II-3 in the transgenic lines. Values are means ± SE of three biological replicates and are normalized to the WT value set at 1. * Statistically significant difference ($p \leqslant 0.05$) from WT. Localization of metacaspase II-3 in the WT, *ZmPgb1.1(A)*, and *ZmPgb1.2(A)* lines. Arrows indicate the apical domain of the embryos. Note that the metacapase-expressing domains coincide with those suppressing *Pgbs* (Figure 3.1C) and accumulating NO and Zn^{2+} (Figure 3.1E). Scale bars = 30

μm. Metacaspase expression and localization studies were conducted in immature (day 7, P medium) embryos.

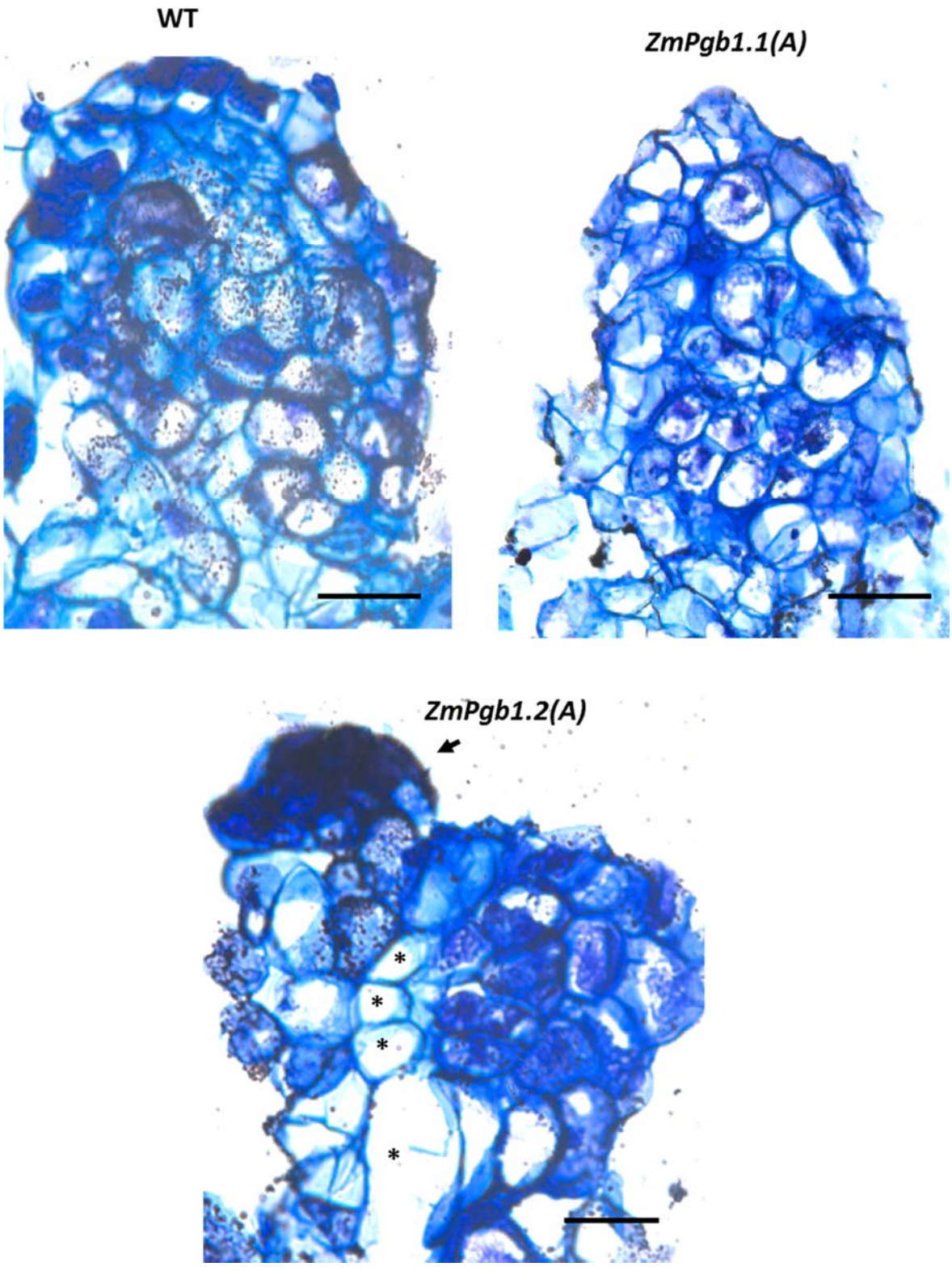

WT
ZmPgb1.1(A)
ZmPgb1.2(A)

Fig. S3.7 Localization of reduced ascorbic acid (ASC) in immature (day 7, P medium) maize embryos produced by the WT line and *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines. Small silver grains denote accumulation of ASC. * show cells devoid of silver grains. Arrow indicates a developing embryo originating from the surface of the embryogenic tissue. Scale bars = 15 μm.

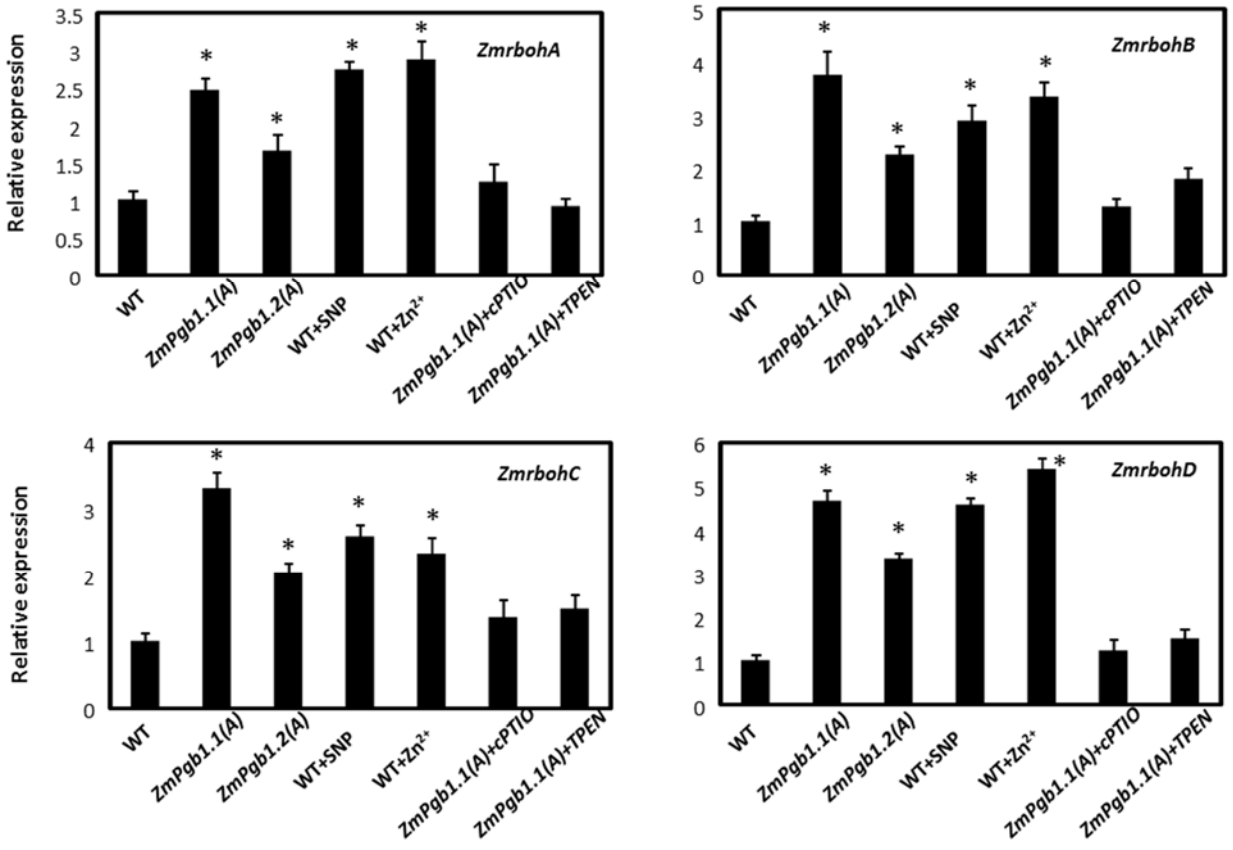


Fig. S3.8 Expression level of the four respiratory burst oxidase homologs (*ZmrbohA-D*) in immature embryos (day 7, P medium) produced from the WT and *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines. The embryos were cultured with different levels of NO and Zn^{2+} using compounds from Figure 3. Values are means ± SE of three independent biological replicates and are normalized to the WT set at 1. * Statistically significant difference ($p \leqslant 0.05$) from WT.

Table S3.1 List of primers used in PCD studies

NO.	Name	Sequence
1	BamHI-ZmPgb1.1-F	GGATCCATGGCACTCGCGGAGGCCGAC
2	BamHI-ZmPgb1.1-R	GGATCCCTAGGCATCGGGCTTCATCTC
3	BamHI-ZmPgb1.2-F	GGATCCATGGGGTTCAGTGAGGCACAG
4	BamHI-ZmPgb1.2-R	GGATCCCTAGGAAGCATCAACAGCCGC
5	KpnI-Ubiquitin-F	GGTACCCTGCAGTGCAGCGTGACCCGGTCG
6	KpnI-Ubiquitin-R	GGTACCCTGCAGAAGTAACACCAAACAAC
7	SacI-Nos-F	GAGCTCGATCGTTCAAACATTTGGCAAT
8	SacI-Nos-R	GAGCTCCCCGATCTAGTAACATAGATGA
9	PZmPgb1.1-F	TGCCAGGCGTTGTCGGAA
10	PZmPgb1.1-R	CTGCTGTCTGCTGATCGGGCT
11	PZmPgb1.2-F	ACGTGGACAACCACCTCTTC
12	PZmPgb1.2-R	CTTGTCTAGCTACTTCTCAGCGCTTC
13	attB1-PZmPgb1.1-F	AAAAAAGCAGGCTTCTGCCAGGCGTTGTCGGAA
14	attB2-PZmPgb1.1-R	CAAGAAAGCTGGGTTCTGCTGTCTGCTGATCGGGCT
15	attB1-PZmPgb1.2-F	AAAAAAGCAGGCTTCACGTGGACAACCACCTCTTC
16	attB2-PZmPgb1.2-R	CAAGAAAGCTGGGTTCTTGTCTAGCTACTTCTCAGCGCTTC
17	attB1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCT
18	attB2-R	GGGGACCACTTTGTACAAGAAAGCTGGGT
19	AtKOD-F	TATGTGGTGGCTAGTTGGACTTACA
20	AtKOD-R	AGCTTTACTTAGAGATGACAGAGACGCT
21	SpeI-AtKOD-F	CAGACTAGTTATGTGGTGGCTAGTTGGACTTACA
22	puc19-M13-R	CAGGAAACAGCTATGAC
23	attB1-AtKOD-F	AAAAAAGCAGGCTTCTATGTGGTGGCTAGTTGGACTTACA
24	attB2-AtKOD-R	CAAGAAAGCTGGGTTAGCTTTACTTAGAGATGACAGAGACGCT
25	RT-ZmPgb1.1-F	ATGGCACTCGCGGAGGCCGAC
26	RT-ZmPgb1.1-R	AGCGGAGGCCCAGGTTGGCGG
27	RT-ZmPgb1.2-F	CCGAGGAGCAGACGAAGAACG
28	RT-ZmPgb1.2-R	CTAGGAAGCATCAACAGCCGC
29	Actin1-F	GATGGTCAGGTCATCACCATTG
30	Actin1-R	AACAAGGGATGGTTGGAACAGC
31	Actin1-HEX	AAGGTTCAGGTGCCCCGAGG
32	AtKOD-FAM	CAGTTGAGTTGATCCATCTTTGCGC
33	ZmMT4-F	ATGGGGTGCGACGACAAG
34	ZmMT4-R	TTAGGCCGAAGCACAGGATGCGCA
35	MT4_fw	CGGGATCGAGGGAAGGATTTCAGGGTGCGACGACAAG
36	MT4_rev	AATTACCTGCAGGGAATTCTTAGGCCGAAGCACAGGATGCGCA
37	ZmrbohA-F	CACACGTGACCTGCGACTTC

38	ZmrbohA-R	CCCCAAGGTGGCCATGA
39	ZmrbohB-F	GGCCAGTACTTCGGTGAAACA
40	ZmrbohB-R	ATTACACCAGTGATGCCTTCCA
41	ZmrbohC-F	TTCTCTTGCCTGTATGCCGC
42	ZmrbohC-R	CTTTCGTATTCCGCAGCCA
43	ZmrbohD-F	CCGGCTGCAGACGTTCTT
44	ZmrbohD-R	CCTGATCCGTGATCTTCGAAA
45	ZmMCII-3-F	GACATTGATGTGGGTTCTATCCGG
46	ZmMCII-3-R	ACTGCCTTGCTCACCTGACTGG

4 CHAPTER TWO: SUPPRESSION OF PHYTOGLOBINS INFLUENCES CELLULAR AUXIN LOCALIZATION PATTERN IN MAIZE SOMATIC EMBRYOGENESIS

4.1 Abstract

Suppression of maize phytoglobin *ZmPgb1.1* significantly decreased mature embryo production while suppression of maize phytoglobin *ZmPgb1.2* significantly increased mature embryo production. This was attributable to indole-3-acetic acid (IAA) localization as there was no change in the total IAA content of either line relative to the wild-type (WT) control. Unlike in the *ZmPgb1.1 (A)* line (the *ZmPgb1.1* anti-sense line), where an obvious decrease or depletion of IAA accumulation was observed in the endoderm (but not protoderm) cells of the apical domain, the decrease of IAA accumulation occurred only in the basal or anchoring cells in the *ZmPgb1.2 (A)* line (the *ZmPgb1.2* anti-sense line). The interference of *Pgbs* in auxin patterning was also evident in auxin biosynthesis genes, especially the IPA pathway where *VT2* and *ZmAO1* responded differently to the suppression of a specific *Pgb*. These alterations of localized IAA in the embryogenic domains could be ascribed to unusual polar auxin transport (PAT) triggered by suppression of phytoglobins, as there were visible differences in the localization of ZmPIN1, an important auxin transporter responsible for endogenous auxin distribution. Microscopic studies indicated that, in the WT line, ZmPIN1 was mainly localized in the basal cells. However, ZmPIN1 was only localized in the protoderm cells of apical domain, but not the endoderm cells or the basal cells in the *ZmPgb1.1 (A)* line, while ZmPIN1 was localized throughout most of the apical domain but not the basal cells in the *ZmPgb1.2 (A)* line. In addition, the requirement of PAT for proper embryogenic development was verified by application of auxin transport inhibitor in the lines. We conclude that endogenous auxin localization mediated by auxin transport is involved in the phytoglobin-regulated embryogenesis.

4.2 Introduction

The initiation of the plant life cycle is primarily achieved via fertilization resulting in the generation of the zygote, which through precise patterns of cell division, differentiation and death forms a zygotic embryo (Yang and Zhang, 2010; Lau et al., 2012). As an alternative process, plant cells are inbuilt with the plasticity to regenerate tissues, organs or the whole plant body by switching their developmental programs. Specifically, cells can de-differentiate and embark in novel differentiation routes. This process is best exemplified by somatic embryogenesis, the ability of somatic cells to generate viable embryos morphologically similar to their zygotic counterparts (Thorpe and Stasolla, 2001). Somatic embryogenesis, one of the morphogenic processes where the fulfillment of cell totipotency is manifested, is largely dependent upon the regulatory signals of plant hormones, especially auxin (Thorpe and Stasolla, 2001). In culture, exogenously supplied auxin, mainly 2,4-dichlorophenoxyacetic acid (2,4-D), represents the inductive signal that triggers the de-differentiation of the somatic cells (Raghavan, 2004). In many systems, including Arabidopsis, applications of 2,4-D is only necessary for the induction phase of embryogenesis, but not for the later stages of embryo development, which is triggered by the removal of (or a decrease in) 2,4-D (Bassuner et al., 2007; Garrocho-Villegas et al., 2012).

In plants, *de novo* synthesis of auxin has been the subject of many investigations resulting in the identification of several pathways (Zhao, 2014). Indole-3-acetic acid (IAA), the well characterized natural auxin, is mainly synthesized through the tryptophan (Trp) dependent pathways (Zhao, 2010). Recently, a simple but complete Trp-dependent IAA biosynthesis pathway has been uncovered in Arabidopsis, in which TAAs (Trp aminotransferases) convert Trp into indole-3-pyruvate (IPA), and IPA is further utilized to produce IAA in a reaction

catalyzed by YUCs (YUCCA flavin-containing monooxygenases) (Zhao, 2012; Zhao, 2014). This two-step auxin biosynthetic pathway has been shown to be highly conserved in both dicots and monocots (Gallavotti et al., 2008; Phillips et al., 2011; Zhao, 2012; Zhao, 2014). In maize for example, the conversion of Trp into IPA is achieved by *VT2* (*vanishing tassel 2*), a co-ortholog of *TAA1/TAR1/TAR2* (Phillips et al., 2011). Furthermore, *SPI1* (*sparse inflorescence 1*), a monocot-specific YUCCA-like gene, is also responsible for auxin biosynthesis and interacts with several auxin transport genes (Gallavotti et al., 2008). Knockout mutants of these genes reduce auxin levels and disturb the correct patterning for normal embryo and inflorescence development (Möller and Weijers, 2009; Phillips et al., 2011).

Auxin maxima within the embryogenic tissue have been reported in several systems and the precise distribution of this hormone is mediated by several classes of auxin transporters including PIN1, the plasma membrane-located proteins that act as auxin efflux carriers (Prasad and Dhonukshe, 2013). In Arabidopsis embryogenesis, AtPIN1 is specifically expressed during the early stages and contributes to the polar auxin transport (PAT) that is required for the embryogenic cell specification (Möller and Weijers, 2009). In maize embryogenesis there is a tight correlation between the ZmPIN1-mediated PAT and the differentiation of embryo tissues resulting in the definition of the embryo organs (Forestan et al., 2010). The requirement of proper PIN expression during embryogenesis has been demonstrated by independent studies. Experimental manipulations of PIN1 expression and/or auxin distribution pattern by the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) severely compromise the embryogenic process both *in vivo* and *in vitro* (Hakman et al., 2009; Su et al., 2009; Elhiti and Stasolla, 2011).

Hemoglobins are a class of iron-containing proteins distributed universally across different kingdoms. In mammalian systems, hemoglobins act mainly as oxygen transporters or as oxygen

storage vehicles. Plant hemoglobins, termed phytoglobins (Pgbs), act mainly as nitric oxide (NO) scavengers (Kundu et al., 2003; Hill, 2012; Vázquez-Limón et al., 2012). From a phylogenetic point of view, phytoglobins can be divided into a group of symbiotic phytoglobins, termed leghemoglobins that are generally limited to plant species forming nitrogen-fixing, symbiotic relationships with microorganisms, and three classes of Pgbs (class 1-3) that are widely present in all plant species. Within the latter group, the first two classes (class 1 and 2) are found in dicots, while only class 1 exists in monocots, for example two copies of *Pgbs* (*ZmPgb1.1* and *ZmPgb1.2*) in maize (Smagghe et al., 2009; Rodríguez-Alonso and Arredondo-Peter, 2013). Pgbs modulate NO homeostasis in a wide range of developmental and stress-related processes (Hill, 2012). For instance, Pgbs may participate widely in abiotic and biotic stresses such as hypoxia, salt, cold and nutrient deficient conditions, as well as pathogen invasions (Hill, 2012). The accumulation of cellular NO in alfalfa root cultures under low oxygen conditions is affected by the expression level of *Pgb1* (Dordas et al., 2003). A similar result was also observed in Arabidopsis and barley where over-expression of *Pgb1* reduces NO emission under hypoxic stress (Perazzolli et al., 2004; Igamberdiev et al., 2006b).

Besides their involvement during stress conditions, Pgbs are also involved in several developmental processes, ranging from embryogenesis, seed germination and dormancy, to post-embryonic development, and *in vitro* organogenesis and embryogenesis (Hebelstrup et al., 2013). During development the action of Pgbs is also mediated by NO (Hebelstrup et al., 2006) and possibly by the NO interaction with several plant hormones. For instance, NO has been identified as a downstream component of auxin signaling during the formation and maintenance of the root apical meristem and adventitious rooting (Lanteri et al., 2008; Fernández-Marcos et al., 2011; Terrile et al., 2012), In addition, through the modulation of NO homeostasis, Pgbs are also active

participants in modulating cytokinin-mediated plant organogenesis (Ross et al., 2004; Wang et al., 2011), as well as ethylene production in maize suspension cultures (Manac'h-Little et al., 2005).

A few studies, besides those presented in Chapter 1, have demostrated the role of NO and Pgbs during *in vitro* plant embryogenesis. Formation of embryogenic tissue in alfalfa and barley is under the control of NO, which promotes cell division and proliferation (Otvös et al., 2005; Rodríguez-Serrano et al., 2012). Similarly, an increase in NO level as a result of suppression of *AtPgb2* promotes embryogenic competence and embryo production in Arabidopsis (Elhiti et al., 2013). The authors further demonstrated that the increased embryo formation in the *Pgb2*-suppressing lines was due to the elevation of NO in specific regions of the explants resulting in the down-regulation of the transcription factor MYC2, a repressor of auxin biosynthesis. The increased auxin synthesis in *Pgb2* suppressing cells was sufficient to promote embryogenesis (Elhiti et al., 2013). From this study it emerges that the relationship between NO, Pgbs, and auxin plays a central role during embryogenesis.

In this context, to further understand the interaction between auxin and Pgbs during somatic embryogenesis, auxin level and localization patterns, as well as the expression of genes involved in auxin synthesis, transport and response were analysed in immature maize embryos generated by the anti-sense *ZmPgb1.1 (A)* and *ZmPgb1.2 (A)* lines.

4.3 Results

4.3.1 Endogenous IAA localization is affected by suppression of phytoglobins

Formation of auxin maxima during embryogenesis is required for the acquisition of polarity, proper histo-differentiation, and formation of the apical meristems (Su et al., 2009; Prasad and

Dhonukshe, 2013). Suppression of maize *ZmPgb1.1* or *ZmPgb1.2* produces different outcomes on the number of somatic embryos (see Chapter 1). While the down-regulation of *ZmPgb1.1* compromises embryogenesis, suppression of *ZmPgb1.2* increases the number of fully developed embryos (Fig. 4.1). To test whether this different behaviour was correlated to endogenous IAA alterations, the localization pattern of IAA was studied in WT, *ZmPgb1.1 (A)* and *ZmPgb1.2 (A)* lines at day 7 on proliferation medium (Fig. 3.1 and Fig. 4.2). Compared with WT tissue, where IAA was detected throughout the immature somatic embryos, the IAA signal was reduced in all embryonic cells produced by the *ZmPgb1.1 (A)* line. In the *ZmPgb1.1 (A)* embryos IAA was mainly detected within the apical cells, i.e. the protoderm layer. Embryos produced by the *ZmPgb1.2 (A)* line exhibited a general IAA signal pattern similar to that observed in WT embryos, however, cells connecting embryos together were depleted of IAA (red inset in Fig. 4.2.4).

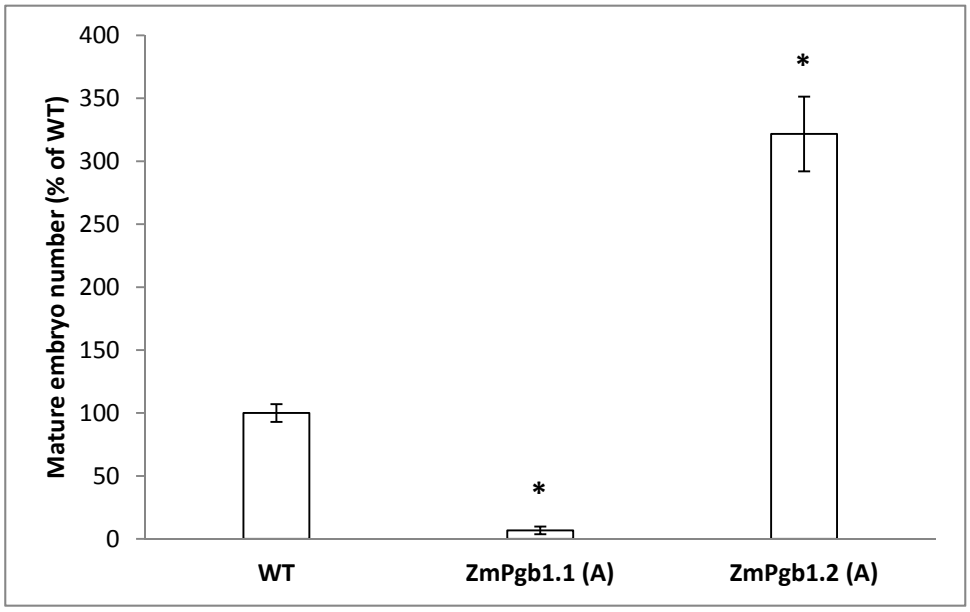


Fig. 4.1 Suppression of phytoglobins affects somatic embryogenesis in maize. Percentage of mature (day 21, D medium) somatic embryos obtained from antisense lines suppressing *ZmPgb1.1* [*ZmPgb1.1 (A)*] or *ZmPgb1.2* [*ZmPgb1.2 (A)*]. Values (WT set at 100%) are means ± SE of four independent biological replicates. Stars indicate values which are statistically significant difference ($p \leqslant 0.05$) from WT.

4.3.2 Nitric oxide (NO) and zinc (Zn^{2+}) mediate the phytoglobin-regulation of IAA localization

In WT tissue, *ZmPgb1.1* and *ZmPgb1.2* are expressed in different domains of the embryogenic cells (Fig. 3.1), and suppression of either of the two *Pgbs* resulted in the accumulation of NO, Zn^{2+}, and induction of PCD (Fig. 3.1. and 3.3). To test whether the Pgb-regulation of IAA localization was mediated by NO or Zn^{2+}, a pharmacological approach was used to manipulate both compounds. Nitric oxide level was experimentally increased with SNP and decreased with cPTIO, while the level of Zn^{2+} was altered using the chelator TPEN or applications of $ZnSO_4$. Inclusions of either SNP or Zn^{2+} reduced the IAA signal in WT embryos (Fig. 4.2), while a reduction of NO by cPTIO or Zn^{2+} by TPEN increased the IAA signal in the *ZmPgb1.1 (A)* embryos.

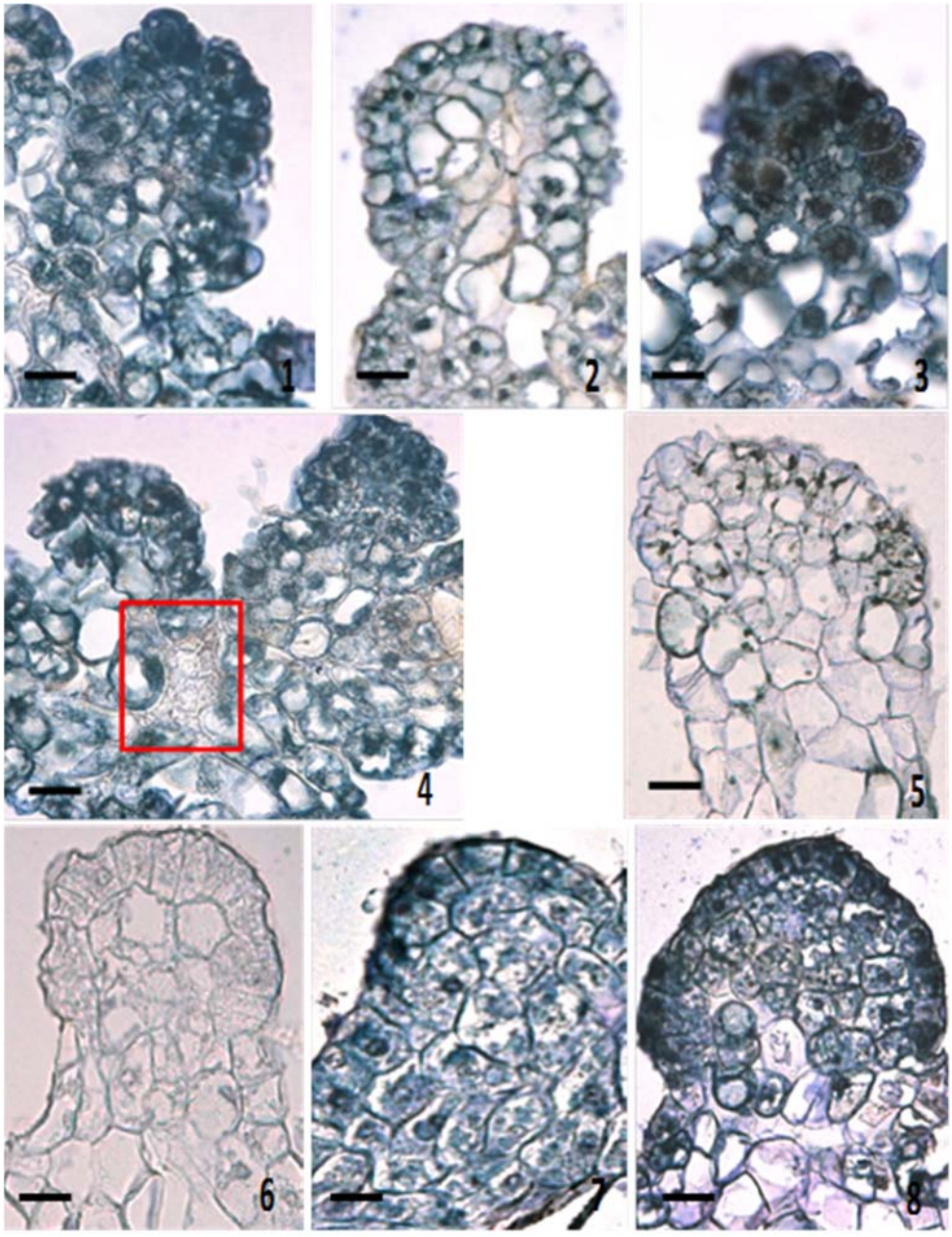


Fig. 4.2 Localization of endogenous IAA in the WT, *ZmPgb1.1 (A)* and *ZmPgb1.2 (A)* lines at day 7 on proliferation medium. 1, WT. **2,** *ZmPgb1.1 (A)*. **3** and **4,** *ZmPgb1.2 (A)*. The red inset indicates the IAA-depleted cells connecting embryos together. **5**. WT+SNP. **6,** WT+Zn^{2+}. **7,** *ZmPgb1.1 (A)*+cPTIO. **8,** *ZmPgb1.1 (A)*+TPEN. Scale bars = 30 μm.

4.3.3 Alteration of Pgbs does not affect the total level of IAA in the embryogenic tissue

To investigate if the difference in IAA localization patterns was the result of quantitative alterations of IAA levels, free IAA was quantified in the WT and the two antisense lines at day 3 and 7 on proliferation medium. The level of IAA in the embryogenic tissue ranged from 1-1.5 ng/g dry weight (DW) and no significant differences were observed among lines (Fig. 4.3).

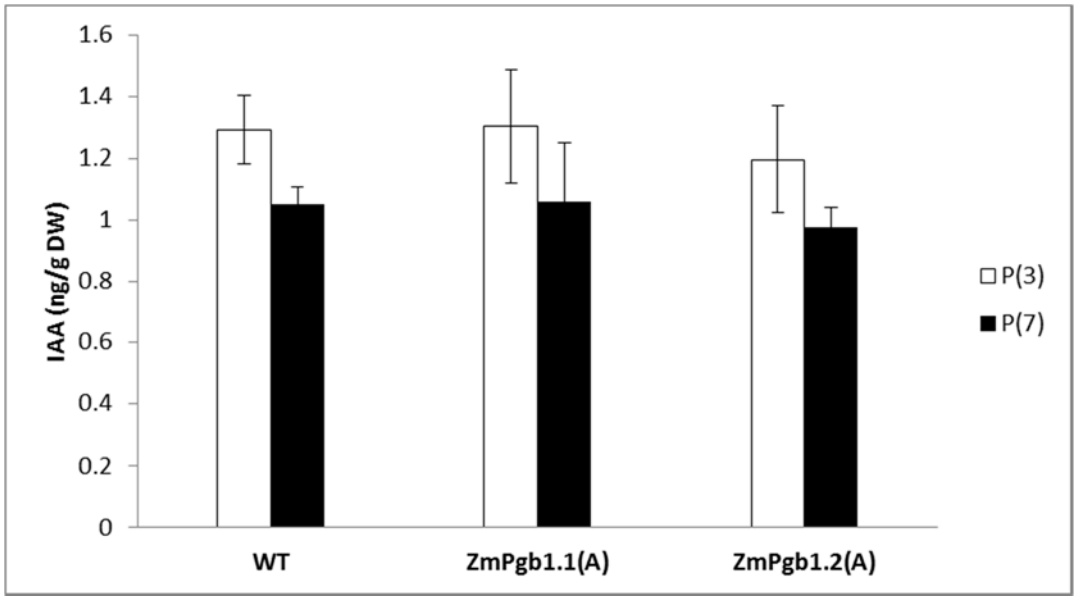


Fig. 4.3 Measurement of free IAA levels in WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* embryogenic tissue at day 3 and 7 on proliferation (P) medium. Values are means ± SE of three independent biological replicates.

4.3.4 Pgbs affect the transcription of genes involved in auxin biosynthesis

The auxin biosynthetic pathways in maize have been presented in previous studies and they include four Trp-dependent pathways and one Trp-independent pathway (Wright et al., 1992; Phillips et al., 2011). As illustrated in Fig. 4.4, the Trp-dependent pathways are (1) The indole-3-acetamide (IAM) pathway, (2) the tryptamine (TAM) pathway, (3) the indole-3-acetaldoxime (IAOx) pathway, and (4) the indole-3-pyruvic acid (IPA) pathway. On the other hand, the Trp-independent pathway is mainly regulated by two tryptophan synthase beta subunits (*TSB1* and *TSB2*). A total of eight genes encoding enzymes catalyzing key reactions in the pathways (Table 4.1) were analyzed by quantitative RT-PCR at day 3 and 7 in proliferation medium.

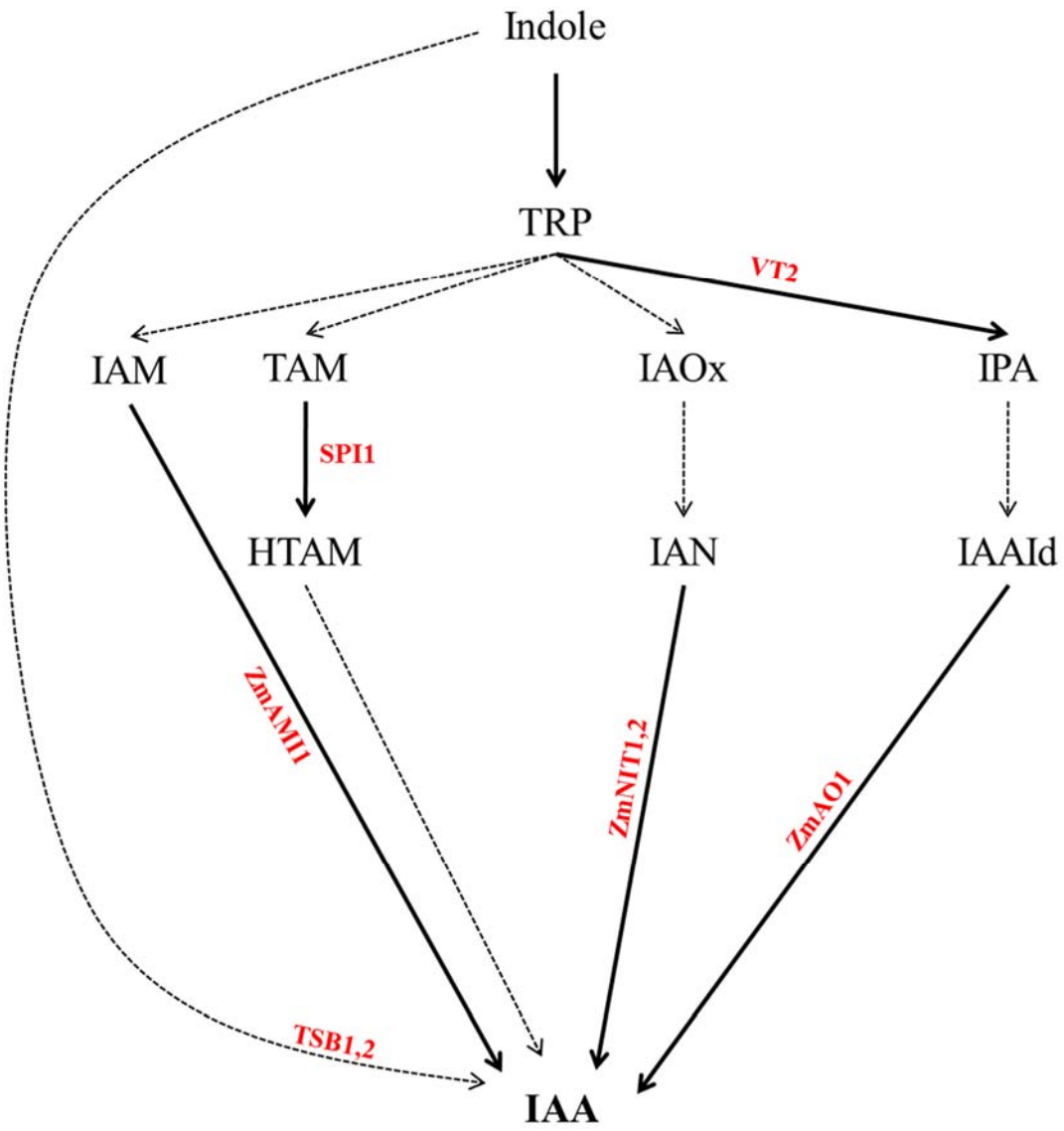


Fig. 4.4 Auxin biosynthesis pathways in maize. Dotted lines indicate poorly characterized steps. Solid lines in bold indicate steps for which genes encoding enzymes catalyzing the reactions have been identified in maize.

Table 4.1 Key genes involved in the auxin biosynthesis pathways of maize

Gene	Function	Reference
ZmAMI1	Orthologous to *AMIDASE 1* in Arabidopsis, which converts IAM to IAA	Pollmann et al., 2003; Lehmann et al., 2010
SPI1	*SPARSE INFLORESCENCE 1*, a monocot-specific YUC gene family member, which converts TAM to HTAM	Gallavotti et al., 2008
ZmNIT1, 2	Orthologous to *NITRILASE1, 2* in Arabidopsis, which possibly converts HTAM to IAA	Park et al., 2003
VT2	*VANISHING TASSEL 2*, co-orthologous to *TAA1/TAR1/TAR2* in Arabidopsis, which converts Typ to IPA	Stepanova et al., 2008; Tao et al., 2008; Yamada et al., 2009; Phillips et al., 2011
ZmAO1	Orthologous to *ALDEHYDE OXIDASE 1* in Arabidopsis, which converts IAAld (N-hydroxyl tryptamine) to IAA	Sekimoto et al., 1997, 1998
TSB1, 2	Tryptophan synthase beta subunits	Last et al., 1991; Wright et al., 1992

In the Trp-independent pathway, two tryptophan synthase beta subunits *TSB1* and *TSB2* were analyzed and their expression level increased significantly in the *ZmPgb1.1(A)* line at day 3 (Fig. 4.5). In the *ZmPgb1.2(A)* line *TSB1* was induced at day 3. No significant differences were detected in the expression of both genes among the three lines at day 7 (Fig. 4.5).

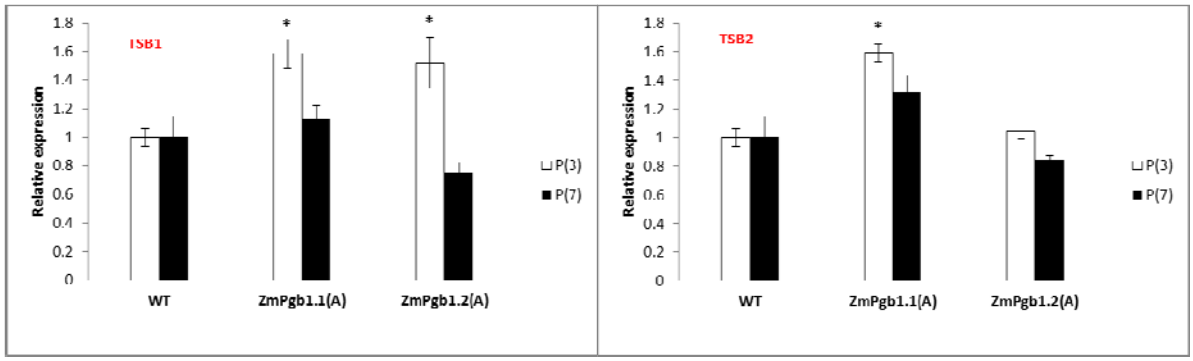


Fig. 4.5 The expression levels of *TSB1* and *TSB2* in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines. Each micro chart represents the expression level of the corresponding gene at day 3 (white bar) and 7 (black bar) on proliferation medium. Values are means ± SE of three independent biological replicates and are normalized to the value of respective WT set at 1. * Statistically significant difference ($p \leqslant 0.05$) from respective WT.

In the indole-3-acetamide (IAM) pathway, the expression level of *AMIDASE* (*ZmAMI1*) was significantly reduced only in the *ZmPgb1.2(A)* line on both day 3 and 7 (Fig. 4.6). In the tryptamine (TAM) pathway, the transcript level of *SPARSE INFLORESCENCE 1* (*SPI1*) was significantly reduced only in the *ZmPgb1.2(A)* line at day 3 (Fig. 4.6). In the indole-3-acetaldoxime (IAOx) pathway, nitrilase *ZmNIT1* was not affected by the suppression of either *Pgb* genes (Fig. 4.6), while the expression of *ZmNIT2* was significantly reduced in the *ZmPgb1.2(A)* line at day 3 and in the *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines at day 7 (Fig. 4.6).

In the indole-3-pyruvic acid (IPA) pathway, *VANISHING TASSEL 2* (*VT2*), a maize co-ortholog of *TAA1/TAR1/TAR2*, was significantly repressed in the *ZmPgb1.1(A)* line at both day 3 and 7 (Fig. 4.7). The expression of *ALDEHYDE OXIDASE 1* (*ZmAO1*) was significantly increased in *ZmPgb1.2(A)* at both day 3 and 7 (Fig. 4.7).

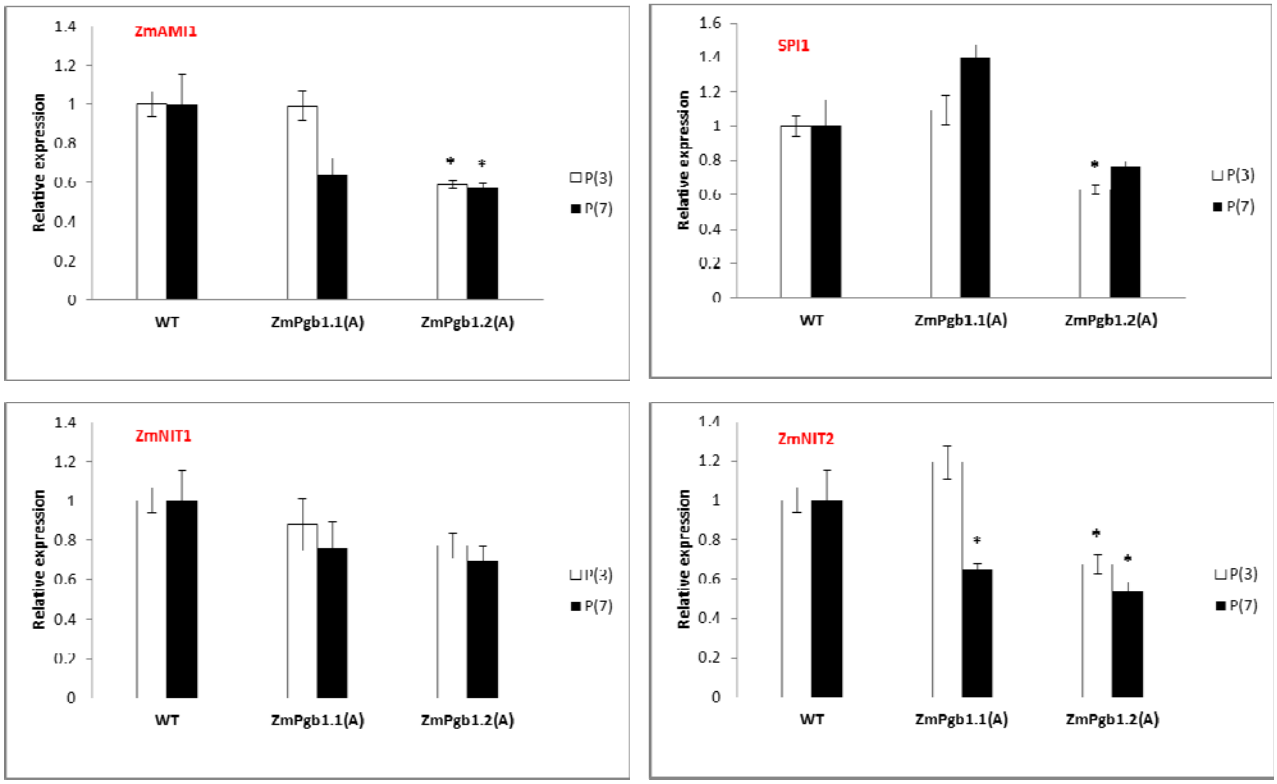


Fig. 4.6 The expression levels of *ZmAMI1*, *SPI1*, and *ZmNIT1,2* in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines. Each micro chart represents the expression level of the corresponding gene at day 3 (white bar) and 7 (black bar) on proliferation medium. Values are means ± SE of three independent biological replicates and are normalized to the value of respective WT set at 1. * Statistically significant difference ($p \leqslant 0.05$) from respective WT.

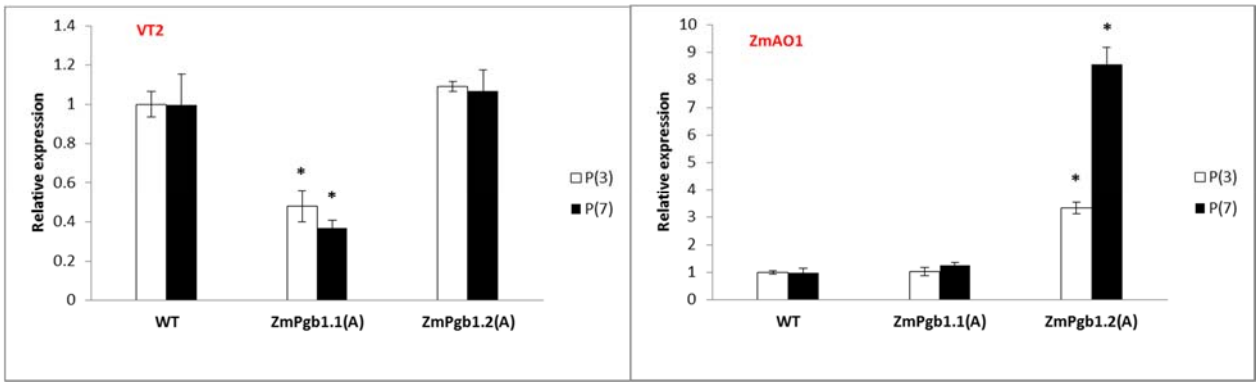


Fig. 4.7 The expression levels of *VT2* and *ZmAO1* in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines. Each micro chart represents the expression level of the corresponding gene at day 3 (white bar) and 7 (black bar) on proliferation medium. Values are means ± SE of three independent biological replicates and are normalized to the value of respective WT set at 1. * Statistically significant difference ($p \leqslant 0.05$) from respective WT.

4.3.5 Localization patterns of *VT2* and *ZmAO1*

Among all the tested biosynthetic genes, the expression of *VT2* and *ZmAO1* showed the most significant difference between the *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines at both day 3 and 7. The expression of *VT2* was repressed in the *ZmPgb1.1(A)* line, while that of *ZmAO1* was induced in the *ZmPgb1.2(A)* line (Fig. 4.7). The localization patterns of *VT2* and *ZmAO1* were further investigated by RNA in situ hybridization. Compared to WT embryos where the expression of *VT2* was localized in many embryonic cells (Fig. 4.8), the *VT2* signal in the *ZmPgb1.1(A)* embryos was almost completely absent (Fig. 4.8). Different localization patterns of *VT2* were observed in the embryos produced by the *ZmPgb1.2(A)* line. While in some embryos the signal was localized in the central domains, in others it was restricted to a few cells connecting the embryos to the embryogenic tissue or in isolated cells of the embryos (Fig. 4.8).

The localization pattern of *ZmAO1* was different from that observed for *VT2*. The *ZmAO1* signal was very weak in WT embryos, limited to a few cells in the central domains of the *ZmPgb1.1(A)* embryos, and extended to the whole embryos produced by the *ZmPgb1.2(A)* line (Fig. 4.9).

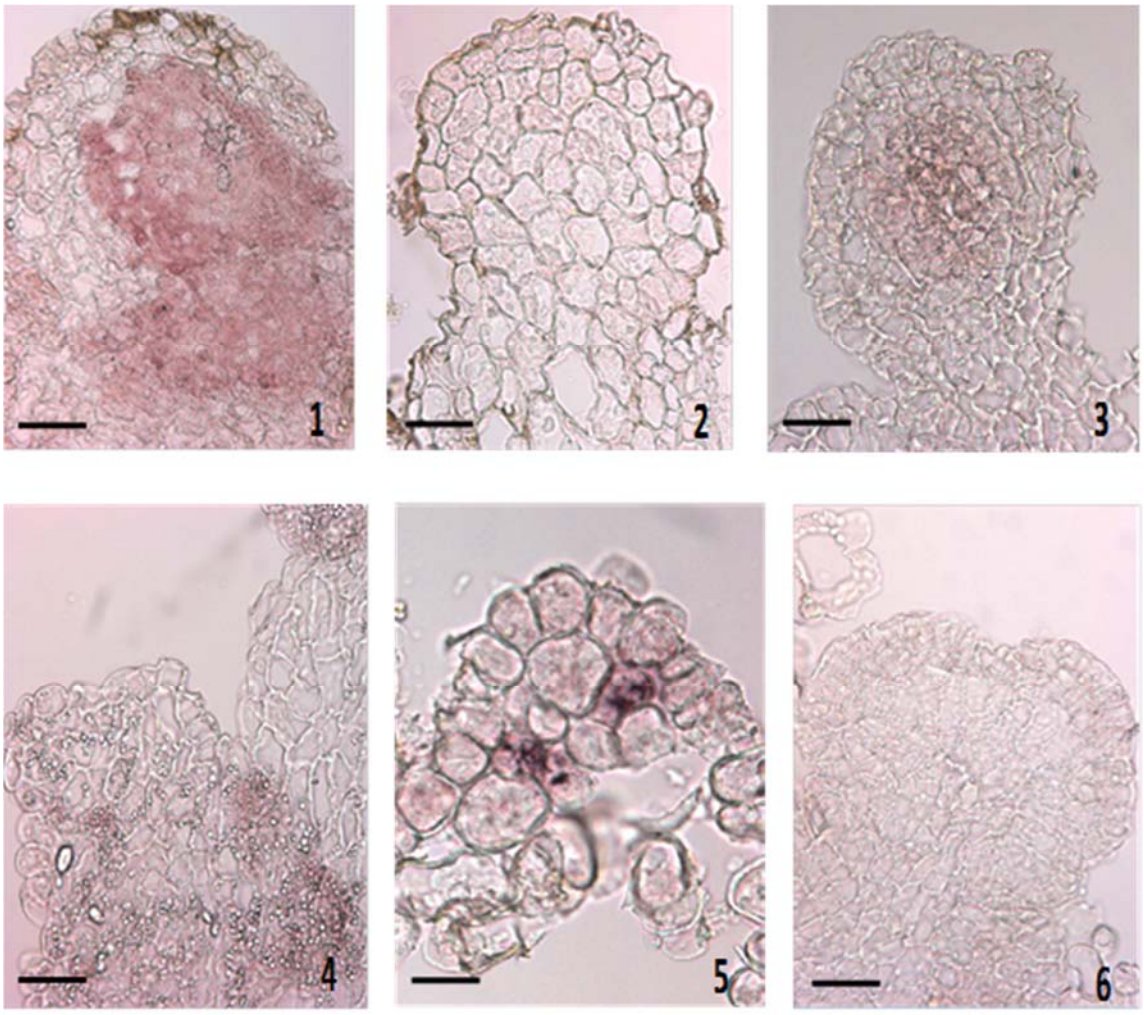


Fig. 4.8 RNA in situ hybridization of *VT2* in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines at day 7 on proliferation medium. *VT2* localization in the WT (**1**), *ZmPgb1.1(A)* (**2**), and *ZmPgb1.2(A)* (**3-5**) embryos. Sense probe was used to verify the validity of the signal (**6**). Scale bars = 30 μm.

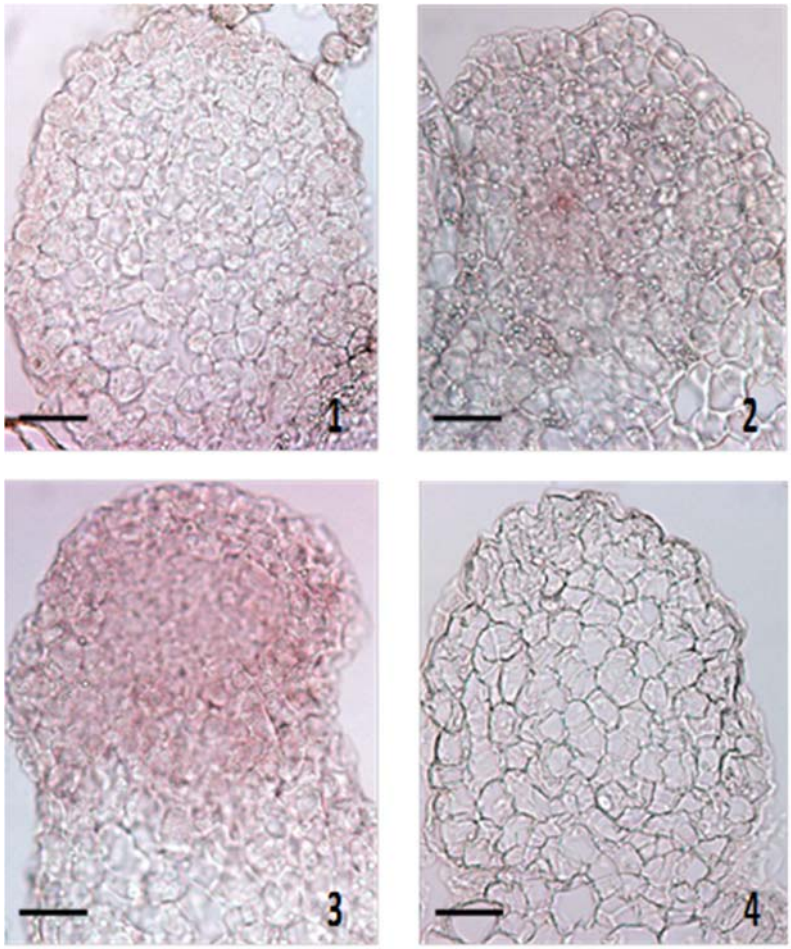


Fig. 4.9 RNA in situ hybridization of *ZmAO1* in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines at day 7 on proliferation medium. *ZmAO1* localization in the WT (**1**), *ZmPgb1.1(A)* (**2**), and *ZmPgb1.2(A)* (**3**) embryos. Sense probe was used to verify the validity of the signal (**4**). Scale bars = 30 µm.

4.3.6 Pgbs affects the expression pattern of IAA transporters

The PIN proteins are plasma membrane located proteins that act as auxin efflux carriers and determine the direction of auxin flow (Prasad and Dhonukshe, 2013). Among the PIN family members, PIN1 proteins have been identified as important mediators of the polar auxin transport (PAT), and have been shown to regulate cellular events during embryogenesis in Arabidopsis and maize (Forestan et al., 2010; Prasad and Dhonukshe, 2013). Transcript levels of three *ZmPIN1* orthologous genes, *ZmPIN1a*, *ZmPIN1b* and *ZmPIN1c* were measured by quantitative RT-PCR in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines at day 3 and 7 on proliferation medium (Fig. 4.10). No significant differences in *ZmPIN1a* expression levels were observed among lines. This was in contrast to the expression of *ZmPIN1b*, which compared to WT increased in the *ZmPgb1.1(A)* embryos at day 7, while it decreased in the *ZmPgb1.2(A)* embryos at both day 3 and 7. A significant suppression in expression level was observed for *ZmPIN1c* in both lines suppressing Pgbs.

Immunolocalization studies were performed to investigate the localization pattern of the ZmPIN1 protein at day 7 on proliferation medium. ZmPIN1 was mainly localized in the basal cells of WT embryos while in the *ZmPgb1.1(A)* embryos the protein was detected within the protoderm, in a pattern consistent with the accumulation of IAA in these embryos (compare Fig. 4.2 and Fig. 4.11). Many cells of the *ZmPgb1.2(A)* embryos expressed the ZmPIN1 proteins.

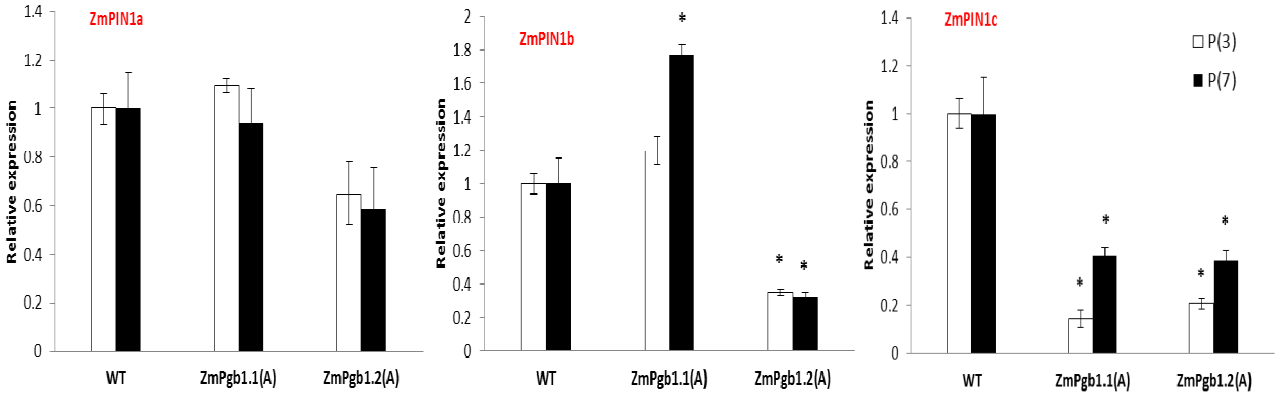


Fig. 4.10 Relative expression levels of *ZmPIN1s* in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines at day 3 and 7 on proliferation medium. Values are means ± SE of three independent biological replicates and are normalized to the value of respective WT set at 1. * Statistically significant difference ($p \leqslant 0.05$) from respective WT.

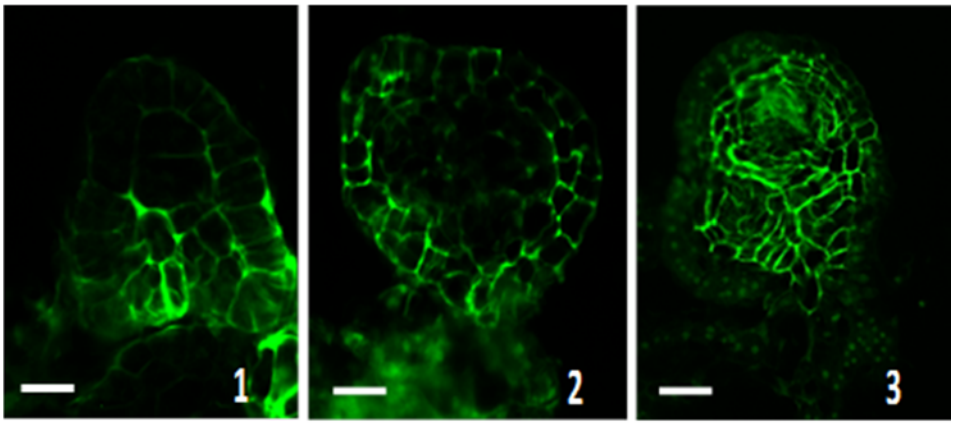


Fig. 4.11 Immunolocalization of the ZmPIN1 protein in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines at day 7 on proliferation medium. 1, WT**. 2,** *ZmPgb1.1(A)*. **3,** *ZmPgb1.2(A)*. Scale bars = 30 µm.

The patterning of PIN-mediated PAT is also regulated by the serine/threonine protein kinase PINOID (PID) (Kleine-Vehn et al., 2009). In maize, *BARREN INFLORESCENCE 2* (*BIF2*) is a co-ortholog of PID and interacts with the basic helix-loop-helix transcription factor *BARREN STALK 1* (*BA1*) (Skirpan t al., 2008). In the *ZmPgb1.2(A)* embryos the expression of *BA1* and *BIF2* increased at both days in culture while the expression of *BA1* decreased in the embryos produced by the *ZmPgb1.1(A)* lines (Fig. 4.12). In addition to the analysis on auxin efflux carriers, the auxin influx carrier *AUXIN-RESISTANT1* (*AUX1*) also contributes to auxin trafficking (Kleine-Vehn et al., 2006), and the expression level of *ZmAUX1* increased slightly in the *ZmPgb1.1(A)* embryos at day 7 (Fig. 4.12).

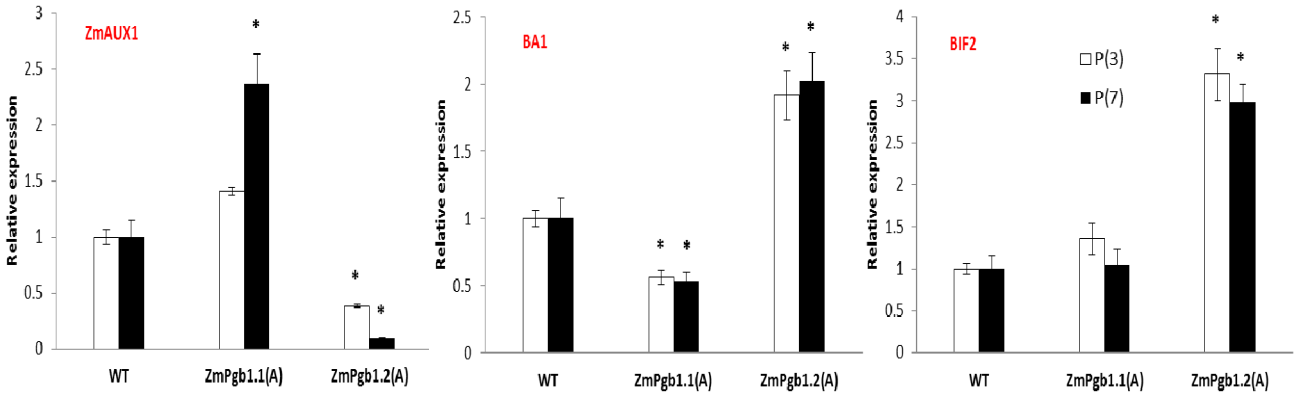


Fig. 4.12 Relative expression levels of auxin transport genes in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines at day 3 and 7 on proliferation medium. Values are means ± SE of three independent biological replicates and are normalized to the value of respective WT set at 1. * Statistically significant difference ($p \leqslant 0.05$) from respective WT.

4.3.7 Pgbs affect the expression pattern of auxin-responsive genes

In the early response auxin induces the rapid expression of a set of specific genes, the early auxin responsive genes which include *small auxin-upregulated RNAs* (*SAURs*) and members of the *Gretchen Hagen 3* (*GH3*) gene family (Hagen and Guilfoyle, 2002). In maize, the expression level of *ZmGH3* and *ZmSAUR2* correlate to IAA distribution (Nishimura et al., 2009; LeClere et al., 2010). During maize embryogenesis the expression of *ZmGH3* was not significantly altered by suppression of either *ZmPgb1.1* or *ZmPgb1.2*. This was in contrast to *ZmSAUR2* which was highly repressed in both transgenic lines at day 3 and 7 on proliferation medium (Fig. 4.13).

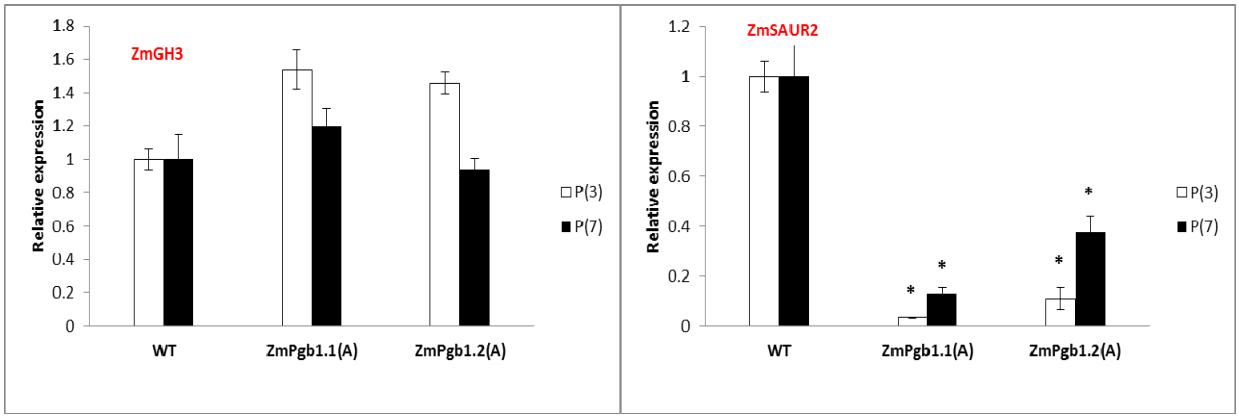


Fig. 4.13 Relative expression levels of two early auxin-responsive genes in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines at day 3 and 7 on proliferation medium. Values are means ± SE of three independent biological replicates and are normalized to the value of respective WT set at 1. * Statistically significant difference ($p \leqslant 0.05$) from respective WT.

4.3.8 Auxin and polar auxin transport are required for somatic embryogenesis

The acquisition of embryogenic competence requires auxin signaling which regulates the transition from somatic cells to embryogenic cells (Karami et al., 2009). During maize somatic embryogenesis the auxin 2,4-D is needed in both maintenance and proliferation media, while its removal from the maturation medium allows the immature embryos to develop further (Fig. 3.1). To test the effect of exogenous auxin on embryo production different levels of 2,4-D were applied in the proliferation medium of the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines. The number of somatic embryos produced by all the three lines was reduced by decreasing levels of 2,4-D and no embryos were produced by omitting 2,4-D (0 μM). However, compared to the WT line, the decreased percentage of embryo production in the *ZmPgb1.1(A)* line was consistently higher at any level of 2,4-D used. A similar, but less pronounced trend was also observed in the *ZmPgb1.2(A)* line (Fig. 4.14).

To further explore the requirement of auxin for somatic embryogenesis, tissue from the different lines was treated with the auxin transport inhibitor TIBA (2,3,5-triiodobenzoic acid) in the proliferation medium. Application of this inhibitor significantly reduced the embryo production in all the lines (Fig. 4.14). Inclusions of TIBA decreased the percentage of embryos by 55% in the WT line, 81% in the *ZmPgb1.1(A)* line, and 47% in the *ZmPgb1.2(A)* line (Fig. 4.14).

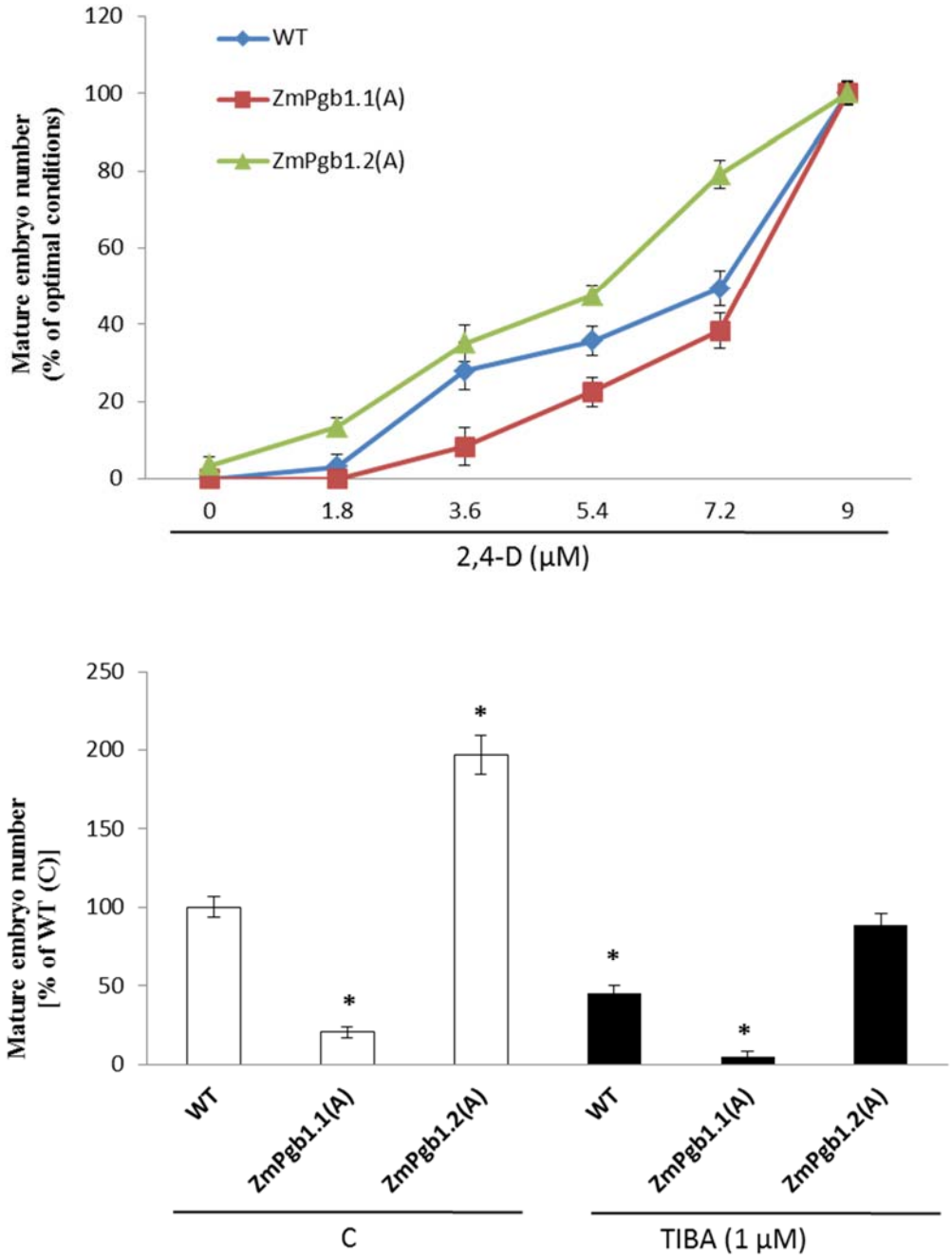


Fig. 4.14 Effects of 2,4-D (Top) and TIBA (Bottom) on mature embryo production in the WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* lines. Values (WT set at 100%) are means ± SE of four independent biological replicates. Stars indicate values which are statistically significant difference ($p \leqslant 0.05$) from WT (C).

4.4 Discussion

Auxin is the inductive signal required for the de-differentiation of somatic cells, which is an obligatory step in several *in vitro* embryogenic systems (Yang and Zhang, 2010). During maize somatic embryogenesis, auxin is required for the proliferation of the embryogenic tissue and its removal triggers the development of the somatic embryos. Given the relevance of this plant hormone for the embryogenic process, this chapter was aimed at investigating whether the different embryogenic responses induced by suppressions of either *ZmPgb1.1* or *ZmPgb1.2* (Fig. 4.1) were related to changes in auxin synthesis and signaling.

There was no effect on the overall IAA levels as a result of suppressing either *Pgbs* (Fig. 4.3). However, the localization of IAA within specific cells was affected in the antisense lines (Fig. 4.2). The decrease in localized IAA accumulation occurred in the endoderm (but not protoderm) cells of the apical domain by *ZmPgb1.1* suppression, while it occurred only in the basal or anchoring cells by *ZmPgb1.2* suppression. In line with this, unusual polar auxin transport (PAT) was evident in the antisense lines. As indicated by ZmPIN1 localization (Fig. 4.11), there was little or no auxin transport in the cells with reduced IAA levels caused by *Pgb* suppression.

Applications of NO or zinc donor obviously decreased the accumulation of IAA in the WT control, while addition of NO scavenger or zinc chelator increased IAA accumulation in the *ZmPgb1.1(A)* line (Fig 4.2). Thus, the role of phytoglobins in the depletion of IAA appears to be mediated by NO and Zn^{2+} (Fig. 4.2), in a pattern consistent with the model described in Chapter 1 (Fig. 3.1), in which the suppression of phytoglobins increases NO and Zn^{2+}, triggering a cascade of events culminating to PCD.

It is not clear from this study whether the decrease in localized IAA accumulation in *ZmPgbs*-suppressing cells is the cause or the result of cell death, although some evidence supporting the causative role of auxin depletion on cell death is available (Xia et al., 2005). The fact that the overall level of IAA was not altered in the transformed line (Fig. 4.3) clearly reinforces the notion that the effects of ZmPgbs on IAA are cell specific, as there was strong evidence of IAA and ZmPIN1 in specific embryogenic domains being influenced by either *ZmPgbs* (Fig. 4.2 and Fig. 4.11). The effect of *ZmPgbs* on auxin biosynthesis pathways should be interpreted with caution in that (1) the auxin biosynthesis pathways in maize is complex and not fully understood, and some of the orthologs need further functional characterizations, and (2) these possible auxin biosynthesis candidate genes may also act differently upon various environmental cues. For example, transcriptional studies on auxin biosynthetic genes showed that, of the eight genes analyzed (Table 4.1), the expression levels of *ZmAMI1*, *VT2* and *ZmAO1* are most affected by altered *ZmPgb* expression (Fig. 4.6 and 4.7). Specifically, suppression of *ZmPgb1.1* repressed *VT2*, while suppression of *ZmPgb1.2* significantly repressed *ZmAMI1* and induced *ZmAO1* (Fig. 4.6 and 4.7). Unlike *VT2*, the well characterized maize ortholog of *TAA1/TAR1/TAR2* (Phillips et al., 2011), the functions of *ZmAO1* and *ZmAMI1* are less known (Sekimoto et al., 1997; Lehmann et al., 2010; Phillips et al., 2011).

Extrapolations from studies on other species suggest that regulation of these genes during IAA biosynthesis might be under the control of diverse stimuli, some of which triggered by developmental events, while others by stress factors. While *AMI1* might play a role in IAA production during development, as its expression is strongly induced during germination (between day 7 and day 14) (Hoffmann et al., 2010), *TAA1* and *AO1* are induced under stress conditions. For example, during over-exposure to aluminum the expression of *TAA1* increases

rapidly at the root-apex transition zone where it mediates the localized auxin biosynthesis to inhibit root growth (Yang et al., 2014). A similar induction was also observed in the expression of *AO1* in roots and leaves of *Pisum sativum* under ammonium and salt stress conditions (Zdunek-Zastocka et al., 2004). Based on these observations, it might be interesting to ascertain whether the control of *Pgbs* on IAA synthesis might also be regulated by diverse stimuli, with some *Pgbs* being induced (and regulating auxin synthesis) during development, while others during stress responses. This diversification of roles would also be in line with the different number of *Pgbs* present in several species and the diversity/distinction of putative regulatory elements in the promoter regions of *ZmPgb1.1* and *ZmPgb1.2* (Hill et al., 2013). In addition to regulatory elements corresponding to different hormonal/environmental cues shared by these two *ZmPgb* promoters, variation in the presence of regulatory elements related to auxin, drought and temperature, and their location in the promoter regions of *ZmPgbs* is an indication that they may have specific cell functions, and act in different ways to cell and environmental perturbations (Hill et al., 2013).

Another relevant point is that the effects of *Pgbs* on IAA synthesis might be species-specific, or most likely event-specific. During Arabidopsis somatic embryogenesis repression of *AtPgb2* increases somatic embryo formation by enhancing the total IAA contents in the embryogenic tissues (Elhiti et al. 2013). However, in maize *ZmPgb* suppression does not have effect on the overall IAA synthesis, rather on the localization of IAA and specific parts of its biosynthetic pathways. In the Arabidopsis system the role of *Pgbs* on IAA production was investigated in relation to the formation of the embryogenic callus, a process requiring auxin. In the present study, the *Pgb*-regulation of IAA is analysed in relation to the behaviour of the already formed embryogenic tissue. It must be noted that embryogenesis in these two plant systems might also

occur through different mechanisms, as there is no indication that the *AtPgb2*-mediated embryogenic development requires programmed cell death (PCD) execution. An interesting future study could involve the investigation of IAA synthesis during the early steps of embryogenic tissue generation in maize using explants with altered Pgb levels. This study will reveal if the role of Pgb in regulating auxin synthesis is different between Arabidopsis and maize.

Auxin transport is important in a number of developmental processes especially during embryogenesis (Friml et al., 2003; Prasad and Dhonukshe, 2013). The direction of auxin transport is mediated by the family members of auxin efflux transport proteins, PIN-formed (PIN) proteins (Prasad and Dhonukshe, 2013). The number of PINs varies in different species, for example, at least eight members are found in Arabidopsis, while 12 are found in rice and 14 in maize (Forestan et al., 2012). In Arabidopsis, PIN1 is possibly the best characterized transporter (Prasad and Dhonukshe, 2013), and during maize *in vivo* embryogenesis, the PIN1 orthologs ZmPIN1a,b,c, account for the majority of auxin mobilized in the developing embryos (Forestan et al., 2010).

Alteration of *ZmPgb* expression level influences *ZmPIN1* expression and protein localization. The differential regulation of *ZmPgb1.1* and *ZmPgb1.2* on PINs (Fig. 4.11), as well as the difference in the localization of PIN1 protein in the developing embryos of the transgenic lines (Fig. 2.8) suggest that the two *ZmPgbs* might affect IAA through diverse mechanisms. The protodermal localization pattern of PIN1 in the *ZmPgb1.1(A)* embryos closely mimics the accumulation of IAA in the same cells. This is also partially true for the *ZmPgb1.2(A)* embryos where the PIN1 protein is visible in the large majority of embryonic cells accumulating IAA (compare Fig. 4.2 and Fig. 4.11). A tight correlation between PIN1 localization and IAA accumulation was also demonstrated in previous studies. For example, during Arabidopsis

somatic embryogenesis a redistribution of PIN1 along the cotyledons of the zygotic embryo contributes to the production of an auxin maximum that is required for the formation of the embryogenic tissue (Elhiti et al., 2013).

As observed for PINs, other auxin transporters are also differentially expressed as a result of altered expression of the two *ZmPgbs* (Fig. 4.12). However, this is not the case of the two auxin responsive genes analysed, *ZmGH3* and *ZmSAUR2* (Fig. 4.13). These should be interpreted cautiously because the auxin-induced expression of *ZmSAUR2* is tissue-specific, for example, in the elongating coleoptile tissue but not in the primary leaves or in roots (Knauss et al., 2003), and that the *small auxin-upregulated RNAs* (*SAURs*) belong to a large multigene family in plants with 87 members in maize (Chen et al., 2014). In addition, the functional roles of GH3 family members in Arabidopsis are highly multifaceted, which are involved in modulating not only auxin, but also JA and SA homeostasis (Park et al., 2007; Zhang et al., 2007b; Gutierrez et al., 2012). In maize ZmGH3 in relation to auxin response is less understood (LeCLere et al., 2010).

Possible mechanisms through which ZmPgbs affect auxin synthesis and distribution might be related to NO, which accumulates in *ZmPgb*-suppressing cells (Fig. 3.1). Higher levels of NO, either through suppression of *ZmPgb1.1* or by application of SNP, reduced localized accumulation of endogenous IAA (Fig. 4.2). The crosstalk between NO and auxin has been documented in several systems during root development (Freschi, 2013) and during the formation of embryogenic tissue in culture (Otvös et al., 2005; Elhiti et al., 2013). In this context, higher levels of NO have been shown to inhibit the PIN1-mediated polar auxin transport (PAT), ultimately leading to defects in the root apical meristem (RAM) in Arabidopsis (Fernández-Marcos et al., 2011). In our study, suppression of *ZmPgb1.1* depletes ZmPIN1 proteins in most of the apical embryogenic domain (Fig. 4.11), corresponding to the NO-enriched cells (Fig. 3.1).

A similar trend was also observed in the *ZmPgb1.2*-suppression line where PIN1 is not present at the base of the developing embryos (Fig. 4.11), where NO accumulates (Fig. 3.1).

Further evidence on the role of Pgbs in regulating auxin localization pattern is apparent from the differential responses exhibited by the lines to different levels of 2,4-D. While the severe decline in embryogenic output exhibited by the *ZmPgb1.1 (A)* line cultured with reduced levels of 2,4-D (Fig. 4.14) could be explained by the low level of IAA present in the developing embryos (Fig. 2.2), the behavior of the *ZmPgb1.2(A)* line is difficult to interpret. What is clear, however, is that auxin plays a crucial role during embryogenesis and that perturbation of auxin transport compromises the formation of the embryos in all the lines utilized (Fig. 4.14).

Collectively these studies reinforce the notion that auxin is an important component of the embryogenic process which can be manipulated by alterations in *Pgb* expression, possibly through the mediation of NO. An important possibility emerging from these findings is that different Pgbs might alter IAA synthesis and/or signaling through diverse mechanisms. This idea might lead to future studies aimed at analysing the endogenous and/or environmental stimuli activating specific Pgbs.

4.5 Materials and methods

4.5.1 Plant material and treatments

The embryogenic cells, culture conditions and treatments were the same as described in Chapter 1. The auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) was applied at the optimal concentration of 1 μM (Elhiti and Stasolla, 2011). Treatments were interrupted at day 7 in the proliferation (P) medium, and the tissue was either cultured on solid development (D) medium to assess embryo yield, or collected for gene expression, immunological, and histological analyses.

4.5.2 Expression studies

RNA extraction cDNA synthesis, and quantitative (q)RT-PCR were conducted as described in Chapter 1. All primers used in this study are listed in Table S4.1.

4.5.3 RNA in situ hybridization

RNA in situ hybridization studies were conducted as described (Elhiti et al., 2010). The partial length cDNAs of VT2 and ZmAO1 were amplified using gene-specific primers (Supplemental Table 1) and cloned into the pGEM-T Easy Vector System (Promega). The cDNAs were subsequently amplified from the vector using T7 and SP6 primers and used for the preparation of digoxigenin (DIG)-labelled sense and antisense riboprobes, following the procedure outlined in the DIG Application Manual (Roche Diagnostics). Slide preparation, hybridization conditions, and color development were performed as documented previously (Elhiti et al., 2010).

4.5.4 Endogenous IAA localization

Immunolocalization of endogenous IAA was carried out as described (Elhiti et al., 2013). Embryogenic cells were first pre-fixed in freshly prepared 4% aqueous 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride for 2 h at 4°C, and then fixed in FAA overnight at 4°C. The fixed tissue was dehydrated in an ethanol series, embedded in paraplast, sectioned (10 μm) and deparaffinised in xylene. The sections were incubated in blocking solution at room temperature for 1 h. A 150 μl portion of monoclonal primary IAA antibodies (Sigma-Aldrich), diluted 1:200 in 10 mM PBS containing 0.8% BSA, was applied to the sections and incubated in a high humidity chamber at room temperature for 4 h. The slides were washed twice, and incubated in 200 μl of secondary antibodies (Promega) overnight in a high-humidity chamber, washed twice in full-strength PBS containing 0.88 g L^{-1} NaCl, 0.1% Tween 20 and 0.8% BSA for 10 min, and then incubated in H_2O for 15 min to remove the excess of secondary antibodies. Samples were stained using 250 μl of Western Blue (Promega) for about 30 min.

4.5.5 Free IAA quantification

Cell samples were collected from the whole tissue of immature embryos of WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* on P(3) and P(7). Cells were weighed, freeze-dried and stored at -80°C. The measurement of free IAA was performed according to previous studies (Yoshimoto et al., 2009).

4.5.6 Immunolocalization of ZmPIN1 proteins

Immunolocalization of ZmPIN1 proteins were conducted as described (Forestan et al., 2010; Elhiti et al., 2013). Immature embryos of WT, *ZmPgb1.1(A)* and *ZmPgb1.2(A)* on P(7) were collected, fixed in a 4% paraformaldehyde, full-strength PBS solution for 1 h, under vacuum conditions at room temperature, and wax embedded (9:1, PEG400 distearate:1-hexadecanol 99%,

Sigma-Aldrich). Embedded tissue was sectioned (10 μm) and incubated with 1:200 anti-AtPIN1 (Santa Cruz Biotechnology) in full-strength PBS, containing 1% BSA overnight at room temperature. The sections were then washed twice for 10 min in full-strength PBS and stained with the secondary antibodies conjugated with Alexa488 (Invitrogen). The slides were washed twice for 10 min with full-strength PBS and visualized with fluorescence microscopy.

4.5.7 Statistical analysis

Statistical analysis was carried out same as described in Chapter 1.

4.6 Supplemental data

Table S4.1 List of primers used in auxin studies

NO.	Name	Sequence
1	ZmAMI1-F	TTTGCCAGCTGAGCATTCCACTTG
2	ZmAMI1-R	ATGAACTCCAGCTCCAGGCTTTCT
3	SPI1-F	ACGGAGGCGACGTGTTCA
4	SPI1-R	TAGAGCCCGTTCTTCCCTTTC
5	ZmNIT1-F	GACGATGACTATGTGCAGACCTAA
6	ZmNIT1-R	CAATCTCGTCCAATCCATGTATA
7	ZmNIT2-F	AGCTGCCAAGAGTGATATCGATACTAAG
8	ZmNIT2-R	CACAAGGAACATAACTGCGGCC
9	VT2-1F	GGTACGTCCGGGTCAGCAT
10	VT2-1R	CATCTTCGTTCAAGCGCCTCT
11	VT2-2F	CTTCTGCAACTTCACCAAGG
12	VT2-2R	CGGTTCAGTCTCACATAGCA
13	ZmAO1-1F	GGGAGGCTGTGTACGTTGAT
14	ZmAO1-1R	TCTCCACCGCTTGGAATATC
15	ZmAO1-2F	GCTACTTGAACTACGGAGCTGG
16	ZmAO1-2R	TCCTTGACGACAGGCATCGTC
17	TSB1-F	TGTACATTCTGGGACAGCAACCCT
18	TSB1-R	AACCTTGTGGAACTCCCTCACCAT
19	TSB2-F	TATATCTTGGGCTCTGTTGCCGGT
20	TSB2-R	AATAAGCCCATGGCGTTTGATCCG
21	ZmPIN1a-F	ATAATCGCGTGCGGGAACAA
22	ZmPIN1a-R	TCCTGCTCCACATCCCCATC

23	ZmPIN1b-F	ATCATCGCGTGCGGGAACAA
24	ZmPIN1b-R	ACCCACGGGTCGGTCACAGG
25	ZmPIN1c-F	TCATCCCCATGGAGTCGAGGATGCCACC
26	ZmPIN1c-R	GGATCCACCCAGACCCAATCCCCATACCTACTTCT
27	ZmAUX1-F	AGTCGAGGGAGAACGCCGTG
28	ZmAUX1-R	GGTGGTGGCGGAGGAAGAAG
29	BA1-F	AGACGCATGCTACTCTGTGTGTGA
30	BA1-R	ACTAGCGTCTAGCAGACACCAACA
31	BIF2-F	CAGCCTGCCGCGCTGCTCCAGC
32	BIF2-R	CGGCGCAGCAGCCTGAAGTCC
33	ZmGH3-F	GCTGCTGTACAGCCTCCAGATG
34	ZmGH3-R	TCTTGTAGTAGCTCGTGAGGAC
35	ZmSAUR2-F	CAAGAAGTGGCAGAGGATGG
36	ZmSAUR2-R	GCTAATATCTTTGCTCCACT

5 GENERAL DISCUSSION AND CONCLUSIONS

The process of plant regeneration is largely controlled by the fine tuning of genetic and epigenetic mechanisms underlying developmental programs like cell division, cell differentiation and programmed cell death (PCD). Plant hormones, such as auxin, play a central role in these processes since they act as versatile signaling molecules to coordinate the proper execution of these events (Pennell and Lamb, 1997; Prasad and Dhonukshe, 2013; Xu and Huang, 2014; Zhao, 2014). Plant regeneration in culture is best exemplified by somatic embryogenesis, a zygotic-like embryogenic process, in which somatic cells grown under appropriate culture conditions de-differentiate and embark in new developmental pathways leading to the formation of fully developed embryos. Somatic embryogenesis is possibly the best characterized example of plant totipotency requiring, at least in various species including dicots and monocots, auxin as the inductive signal for the de-differentiation step. For many years cell division and differentiation were considered as major events occurring during the production of somatic embryos. It is only recently that PCD has emerged as a process required to control the early developmental stages of *in vitro* plant embryogenesis (Thorpe and Stasolla, 2001; Yang and Zhang, 2010; Smertenko and Bozhkov, 2014).

Together with auxin, nitric oxide (NO) has been identified as a versatile molecule orchestrating the initiation, transition and progression of the embryogenic development (Otvös et al., 2005; Rodríguez-Serrano et al., 2012; Freschi, 2013). Therefore, regulation of cellular NO homeostasis, through its synthesis and/or degradation, is important for the proper development of the embryos. Depletion of cellular NO can occur through several processes and phytoglobins (Pgbs) have been identified as effective NO scavengers (Hill, 2012). It is therefore plausible to assume that alterations in *Pgbs* expression would modulate NO availability and ultimately

regulate all those NO-mediated processes, including embryogenesis. To test this hypothesis, the expression of two maize phytoglobins *ZmPgb1.1* and *ZmPgb1.2* was manipulated during maize somatic embryogenesis.

In the first chapter, the embryogenic competence of maize cells suppressing either *ZmPgb1.1* or *ZmPgb1.2* was analysed. While repression of *ZmPgb1.1* decreased embryo production, repressing *ZmPgb1.2* enhanced the formation of maize somatic embryos. This opposite outcome was due to the fact that while fulfilling similar functions, the two *Pgbs* were expressed in different embryonic domains. It is demonstrated that suppression of the two *Pgbs* induces PCD through a process mediated by NO and Zn^{2+}. Specifically, cells suppressing one of the two *Pgbs* accumulate NO which release Zn^{2+} from metallothioneins (MTs). Elevation in Zn^{2+} levels trigger MAPK-dependent responses resulting in the accumulation of reactive oxygen species (ROS), possibly the final executors of the cell death program. While suppression of *ZmPgb1.1*, which is expressed throughout the developing embryos, causes massive PCD leading to embryo abortion, suppression of *ZmPgb1.2* increases the number of embryos due to its restricted localization pattern. *ZmPgb1.2* is in fact expressed only in the cells anchoring the developing embryos to the embryogenic tissue (here defined as “anchor cells”) and in cells connecting two embryos. Death and removal of these cells as a result of suppression of *ZmPgb1.2* releases the embryos in the culture medium and favors their further development and maturation.

In the second chapter, mechanisms underlying the opposite embryogenic response controlled by *ZmPgb1.1* or *ZmPgb1.2* were further analyzed in relation to auxin, the inductive signal of maize somatic embryogenesis. The opposite embyogenic effect caused by suppressing either of these two *Pgbs* is attributable to the localization pattern of the endogenous IAA, as

there is no significant difference in the total IAA content. Microscopic studies indicated that, when the expression of *ZmPgb1.1* is suppressed, an obvious decrease in localized IAA accumulation was observed in the endoderm (but not protoderm) cells of the apical domain in the developing embryo. When the expression of *ZmPgb1.2* is suppressed, decrease in the localized accumulation of IAA occurred only in the basal or anchoring cells of the developing embryo. It is visible that, cells with less IAA localized accumulation are most likely those enriched with NO due to the suppression of *ZmPgb1.1* or *ZmPgb1.2*. The interference of *Pgbs* in cellular auxin localization pattern is also evident in specific auxin biosynthesis genes, especially in the IPA pathway, where VT2 and ZmAO1 exhibit different responses, including their transcriptional levels and localization patterns, to the suppression of a specific *Pgb*. Without changing total endogenous auxin content in the embryogenic tissue, the *ZmPgb* expression/suppression controlled endogenous auxin accumulation/depletion in specific embryogenic cells is most likely established by polar auxin transport (PAT), as similar localization patterns of ZmPIN1, the most important auxin transporter responsible for endogenous auxin distribution/redistribution, are observed. ZmPIN1 is only localized in the protoderm cells of apical domain, but not in the endoderm cells or the basal cells in the *ZmPgb1.1 (A)* line, while it is localized throughout most of the apical domain but not the basal cells in the *ZmPgb1.2 (A)* line. Variations in the expressions of other auxin transport genes and early auxin response genes imply the complexity of auxin-Pgbs interactions in somatic embryogenesis. However, the requirement of PAT for proper embryogenic development was verified by the application of auxin transport inhibitor in the lines. Thus, endogenous auxin patterning mediated by auxin transport is involved in the phytoglobin-regulated embryogenesis.

Taken together the results from these studies support the original hypothesis that by modulating NO levels, Pgbs are key regulators of plant somatic embryogenesis. In the model proposed from these studies, cells suppressing *ZmPgb1.1* or *ZmPgb1.2* accumulate NO, have reduced localized accumulation of IAA, and undergo PCD. While still preliminary, studies from the second chapter suggest a possible correlation between reduced localized IAA accumulation and PCD during *in vitro* embryogenesis, a correlation which has been established primarily in plant post-embryonic growth, especially during biotic and abiotic stress conditions (Gopalan, 2008; Krishnan et al., 2009; Blomster et al., 2011; Abrahamsson et al., 2012).

It cannot be excluded, however that, Pgbs might regulate plant embryogenesis by interacting with other plant hormones. Cell suspension cultures down-regulating *Pgbs* have increased ET production (Manac'h-Little et al., 2005), and over-expression of *Pgbs* favours shoot organogenesis by increasing CK sensitivity (Wang et al., 2011). Collectively, these observations can lead to future studies aimed at investigating (1) the interactions between plant Pgbs and plant hormones during fundamental developmental processes, and (2) the causes of the differential localization patterns of different *Pgbs*, as also suggested by the diversity of putative cis-regulatory elements in the maize *Pgbs* promoters (Hill et al., 2013).

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