Suppression of Phytoglobin Proteins (Pgb) Increase Corn Seedling Tolerance to Goss's Bacterial Wilt Infection

by

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DEDICATION

I dedicate this thesis to my husband (Enock Aidoo) and my children (Anas and Edwin), whose love and support always kept me going. I love you all.

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ABBREVIATIONS

ABA, Abscisic Acid

ACC, 1-aminocyclopropane-1-carboxylic acid

ACO, 1-aminocyclopropane-1-carboxylic acid oxidase

AOA, Aminooxyacetic acid

AOC, Allene oxide cyclase

AOS, Allene oxide synthase

AdoMet, S-adenosylmethionine

AvrPto, Avirulence protein

celA, Cellulose gene

cGMP, Cyclic Guanosine Monophosphate

CK, Cytokinin

CN, *Clavibacter Nebraskensis*

COI1, Coronatine insensitive 1 cPTIO, 2-(4-Carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt

DAF-2DA, 4,5-diaminofluorescein diacetate

ERF, Ethylene Response Factor

ET, Ethylene

ETH, Ethephon

ETI, Effectors-Triggered Immunity

ETS, Effectors-Triggered susceptibility

FLS2, Flagellin sensitive 2

GA, Gibberellic acid

Hb, Hemoglobin

HR, Hypersensitive Response

Hpi, hours post inoculation

IAA, Indole-3-acetic acid

JA, Jasmonic Acid

JAR1, Jasmonate resistant 1

LAR, local acquired resistance

LegHb, Leghemoglobin

LOX, Lipoxygenase

MAPK, Mitogen-activated protein kinase

MC, Matacaspase

MYC2, Jasmonate insensitive 1

Nr, Never ripe

NBY, Nutrient broth yeast extract

NOS, Nitric oxide synthase

NPR1, Nonexpressor of pathogenesis-related 1

NR, Nitrite reductase

OPR, 12-oxophytodienoic acid reductase

PAMP, Pathogenesis-Associated Molecular Pattern

PAL, Phenylalanine ammonia lyase

pat-1, Pathogenicity factor 1

PCD, Programmed Cell Death

pCM2, Plasmid 2

PDF1.2, Plant defensin gene

PEG, Polyethylene glycol

PEM, Pro-Embryogenic Mass

Pgb, Phytoglobin

PM, Plasma membrane

PR, Pathogenesis-related proteins

PI, Pathogenesis inducer

PRR, Pathogen-Recognition Receptor

PTI, PAMPs-Triggered Immunity

RBOH, Respiratory burst oxidase homolog

RLP, Receptor-like proteins

ROS, Reactive Oxygen Species

SA, Salicylic Acid

SAR, Systemic acquired resistance

SNP, Sodium nitroprusside

T3SS, Type III secretion system

TUNEL, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling

WT, Wild Type

ZmPgb, *Zea mays* phytoglobin

ABSTRACT

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Suppression of Phytoglobin Proteins (Pgb) Increase Maize Seedling Tolerance to Goss's Bacterial Wilt Infection

Supervisor: Dr. Claudio Stasolla

The effects of suppression of the *Zea maize* Phytoglobin 1 (*ZmPgb1*) on plant response to infection with *Clavibacter nebraskensis* (isolate Cmn14-5-1), were evaluated by measuring leaf lesion and physiological events associated with biotic stress. Relative to wild type (WT), suppression of *ZmPgb1* reduced the size of leaf lesions and elevated the expression of genes involved in ethylene synthesis and response, as well as those contributing to the generation of reactive oxygen species (ROS). The same plants displayed a more pronounced accumulation of ROS and programmed cell death (PCD) in proximity of the inoculation site. Similar transcriptional responses were also observed in cultured maize cells infected with isolate Cmn 14-5-1. Pharmacological treatments were performed in culture to alter the levels of NO, and/or ethylene (ETH). An experimental increment in NO with the NO donor sodium nitroprusside (SNP), or ethylene with the ethylene precursor ethephon (ETH), increased the production of ROS and elevated the number of cells undergoing PCD in WT cells. An opposite response was observed in cells suppressing *ZmPgb1* when the level of NO or ethylene was reduced with the respective utilization of 4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) or aminooxyacetic acid (AOA). Collectively these findings suggest that suppression of *ZmPgb1* enhances tolerance to *Clavibacter nebraskensis* through a mechanism initiated by the

accumulation of NO, mediated by ethylene, and culminating with the production of ROS and the execution of PCD.

FORWARD

This thesis follows the paper style outlined by the Department of Plant Science and Faculty of Graduate Studies at the University of Manitoba. The manuscript follows the style recommended by the Journal of Experimental Botany. This thesis is presented as a single manuscript, comprised of an abstract, introduction, materials and methods, results, and a discussion section. Supplementary tables follow the results section of the manuscript. A general introduction and literature review precedes the manuscript and general discussion and conclusions follows the manuscript.

GENERAL INTRODUCTION

Goss's bacterial wilt and leaf blight is a devasting disease of corn caused by the bacteria pathogen *Clavibacter michiganensis* subsp. *nebraskensis* (Cmn) (Vidaver and Mandel, 1974), which has recently been renamed as *Clavibacter nebraskensis* (Cn) (Li et al., 2018).There are five other subspecies of *Clavibacter* responsible for causing infections in their corresponding host plants cells (Carlson and Vidaver, 1982; Davis et al., 1984; Riley and Ophel, 1992). However, recent studies by Li et al. (2018), have re-classified and moved two of the five subspecies of *Clavibacter michiganensis* (*Clavibacter michiganensis* subsp. *nebraskensis* and *Clavibacter michiganensis* subsp. *sepedonicus*) to species level based on the whole genome and multi-locus sequence analysis (MLSA). Therefore, *Clavibacter michiganensis* subsp. *nebraskensis* is currently referred to as *Clavibacter nebraskensis* (Cn) (Li et al., 2018). Goss's wilt is an economically important disease of corn which may affect plants at any developmental stage causing yield loss of 50% or more (Wysong and Doupink, 1984). The pathogen usually resides in previously infected corn stubble and is able to enter host cells following wounding by hail, heavy wind and sandblasting, or through stomatal openings and insect feeding (Załuga et al., 2014). Characteristic symptoms of the disease are mainly associated with water-soaked spots commonly known as freckles along the margins or ends of developing lesions (Schuster et al., 1972).

Since its discovery in the early 21st century, Goss's wilt has spread to previously uninfected regions across Midwest U.S. and the Canadian provinces of Alberta (Howard et al., 2015), Manitoba and Ontario (Desjardins, 2010). Disease persistence and re-emergence have been associated to tight corn rotation, changes in pathogen diversity and virulence, high relative

humidity, minimal tillage practices, and inadequate resistance varieties (Jackson et al., 2007; Załuga et al., 2014).

Management of the disease over the past years has greatly relied on the use of resistant hybrid varieties, as well as good agronomy practices such as rotating to non-hosts crops, and tillage practices that enhance decomposition of infected residues (Jackson et al., 2007a; Jackson 2009). Unfortunately, fungicide applications are not effective in controlling the disease and only a few Goss's wilt chemical efficacy experiments have been documented (Agarkova et al., 2011; Oser et al., 2013). Mehl et al. (2015) reported that the use of copper hydroxide or citric acid was ineffective for Goss's wilt management, and it was suggested that farmers should still depend on cultural strategies and planting of resistant cultivars (Mehl et al., 2015).

The interactions between plants and pathogens are modulated through a series of signaling transduction pathways (Veronese et al., 2003). The molecular mechanisms participating in the activation of defense responses are not fully understood and depend upon the ability of the plant to recognize invading pathogens. For instance, diverse pathogens are recognized by the plant cell surface-localized Pathogen-Recognition Receptors (PRRs), which then interfere with the Pathogenesis-Associated Molecular Pattern (PAMP) and pathogenesis elicitors (Boller and Felix, 2009). This process triggers the activation of the plant's innate PAMPs-triggered immunity (PTI) or the inducible effectors-triggered immunity (ETI) (Ausubel, 2005; Dodds and Rathjen, 2010).

Activation and regulation of the various defense responses are modulated by a complex network signaling of interconnected endogenous hormones (Pieterse et al., 2009). This signaling

usually involves plant hormones such as ethylene (ET), jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA) (Bari and Jones, 2009; Pieterse et al., 2009). The crosstalk of plant hormones which includes antagonistic and synergistic interactions may result in the induction of antimicrobial pathogenesis-related protein (PR) genes in challenged cells, tissues and organs (Fu and Dong, 2013), and the activation of the hypersensitivity response (HR), a process to starve the pathogen of water and nutrients at the infection site (Caplan et al., 2008).

Hypersensitivity response is a form of rapid localized programmed cell death (PCD) following infection (Mur et al., 2008). At a morphological level, HR shares characteristics common to necrosis and vascular plant cell death (Van Doorn et al., 2011). The HR process results from incompatible plant-pathogen interaction. Hence, the HR is determined by the mutual association of both the plant and pathogen gene products (Peart et al., 2005). The HR is effective against many hemibiotrophic bacterial, fungal and viral pathogens that depend on the active host for survival (Spoel and Dong, 2012). Some hemibiotrophs utilize their effectors to inhibit HR and keep the cells of the host plants alive in order to complete the infection process (Coll et al., 2011). Hypersensitive responses are triggered by ROS and mediated by NO (Mur et al., 2013). Other key elements in the induction of HR are SA, Ca^{2+} and mitogen-activated protein kinases (MAPKs) (Oliver et al., 2009; Kombrink et al., 2001). For example, some plant cells exposed to pathogens accumulate Ca^{2+} in the mitochondria leading to the over-production of ROS, compromising membrane function and ultimately triggering PCD (Green and Kroemer, 2004). Over-production of ROS is mainly mediated by the activity of NADPH oxidases (Yun et al., 2011). Thus factors influencing the level of NO, ROS, and PCD can alter the plant's response to pathogens.

Phytoglobins (Pgbs) are heme-containing globular protein found in plants. Present in both dicot, monocot and gymnosperms, Pgbs play a fundamental role during normal growth and development, as well as during stress conditions (Hill, 2012). It is generally accepted that Pgbs can be divided into four classes (Vinogradov et al., 2011). Besides symbiotic Pgbs, three classes of non-symbiotic Pgbs have been identified (Stasolla and Hill, 2017). One of the known functions of Pgbs is to scavenge NO (Gupta et al., 2011; Hill, 2012), thus, influencing cellular NO homeostasis and all those processes influenced by NO. For example, by removing NO, Pgbs reduce the levels of ROS and ethylene which are elevated under several types of stress, including hypoxia (Mira et al., 2016a) and drought (Mira et al., 2017). Reduction of both ROS and ethylene in cells expressing Pgbs protects cells from dying under both types of stress (Mira et al., 2016a; 2017). During embryogenesis Pgbs also interfere with JA and Indole-3-acetic acid (IAA) (Mira et al., 2016b), and influence cell fate acquisition. This is apparent during corn somatic embryogenesis where cells destined to die by PCD have reduced expression of Pgbs and the tissue-specific localization of Pgbs determines which cells will survive (Huang et al., 2014).

It has also been demonstrated that Pgbs might regulate plant-pathogen interaction. Activation of Pgbs usually occurs at the initial stages of infection. This was demonstrated by Qu et al. (2005) who observed an elevation in Pgb1 expression in cotton cells infected with *Verticillium dhaliae* (Qu et al., 2005). Additionally, in Arabidopsis, controlling *Pgb1* levels alters the resistance to both *Botrytis cinerea* and *Pseudomonas syringae* and this effect was mediated by regulating NO, ROS and JA/ET levels (Mur et al., 2012b).

Based on the evidence above, the hypothesis tested in this thesis is that alterations in Pgb expression influence the response of corn plants to *Clavibacter nebraskensis* infection through mechanisms involving ethylene, ROS, and PCD.

1 LITERATURE REVIEW

1.1 Corn production

Corn (*Zea mays* L.), also referred to as maize belongs to the family of *Poaceae*, subspecies *mays*. Corn, due to its economic significance, is one of the most essential cereal crops planted worldwide. Corn is Canadian 3rd most valuable crop. Canada is ranked as the 10th largest growers of corn globally. In 2000, the area cultivated to corn was estimated to be about 1.12 million ha, whereas in 1995 the corn area was 1.0 million ha (FAOSTAT 2000). Within Canada, Ontario and Quebec are known to be the largest corn-growing prairies. Following 2011 agriculture census, Ontario accounted for 61.7% of planted area, followed by Quebec at 30.2% and Manitoba at 6.4 % (Statistics Canada, Census of Agriculture, 2011). Canadian land planted to corn reached 1.25 x 106 ha in 2000, in response to increased request generated by the ethanol and animal industries (Statistics Canada, 2002).

Corn cultivation is predominant in the Northern Central parts of American prairies (Iowa, Wisconsin, Illinois, Minnesota, Eastern South Dakota and Indiana Nebraska) (USDA Economic Research Service 2014).There are many categories of corn, namely sweet corn, popcorn, dent corn, waxy corn, flint corn, and flour corn (Gibson and Benson, 2002). However, the major types of corn grown in the U.S and other regions are the sweet corn, popcorn and grain corn (USDA Natural Resource Conservation Service 2014). According to Gibson and Benson (2002), yield potential was low when it was first planted in the U.S. However, in recent years modern technological advances and comprehensive research have contributed to the mass production of

corn with a higher yield than the past. A report by the U.S. Department of Agriculture–National Agricultural Statistics Service (USDA-NASS 2014) indicated that 35.8 million hectares of corn were planted in 2015.

1.1.1 Corn diseases and management

Plants are often challenged by a variety of pathogens and pests influencing yield potential and economic value. Corn production is not an exception to the rule; this crop is challenged with different range of pests and pathogens. Several fungal diseases and a few bacterial diseases in most of the corn growing prairies have been reported. Northern leaf blight caused by fungi has currently been among those diseases causing a drastic loss in corn yield (Wise et al., 2010; Wise et al., 2015). Besides Northern leaf blight, other important diseases in predominant corn growing areas include Fusarium stalk rot, common rust, seedling blights, Gibberella stalk and ear rot, Goss's leaf blight, wilt and gray leaf spot (Wise et al., 2015).

Common disease management techniques for corn diseases include the use of fungicides, resistant hybrids, and cultural practices (Stuckey et al., 1991). For example, the use of *Bacillus thuringiensis* against insect attack has minimized the application of insecticide (Hellmich et al., 2012). Fungicide application has enabled an increase in corn yield, by suppressing disease severity and changing the physiological functions of the plant in some instances (Wise et al., 2011). Combined applications of herbicides, insecticides, and fungicides are often required to ensure high yield (Miller et al., 2002). One of the most dominant and detrimental bacterial

diseases in corn is Goss's wilt (Wise et al., 2015). The rapid spread of this disease is attracting a lot of attention and research to develop resistant plants is ongoing.

1.2 Goss's bacterial wilt infection in corn

1.2.1 Origin and distribution of Goss's wilt

Goss's wilt was initially detected in the southern-central region of Nebraska in 1969 (Vidaver and Mandel, 1974), the lesions observed on the leaves were similar to those induced by the corn bacteria pathogen *Pantoea stewartii*. The causal agent of the disease was subsequently isolated and characterized as a new species of *Corynebacterium nebraskense* (Vidaver and Mandel, 1974). The original name of the disease was Nebraska leaf freckles and wilt but has also been referred to as bacterial leaf blight and wilt, bacterial leaf freckles and wilt (Schuster, 1975; Jackson et al., 2007a), and leaf freckles and wilt (Jackson et al., 2007a). The current name, Goss's bacterial leaf blight and wilt of corn, originated from Dr. R.W. Goss, a plant pathologist at the University of Nebraska (Jackson et al., 2007a).

Since its discovery, Goss's wilt disseminated to about nine different counties throughout Nebraska in the subsequent growing season and there was further confirmation of the disease in several fields and in about 100 different corn varieties from seed corn industries (Wysong et al., 1973). The disease spread rapidly, reaching Indiana in 2008 (Ruhl et al., 2009) and Minnesota and Texas in 2009 (Korus et al, 2010; Malvick et al., 2010). Goss's wilt was first reported in Manitoba in 2009 near Roland. During the 2016 growing season, a field survey in Manitoba

detected the presence of Goss's wilt in about 59 fields (www.manitoba.ca/agriculture/crops/plant-diseases/print, goss-wilt.html)

The causal agent of Goss's wilt belong to *Clavibacter michiganensis*, a species further classified into five subspecies according to their diverse host range (Jahr et al., 1999). It belongs to the genus *Clavibacter* and placed in *Microbacteriaceae* family (Tambong, 2017). The newly classified species are *Clavibacter nebraskensis* (Cn) is the causal agent of wilt and leaf blight of corn and *Clavibacter sepedonicus* (Cs) causes ring rot of potato (Li et al., 2018; Carlson and Vidaver, 1982; Davis et al., 1984). The subspecies are known to cause diseases in diverse hosts. *Clavibacter michiganensis* subsp. *insidiosus* is responsible for wilting and stunting in alfalfa, *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) causes bacterial wilt and canker of tomato and potato, *Clavibacter michiganensis* subsp. *tessellarius* (Cmt) is responsible for leaf freckles and leaf spots in wheat (Carlson and Vidaver,1982; Davis et al., 1984; Riley and Ophel, 1992), *Clavibacter michiganensis* subsp. *capsici* (Cmc) is responsible for bacterial canker disease in pepper and was recently discovered by (Oh et al., 2016).

Clavibacter michiganensis (Cn) is a rod or coryneform-shaped gram-positive bacterium, non-flagellate and non-motile with an average size of 0.5 to 2.5 µm (Li et al., 2018); Davis et al., 1984; Claflin, 1999). *Clavibacter nebraskensis* contains a small genome, with fewer protein-coding genes relative to other subspecies (Tambong, 2017).

On nutrient broth yeast extract (NBY), Cn produces mucoid orange and yellow colonies, due to the presence of carotenoid pigmentation (Ahmad et al., 2015; Takeuchi, 2006). The most favorable temperature for growth is between 24°C and 28°C under controlled environment (Vidaver and Mandel, 1974), but the bacteria are unable to survive once the mean temperature is 38° C and dies within 24 hours (Smidt and Vidaver, 1986).

1.2.2 Symptoms and Epidemiology of Goss's wilt

Goss's wilt is characterized by two distinct phases. The early infection phase, causing wilting in younger plants, is caused by the production of extracellular polysaccharides (EPS) by the pathogen, depriving the plant of nutrients by blocking the water supply. This results in wilting symptoms and, in most instances, death before plant maturity (Schuster, 1975).

The second phase is usually observed in adult plants. Movement of the bacterium in the vascular system can cause systemic infection leading to severe damage and premature death of the host plant (Jackson et al., 2007a; Jackson, 2008; Robertson and Jesse, 2008). The pathogen resides inside the vascular tissue and parenchyma cells. As the infection progresses, long curly lesions with uneven, dark irregular water-soaked spots occur on the margins of the lesions, referred to as freckles (Fig.1). Infection can occur in every part of the plant with shiny bacterial exudates released from the top or bottom of the leaf surface (Schuster, 1975). The leaf blight symptoms resemble those produced by Northern leaf blight and Stewart's wilt. However, the existence of freckles on Goss's wilt lesions (Raven and Neu.,1945) and the lack of shiny

exudates and absence of discontinuous water-soaked spots on Stewart's wilt are the key distinguishing features between the two diseases (Carson, 1991).

The epidemiological background of the disease is not well understood. However, agricultural practices such as planting of susceptible hybrids, wet weather and high relative humidity, tight corn rotation, alteration in pathogen diversity and virulence and fewer tillage practices might be contributing factors (Jackson et al.,2007; Załuga et al., 2014). Higher relative humidity may influence the survival rate of the pathogen. Mallowa et al. (2016) showed that increased relative humidity favors the survival of an epiphytic population of the bacterium under greenhouse conditions.

Clavibacter nebraskensis overwinters on previously infected corn residues. Wounding by external factors, like insect feeding, leaf litter from the wind, hail damage, and abrasion by blowing sand and/or stomal openings enhances pathogen penetration into the host tissue (Załuga et al., 2014) (Fig.1.1). Infection becomes predominant following hailstorm and rain events but disease development can be observed in both irrigated and dryland fields (Jackson et al., 2007a). Wounding activates a xylem-inhibiting response producing phenolic compounds and mucilage into the xylem in an attempt to delay the spread of bacteria (Crews et al., 2003). In some instances, infection also occurs in the absence of intense wounding but physical injury and stomata may serve as alternative entry points for the bacteria (Mallow et al., 2011; Mallowa et al., 2015).

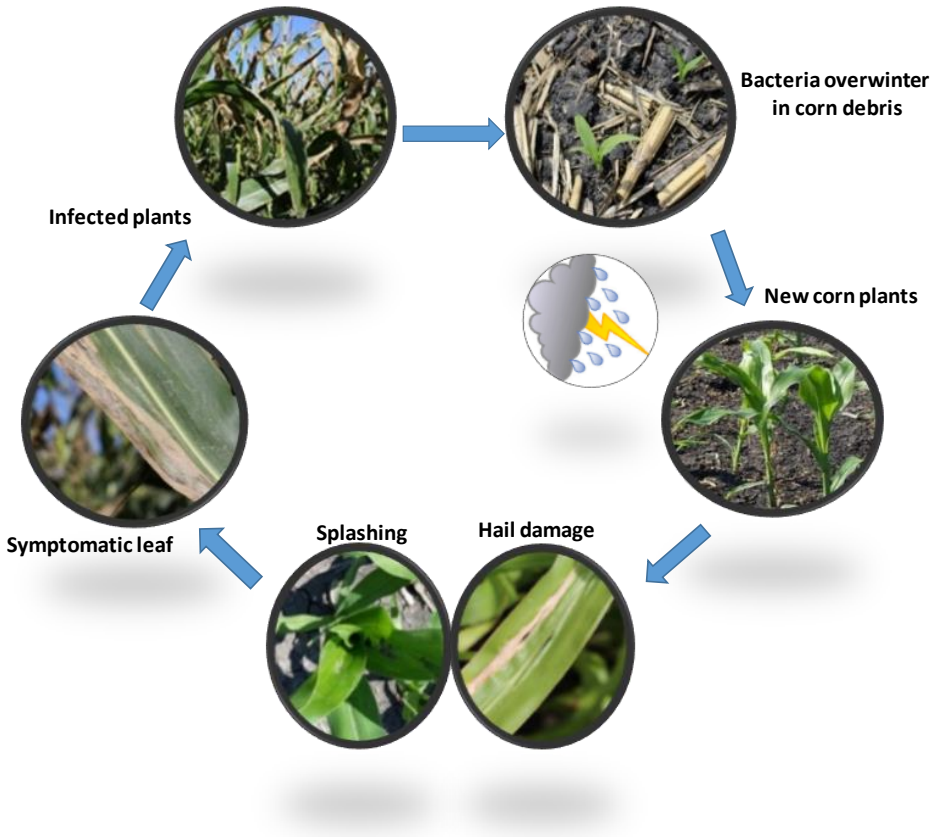

Figure 1.1. Infection process of Goss's bacterial wilt of corn. The pathogen overwinters in old corn stubble infected with inoculum. With wounding, which is the primary point of entry or by stomatal openings, the pathogen can penetrate the tissues to begin infection. Environmental conditions such as wind, hail, and rain splash influence infection and disease severity in a particular growing season which are normally seen on the leaf as freckles.

Bacterial spread can occur through infected stubble from previous cultivation and seed transmission is also possible, although minimal (0.1-0.4%) (Biddle et al., 1990). Other inoculum sources include insects and alternative hosts. *Clavibacter nebraskensis* adheres to leaf surfaces

and survives as an epiphytic pathogen (Li et al., 2018; Marcell and Beattie, 2002). This epiphytic strategy of Cn is crucial for disease development. Epiphyte on uninfected corn leaf and rainfall event can lead to disease outbreak and spread to new areas (Eggenberger et al., 2015).

A multistate survey on the re-occurrence of the Goss's wilt pathogen in the Midwest U.S.A., relative to disease incidence, severity and re-emergence suggested that hybrid resistance and plant population density are the key predictors of the disease (Langemeier et al., 2015).

Yield loss due to Goss's wilt in susceptible corn can be high when infection is severe. Recent studies by Mbofung et al. (2016), demonstrated the colonization ability of Cn in both susceptible and resistance corn hybrids. Their results indicated that leaf blight was more severe in susceptible hybrids. Visible blight lesion symptoms were seen on both susceptible and resistant hybrids, suggesting that the bacteria are able to overcome the basal defenses in the host plant (Mbofung et al., 2015). Also, a 63% yield loss difference between susceptible and resistant hybrid in the same plot has been reported (Jackson et al., 2007). The pathogen can also cause infection at any stage of plant development but economic yield losses of about 50% can occur when the infection starts at the early stages of plant growth (Ahmad et al., 2015). Mehl et al. (2015) also confirmed that about 50% yield losses can occur in young seedlings under severe epidemics and at systemic infection stage.

1.2.3 Host range of Cn

Monocots including sweet corn and dent corn are the primary host of Cn (Jackson et al., 2007a). *Clavibacter nebraskensis* has the ability to proliferate on other plants and develop an infection (Vidaver and Lambrecht, 2004). Several plant species, including grain sorghum (*Sorghum bicolor*), teosinte (*Euchlaena Mexicana*), shattercane (*Sorghum bicolor*), Eastern gamagrass (*Tripsacum dactyloides*), sudangrass (*Sorghum vulgare sudanse*), sugarcane (*Saccharum officinarum*) (Schuster, 1975), and barnyard grass, (*Echinochloa crus-galli*) have been reported as hosts of Cn (Wysong et al., 1981; Jackson et al., 2007a; Robertson and Jesse, 2008). Experiments under greenhouse conditions also confirmed that four *Setaria* species inoculated with Cn can act as alternative hosts (Langemeier et al., 2014). Ikley et a. (2015), also reported that annual ryegrass (*Lolium multiflorum*), Johnsongrass (*Sorghum halepense*), and large crabgrass (*Digitaria sanguinalis*) can be infected by Goss's wilt.

1.2.4 Management strategies

The use of resistant hybrids and good agronomic practices over the years have been effective Goss's wilt management techniques. Agronomic practices such as tilling to break the inoculum cycle and to increase corn residue decomposition and rotating to nonhost crops have contributed to decreased persistence and spread of the pathogen (Schuster 1975; Jackson et al., 2007a; Jackson, 2009). Since the bacteria overwinter in infected crop debris, infection in the subsequent growing season may be high if infected debris is not properly managed (Jackson et al., 2007a; Jackson, 2009).

No chemical agent is available to effectively reduce infection by the pathogen. Limited control of the disease has been reported in a few studies with copper hydroxide and citric acid (Mehl etal., 2015). Schlundt (2015) showed that spray adjuvants usually used in corn production do not influence Goss's wilt severity. However, increased adjuvants spray at higher rates might decrease the population of epiphytic Cn below the levels required for infection (Schlund, 2015). Even though not effective on Goss's wilt bacteria, fungicides are often used by growers. Fungicide application is directed towards yield increase rather than disease control (Wise and Mueller, 2011). According to Wise and Mueller (2011), in 2011 over four million-corn acres cultivated in the U.S were sprayed with fungicides, with some growers applying fungicides even in the absence of disease, probably as a prophylactic measure.

2.0 General mechanisms of plant responses to pathogen infection

2.1 Hypersensitive response

Plants consistently defend themselves against pathogenic infections from bacteria, fungi, and viruses. One means plants have evolved to combat pathogen attacks involves the activation of the hypersensitive response (HR), a process triggered upon pathogen recognition by plants. The HR occurs when a resistance gene (R gene) of the host plant binds with matching avirulent gene products secreted by the pathogen (gene-for-gene interaction) (Jia et al., 2000). The receptor proteins, that is the R proteins produced by the plant, have specificity to certain pathogen antigens. When an R protein binds to a pathogenic antigen, induction of signal transduction occurs within the cell triggering the activation of HR (Jia et al., 2000). The HR is considered a plant's immediate defense response, to arrest pathogen in the local infection zone thus preventing subsequent spread of the infection (Greenberg, 1997). The HR also serves to

starve and prevent the development of invading pathogens by intentionally killing the infected and surrounding cells by programmed cell death (PCD), resulting in a physical barrier composed of dead cells. In the HR process, the wall of dying plant cells becomes stronger and accumulates toxic phenolic compounds and phytoalexins (Dangl, 1996). The HR response in pathogen-invaded cells occurs within a few minutes and is rapidly accompanied by localized gene activation resulting in the accumulation of reactive oxygen species (ROS) and cellulose, which increases cell wall thickness (Kombrink and Schmelzer, 2001). Some visible features of HR include the collapse of challenged cells, membrane damage, necrosis, thickening of cell walls and browning of the cytoplasm (Greenberg et al., 1994; Ponce De León et al., 2012).

Biochemical and molecular studies suggest that activation of caspase-like enzymes are implicated in the HR. Reduction of HR can indeed be achieved using artificial peptide caspase inhibitors (Lam et al., 2001). Key elements in the induction of HR are nitric oxide (NO), ROS, salicylic acid (SA) and calcium, mitogen-activated protein kinases (MAPKs) (Oliver et al., 2009; Kombrink et al., 2001). Early detection of the pathogen by plant receptors stimulates signal transduction pathways, which translocate Ca^{2+} and protons across the membrane into the cytosol. This translocation results in the activation of enzymes such as NADPH-oxidase and peroxidases which generate ROS. A concomitant increase of NO and SA occurs. The diffusion of stress hormones such SA, Jasmonic acid, and ethylene generated in HR-developing cells, activates both local acquired resistance (LAR) and systemic acquired resistance (SAR) (Oliver et al., 2009; Kombrink et al., 2001).

2.1.1 Bacterial effector secretion system

During plant-pathogen interactions, both the host plant and pathogen have adopted many mechanisms to distribute their effectors during the interaction process. Several gram-negative plant bacterial pathogens manipulate their host cell functions through the secretion of proteins, referred to as effectors into the plant cell cytosol by means of the type III secretion system (T3SS) (Mudgett, 2005). The T3SS, also known as injectisome, is a needle-like attachment that enables ejection of proteins directly into the host cells. Allocation of bacterial effectors across the plant cell wall through the T3SS can efficiently reduce the plant mechanisms of immunity, enabling bacteria invaders to propagate unrestricted (Mudgett, 2005). When a plant receptor recognizes a pathogen-associated molecular pattern (PAMP) it induces plant defense response that often leads to the generation of antimicrobial compounds that hinder the development and spread of attacking bacteria, a process that can eventually lead to HR.

Clavibacter is a gram-positive bacterium, which does not possess the T3SS. Hence, not much is known about its pathogenicity. Eichenlaub and Gartemann (2011) demonstrated that Cmm and Cms are able to inject effectors that lead to pathogenicity and establishment of a colony in the plant, but their targets and mechanisms of translocation into the host cells remain elusive. Previous research has also indicated that Cms produce effectors that can induce an HR when the effector is localized to the apoplast of plant cells, implying that certain disease causing effectors can cause infection without penetrating plants cells (Lu et al., 2015).

2.1.2 Bacterial pathogenicity

Comprehensive studies on molecular mechanisms of disease development have been conducted in Cmm and Cms. However, Cmm, responsible for bacterial canker and wilt of tomato (*Lycopersicon esculentum*), is preferentially utilized as a model system (Davis et al.,1984). The virulence factors for this pathogen are encoded by a serine protease gene (*pat-1),* and a cellulase gene (*celA).* The *celA* gene is required to initiate bacterial canker in tomatoes and potato (Laine et al., 2000).

The second virulence factor, *pat-1,* belongs to the family of serine proteases and it is located on plasmid pCM2 in Cmm (Dreier et al., 1997). Homologs of *pat-1* are also found in some of the subspecies of *C. michiganensis*. In Cmm, the homolog of *pat-1* is known as the chromosomal homolog of *pat-1 9* genes *(chp),* when present on chromosomal DNA and plasmid homolog of the *pat-1* gene (*php)* when located on plasmid DNA. There are 9 *pat-1* in Cmm: 7 *chps* and 2 *phps* (Gartemann et al., 2008). *Clavibacter michiganensis* subsp. *michiganensis* contains a 129 kb region that is necessary for causing disease; this region is usually referred to as Pathogenicity Island (Gartmemann et al., 2008).

Plasmids can be translocated from one bacterial cell to the other through the process of horizontal gene transfer. Therefore, a plasmid with a virulence factor can cause an avirulent isolate to become virulent (Jahr et al., 2000; Laine et al., 2000). The role of plasmids in virulence of tomato and potato pathogens is clear, while in Cn the presence of a plasmid is not necessarily associated with virulence (Gross and Vidaver, 1979). Virulent isolates can be devoid

of plasmids, whereas the plasmids can be present in avirulent isolates. The work of Ahmad et al. (2015), compared 33 sequenced virulence genes in the grouping of pathogenic and non-pathogenic isolates of Cn. Single nucleotide polymorphisms marker (SNPs) discovered five of the genes tested, including cellulase A (*celA*), but neither the single, and or combined potential virulence genes were enough to differentiate between infectious and non-infectious isolates (Ahmad et al., 2015).

2.1.3 Host targets of plant pathogen effectors

Once the pathogen is inside the host cell, the plant defense system is equipped with receptors known as Pattern recognition receptors (PRRs). These PRRs are usually receptor-like proteins (RLPs) or receptor-like kinases (RLKs) localized within the plant plasma membrane. They play a vital role in the intrinsic defense response by detecting Pathogen Associated Molecular Patterns (PAMPs) and induce the main defense layers utilized by plants: Effector-triggered immunity (ETI) and Pattern-Triggered Immunity (PTI) (Göhre et al., 2008) (Fig 2.1).

Pattern recognition receptors (PRRs) are usually the suitable targets for pathogen effectors to suppress innate immunity. For instance, in tomatoes, Arabidopsis and other higher plants, flagellin sensitive 2 (FLS2) is the corresponding PRR for bacterial flagellin (flg22) (a stretch of 22 amino acid in the N-terminal of flagellin) and convenient target of *Pseudomonas syringae* effectors AvrPto and AvrPtoB. AvrPto is delivered into the plant cell via the T3SS to disrupt PTI by preventing the activity of FLS2 or interrupting the formation of FLS2-BAK1 complexes (Xiang et al., 2008). Even though its main purpose is to target PRRs for degradation, AvrPtoB

is able to overcome ETI by physically interacting with a protein kinase Fen, which results in disease susceptibility in Fen-expressing tomato lines (Rosebrock et al., 2007).

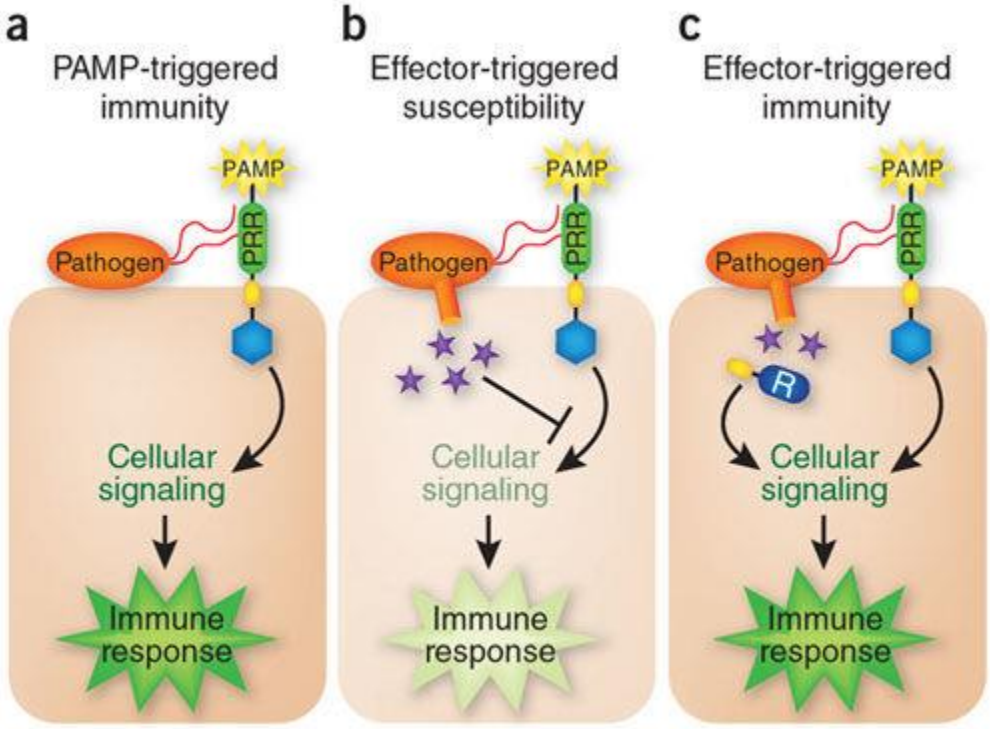

Figure 2.1. Plant-pathogen interaction processes. (a) Pathogen-associated molecular patterns (PAMPs) trigger pattern-recognition receptors (PRRs) in the host; enhancing the activation of downstream signaling cascade that induces PAMP-triggered immunity (PTI) (b) Virulent pathogens have developed effectors that repress PTI, stimulating effector-triggered susceptibility (ETS). (c) plants have also evolved acquired resistance (R) proteins that are able to detect these pathogen-specific effectors, ensuing in a secondary immune response known as effector-triggered immunity (ETI) (Pieterse et al., 2009).

2.2. Plant hormone involvement in defense response

During plant defense responses plant hormone signaling is involved in the induction of active defense mechanisms such as the expression of pathogenesis-related proteins (PR proteins), oxidative burst and/or PCD in the host systems (Glazebrook, 2005). Key plant hormones such as ethylene (ET), jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA) participate in plant defense signaling pathways (Bari and Jones, 2009). Activation of defense signaling is specific and dependant on the kind of pathogen involved in the infection process. Salicylic acid is usually associated with mounting resistance against biotrophic pathogens, while JA and ET normally provide protection against necrotrophic pathogens (Bari and Jones, 2009).

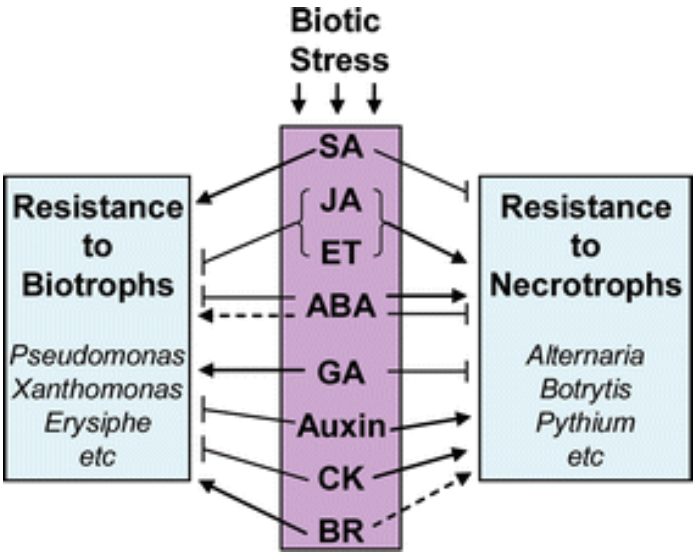

Figure 2.2. Hormonal involvement in the modulation of plant resistance against various pathogens. The arrows show induction or positive interactions and blocked lines represent suppression or negative interactions (Bari and Jones, 2009).

2.2.1 Plant hormone biosynthesis and signaling pathways

Biosynthesis of JA usually occurs in the chloroplasts. Conversion of linolenic acid to 12-oxophytodienoic acid is mediated by the activities of lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC). This is followed by the reduction of 12-oxophytodienoic acid by 12-oxophytodienoic acid reductase (OPR) in the peroxisomes, and the activation of a β-oxidation phase leading to the formation of JA (Devoto et al., 2002; Bosch et al., 2014).

The three major JA signaling components are coronatine insensitive 1 (*COI1*), jasmonate resistant 1 (JAR1) and jasmonate insensitive 1/MYC2 (JIN1/MYC2). *Coronatine insensitive 1* encodes an F-box protein that regulates the protein complex, essential for most JA dependant responses (Chini et al., 2009). *Jasmonate resistant 1* encodes an amino acid synthase that can catalyze the conjugation of isoleucine to JA, an active signal that can activate defense responses in plants (Kombrink, 2012), while MYC2/JIN1 is a transcription factor that modulates JA responsive gene transcription (Kazan and Manners, 2008).

Build-up of JA in plant cells has been shown to trigger the *COI1* receptor involved in modifying and degrading protein (Devoto et al., 2002). This eventually leads to the elevation of JA-triggering genes such as *PROTEINASE INHIBITORS* and *DEFENSIN* (Chini et al., 2009). In addition to providing defense against several pathogens as shown in (Fig. 2.3 Creelman and Mullet, 1995), JA signaling plays an essential role in triggering plant protection against herbivores such as spider mites and leafhoppers (Howe and Jander, 2008).

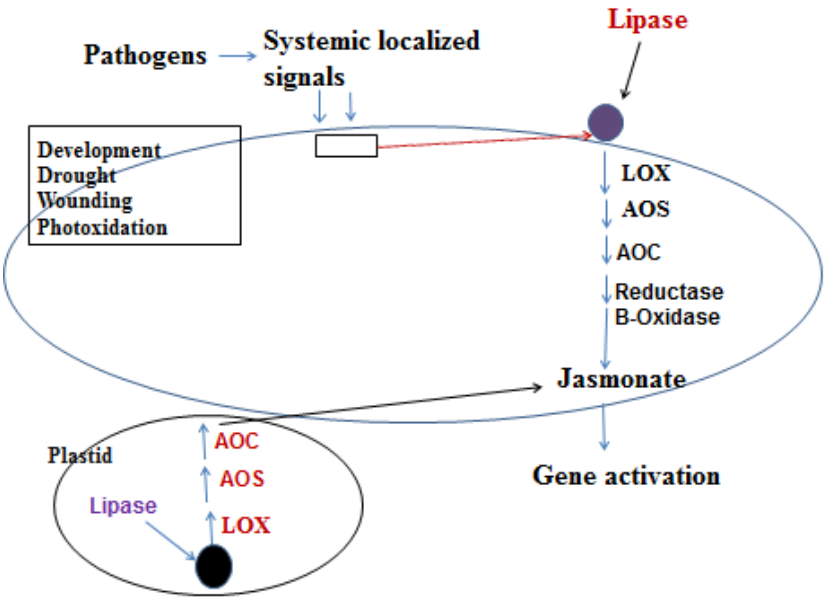

Figure 2.3. Jasmonic acid pathway during pathogen invasion. Localized and systemic elicitors are known to be produced by insects and pathogens that interact with plasma membrane receptors. The activation of receptors induces the lipase-mediated formation of linolenic acid followed by conversion to JA. These reactions require the activities of lipoxygenase (LOX), (AOS), (AOC), 12-oxo-PDA reductase, and X3-oxidation. Adapted from (Creelman and Mullet, 1995).

Ethylene is a gaseous plant hormone, which participates in stress responses such as injury, osmotic stress, hypoxia, and pathogen attack. Its role in plant-pathogen interaction and disease resistance is well recognized (Yang et al., 2015). Ethylene is a signaling molecule in plants and can diffuse rapidly from cell to cell across membranes (Wang et al., 2016; Geisler-Lee et al., 2010).

Ethylene biosynthesis begins with the conversion of methionine into S-adenosylmethionine (AdoMet), a reaction catalyzed by AdoMet synthetase, followed by synthesis of ACC, 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase 1-aminocyclopropane-1-carboxylic acid synthase (ACS). 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) then produces ethylene (Geisler-Lee et al., 2010) (Fig 2.4). In maize, the ACS multigene family is comprised of three members, *ZmACS2, ZmACS6,* and *ZmACS7* while *ACS* in *Arabidopsis* has nine members. The maize ACO family contains four members: *ZmACO15, ZmACO20, ZmACO31,* and *ZmACO35*. *ZmACS6* is known to be responsible for most ethylene synthesis in the leaves (Geisler-Lee et al., 2010).

Ethylene receptors have been characterized in several plants such as rice and *Arabidopsis*. In rice five ethylene receptors have been described: *ERS1* and *ERS2* from subfamily I, and *ETR2*-like (*ERL1*), *ETR4* and ETR5 from subfamily II (Wuriyanghan et al., 2009). The same number of ethylene receptors have also been identified in *Arabidopsis*: namely *ETR1, ETR2, ERS1, ERS2,* and *EIN4* (Hua and Meyerowitz, 1998). These receptors act upstream of the *constitutive triple response* (*CTR*) gene. *CTR* triggers ethylene insensitive gene which is a constituent of a signaling cascade that activates an ethylene-response protein, encoded by the gene *EIN3 binding f-box protein* or *EBF* (Potuschak et al., 2003).

Ethylene response during abiotic stress has shown that the absence or reduction of ethylene signaling in the plant might result in either increased in resistance or susceptibility in susceptible plants. This confirmation was established by the *Arabidopsis* ethylene *insensitive ein2* mutant

infected with *Xanthomonas campestris* pv. *campestris* and *P. syringae* pv. tomato (Pst), showed a greater decrease in symptoms expression of the disease (Bent et al.,1992). Similarly, infection of the ethylene-insensitive tomato line, Never ripe (Nr) led to reduced symptom development (Lund et al., 1998). In contrast to these results, ethylene insensitivity increased susceptibility to pathogens in several crops such as tobacco, soybean, and Arabidopsis (Hoffman et al., 1999; Thomma et al., 1999; Knoester et al., 2018;). Leaf senescence and fruit ripening are enhanced by ethylene, which in some pathogen interactions might lead to an increased disease susceptibility (van Loon et al., 2006).

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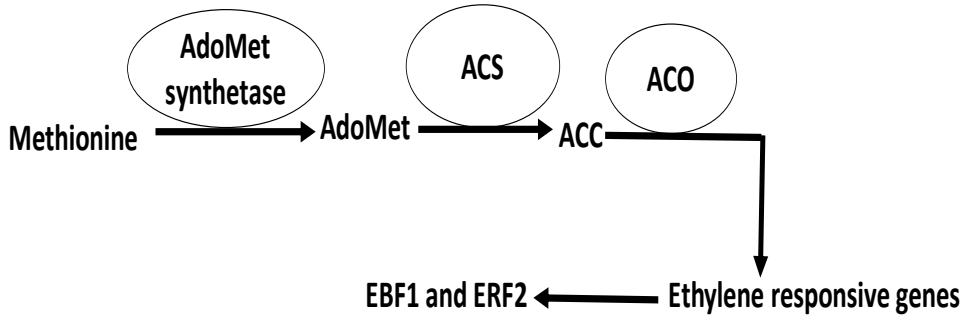


Figure 2.4. Ethylene biosynthetic pathway. Methionine (Met) is the precursor in ethylene biosynthesis, which is converted to AdoMet (S-adenosylmethionine) by AdoMet synthase, following by production of ACC by ACS. 1-aminocyclopropane-1-carboxylic acid (ACC) undergoes oxidative cleavage to form ethylene; a reaction catalyzed by ACO (adapted from (Muday et al., 2015).

Salicylic acid (SA) signaling is implicated in triggering SAR which eventually activates the HR process. Accumulation of SA is associated with the initiation of localized PCD that inhibits biotrophic pathogens from feeding on living plant cells (Glazebrook, 2005). Lawton et al. (1995)showed that enhanced resistance to further pathogen infections in *Arabidopsis* plants requires SA, while Gaffney et al. (2008) demonstrated that induction of SAR, in tobacco is activated by SA. Salicylic acid signaling transduction has been reported to be essential for resistance against *Puccinia graminis,* a biotrophic pathogen that causes stem rust of wheat (Crampton et al.,2009).

2.2.2 Crosstalk between hormone signaling networks

Responses to abiotic stress are often mediated by the interaction of two or more hormones. For example, while an antagonistic association between SA and JA/ET has been documented, there is also evidence that the same hormones might act synergistically (Spoel and Dong, 2008). An example of antagonistic relation was demonstrated in the defense responses of Arabidopsis challenged with silver leaf whitefly (*Bemisia tabaci*) where the activation of SA response blocked JA synthesis (Kempema et al., 2006). Interestingly, ET is also implicated in the SA-JA signaling interaction in a nonexpressor of pathogenesis-related 1 NPR1-dependent fashion (Leon-Reyes et al., 2009). In *Arabidopsis,* the *npr1-1/ein2-1* double mutant was utilized to establish the link between ET and NPR1 in modulating the JA-SA crosstalk. It was reported that ET mediates the antagonistic roles of JA and SA through NPR1 and that this mediation led to the SA-dependent induction of PR genes (Leon-Reyes et al., 2009). The *WRKY* gene transcription factors also modulate SA and JA responses (Chen et al., 2012). Upregulation of *WRKY70* promoted the synthesis of SA-responsive *PR* genes, such as *PR*-1, and increased protection

against the biotrophic pathogen. However, an elevation in *WRKY70* expression also decreased the transcription of JA-responsive *PR* genes, such as *PR-9,* that increased necrotrophic pathogen vulnerability (Li et al., 2004). Thus it is evident that *WRKY70* induces the SA-dependent pathway but acts as a negative regulator of JA-dependent defense signals (Li et al.,2004).

Jasmonic acid and ethylene interact synergistically to activate inducible defense response signals against pathogen attack (Kim et al., 2015; He et al., 2017). Studies have shown that several defense genes, such as pathogenesis-related genes (*PR*) typically induced by JA or ET, are expressed at higher levels following pharmacological treatments elevating both JA and ET at the same time (Schenk et al., 2000). Based on the above evidence, it is apparent that plant defense mechanisms are often controlled/regulated by the interaction of more than one hormone.

2.2.3 Nitric oxide

Nitric oxide (NO) is a highly reactive signaling molecule, a gaseous free radical that can quickly diffuse into the lipid membranes (Romero-Puertas et al., 2004; Wilson et al., 2008). Currently, NO has emerged as one of the most important plants signaling molecules during both development and stress responses. The effects of NO in signaling is generally dose-dependent. In tomatoes, for examples, high concentrations of NO inhibited growth and development, whereas lower levels enhanced growth (Palavan-Unsal and Arisan, 2009). Thus, factors affecting NO synthesis and homeostasis are critical in the mediation of NO responses.

2.2.3.1. Synthesis of NO in plants

In a plant cell, NO can be produced via the inorganic nitrogen pathway or by enzymatic activity (Romero-Puertas et al., 2004). The inorganic NO generation pathway involves the non-enzymatic synthesis of NO from nitrite (NO_2^-); this process occurs under low pH and requires a reductant (Caro and Puntarulo, 1998). Enzymatic production of NO requires nitrate reductase (NR). The NR production of NO has been established both during pathogen infection (Wilson et al., 2008; Palavan-Unsal and Arisan, 2009) and as a consequence of several types of abiotic stress (Wilson et al., 2008).

Nitric oxide synthase (NOS) is the key enzyme that regulates NO production in animals (Palavan-Unsal and Arisan, 2009), and mounting evidence suggests the presence of NOS-like activity in plants (Wilson et al., 2008). Nitric oxide synthase-like activity in plants have been found to be present in the cytoplasm, nucleus, and in the matrix of peroxisomes and chloroplasts (Palavan-Unsal and Arisan, 2009). Interestingly, NOS activity has been documented during plant-pathogen interaction in tobacco and *Arabidopsis* plants (Chandok et al., 2003).

2.2.4 Nitric Oxide and plant-pathogen interactions

Nitric oxide detection during plant-pathogen interaction has been associated with infection by biotrophic pathogens (Romero-Puertas et al., 2004), and more specifically linked to avirulent pathogens. This was demonstrated in both soybean plants and *Arabidopsis* cells challenged with avirulent bacteria (Delledonne et al., 1998; Zhang et al., 2003). Oat plants challenged with crown rust fungus showed a rapid elevation in NO at the onset of defense response, and a similar

observation was documented in tobacco leaves pretreated with fungal elicitor cryptogein (Foissner et al., 2000; Tada et al., 2004).

Nitric oxide influences plant responses to biotic stress by interfering with hormonal signaling through complex mechanisms. For example, NO interferes with SA and JA/ET signaling pathways in the activation of pathogenesis-related proteins, that provide effective protection against pathogens by establishing SAR (Van Der Biezen and Jones, 1998; Glazebrook, 2005; Mur et al., 2013). The NO-dependent SAR induction, mediated by PR-1, is further enhanced by applications of SA (Durner et al., 1998). During the antagonistic interaction of SA and JA/ET pathways NO has been suggested to play a downstream component influencing the production of ROS (Glazebrook, 2005; Mur et al., 2013).

The interaction between NO and ET is also complex, but independent evidence suggests NO might modulate ethylene level and response. For example, during the ET-mediated HR, NO induces the expression of ET biosynthesis through nitrosylation mechanisms (Mur et al., 2013). Furthermore, pharmacological treatments with the NO donor (SNP) increase the expression of ACC oxidase and ethylene responsive genes (Chun et al., 2012). The initial phases of HR during plant-pathogen interaction are influenced by the level of both NO and ROS (Keller et al., 1998; Delledonne et al., 1998). The simultaneous accumulation of NO and ROS is sufficient to trigger the HR in several systems, such as soybean and tobacco, while the independent rise of either NO or ROS is less effective in activating the death program (Delledonne et al., 1998; Pinto et al., 2002). These observations, along with the requirement of NO for the induction of several

defense-related genes such as phenylalanine ammonia lyase (*PAL*) and *PR1*, suggest that NO participates in cell fate determination during the plant-pathogen interaction, and more specifically influences processes related to the death program by interfering with hormones and other signal molecules.

2.2.5 Programmed Cell Death in plants (PCD)

Programmed cell death (PCD), a genetically controlled sequence of activities leading to cell suicide, is an integral component of plant development and responses to environmental stresses (Van Doorn et al., 2011). The process is characterized by chromatin condensation and fragmentation, cytoplasmic condensation and vacuolization (Van Doorn and Woltering, 2004 ; Olvera-Carrillo et al., 2012). Overall, there are two types of PCD: developmental-related PCD and environmental-related PCD. Developmental related PCD usually takes place under unstressed conditions, as in the formation of treachery elements and the elimination of senescent old tissues and organs. These are the most cited examples (Greenberg et al., 1996; Olvera-Carrillo et al., 2012). Environmental-related PCD is generally activated by biotic and abiotic factors, as in the HR occurring during plant-pathogen interactions leading to the death of cells at infection zones to limit the spreading of the pathogen (Drew et al., 2000; Greenberg, 1996).

The mechanisms leading to cell suicide are well characterized in animals (Liu et al., 2005) but poorly understood in plants (Dangl and Jones, 2001). Animal cells undergo apoptosis via two main pathways regulated by a complicated controlling network: the external pathway (death receptor pathway) or the internal pathway (mitochondrial pathway). Caspase enzymes are the

key executioners of apoptosis in animals (Lam, 2004). Plants might share similar mechanisms and components, such as metacaspases, caspase-like proteins (Lam et al., 2001). In plants, PCD can be activated by toxins or elicitors generated by a number of pathogens that include the fungal toxin victorin, xylanase from *Trichoderma viridae*, and '*harpins*' produced from *Pseudomonas syringae*, *Erwinia amylovora*, and *Xanthomonas campestris* (Lam et al.,1999). Also, plant viruses such as tobacco mosaic virus (TMV) have been suggested to activate PCD (Del Pozo and Lam, 2003).

Several events have been associated with the induction of the death program including alterations in the mitochondria functions linked to the production of ROS, reduction in ATP synthesis and the interruption of electron transport chain (Ferri and Kroemer, 2009; Bras et al., 2005). The ATP synthesis levels during the cell death process could determine the fate of the cell to either undergo PCD or necrosis (Formigli et al., 2000). Calcium is also crucial in regulating mitochondrial activities and ATP synthesis. For example, the accumulation of Ca^{2+} in the mitochondria leads to the over-production of ROS, compromising membrane function and ultimately triggering PCD (Green and Kroemer, 2004). The involvement of ROS in the death program has been well established (Jin and El-Deiry, 2005).

2.2.6. Reactive oxygen species

Reactive oxygen species (ROS) are signaling molecules that regulate various biological activities especially in response to biotic and/or abiotic cues (Mittler et al., 2011). They include free radicals such as the superoxide anion (O_2^-) and non-radical molecules like hydrogen

peroxide (H_2O_2) and singlet oxygen ($^1O_2$). In plants, production of ROS can occur in several compartments, namely chloroplasts, mitochondria, and peroxisomes (Mittler et al., 2004). The major source of ROS in plants is the NADPH oxidase enzyme complex composed of several subunits, some of which are referred to as respiratory burst oxidase homologs (RBOH) (Miller et al., 2010; Suzuki et al., 2011). The plant NADPH oxidase enzyme complex is induced under many biotic and abiotic stress conditions, and especially in cells challenged with a pathogen (Torres and Dangl, 2005).

Reactive oxygen species act as signal molecules in diverse pathways (Suzuki et al., 2011), but they can be extremely damaging to cells if produced at high levels. This can lead to detrimental effects like lipid peroxidation, protein unfolding, enzyme inactivation and DNA damage (Miller et al., 2010). All these types of damage are extremely harmful and can ultimately cause death (You and Chan, 2015). The level of ROS is dependent not only on their synthesis but also by the activity of the cellular antioxidant machinery able to scavenge ROS, thus alleviating their damaging effects. Several antioxidants such as ascorbic acid, glutathione, as well as ROS scavenging enzymes such as superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase, and peroxiredoxin are crucial for ROS detoxification (Miller et al., 2010). Hence, the ability of plant cells to detoxify ROS is equally important as their synthesis for cell survival (Miller et al., 2010).

During plant-pathogen interactions, the execution of PCD in the HR has been linked to the activation of RBOHs and the over-production of ROS (Torres and Dangl, 2005). In *Arabidopsis*, it has been found that *AtRbohD* and *AtRbohF* are the main ROS producers that mediate the

activation of defense genes to various pathogens (Torres and Dangl, 2005). In tobacco, the *NtRbohD* is induced and responsible for the generation of ROS in cells challenged with the fungal elicitor cryptogein (Simon-Plas et al., 2002). Silencing of *NbRbohA* and *NbRbohB* in *Nicotiana benthamiana* leaves resulted in lower ROS production and limited resistance to *Phytophthora infestans* infection, suggesting the need of these genes for ROS accumulation (Yoshioka et al., 2003). Reduction or suppression of Rboh also influences HR mechanisms. This is the case in tobacco *Nbrboh*-silenced plants which, relative to wild-type plants exhibited reduced HR when infected with *P. infestans* (Yoshioka et al., 2003). It is worth mentioning that the Rboh-production of ROS might also modulate SAR, as demonstrated in the Arabidopsis *rbohD* and *rbohF* double mutant showing increased resistance against parasitic nematodes ((M. A. Torres et al., 2002); Siddique et al., 2014).

2.2.7 Phytoglobins (Pgbs)

Hemoglobins are globular proteins characterized in animals, plants, and bacteria with a secondary structure consisting of a myoglobin–fold and a heme prosthetic group with A-H helices that is settled in a hydrophobic cavity configuring a 3 on 3 sandwiches of helices above each other (Vázquez-Limón et al., 2012). The heme prosthetic group is capable of reversibly binding with oxygen and the rate in which hemoglobins bind to or release oxygen is important for their classification (Carl-vilhelm, 2016). Hemoglobins can also bind to other ligands such as NO, hydrogen sulfide and carbon monoxide (Gupta et al., 2011).

Extensive studies including genomic sequencing in a wide range of organism from bacteria to higher plants have been utilized to ascertain the various biological functions of these proteins (Hill, 2012). The first plant hemoglobin, named leghemoglobin, was originally found in soybean root nodules where it is involved in the symbiotic relationship between plant cells and different Rhizobia species during nitrogen fixation processes (Kubo, 1939; Hoy and Hargrove, 2008). Other non-symbiotic types of hemoglobins were subsequently identified in plants. To distinguish plant hemoglobins from hemoglobins in other species, the term Phytoglobin (Pgb) has been recently adopted (Hill et al., 2016).

2.2.7.1 Classification of Pgbs

It is generally accepted that Pgbs can be divided into four classes (Vinogradov et al., 2011). Besides symbiotic Pgbs, three classes of non-symbiotic Pgbs with specific roles in growth, development, and stress responses, have been identified (Stasolla and Hill, 2017). Their characteristics will be discussed below.

Symbiotic Pgbs

Leghemoglobins or symbiotic hemoglobins are expressed in root nodules of plants like soybeans, where they are involved in nitrogen (N) fixation by facilitating oxygen transport to symbiotic nitrogen-fixing bacteria (Kubo 1939; Hoy et al., 2007). Additional Pgbs in non-nodulating plants, like *Ulmaceae Parasponia, andersonii* in symbiosis with Rhizobium, have also been identified (Hoy and Hargrove, 2008). Symbiotic hemoglobin (sHb) can also be found in root

nodules of non-legumes like *Casuarina glauca* in symbiotic association with the actinomycete, Frankia (Hoy and Hargrove 2008; Hill 2012).

Class 1 non-symbiotic Pgbs

Class 1 non-symbiotic possess high oxygen affinities with low dissociation constants. The high oxygen affinity of class 1 Pgbs enables them to remain oxygenated even under very low oxygen concentrations (Hill, 1998; Dordas, 2009). The major role of class 1 Pgbs is to scavenge NO by oxygenating NO to nitrate in a process forming methemoglobin (Fig.2.5) (Igamberdiev and Hill, 2004; Gupta et al.; 2011). Expression of class 1 Pgbs is not confined to specific organs but rather found in seeds, embryos, root, leaves, and especially in young meristematic cells (reviewed by Hill et al., 2012). Class 1 Pgbs are involved in hypoxic responses (Igamberdiev and Hill, 2004), as well as different types of stress such as drought (Mira et al., 2017), flooding (Mira et al., 2016; Youssef et al., 2016), and biotic stress responses. For instance, increased resistance to pathogen was observed in Arabidopsis plants over-expressing a cotton class 1 Pgb (Qu et al., 2005; 2006). The role of Pgb during pathogenesis has been well studied. Specifically, class 1 Pgb expression appears to be more directly involved in plant-pathogen interactions (Mur et al., 2012b). Mur et al. (2013) examined the Pgb role in the interaction between Arabidopsis challenged with hemibiotrophic and necrotrophic fungi isolates. They indicated that suppressing Pgb1 levels elevated disease resistance against the pathogenic isolates, and the responses observed were mediated by increased NO levels (Mur et al., 2013). Similarly, in Arabidopsis altering Pgb1 levels enhanced changes in resistance towards both *Pseudomonas syringae* and *Botrytis cinerea*, and these effects were attributed to NO, ROS and

hormone modulation (Mur et al., 2013b). The activation of pathogenesis-related protein is also influenced by Pgb expression. An example is the induction of pathogenesis-related genes *PR-1* and *PDF1.2* in *GhHb1* transgenic plants elevated the level of resistance in Arabidopsis against *P. syringae* (Qu et al., 2006).

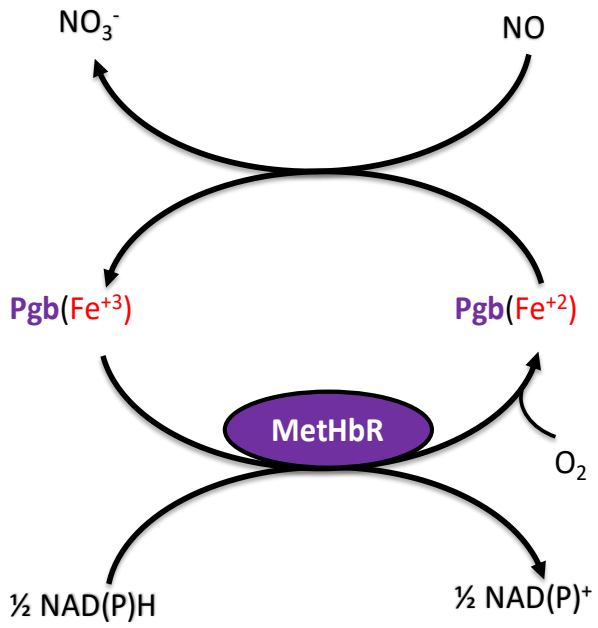


Figure 2.5. The Scavenging of nitric oxide (NO) by Pgbs. Nitric oxide is converted to nitrate through an interaction with oxygenated ferrous hemoglobin. Adapted from (Igamberdiev et al., 2005; Gupta et al., 2011).

Class 2 non-symbiotic Pgbs

Class 2 phytoglobins show tighter hexa-coordination than class1 phytoglobins. They have low affinity to bind oxygen and less sensitive to scavenge NO (Kakar et al., 2010). Class 2 phytoglobins are usually not induced under low oxygen deficit (Garrocho-Villegas et al.,2008) although their overexpression may enhance the survival under hypoxic conditions (Kakar et al., 2010). It has been found out that Class 2 Pgbs are triggered under specific conditions such as treatments with cytokinins or chilling, while nutrient deficiency does not influence their expression pattern (Hill 2012). Class 2 Pgbs are abundant in the vascular tissue, leaves and in cells connecting the pedicel with the stem (Hill 2012).

Class 3 non-symbiotic Pgbs

Class 3 Pgbs, also referred as truncated hemoglobin (trHbs) consist of a group of lower oxygen – binding heme proteins that exist within eubacteria, cyanobacteria, protozoa, and plants (Watts et al., 2001). They have a different structure from the other classes of Pgbs. Unlike other Pgbs possessing a ‘three on three’ alpha-helical fold, trHbs contain a ‘two on two’ alpha-helical fold (Hoy and Hargrove, 200). Watts et al. (2001) described the presence of these proteins in Arabidopsis. Truncated Pgbs are generally expressed throughout the plant but are the least investigated among all classes of Pgbs. They are suppressed under low oxygen concentrations (Gupta et al., 2011) and are generally not induced by conditions upregulating the other classes of Pgbs (Hill, 2012).

2.2.7.2 Phytoglobin signaling during development and under conditions of stress

Phytoglobin signaling is rapidly activated in cells under stress environments, and alteration in their expression level influences plant response to stress (Silva-Cardenas et al., 2003). It has been documented that the primary function of Pgbs is to scavenge NO, thus influencing NO homeostasis in the tissue (Igamberdiev et al.,2005). As a result, the cellular level of Pgbs influences all NO-mediated responses during plant development or under conditions of stress.

By modulating NO, Pgbs influence cell fate during maize somatic embryogenesis (Huang et al., 2014). Cells suppressing Pgbs accumulate NO which elevates the level of Zn^{2+}. An increase in Zn^{2+} triggers MAPK cascades activating NADPH oxidase activity and ROS production leading to PCD. Thus, cells suppressing Pgbs die while those expressing Pgbs remain alive. The activation of PCD as a result of Pgb suppression is important for removing cells during embryogenesis and influencing the number of somatic embryos produced. That is, suppression of *ZmPgb1.1*, expressed in many cells results in the death of large embryonic domains causing embryo abortion. Suppression of *ZmPgb1.2*, expressed in cells anchoring the immature embryos to the embryogenic tissue causes the death of the anchoring cells allowing the embryos to disperse into the medium and develop further at high frequency (Huang et al., 2014). This observation suggests that the expression domains of Pgbs are important in the final outcome of the response.

Phytoglobin-mediated responses also involve plant growth regulators. For instance, downregulation of Pgb levels induces the synthesis of ethylene (Manac'h-Little et al., 2005). The participation of ethylene in Pgb signaling is apparent during conditions of flooding. Corn plants exposed to flooding showed reduced photosynthesis and root death (Youssef et al.,2016; Mira et al., 2016). These conditions were alleviated when the levels of Pgbs was increased and aggravated when the level of Pgbs was suppressed. Suppression of Pgb resulted in a rapid increase in ethylene responsible for the accumulation of ROS and the death of the root meristematic cells. These effects were mediated by NO (Mira et al., 2016). Through ethylene, Pgbs also influence plant response to drought which causes the death of the root meristematic cells. These effects are more apparent in plants suppressing *Pgbs* which accumulate more ethylene and ROS (Mira et al., 2017).

Auxin and JA are also hormones indirectly affected by Pgbs. During Arabidopsis, somatic embryogenesis suppression of class 2 Pgb increases the level of JA which through the suppression of the transcription factor MYC2 elevates the level of auxin in the tissue ((Elhiti et al., 2013; Mira et al., 2016). High concentrations of auxin in the explants favor the formation of the embryogenic tissue and increase the number of somatic embryos produced (Elhiti et al., 2013; Mira et al., 2016). All these observations suggest that changes in hormone synthesis and/or response are governed by the level of Pgbs.

THESIS HYPOTHESIS

Based on the above information, the hypothesis tested in this thesis is that alterations in Pgb expression influence the response of corn plants to *Clavibacter nebraskensis* infection through mechanisms involving ethylene, ROS, and PCD.

OBJECTIVES

The hypothesis will be tested with three objectives:

1) Measure the lesion size and expression of genes involved in ethylene and ROS synthesis in corn plants over-expressing or down-regulating the maize Pgb *ZmPgb1.1*.
2) Develop a cell culture system where the responses observed in objective 1 can be duplicated.
3) Use the cell culture system to understand the link between Pgb, ethylene, ROS, and PCD using pharmacological studies.

3 CHAPTER: Suppression of Phytoglobin Proteins (Pgb) Increase Maize Seedling Tolerance to Goss's Bacterial Wilt Infection

3.1 Abstract

The effects of suppression of the *Zea maize* Phytoglobin 1 (*ZmPgb1*) on plant response to infection with *Clavibacter nebraskensis* (isolate Cmn14-5-1), were evaluated by measuring leaf lesion and physiological events associated to biotic stress. Relative to wild type (WT), suppression of *ZmPgb1* reduced the size of leaf lesions, and elevated the expression of genes involved in ethylene synthesis and response, as well as those contributing to the generation of reactive oxygen species (ROS). The same plants displayed a more pronounced accumulation of ROS and programmed cell death (PCD) in proximity of the inoculation site. Similar transcriptional responses were also observed in cultured maize cells infected with isolate Cmn 14-5-1. Pharmacological treatments were performed in culture to alter the levels of nitric oxide (NO), and/or ethylene. An experimental increment in NO with the NO donor sodium nitroprusside (SNP), or ethylene with the ethylene donor, ethephon (ET), increased the production of ROS and elevated the number of cells undergoing PCD in WT cells. An opposite response was observed in cells suppressing *ZmPgb1* when the level of NO or ethylene was reduced with the respective utilization of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) or aminooxyacetic acid (AOA). Collectively these findings suggest that suppression of *ZmPgb1* enhances tolerance to *Clavibacter nebraskensis* through a mechanism initiated by the accumulation of NO, mediated by ethylene, and culminating with the production of ROS and the execution of PCD.

3.2 Introduction

Goss's bacterial wilt, one of the most important diseases of corn, is caused by the gram-positive bacterium pathogen *Clavibacter nebraskensis* (Cn) (Li et al., 2018; Vidaver and Mandel, 1974). The disease affects plants at any developmental stage and can reduce yield by 50% or even more (Wysong and Doupink, 1984; Clafflin et al., 1999). *Clavibacter nebraskensis* usually overwinters in previously infected corn debris and is able to penetrate the host cell following physical wounding such as hail, heavy wind and sandblasting, or through stomatal openings and insect feeding (Załuga et al., 2014). The main characteristic symptoms associated with the disease include water-soaked spots and dark black spot commonly known as freckles along the margins or ends of developing lesions (Schuster et al., 1972). Persistence and recurrence of the disease have been linked to continuous corn production, changes in pathogen virulence, high relative humidity, reduced tillage practices, and varieties with inadequate resistance (Jackson et al., 2007; Załuga et al., 2014).

Disease management over the past decade has greatly depended on the use of resistant hybrid varieties, as well as good agronomic practices such as rotating to non-host crops, and tillage practices that enhance decomposition of infected residues (Jackson et al., 2007a; Jackson, 2009). Fungicide applications have been reported as ineffective in controlling the disease (Agarkova et al., 2011; Oser et al., 2013). The work of Mehl et al. (2015) demonstrated that the use of copper hydroxide or citric acid was unsuccessful for Goss's wilt management, and it was recommended that farmers should still depend on cultural strategies and planting of cultivars with some levels of resistance (Mehl et al., 2015).

The interaction between plants and pathogens is modulated by a complex network of signal molecules and plant growth regulators such as ethylene (ET), jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA) (Bari and Jones, 2009; Pieterse et al., 2009). Antagonistic and synergistic interactions among two or more of these hormones can lead to either induction or suppression of antimicrobial pathogenesis-related (PR) proteins (Fu and Dong, 2013), and potentially to the hypersensitivity response (HR), a process that deprives pathogens of water and nutrients at the infection site (Caplan et al., 2008).

The hypersensitive response (HR) is characterized by rapid and localized programmed cell death (PCD) following infection (Mur et al., 2008). At a morphological level, HR shares observable features common to necrosis and vacuolar plant cell death (Van Doorn et al., 2011). The HR process results from an incompatible plant-pathogen interaction. Hence, the HR is determined by the mutual association of both the plant and pathogen gene products (Peart et al., 2005). The HR is effective against many hemibiotrophic bacterial, fungal and viral pathogens that depend on the active host for survival (Spoel and Dong, 2012). Some hemibiotrophs use their effectors to prevent HR and keep the cells of the host plants alive in order to complete the infection process (Coll et al., 2011). Hypersensitive responses are activated by ROS and mediated by NO (Mur et al., 2013). Other key elements in the induction of HR are SA, Ca^{2+} and mitogen-activated protein kinases (MAPKs) (Oliver et al., 2009; Kombrink et al., 2001). For instance, some plant cells exposed to pathogens accumulate Ca^{2+} in the mitochondria leading to the over-production of ROS, compromising membrane function and ultimately triggering PCD (Green and Kroemer, 2004). Over-production of ROS is mainly mediated by the activity of

NADPH oxidases (Yun et al., 2011). Thus, factors influencing the level of NO, ROS, and PCD can alter the plant's response to pathogens.

Phytoglobins (Pgbs) are plant hemoglobin proteins. Present in both dicots and monocots, Pgbs play a fundamental role during normal growth and development, as well as during stress conditions (Hill, 2012). Pgbs can be categorized into four classes (Vinogradov et al., 2011). Besides symbiotic Pgbs, three classes of non-symbiotic Pgbs have been characterized (Stasolla and Hill, 2017). The major function of Pgbs is to scavenge NO (Gupta et al.,2011; Hill, 2012), thus influencing cellular NO homeostasis and all those processes regulated by NO. For example, by removing NO, Pgbs reduce the levels of ROS and ethylene which are raised under several types of stress, including hypoxia (Mira et al., 2016a) and drought (Mira et al., 2017). Decreasing both ROS and ethylene in cells expressing Pgbs protects cells from dying under both types of stress (Mira et al., 2016a; 2017). During embryogenesis Pgbs also interfere with JA and auxin (Mira et al., 2016b), and influence cell death acquisition. This is apparent during corn somatic embryogenesis where cells destined to die by PCD have reduced expression of Pgbs and the tissue-specific localization of Pgbs determines which cells will survive (Huang et al., 2014).

It has also been documented that Pgbs may influence plant-pathogen interaction. Activation of *Pgbs* usually occurs at the early phases of infection. This was demonstrated by Qu et al. (2005) who demonstrated an increase in *Pgb1* expression in cotton cells challenged with *Verticillium dahliae* (Qu et al., 2005). Additionally, experimental manipulations in *Pgb*

expression alter the resistance of Arabidopsis plants to both *Botrytis cinerea* and *Pseudomonas syringae*, through changes in NO, ROS and JA/ET content (Mur et al., 2012b).

Based on the role of Pgbs and their influence on downstream components implicated in plant-pathogen interactions, it is hypothesized that the level of Pgb influences the response of corn plants to *Clavibacter nebraskensis* infection through mechanisms involving NO, ethylene, ROS, and PCD. This would be the first evidence of Pgb effects on Goss's wilt of corn interactions, and would provide great insight into dealing with bacterial plant diseases, as no cost-effective solution exists other than genetic resistance.

3.3 MATERIALS AND METHODS

3.3.1 Plant materials

The WT and Pgb-downregulating (ZmPgb1:RNAi) Maize (Zea mays *L*.) lines were characterized previously by Huang et al. (2014). Plants were grown in soilless medium under controlled conditions in a growth chamber at a temperature of 22/18^{o}C day/night with a photoperiod of 16/8 hrs light/darkness.

3.3.2 Bacterial isolate inoculum preparation and growth conditions

The Goss's bacterial wilt isolate Cmn 14-5-1 was grown on a nutrient broth NBY containing 8 g/L nutrient Broth, 2 g/L yeast extract, 15g/L agar, 2g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 5g/L glucose, and 2.5 x10^{-4} mg/L $MgSO_4.7H_2O$ for 2-3 days at 23^{o}C (Goss and Vidaver, 1979). Loops of bacterial colonies were suspended in phosphate buffer (2.5 x10^{-3} g/L K_2HPO_4 and 2.5 x10^{-3} g/L KH_2PO_4, pH 6.7) to prepare the isolate inoculum. Cell concentration was measured by

spectrophotometer at OD_{600} = 1.0 (Agrakova et al.,2011) and adjusted to approximately 1×10^7 cfu/ml for inoculation.

3.3.3 Leaf inoculation process

Maize plants at the five leaf stage (four collar) were mechanically wounded using a 1 mL disposable syringe plunger mounted with a 5-mm sand paper disk. The wounds were induced on the 3rd and 4th leaves on either side of the middle vein (Soliman et al., 2018). Twenty microliters of the phosphate buffer solution was added on the wounds of the control (mock) plants, and 20µl of isolate Cmn 14-5-1 inoculum was added as a treatment on the three wounded leaves. Experiments were conducted in three biological replicates each consisting of three plants. After inoculation, plants were placed in a humidity chamber (100% relative humidity) using an ultrasonic humidifier overnight and then transferred into a growth room for 14 days. All measurements and gene expression studies presented in this work refer to the 4th leaf, as very similar results were observed for the 3rd leaf.

3.3.4 Lesion length measurement, disease rating, and sampling

The lesion length was measured upward and downward from the site of infection using a millimeter ruler at 5, 7, 10 and 14 days post-inoculation (DPI). The mean of eight measurements was used to calculate the total area under the disease progress curve (Total AUDPC) for each replicate. The disease severity was measured using the disease severity scale developed by Soliman et al. (2016). In brief, infected leaves were rated using the following scale: 0- Control – no leaf lesion other than wound; 1- chlorosis or reddening, no necrosis; 2- chlorosis or reddening with freckling, with about 10% necrosis; 3- chlorosis or reddening with freckling with about 10-25% necrosis or wilting; 4- 26-50% necrosis; 5- 51-75% necrosis and 6- 76-100% necrosis. Segments (2 cm) of leaves were excised around the inoculated area at 0h, 0.5h, 1h, 2h,

6h, 12h, 24h, 48h, and 216h after inoculation and immediately frozen in liquid nitrogen to limit the degradation of RNA. A schematic diagram of Goss's wilt infection process is shown in Fig. 3.1 below.

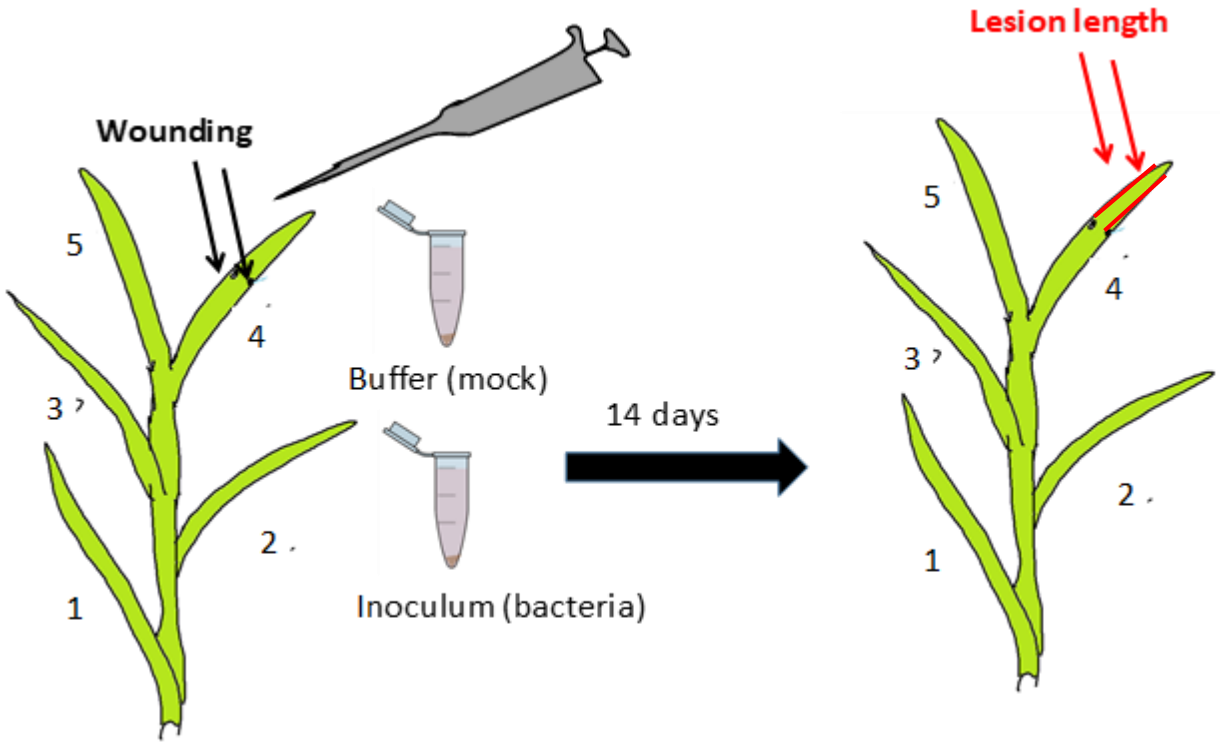


Figure 3.1. Schematic diagram representing the experimental procedure for the leaf inoculation process and lesion length measurement of Goss's wilt infection. Leaves were wounded and inoculated with 20µl of isolate Cmn 14-5-1 inoculum. Control or mock leaves were inoculated with 20µl of buffer. Lesion length was recorded from day 5 to day 14 after inoculation.

3.3.5 RNA extraction

RNA was extracted using the TRI Reagent Solution following the manufacturer's instructions (Invitrogen). Excised tissues about 2cm segment were ground in liquid nitrogen in a

mortar and pestle with 1 mL of TRI Reagent, and the solution was pipetted into a microcentrifuge tube and incubated at RT for 10 min. Samples were centrifuged at 12,000xg at 4°C for 10 min. The supernatant was transferred into a clean tube and 200 µL chloroform was added. The solution was gently inverted and incubated at RT for 10 min. The tubes were centrifuged at 12,000xg, and then the top layer was pipetted into a clean tube. RNA was precipitated by addition of 500 µL of 2-propanol, and samples were gently inverted and left to incubate for another 10 minutes. Samples were centrifuged at 12,000xg at 4°C. The RNA pellets were washed with 1 mL 70% ethanol/DEPC water, vortexed, and then centrifuged at 7500xg at 4°C for 5 minutes. The RNA pellets were air-dried for 2-5 min and then dissolved in DEPC water. The DNase I recombinant RNase-free kit from Roche (Sigma) was used to degrade DNA. With the samples on ice, 2 µL of DNase I and 10 µL of buffer were added to each sample, and the tubes were vortexed. The samples were incubated for 30 minutes at 37°C, 5 minutes at 10°C, and then for 10 minutes at 75°C. Prior to the 10°C step, 4 µL of EDTA per 100 µL sample was added. After incubation, 10 µL 3M NaOAc pH 5.5 and 200 µL 100% ethanol were added to each sample to precipitate RNA and the tubes were vortexed. Samples were then placed at -20°C overnight and subsequently centrifuged at 21,000 xg for 30 minutes at 4°C. The pellet was washed with 500 µL of 70% ethanol and dissolved in 88-100 µL DEPC water.

3.3.6 cDNA preparation and gene expression studies

The concentration of RNA was measured using a Nanodrop spectrophotometer and diluted to 1 mg/ µL for cDNA synthesis assay. The cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis. From the cDNA kit, 2 µL buffer, 2 µL primer, 0.8 µL dNPTs, 4.2 µL DEPC water, and 1 µL enzyme were added to 10 µL sample containing 1 mg RNA. Tubes were incubated for 15 minutes at 25°C, 2 hours at 37°C, and then 85°C for 5

minutes. The total cDNA was further used for measuring gene expression by quantitative (q)RT-PCR. All primers used for gene expression studies are listed in Supplementary Table 1. The relative gene expression level was analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using actin as the reference gene (Huang et al., 2014). For gene expression studies, three biological replicates were used each consisting of 3 different plants.

3.4 Cell culture experiments

Cultured cells of *Zea mays* (Hi II Type II) were generated from zygotic embryos, as reported by Huang et al. (2014). The cells were maintained for 2 weeks on solid medium composed of 4.6 g/L N6 salts, 1ml/L N6 vitamins, 100mg/L myo-inositol, 2mg/L 2,4-D, 30g/L sucrose and 3g/L phytogel at pH 5.8, proliferation was encouraged in liquid medium of similar composition but devoid of phytogel. Cells cultured for 1 week in liquid medium were exposed to induced (containing bacteria extracts) or un-induced (devoid of bacteria extracts, control) xylem sap.

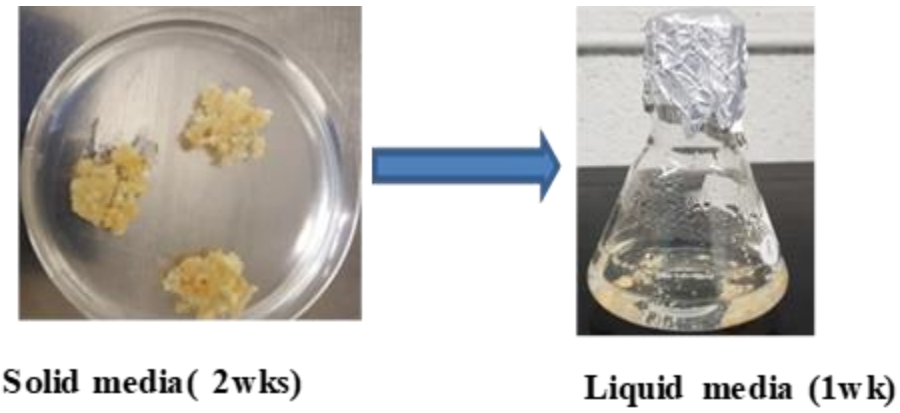


Figure 3.2. Maize callus maintained on a solid media for 2 weeks and proliferated in liquid medium for 1 week.

3.4.1 Preparation of xylem sap

Seeds of the commercial corn lines (CO447) were grown for 10 weeks in a growth room under controlled conditions as previously described. The stems were excised at the stem/root junction, and the cut surface was cleaned using Whatman filter papers for 5 minutes. The xylem sap was then collected for approximately 1 hr. The collected xylem sap was filtered through a 0.22 µm syringe filter and stored at -20ºC. The highly aggressive isolate Cmn 14-5-1 was cultured for 3-5 days on an NBY medium, and then 3-5 mL phosphate buffer was added to the plate and the bacterial lawn was harvested using a loop. The bacteria solution was centrifuged at 4000xg to collect the cells and washed two times with sterilized water. The bacterial pellet was re-suspended in the harvested corn xylem sap and incubated for 12 hrs at RT. The cells were separated by centrifugation at 6000xg for 20 minutes and the supernatant (induced xylem sap) was filtered through 0.22 µm syringe filter to ensure the complete removal of bacterial cells, and then stored at -20°C until used. Ten milliliters of the induced xylem sap was added to 50 ml suspension culture containing 1500 mg (fresh weight) of maize cells. Un-induced sap was used as a control. After the addition of the xylem sap, the maize cells were harvested at 0h, 3h, 6h, 9h and 24h for gene expression analysis and ethylene measurements.

3.4.2 Pharmacological treatments

Pharmacological treatments were applied after a week in proliferation medium prior to the addition of the induced or un-induced (control) xylem sap. The NO scavenger 2-(4-carboxyphenyl)-4, 4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) and the NO donor sodium nitroprusside (SNP) were applied at concentrations of 60 µM and 150 µM, respectively

(Elhiti et al., 2013). The ethylene donor ethephon (ETH) and the ethylene biosynthetic inhibitor aminooxyacetic acid (AOA) were both applied at 80 μM (Kapoor et al., 2018).

3.4.3 Ethylene measurements

Ethylene measurements were performed according to Geisler-Lee et al. (2010). About 1g of cells was pre-incubated in an unsealed 3-mL syringe for 30 min. The syringe was then sealed and incubated in the dark for 2h at 22°C. The gas accumulated in the headspace (1 mL) was analyzed with a Bruker 450-GC gas chromatograph (Mira et al., 2016). Data analysis was carried out using the Bruker Compass Data analysis 3.0 software.

3.4.4 Localization of programmed cell death (PCD), reactive oxygen species (ROS), and nitric oxide (NO)

Programmed cell death (PCD) was measured by nuclear DNA fragmentation using the In Situ Cell Death Detection Kit-Fluorescein (TUNEL assay) (Roche) as described by Huang et al. (2014). Leaf tissue or cells were fixed in 4% (w/v) paraformaldehyde, dehydrated in an ethanol series and embedded in wax. Sections (10μm) were dewaxed in xylene and labeled with the In Situ Cell Death Detection Kit according to the manufacturer's protocol, eliminating the permeabilization step by proteinase K (Huang et al., 2014).

To visualize superoxide, excised leaves or cells were placed in a 0.5mg/mL nitroblue tetrazolium (NBT) solution in 10mM potassium phosphate buffer (pH 7.6) in the dark at 25°C for 3 hours (Campbell et al., 2015). For hydrogen peroxide, excised leaves or cells were placed in a 2 mg/ L diaminobenzidine tetrahydrochloride (DAB) solution in 50mM Tris-acetate buffer (pH 5.0) and incubated in the dark at 25°C for 24h (Campbell et al., 2015). For both procedures, chlorophyll was removed from the leaves by boiling in 95% ethanol, followed by

rehydration in 10% glycerol for 24 hours (Campbell et al., 2015). Localization of NO was performed using 4, 5-diaminofluorescein diacetate (DAF-2DA) exactly as described by Mira et al. (2016 a).

3.4.5 Statistical analysis

Analysis of variance of the data was conducted using the GLMMIX procedure (SAS Institute, 2005) of SAS University Edition Version 9.04.01. Treatment means were compared using the Tukey test ($\alpha = 0.05$). Each experiment was replicated three times and each replicate consisted of six plants or flasks (for cultured cells).

3.5 Results

3.5.1 Suppression of *ZmPgb1* reduces lesion size in plants inoculated with Clavibacter nebraskensis isolate

The transcript levels of *ZmPgb1* were measured in WT leaves inoculated with isolate Cmn 14-5-1. The *ZmPgb1* levels showed two distinct peaks. The first, a minor peak, was observed 30 min after inoculation while a second, more pronounced peak, occurred around 48h after inoculation. A decline in *ZmPgb1* expression level was observed at later time (Fig.3.3). To assess the influence of *ZmPgb1* expression during the infection process, plants downregulating the same gene (ZmPgb1:RNAi line) and extensively characterized in previous studies (Huang et al., 2014) were utilized. Relative to inoculated WT plants where the lesion from the wounded area progressed rapidly towards the leaf base (Fig.3.4A), suppression of *ZmPgb1* reduced the length of the lesion (Fig. 3.4A and 3.4B).

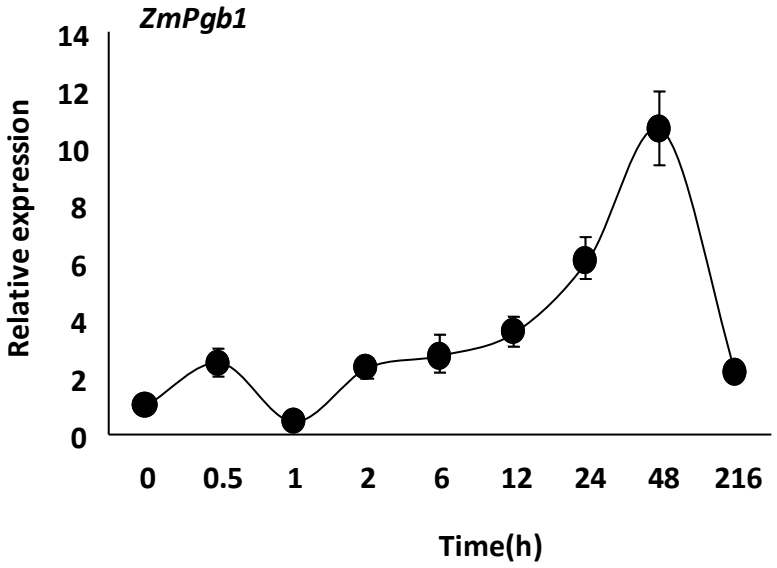


Figure 3.3. Relative expression of *ZmPgb1* in WT leaves over time inoculated with isolate Cmn 14-5-1. Expression values are the means of three biological replicates ± SE and are normalized to the WT mock (absence of bacterium) values at each respective time point.

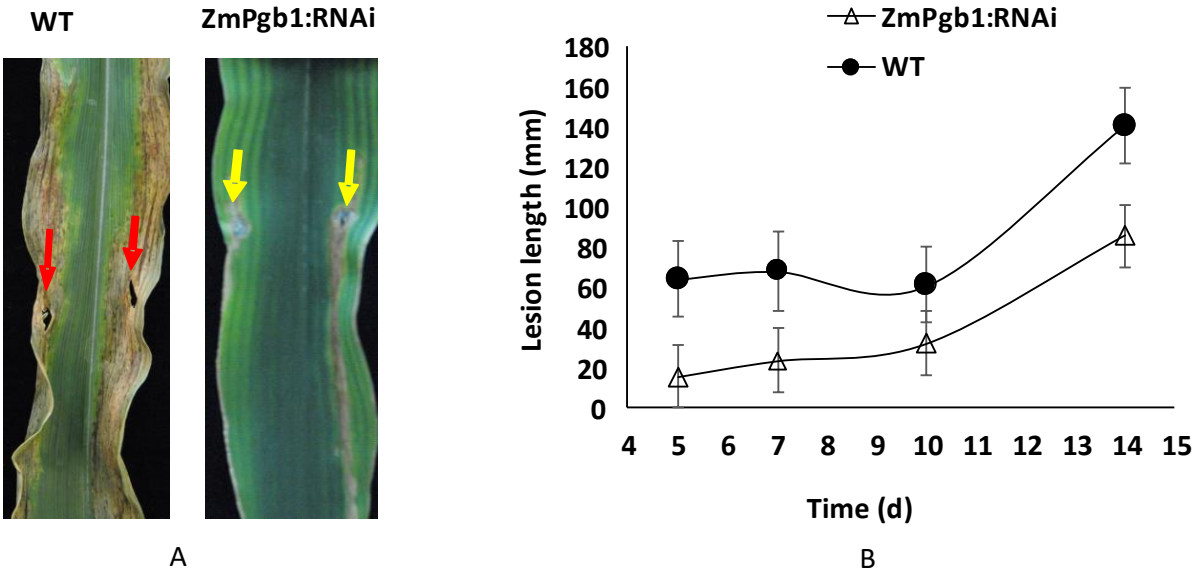


Figure 3.4. (A) Morphology of WT and ZmPgb1:RNAi leaves 14 days after inoculation with isolate Cmn 14-5-1. Arrows indicate inoculation sites. (B) Lesion length measurement in WT and *ZmPgb1:RNAi* leaves inoculated with the isolate Cmn 14-5-1.Values represent the means of three biological replicates ± SE.

3.5.2 Suppression of *ZmPgb1* induces transcriptional changes in ethylene biosynthesis and response

The involvement of *ZmPgb1* in the transcription of ethylene biosynthetic and response genes during the infection process was evaluated by measuring the transcript levels of ACC synthase (*ACS2, 6,* and *7*), and *ACC oxidase* (*ACO15, 20, 31,* and *35*), as well as the ethylene response factors *EIN3* Binding F-Box Protein 1 (*EBF1*) and Ethylene Response Factor 2 (*ERF2*). Relative to inoculated WT leaves, suppression of *ZmPgb1* increased the transcript levels of *ACS2* between 12 and 216h, *ACS6* after 12h, and *ACS7* between 6h and 24h (Fig.3.5). A similar induction in ZmPgb1:RNAi leaves was observed for *ACO15* between 24h and 216h, and *ACO20* between 12h and 48h (Fig.3.6). No major differences between WT and ZmPgb1:RNAi leaves were observed for *ACO31*, while a small increase in expression was measured for *ACO35* in WT leaves (Fig.3.6).

The two ethylene response genes, *EBF1* and *ERF2* showed a distinct expression peak between 6h and 24h in the ZmPgb1:RNAi leaves. These peaks were delayed by 12h in the WT leaves (Fig.3.7).

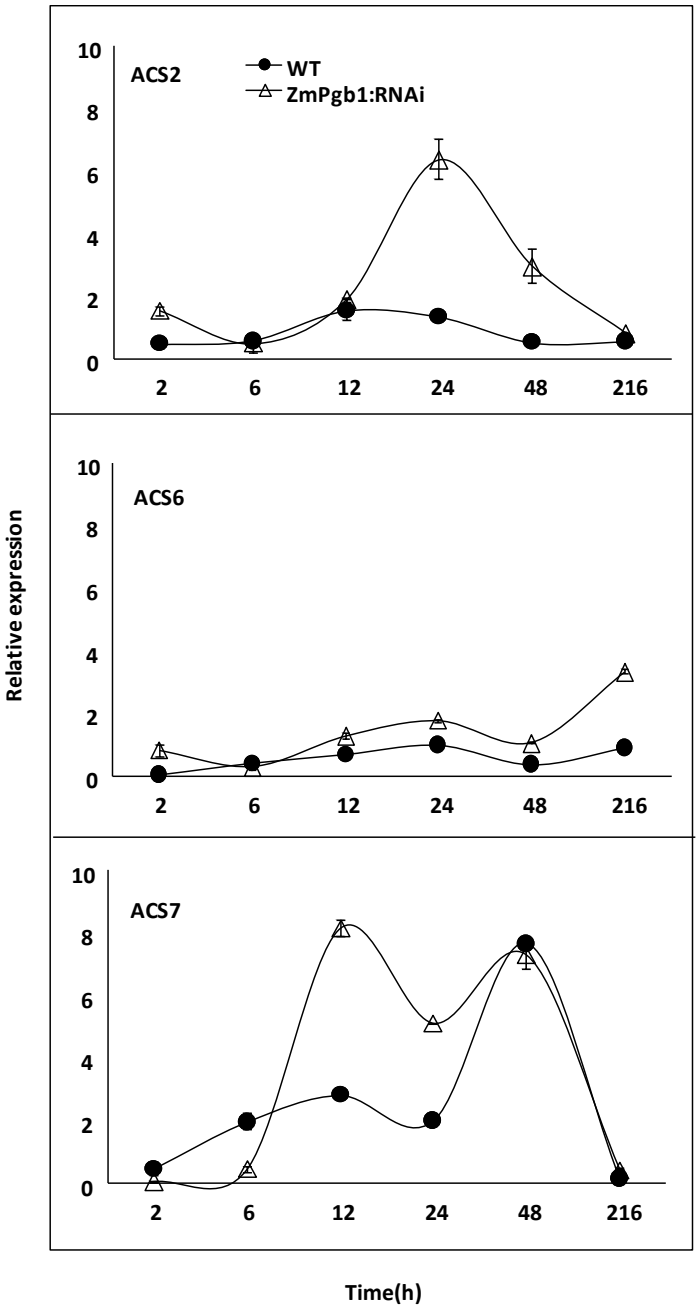


Figure 3.5. Relative expression levels of the ethylene biosynthetic genes *ACS synthase* (*ACS2*, *6*, and *7*) in WT and *ZmPgb1:RNAi* leaves inoculated with the isolate Cmn 14-5-1. Values represent the means of three biological replicates ± SE and are normalized to the mock (absence of bacterium) values at each respective time point.

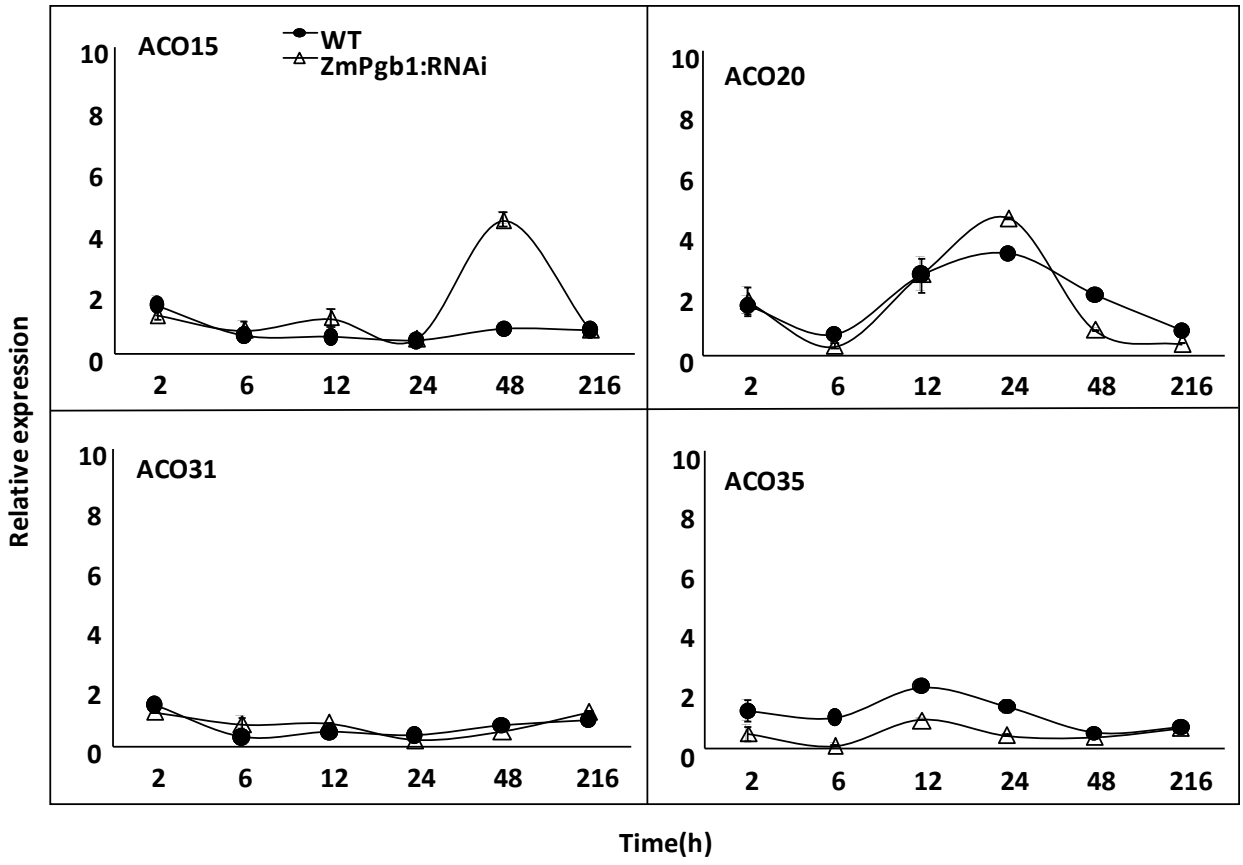


Figure 3.6. Relative expression levels of the ethylene biosynthetic genes *ACO Oxidase* (*ACO15, 20, 31* and *35*) in WT and *ZmPgb1:RNAi* leaves inoculated with the isolate Cmn 14-5-1. Values represent the means of three biological replicates ± SE and are normalized to the mock (absence of bacterium) values at each respective time point.

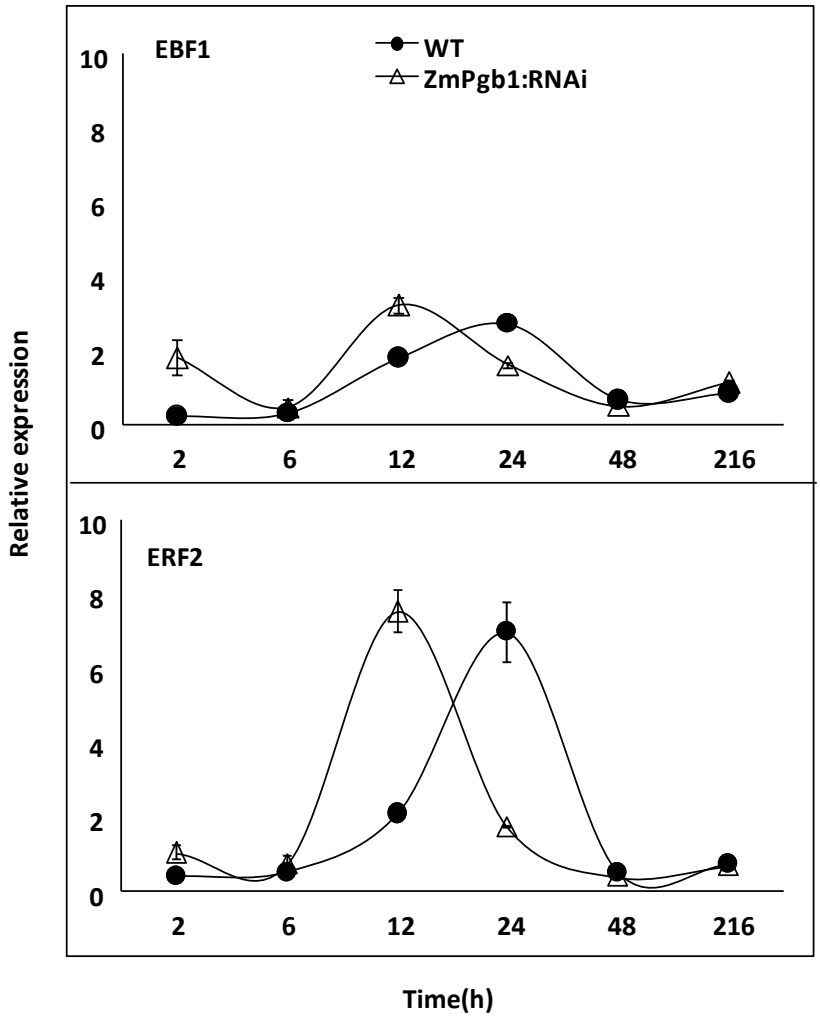


Figure 3.7. Relative expression levels of the ethylene-responsive genes *EIN3 Binding F-Box Protein 1* (*EBF1*) and *Ethylene Response Factor 2* (*ERF2*) in WT and *ZmPgb1:RNAi* leaves inoculated with the isolate Cmn 14-5-1. Values represent the means of three biological replicates ± SE and are normalized to the mock (absence of bacterium) values at each respective time point.

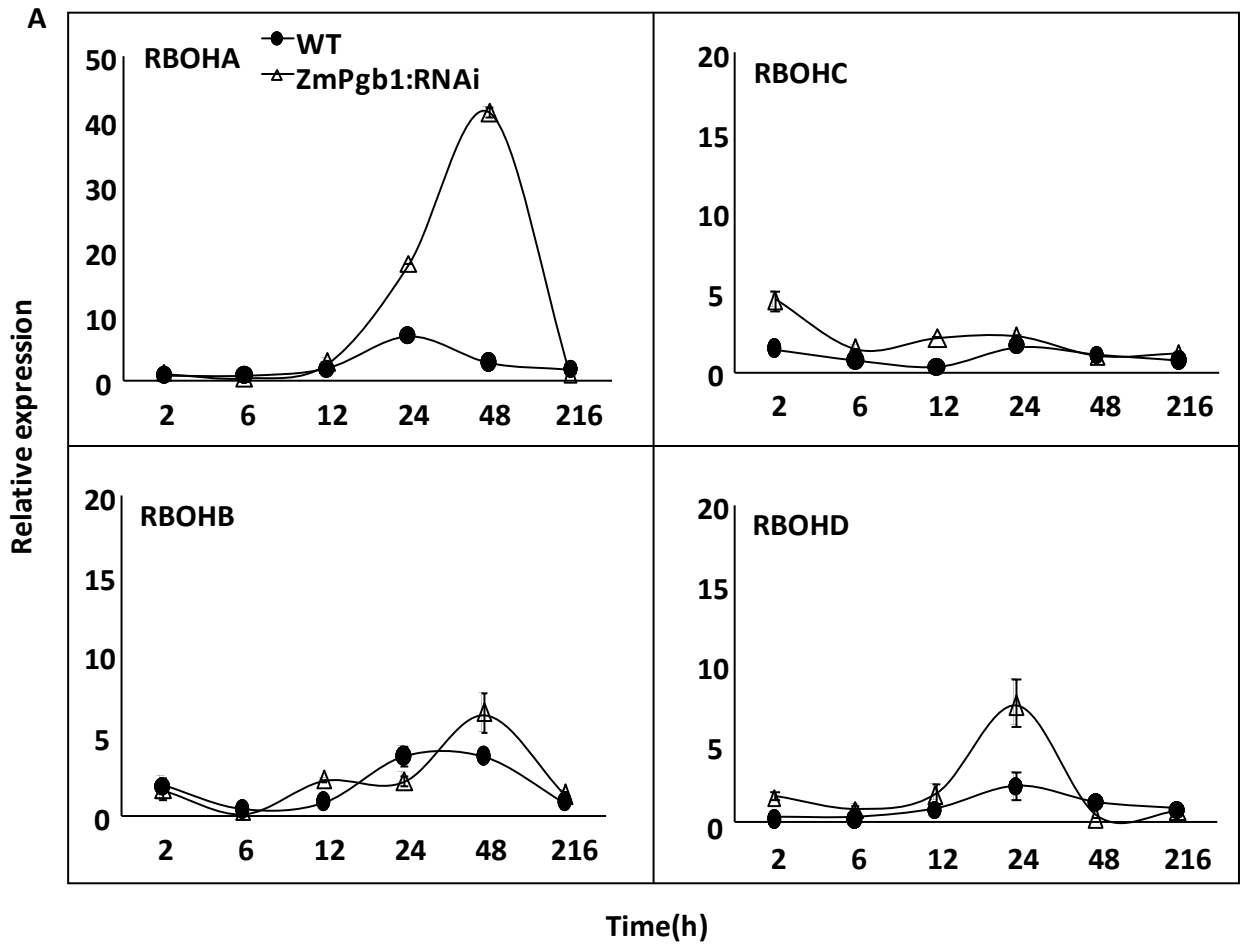
3.5.3 Production of reactive oxygen species (ROS) and PCD are influenced by ZmPgb1

The generation of ROS has been reported under stress conditions (Apel and Hirt, 2004).

The relative expression of the four *ZmRBOH*s genes was measured in WT and ZmPgb1:RNAi treated plants (Fig. 3.8A). Relative to WT, the expression of *ZmRBOHA,* and to a lesser extent *ZmRBOHB*, peaked after 24h in leaves suppressing *ZmPgb*. A small increase in transcript levels, between 12 and 48h, was also recorded for *ZmRBOHD*. The transcript abundance of *RBOHC* was generally higher in the ZmPgb1:RNAi plants especially during the first 24h following inoculation (Fig. 3.8A).

Reactive oxygen species were localized in leaves at 24h after inoculation. Relative to WT, a stronger signal with DAB and NBT (staining hydrogen peroxide and superoxide respectively) occurred around the wounding site of the inoculated ZmPgb1:RNAi leaves (Fig. 3.8B).

During biotic stress conditions, ROS production is closely linked to programmed cell death (PCD), which was monitored by TUNEL assays 24h after inoculation. Compared to WT, several TUNEL-positive nuclei were detected adjacent to the wounding site of the inoculated ZmPgb1:RNAi leaves (Fig. 3.9).


A
RBOHA
WT
ZmPgb1:RNAi
RBOHC
RBOHB
RBOHD
Relative expression
Time(h)
2
6
12
24
48
216

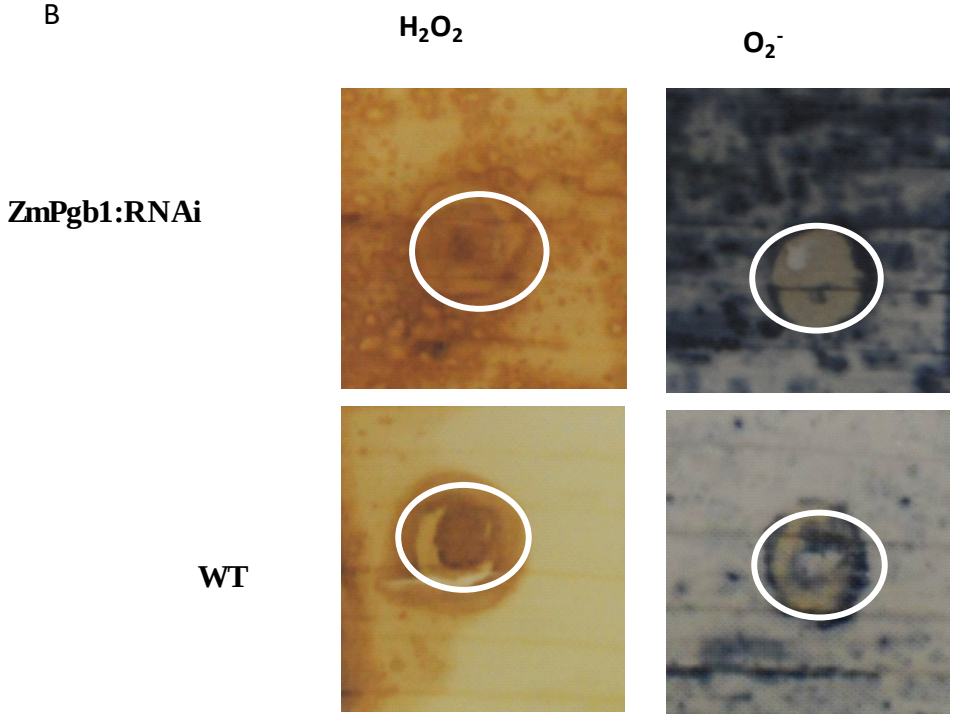

B
H_2O_2
O_2^-
ZmPgb1:RNAi
WT

Figure 3.8. (A) Relative expression levels of the maize Respiratory Burst Oxidase Homologs (*ZmRBOHA-D*) in WT and *ZmPgb1:RNAi* leaves inoculated with the isolate Cmn 14-5-1. Values represent the means of three biological replicates ± SE and are normalized to the mock (absence of bacterium) values at each respective time point. (B) ROS localization of hydrogen peroxide by 3,3'-diaminobenzidine (DAB) and superoxide by nitroblue tetrazolium (NBT) in proximity of the inoculation size in WT and *ZmPgb1-RNAi* leaves 24h after inoculation the with isolate Cmn 14-5-1. Arrows represent the inoculated site where the biotic stress was induced.

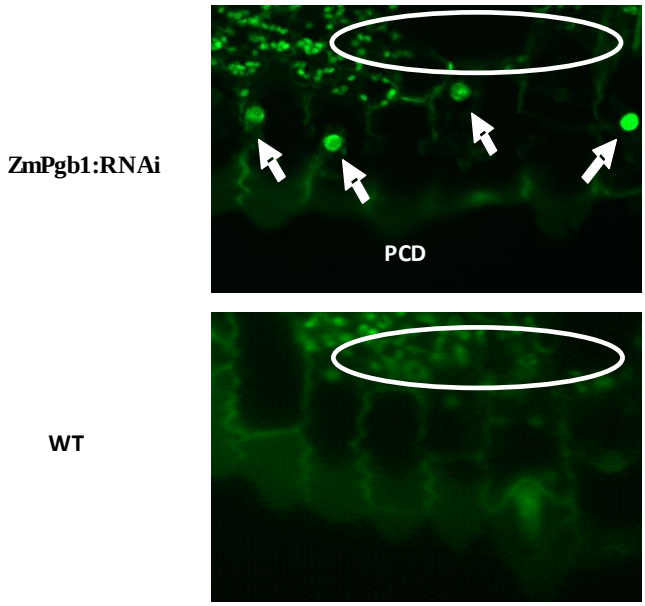


Figure 3.9. Programmed cell death (PCD) analysis by TUNEL in WT and *ZmPgb1-RNAi* leaves 24h after inoculation with the isolate Cmn 14-5-1. Arrows represent TUNEL-positive nuclei of cells undergoing PCD in proximity of the inoculation site (circled area).

3.5.4 Suppression of *ZmPgb1* alters the transcription of pathogenesis-related proteins

Participation of *ZmPgb1* in the transcription of pathogenesis-related proteins during the infection process was assessed by measuring the transcript levels of *PI* and *PR1* genes. The expression level of *PI* was highest in leaves suppressing *ZmPgb1* between 12h and 48h after inoculation. A similar peak in *PI* expression, delayed by 24h was also observed in WT leaves, compared to ZmPgb1:RNAi inoculated leaves (Fig. 3.10). A gradual rise in *PRI* transcripts occurred in both WT and ZmPgb1:RNAi leaves during the infection period, before declining after 48h (Fig. 3.10).

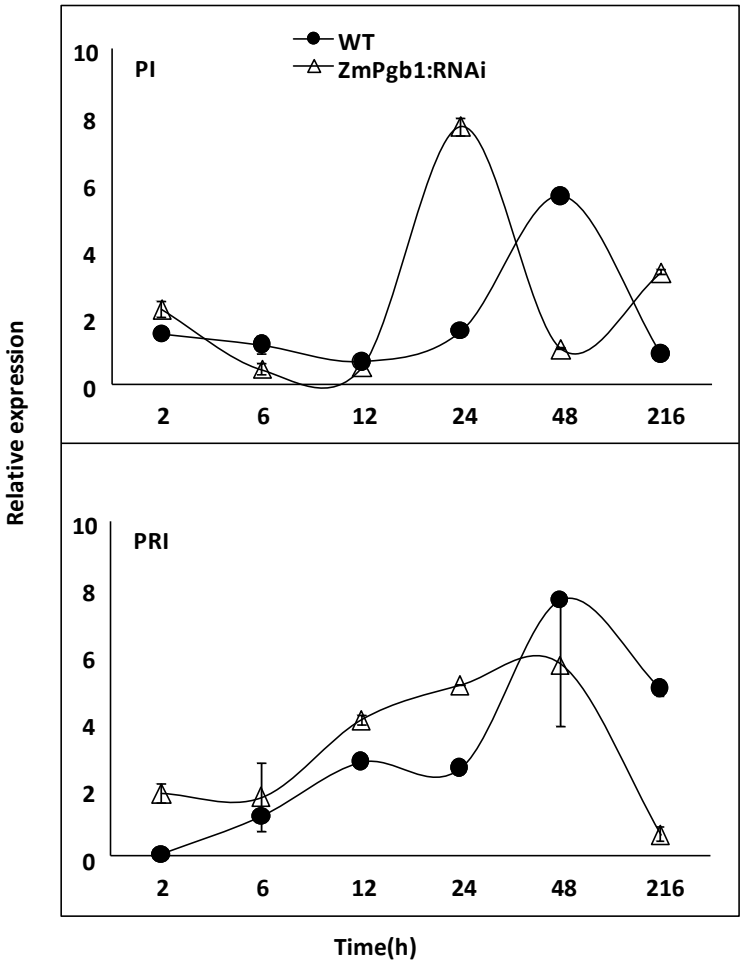


Figure 3.10. Relative expression levels of pathogenesis-related genes (*PI* and *PR1*) in WT and *ZmPgb1:RNAi* leaves inoculated with the isolate Cmn 14-5-1.Values represent the means of three biological replicates ± SE and are normalized to the mock (absence of bacterium) values at each respective time point.

3.5.5 Measurement of ZmPgb1 in Cn-infected cultured cells

To assess if the Pgb-mediated responses observed *in planta* were reproducible *in vitro*, maize cultured cells produced from zygotic embryos were exposed to induced (containing the isolate Cmn 14-5-1) or un-induced (devoid of the isolate Cmn 14-5-1) xylem sap.

The *ZmPgb1* expression level in induced WT cells showed two separate peaks. A major sharp peak was observed between 0h post inoculation (hpi) and 1h after infection while a second increment of transcript level occurred between 3hpi and 9hpi (Fig. 3.11).

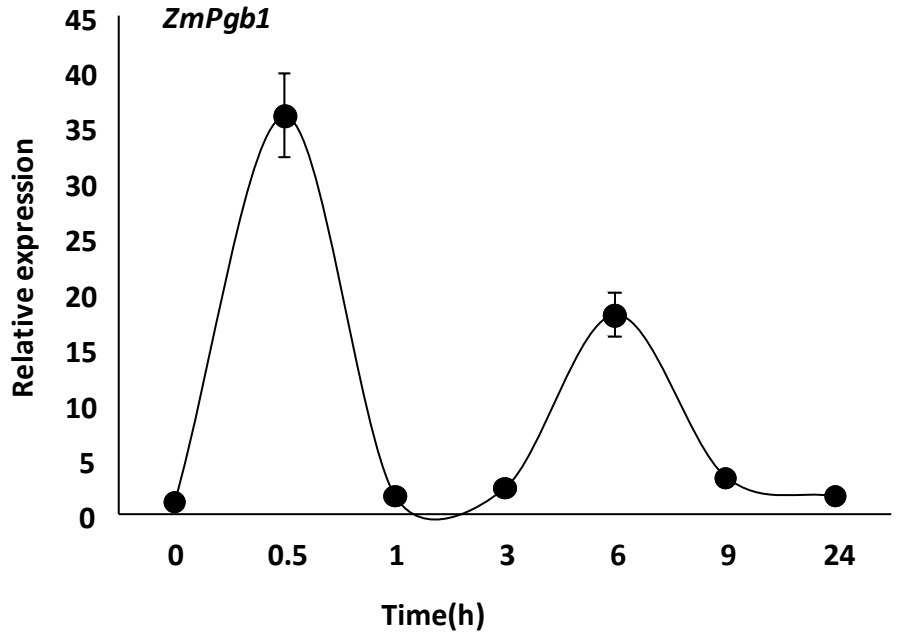


Figure 3.11. Gene expression levels of *ZmPgb1* in WT cultured cells over time exposed to induced xylem sap (containing the isolate Cmn 14-5-1). Expression values are the means of three biological replicates ± SE and are normalized to the WT un-induced (devoid of the isolate Cmn 14-5-1) values at each respective time point.

3.5.6 Suppression of *ZmPgb1* elevates NO levels

Since a major role of Pgbs is to scavenge NO, we tried to localize NO in untreated (C) cells, and cells exposed to un-induced (X) or induced (B) xylem sap. In C and X cells generated from WT explants, a weak NO signal was observed. The intensity of the signal increased in presence of the induced xylem sap (B). A similar staining pattern, albeit more pronounced, was observed in cells down-regulating ZmPgb1:RNAi (Fig. 3.12). These data confirmed the previous observations that down-regulation of *ZmPgb1* elevates NO level (Huang et al., 2014).

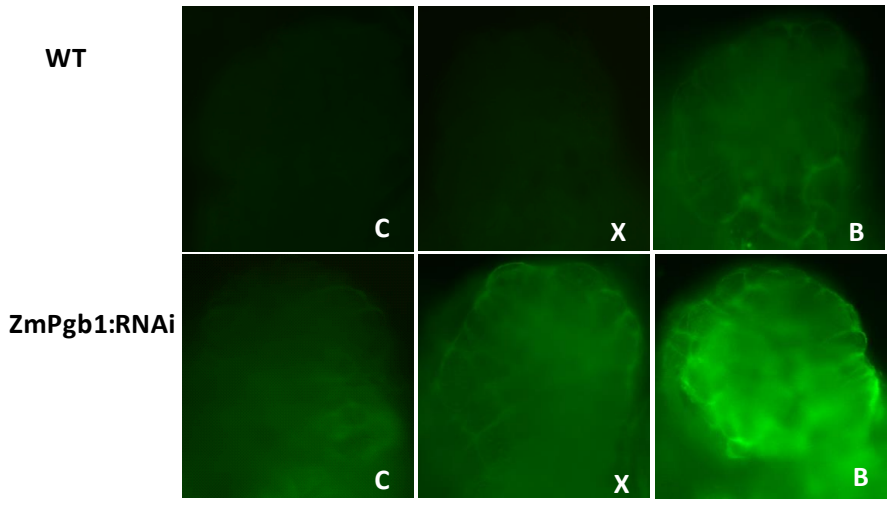


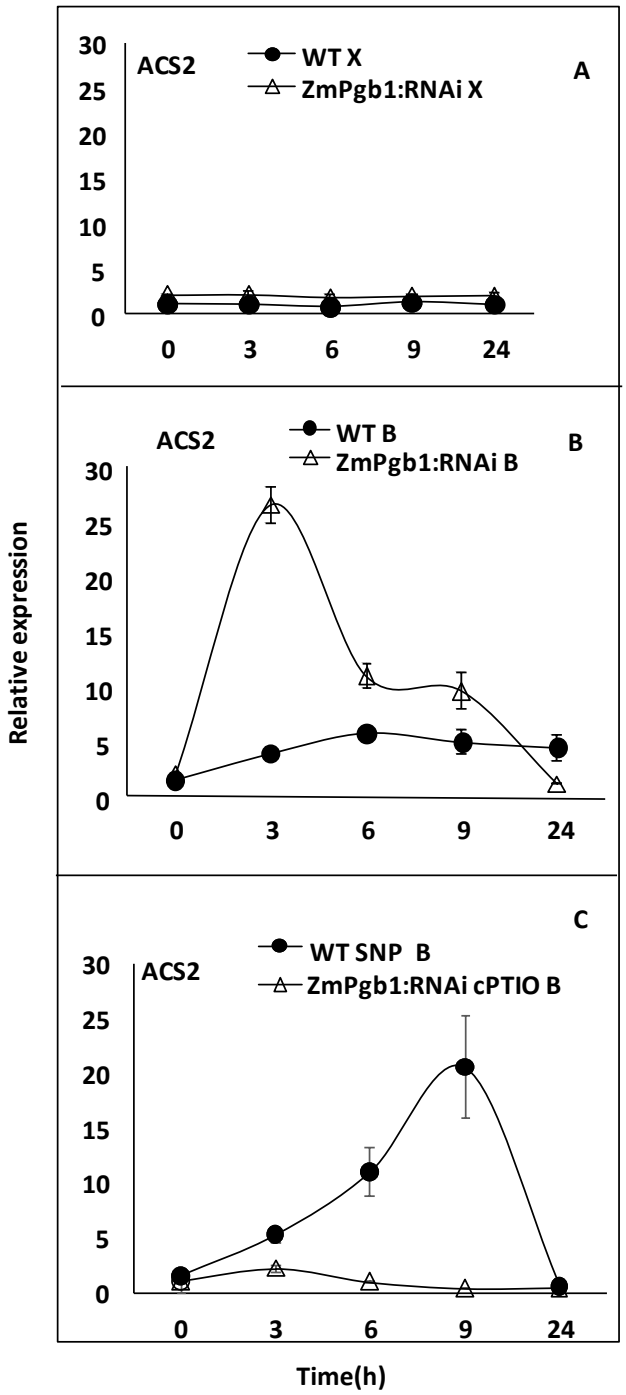
Figure 3.12. Localization of Nitric Oxide (NO) with DAF-2DA in WT and *ZmPgb1:RNAi* maize cultured cells untreated (C), or exposed to un-induced (X) or induced (B) xylem sap. Localization was performed after 3h.

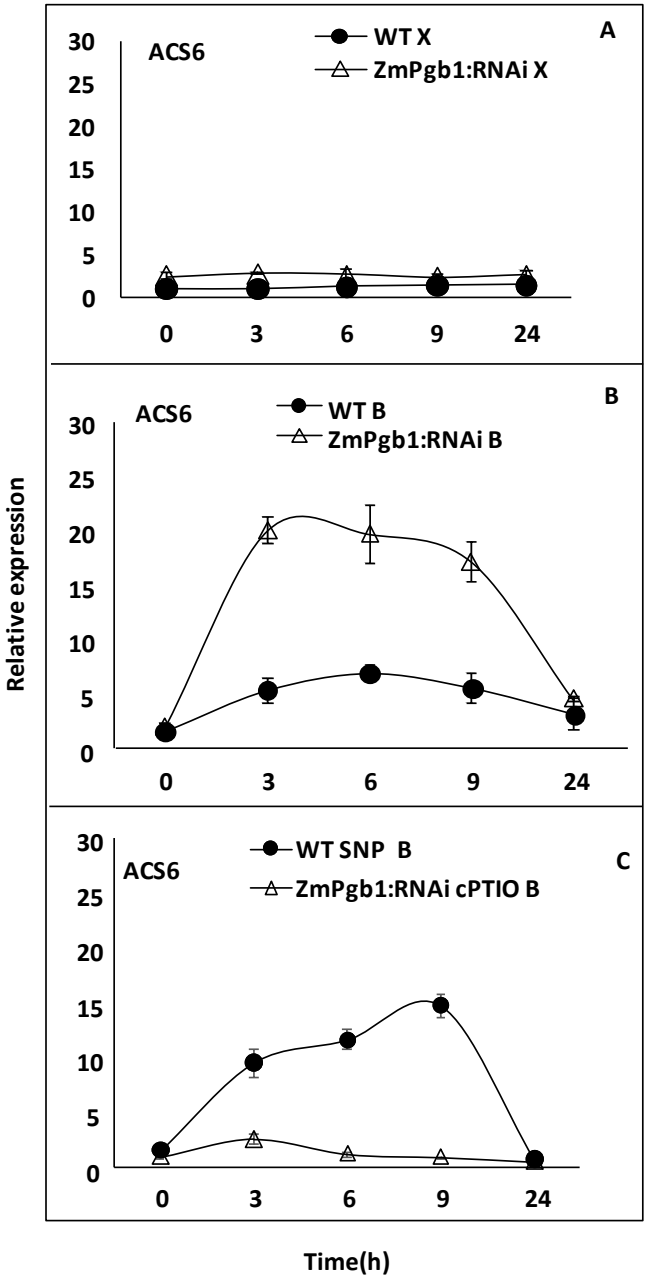
3.5.7 Transcription of ethylene synthesis and response genes is influenced by Pgb through NO

Given the established interaction between NO and ethylene in stress responses, the transcript levels of *ACS* and *ACO* genes, as well as *EBF1* and *ERF2* were measured in cells exposed to un-induced (X) or induced (B) xylem sap. Un-induced (X) cells generated from WT and ZmPgb1:RNAi explants had similar levels of *ACS2*, *ACS6*, and *ACS7* transcripts (Fig. 3.13A). A rapid increase in transcript levels of the three genes was observed following exposure to the induced xylem (B), especially in the cells suppressing *ZmPgb1 (*ZmPgb1:RNAi) (Fig. 3.13B). To evaluate the participation of NO in this response, pharmacological experiments were conducted. The level of NO was elevated with SNP in induced WT cells (characterized by a lower NO signal, Fig. 3.12), and reduced with cPTIO in induced ZmPgb1:RNAi cells (characterized by a pronounced NO signal, Fig. 3.12). For all the *ACS* genes analyzed, applications of SNP in WT cells raised the transcript levels, while applications of cPTIO in the ZmPgb1:RNAi cells reduced the transcript levels (Fig. 3.13C).

Of the *ACO* genes analyzed, the expression of *ACO15* and *ACO20* followed similar profiles of the *ACS* genes, with no remarkable differences in un-induced (X) cells (Fig. 3.14A), and a sharp increase in expression level in induced (B) ZmPgb1:RNAi cells relative to WT cells (Fig. 3.13B). The expression of *ACO31* and *35* were generally slightly higher in both un-induced (X)

and induced (B) ZmPgb1:RNAi cells (Fig. 3.13B). All ACO genes showed an induction in WT cells treated with SNP and a repression in ZmPgb1:RNAi cells treated with cPTIO (Fig. 3.13C).




A
ACS6
WT X
ZmPgb1:RNAi X
30
25
20
15
10
5
0
0
3
6
9
24
B
ACS6
WT B
ZmPgb1:RNAi B
C
ACS6
WT SNP B
ZmPgb1:RNAi cPTIO B
Relative expression
Time(h)

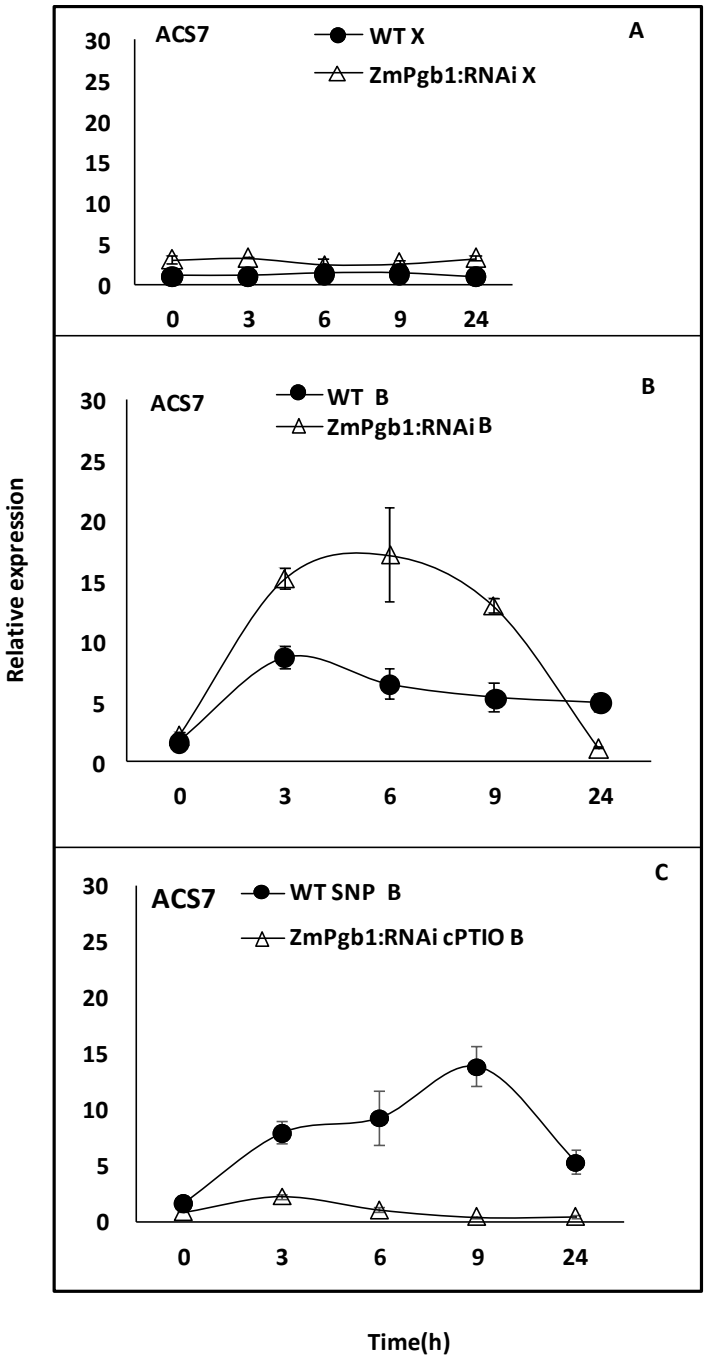
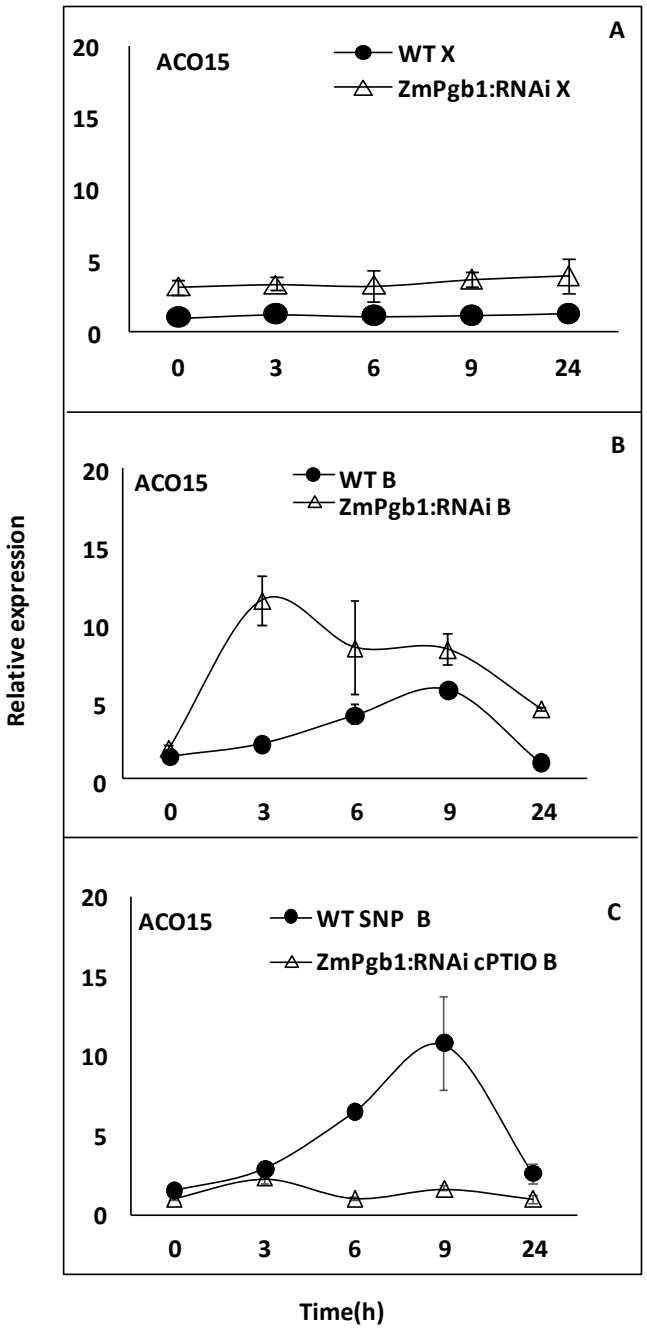
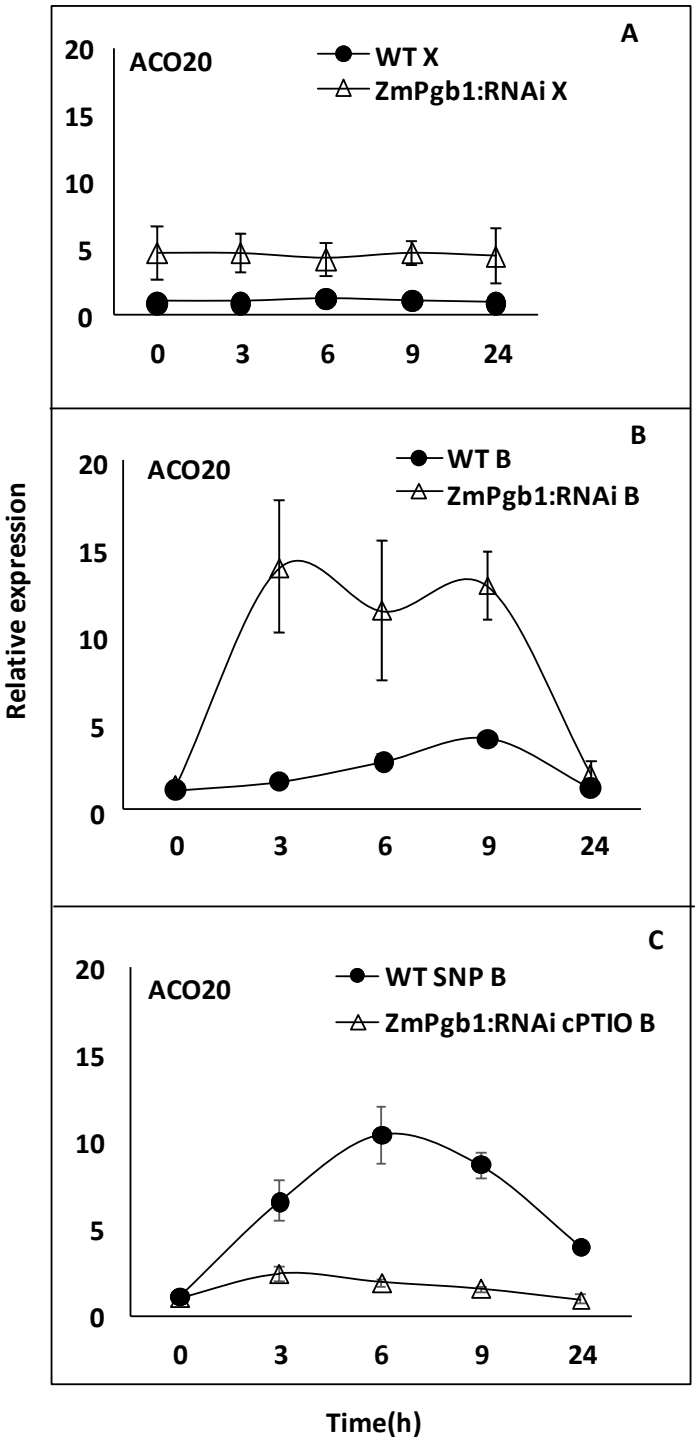
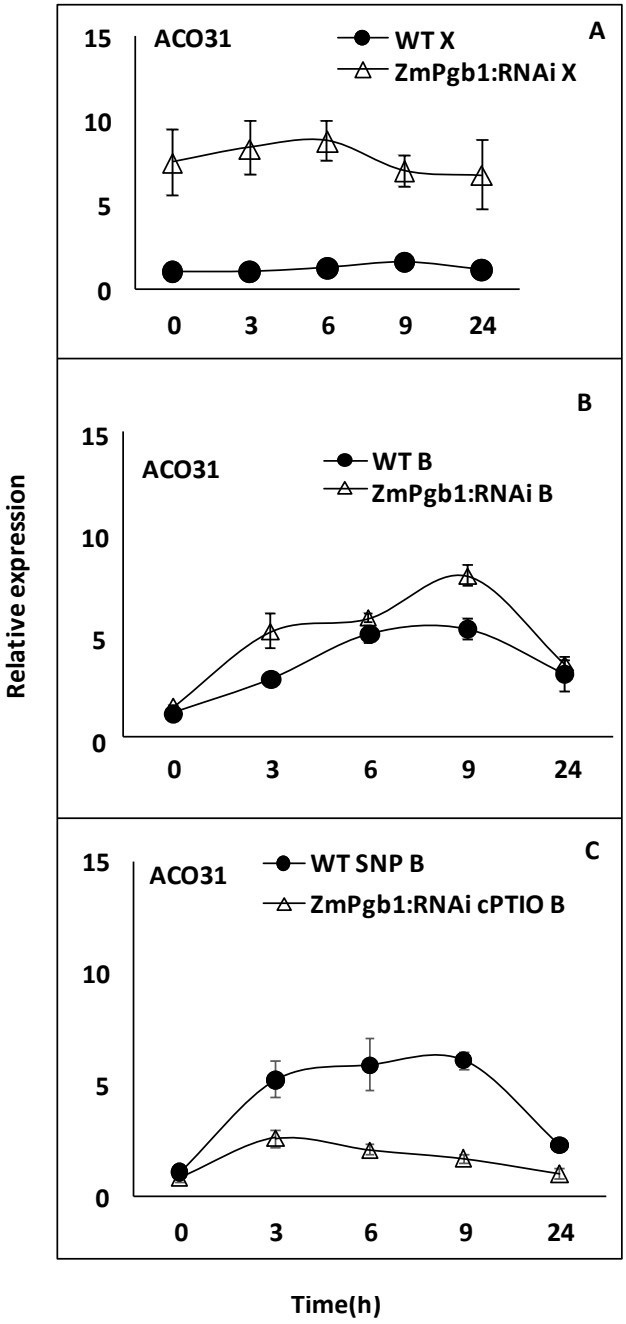


Figure 3.13. Relative expression levels of the ethylene biosynthetic genes *ACS synthase* (*ACS2, 6*, and *7*) in WT and *ZmPgb1:RNAi* cells exposed to un-induced (X) (A) or induced (B) xylem sap (B). The expression of the same genes was also measured in WT cells where the level of NO was elevated by SNP, and in ZmPgb1:RNAi cells where the level of NO was reduced by cPTIO

(C). Expression values are the means of three biological replicates ± SE and are normalized to the WT values at 0 h.




A
ACO20
WT X
ZmPgb1:RNAi X
20
15
10
5
0
0
3
6
9
24
B
ACO20
WT B
ZmPgb1:RNAi B
C
ACO20
WT SNP B
ZmPgb1:RNAi cPTIO B
Relative expression
Time(h)


A
ACO31
WT X
ZmPgb1:RNAi X
15
10
5
0
0
3
6
9
24
B
ACO31
WT B
ZmPgb1:RNAi B
C
ACO31
WT SNP B
ZmPgb1:RNAi cPTIO B
Relative expression
Time(h)

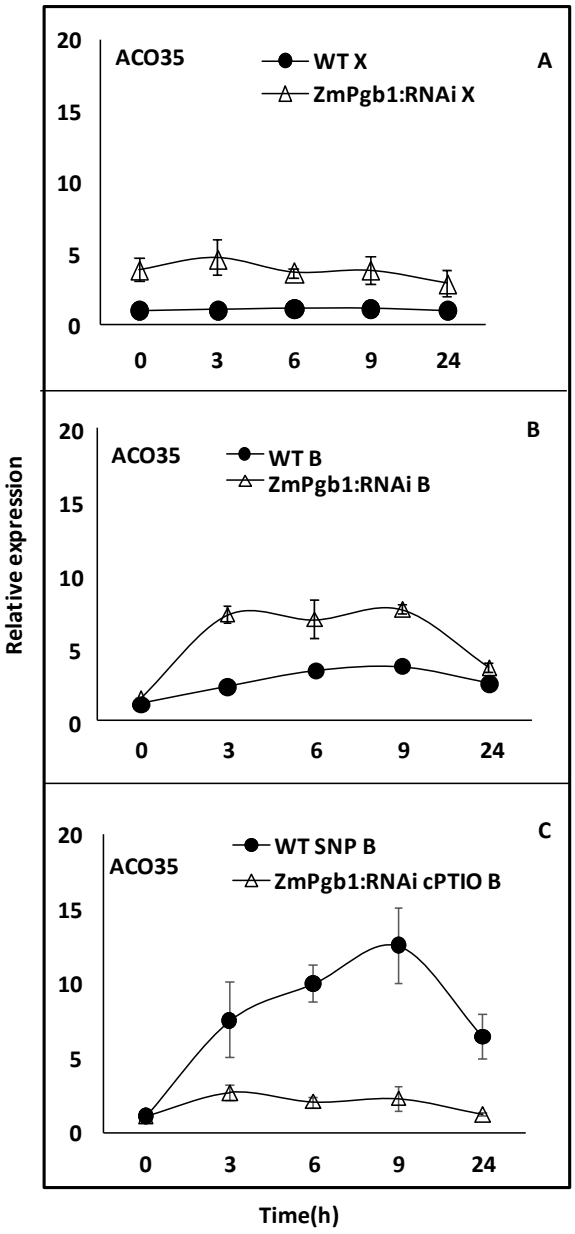


Figure 3.14. Relative expression levels of the ethylene biosynthetic genes *ACC oxidase* (*ACO15*, *20*, *31* and *35*) in WT and *ZmPgb1:RNAi* cells exposed to un-induced (X) (A) or induced (B) xylem sap (B). The expression of the same genes was also measured in WT cells where the level of NO was elevated by SNP, and in ZmPgb1:RNAi cells where the level of NO was reduced by cPTIO (C). Expression values are the means of three biological replicates ± SE and are normalized to the WT values at 0 h.

The observed regulation of Pgb (through NO) on the transcription of ethylene biosynthetic genes was also supported by measurements of ethylene. The level of ethylene increased in induced (B) cells, especially in those where the level of *ZmPgb1* was suppressed (ZmPgb1:RNAi cell) (Fig. 3.15). Ethylene accumulation also increased in WT cells treated with SNP and decreased in ZmPgb1:RNAi cells treated with cPTIO (Fig.3.15).

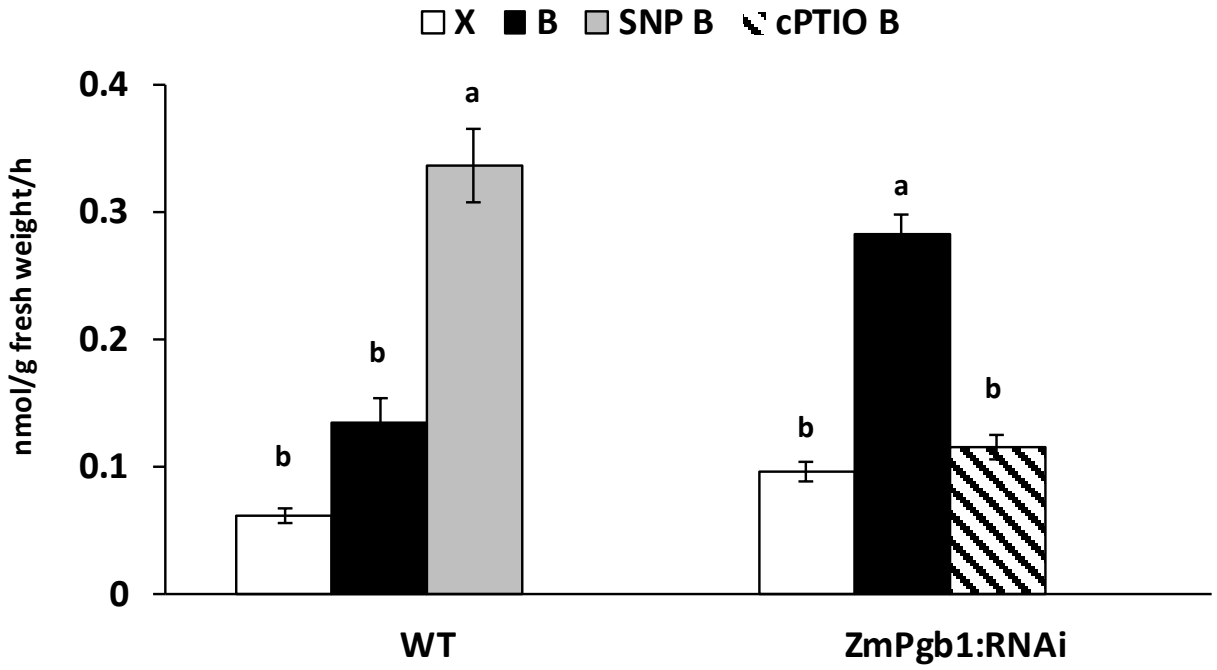
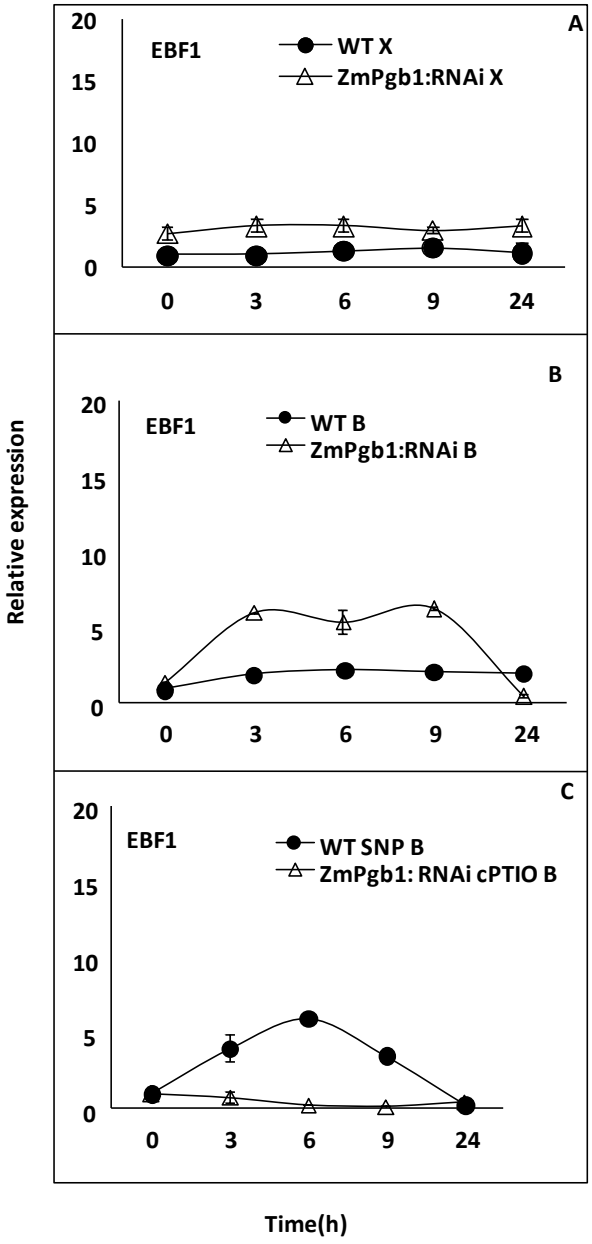


Figure 3.15. Ethylene in WT and *ZmPgb1:RNAi* un-induced (X) and induced (B) cells after 3 hours. Cells were also treated with SNP to elevate the level of NO in WT, and cPTIO to reduce the level of NO in the ZmPgb1:RNAi cells. Values ± SE are the means of three biological replicates. Letters represent statistically significant differences ($P<0.05$).

Ethylene response in cultured cells was assessed by measuring the transcript levels of *EBF1* and *ERF2*. No marked differences in the expression of either gene were observed between WT and ZmPgb1:RNAi cells under un-induced (X) conditions (Fig.3.16A). Exposure to induced (B) xylem sap elevated the expression of *ERF2*, and to a lesser extent that of *EBF1*, in cells with reduced levels of *ZmPgb1* (ZmPgb1:RNAi) (Fig. 3.16B). The transcript levels of both genes were elevated by SNP in WT cells, and repressed by cPTIO in ZmPgb1:RNAi cells (Fig.3.16C). Collectively these results suggest an increase in ethylene synthesis and response in induced ZmPgb1:RNAi cells relative to induced WT cells. Also, the *ZmPgb1* regulation of ethylene is mediated by NO.


A
EBF1
WT X
ZmPgb1:RNAi X
20
15
10
5
0
0
3
6
9
24
B
EBF1
WT B
ZmPgb1:RNAi B
C
EBF1
WT SNP B
ZmPgb1: RNAi cPTIO B
Relative expression
Time(h)

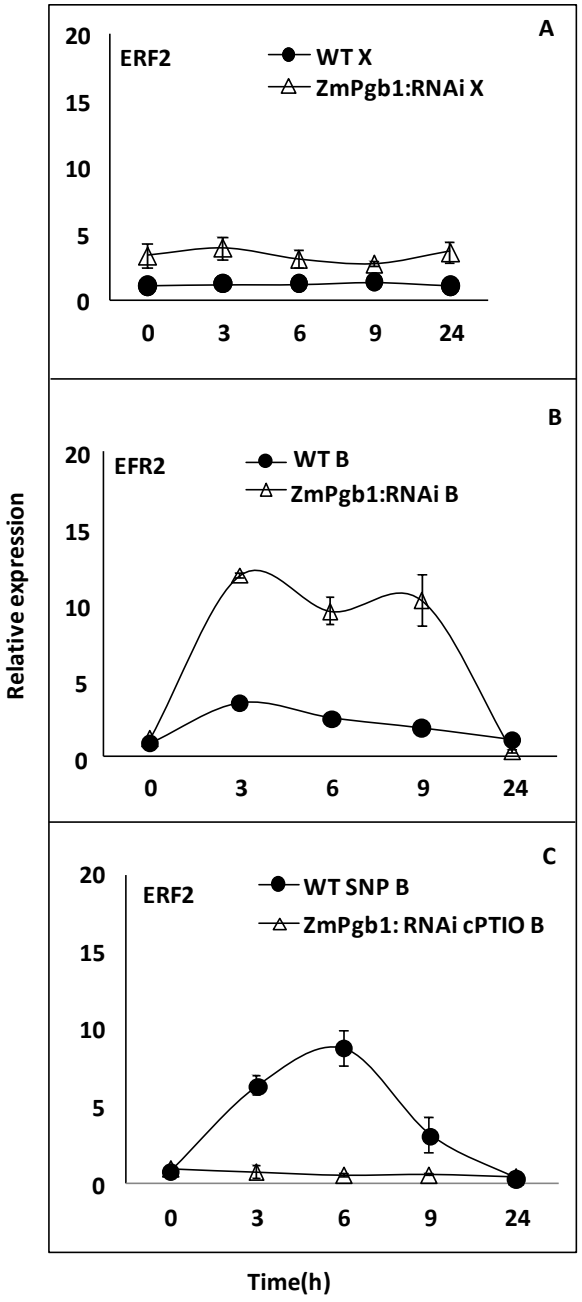


Figure 3.16. Relative expression levels of the ethylene response genes *EBF1* and *ERF2* in WT and *ZmPgb1:RNAi* cells exposed to un-induced (X) (A) or induced (B) xylem sap (B). The expression of the same genes was also measured in WT cells where the level of NO was elevated by SNP, and in ZmPgb1:RNAi cells where the level of NO was reduced by cPTIO (C).

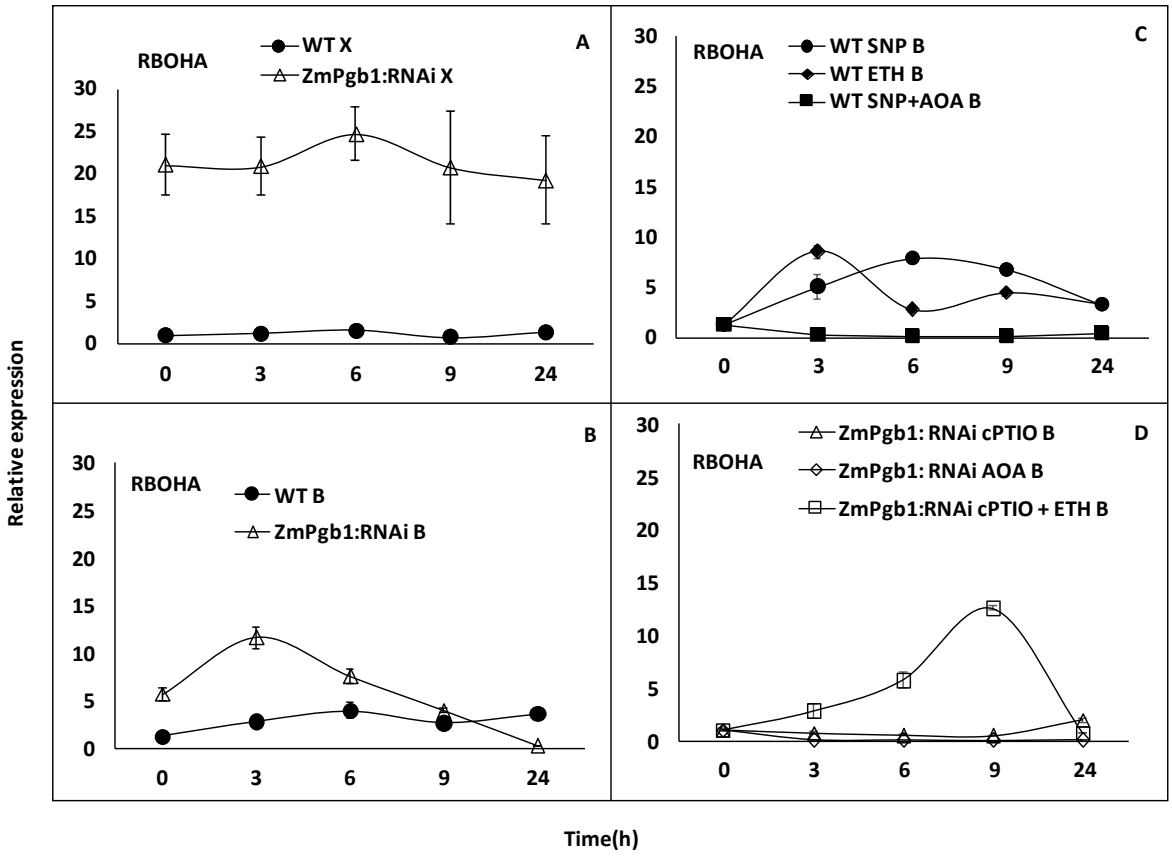
Expression values are the means of three biological replicates ± SE and are normalized to the WT values at 0 h.

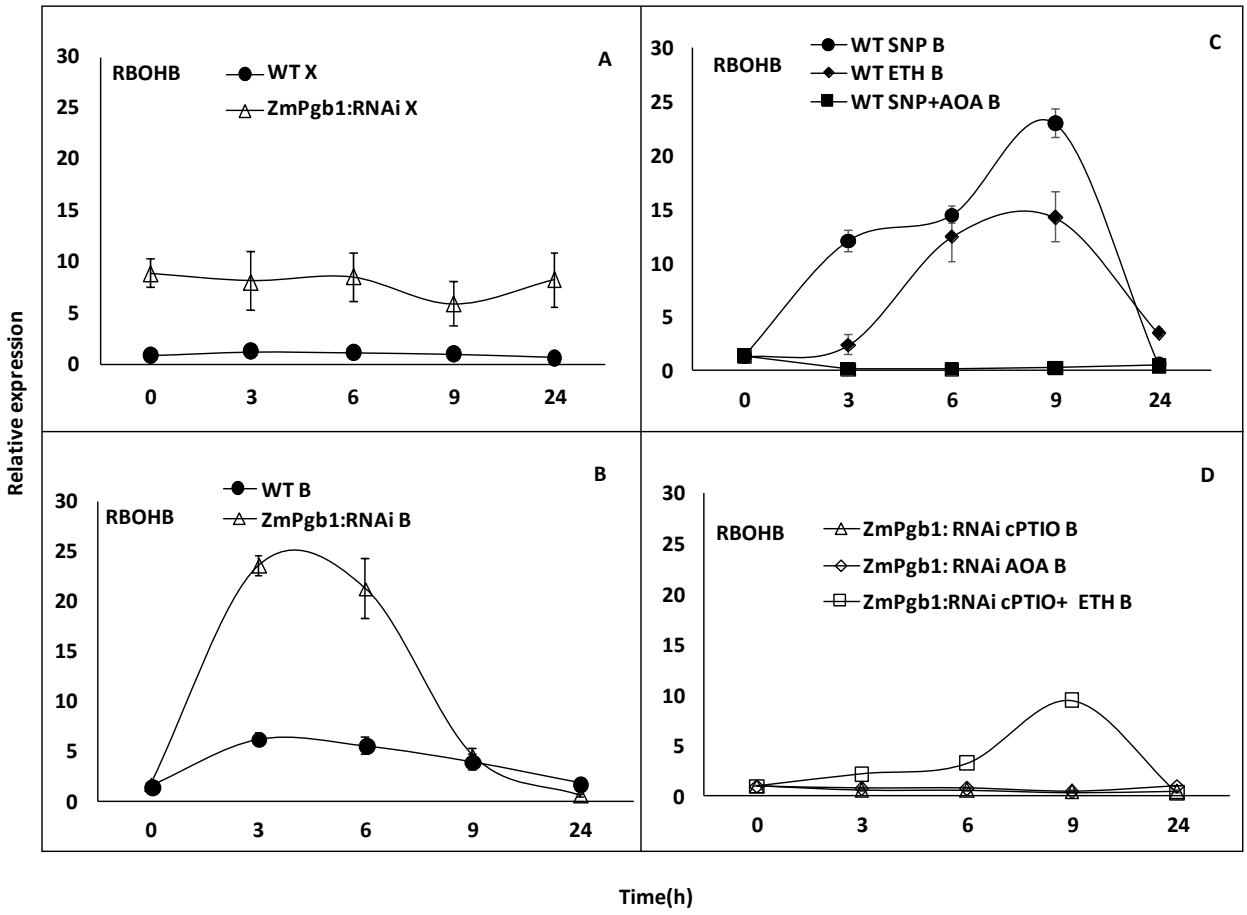
3.5.8 Suppression of ZmPgb1 influences the expression levels of ROS

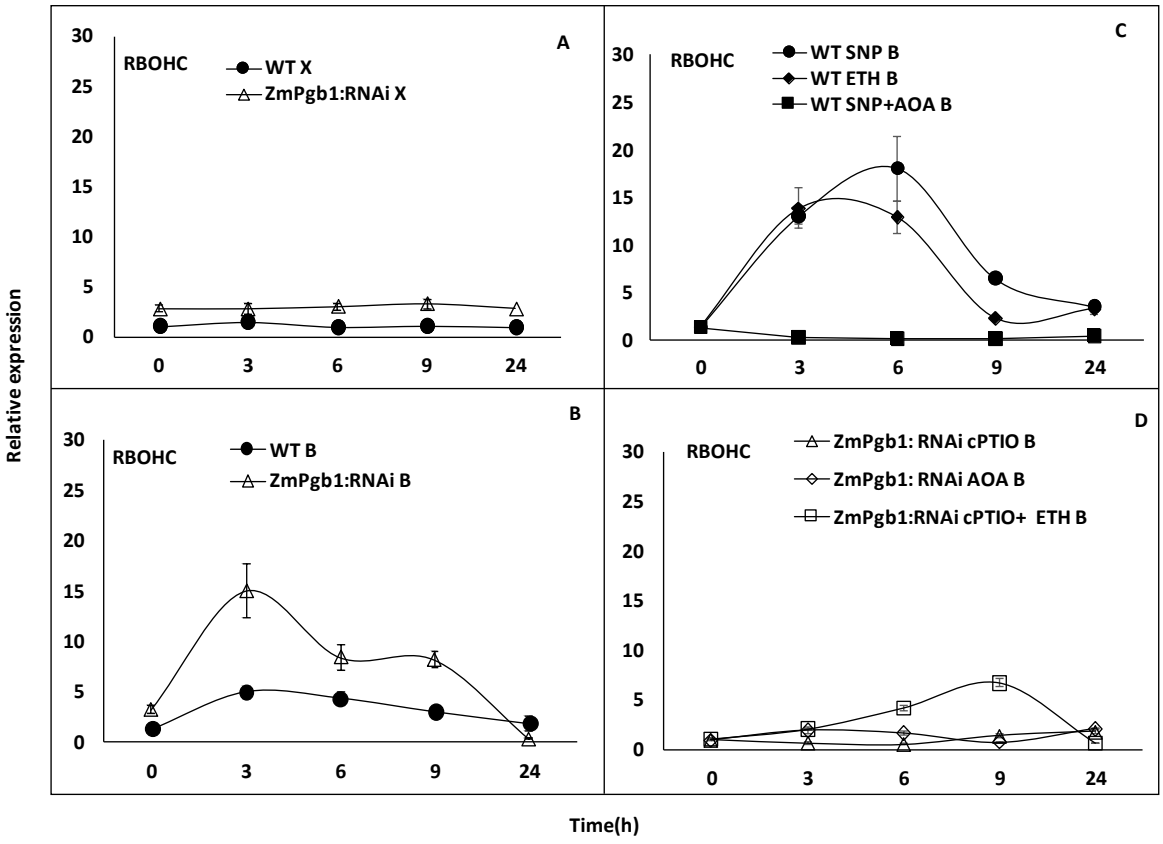
Previous work demonstrated the influence of *ZmPgb1* expression on the production of ROS under abiotic stress conditions. To further explore the link between Pgb, NO and ethylene and their effects on the production of ROS, the level of the four maize respiratory burst oxidase homologs [*ZmRBOH* (A-D)] was measured in cultured cells. Under un-induced (X) conditions, the transcript levels of all *ZmRBOHs* were higher in the ZmPgb1:RNAi cells relative to WT (Fig.3.17A). Under induced (B) conditions, a sharp increase in expression levels occurred within the first 9h in the cells where *ZmPgb1* was repressed (Fig.3.17B). Treatments elevating the amount of NO (by SNP) or ethylene (ETH) in WT cells [characterized by low NO (Fig.3.12) and ethylene (Fig. 3.15)] increased the transcript levels of all *ZmRBOHs* (Fig. 3.17C), while treatments reducing the level of NO (by cPTIO) and ethylene (AOA) in ZmPgb1:RNAi cells [characterized by high NO (Fig.3.12) and ethylene (Fig.3.15)] repressed the same genes (Fig.3.17D). This expression trend was also supported by ROS localization studies (Fig. 3.18).

To establish a temporal relationship between NO and ethylene in the regulation of ROS, the amounts of NO and ethylene were altered at the same time. In WT cells the promotive effect of NO on the expression of all *ZmRBOHs* was abolished by reducing ethylene (compare SNP and SNP+AOA in Fig. 3.17C). Similarly, in the ZmPgb1:RNAi cells the inhibitory effect of low NO was reverted by elevating ethylene (compared cPTIO and cPTIO+ETH in Fig. 3.17D).

Collectively, these results indicate that the level of ROS in induced cells is increased under the condition of low *ZmPgb1* expression. This increase is regulated by ethylene though NO, thus placing NO upstream of ethylene in the Pgb response.




A
RBOHB
WT X
ZmPgb1:RNAi X
B
RBOHB
WT B
ZmPgb1:RNAi B
C
RBOHB
WT SNP B
WT ETH B
WT SNP+AOA B
D
RBOHB
ZmPgb1: RNAi cPTIO B
ZmPgb1: RNAi AOA B
ZmPgb1:RNAi cPTIO+ ETH B
Relative expression
Time(h)
0
3
6
9
24
5
10
15
20
25
30


A
RBOHC
WT X
ZmPgb1:RNAi X
B
RBOHC
WT B
ZmPgb1:RNAi B
C
RBOHC
WT SNP B
WT ETH B
WT SNP+AOA B
D
RBOHC
ZmPgb1: RNAi cPTIO B
ZmPgb1: RNAi AOA B
ZmPgb1:RNAi cPTIO+ ETH B
Relative expression
Time(h)
0
3
6
9
24
5
10
15
20
25
30

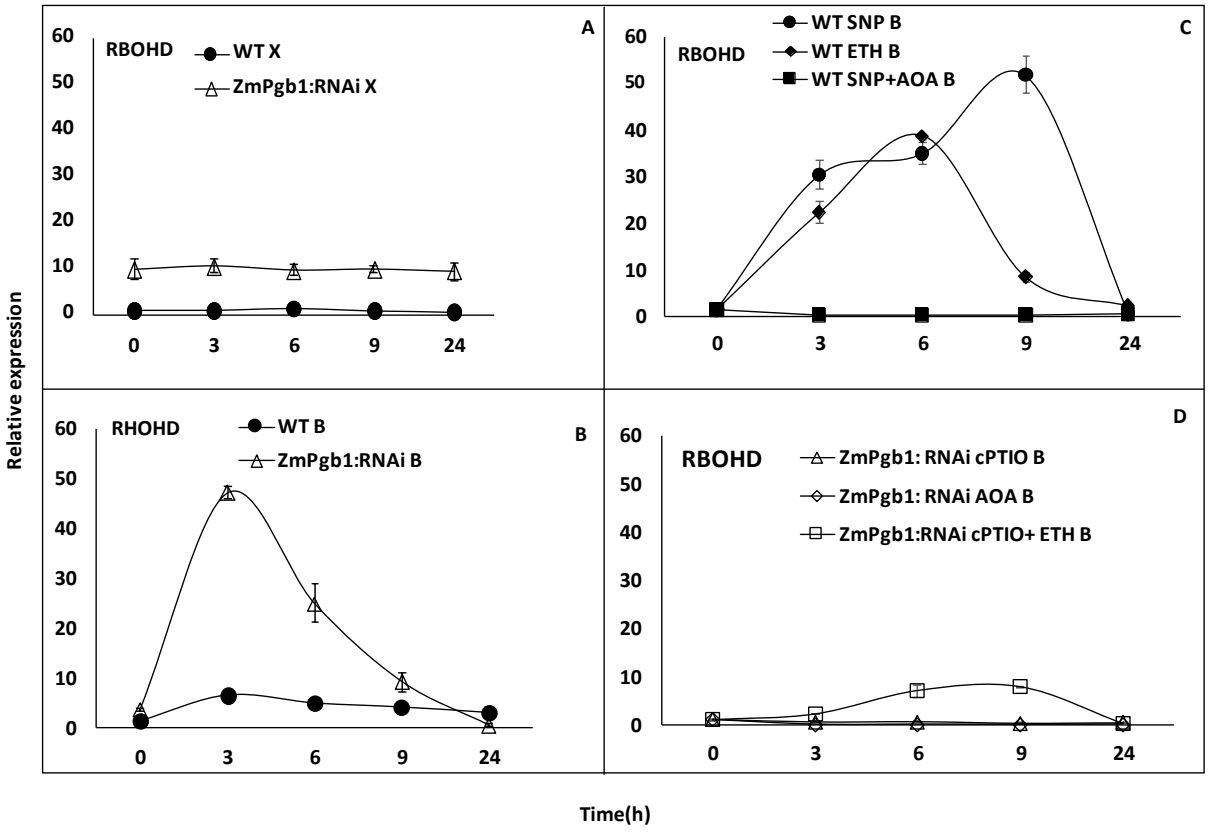


Figure 3.17. Relative expression levels of the maize Respiratory Burst Oxidase Homologs (*ZmRBOHA-D*) in WT and *ZmPgb1:RNAi* cells exposed to un-induced (X) (A) or induced (B) xylem sap (B). The expression of the same genes were also measured in WT (C) and ZmPgb1:RNAi cells (D) where the level of NO and ethylene were altered by application of SNP, cPTIO, ETH, and AOA. Expression values are the means of three biological replicates ± SE and are normalized to the WT values at 0 h (A-C) and *ZmPgb1:RNAi* values at 0h (D).

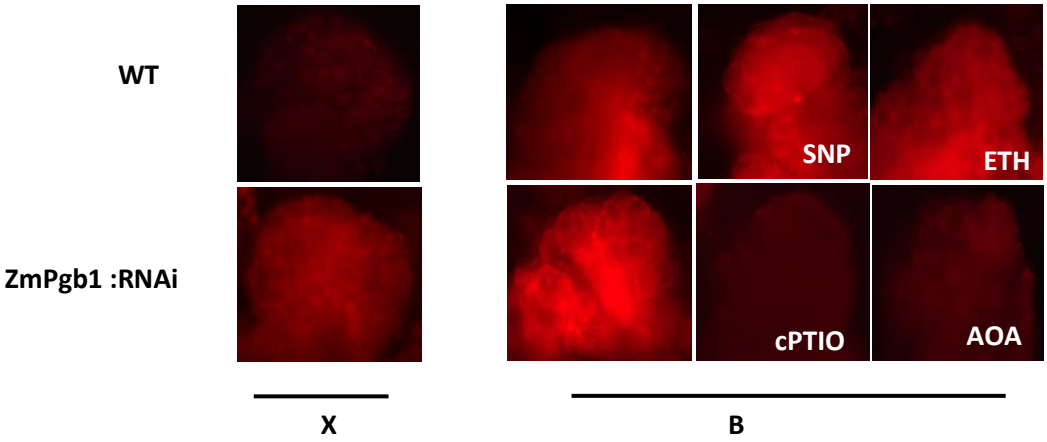
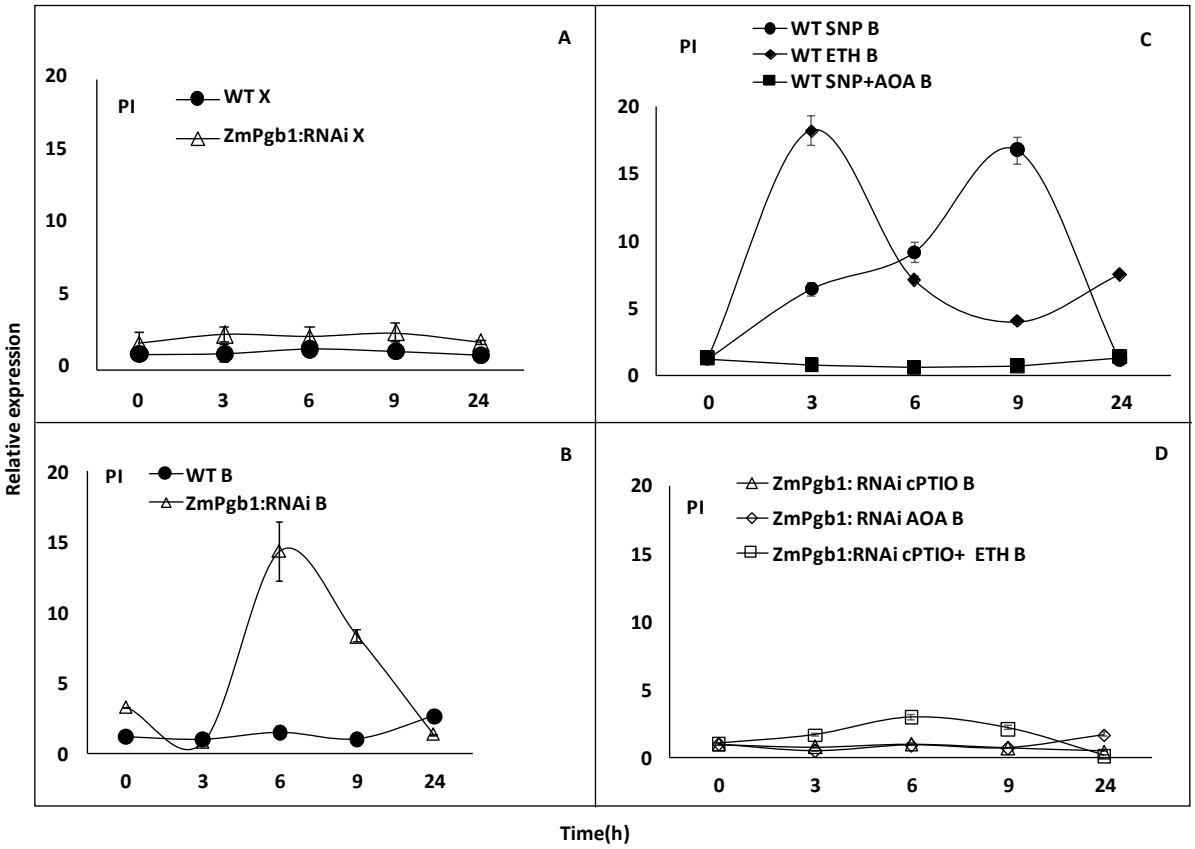


Figure 3.18. Localization of reactive oxygen species (ROS) with dihydroethidium in WT and *ZmPgb1:RNAi* cells exposed to un-induced (X) or induced (B) xylem sap for 6h. Cells were also treated with SNP or cPTIO to alter the levels of NO, and with ETH and AOA to alter the level of ethylene.

3.5.9 Suppression of ZmPgb1 induces transcription of pathogenesis-related proteins

The involvement of *ZmPgb1* in the transcription of Pathogenesis-related proteins during the *in vitro* infection process was assessed by measuring the transcript levels of *PI* and *PR1* genes. The expression level of both genes did not fluctuate in the WT or ZmPgb1:RNAi cells under un-induced (X) conditions (Fig. 3.19A), but increased in induced (B) ZmPgb1:RNAi cells (Fig. 3.19B). An experimental elevation of NO (by SNP) or ethylene (by ETH) increased the transcript levels of both *PI* and *PR1* in the WT cells (Fig. 3.19C), while a reduction of NO (by cPTIO) or ethylene (by AOA) in the ZmPgb1:RNAi cells had a repressive effect (Fig. 3.19D). Suppression of ethylene in the WT cells was sufficient to reverse the inductive effect of high NO on the expression of both *PI* and *PR1* (compare SNP and SNP+AOA in Fig. 3.19C). A rise in ethylene

in the ZmPgb1:RNAi cells partially reversed the inhibitory effect of low NO levels (compared cPTIO and cPTIO+ETH in Fig. 3.19D).



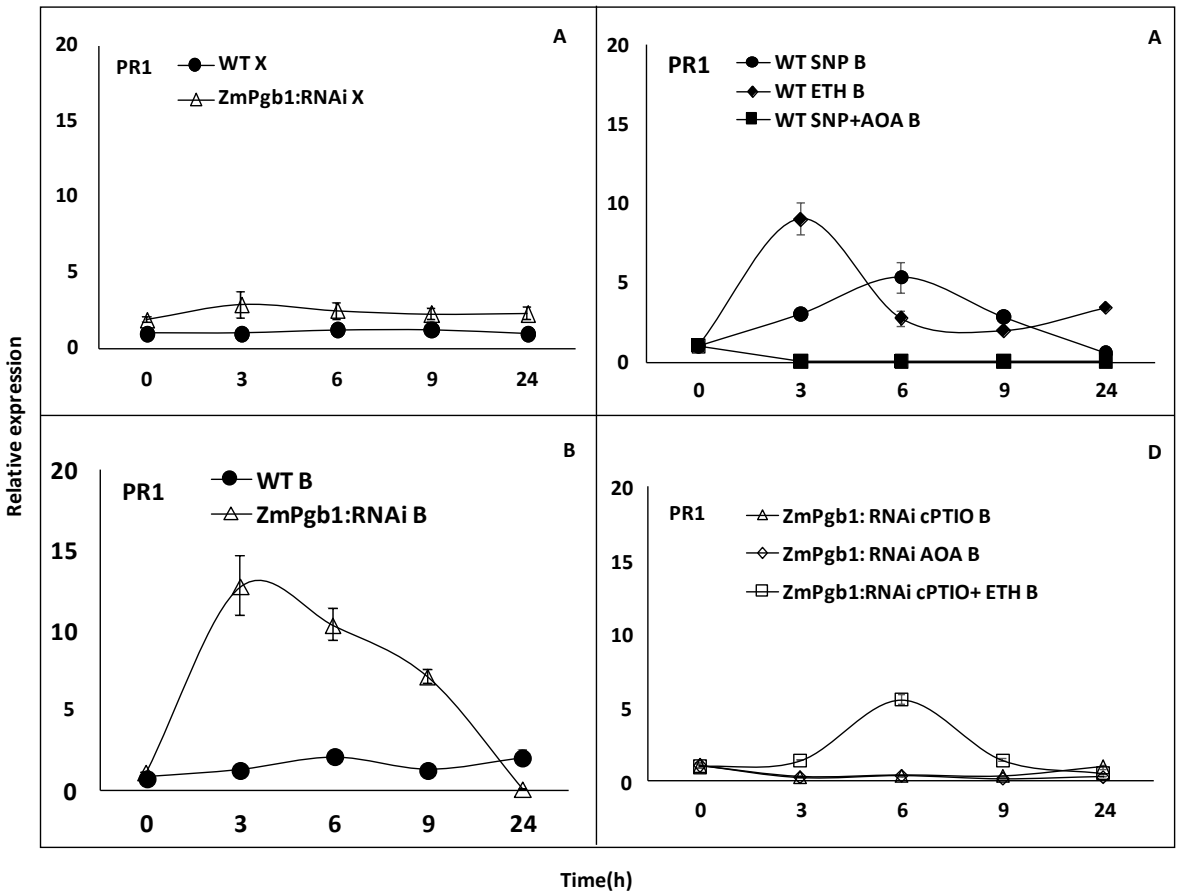
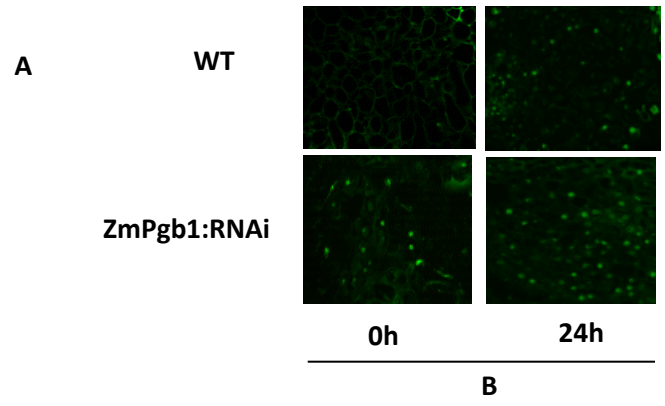


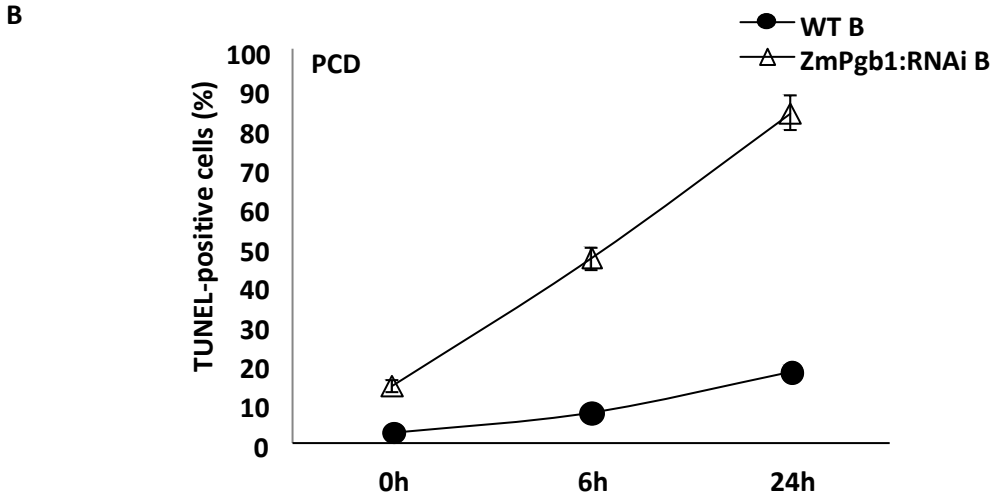
Figure 3.19. Relative expression levels of Pathogenesis-related protein1 (*PI*) and (*PR1*) in WT and *ZmPgb1:RNAi* cells exposed to un-induced (X) (A) or induced (B) xylem sap (B). The expression of the same genes was also measured in WT (C) and ZmPgb1:RNAi cells (D) where the levels of NO and ethylene were altered by application of SNP, cPTIO, ETH, and AOA. Expression values are the means of three biological replicates ± SE and are normalized to the WT values at 0 h (A-C) and ZmPgb1:RNAi values at 0h (D).

3.5.10 *ZmPgb1* regulates PCD through NO and ethylene

During maize somatic embryogenesis and conditions of abiotic stress, the occurrence of PCD is regulated by the level of Zm*Pgb1* (Hang et al., 2014; Mira et al., 2017). Programmed cell death (PCD) in cultured cells was monitored by TUNEL. Prior to the induction (0h) cells suppressing *ZmPgb1* showed a slightly higher number of TUNEL-positive nuclei relative to WT cells (Fig. 3.20A); an observation agreeing with previous studies (Huang et al., 2014). Exposure to the induced xylem sap (B) caused an increase in PCD in both cell types, but especially in the ZmPgb1:RNAi cells (Fig. 3.20B).

To evaluate the requirement of NO and ethylene on PCD, pharmacological treatments were applied. An elevation in NO (by SNP) or ethylene (by ETH) in the WT cells [characterized by low NO (Fig. 3.12) and ethylene (Fig. 3.15)] increased the number of TUNEL-positive nuclei (Fig. 3.20C). A reduction of NO (by cPTIO) or ethylene (by AOA) in the ZmPgb1:RNAi cells [characterized by high NO (Fig. 3.12) and ethylene (Fig. 3.15)] reduced the incidence of PCD (Fig. 3.20C).


A
WT
ZmPgb1:RNAi
0h
24h
B


B
PCD
WT B
ZmPgb1:RNAi B
TUNEL-positive cells (%)
100
90
80
70
60
50
40
30
20
10
0
0h
6h
24h

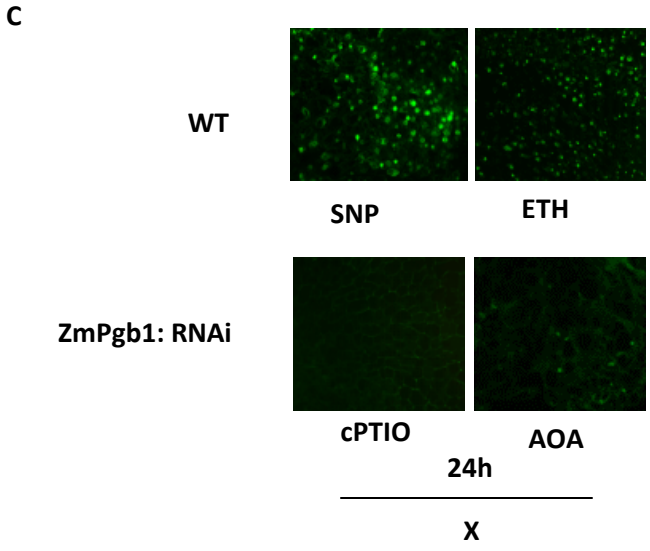

C
WT
SNP
ETH
ZmPgb1: RNAi
cPTIO
AOA
24h
X

Figure 3.20. Programmed cell death (PCD) pattern measured by TUNEL in WT and *ZmPgb1:RNAi* callus challenged with isolate Cmn 14-5-1. (A) TUNEL assay on induced cells. (B) Time-course of PCD on induced cells during 24h in culture. (C) Effects of alterations of NO (by SNP or cPTIO) and ethylene (by cPTIO and AOA) in WT cells and cells with reduced levels of *ZmPgb1*.

3.6 Supplementary Data

Supplementary Table 1. Primers used in gene expression

Name	Sequence
ZmEBF1 – F	CTGTCCGGCTGTATGAAGGT
ZmEBF1 – R	TGGTTGCCAATGAAGTTGAA
ZmERF2 – F	AGACAATGAGGCGTGCAAGT
ZmERF2 – R	AATTGCTCCCGAGCTTATCG
ZmACS2 – F	ATCGCGTACAGCCTCTCCAAGGA
ZmACS2 – R	GGCCATGAACTCCGCGTCC
ZmACS6 – F	CGCGCCGCCACGGACGACG
ZmACS6 – R	ATCTTGGTGGCCGCGGAGAC
ZmACS7 – F	ATCGCGTACAGCCTCTCCAAGGA
ZmACS7 – R	TGCCATGAACTCCGCGTCGG
ZmACO15 – F	AGCGGCGGCGACGCATACC
ZmACO15 – R	GGAGATGACTTGGGCGCTGCAA
ZmACO20 – F	CGTTCGGCACCAAGGTGAGC
ZmACO20 – R	ACGTCCACCCACTCCCCGC
ZmACO31 – F	AGCGGCGGCGACGCATACC
ZmACO31 – R	GGAGATGACTTTGGCGCCCC
ZmACO35 – F	CGTTCGGCACCAAGGTGAGC
ZmACO35 – R	CACGTCCACCCACTCCCCG
ZmRBOHA – F	CACACGTGACCTGCGACTTC
ZmRBOHA – R	CCCCAAGGTGGCCATGA
ZmRBOHB – F	GGCCAGTACTTCGGTGAAACA
ZmRBOHB – R	ATTACACCAGTGATGCCTTCCA
ZmRBOHC – F	TTCTCTTGCCTGTATGCCGC
ZmRBOHC – R	CTTTCGTATTCCGCAGCCA
ZmRBOHD – F	CCGGCTGCAGACGTTCTT
ZmRBOHD – R	CCTGATCCGTGATCTTCGAAA
ZmPI-F	GCGGATTATCGCCCTAACC
ZmPI-R	CGTCTGGGCGACGATGTC
ZmPR1-F	TACGGCGAGAACCTCTTCTG
ZmPR1-R	GTTGGTGTCGTGGTCGTAGT
ZmPgb1 – F	TCCGCTTCTTTCTCAAGGTCTTCG
ZmPgb1 – R	AGGTCATGACGAAGACGGACAT
Actin – F	GATGGTCAGGTCATCACCATTG
Actin – R	AACAAGGGATGGTTGGAACAAC

4.0 DISCUSSION

We showed that suppression of *ZmPgb1* is sufficient to reduce lesion size in maize infected with *Clavibacter nebraskensis*, and provided a mechanistic model suggesting that down-regulation of *ZmPgb1* induces PCD through an increase in NO, ethylene, and ROS. Phytoglobins (Pgbs) are effective NO scavengers (Hebelstrup et al., 2006) and thus influence all those processes requiring NO as a signal molecule. This is apparent under several conditions of abiotic stress, where Pgbs, through the modulation of NO level, alter cell behavior and plant responses by regulating ethylene and ROS production (Mira et al., 2016). Specifically, during drought and flooding stress, *Pgbs* provide a protective role in the plant by preventing cell death through a reduction in NO, ethylene and ROS (Youssef et al., 2016; Mira et al. 2017).

The participation of *Pgbs* during biotic stress conditions has been recently documented, although the mechanisms in the observed responses are not fully understood. The expression levels of *Pgb1* increased in cotton cells infected with *Verticillium dahliae* (Qu et al., 2005), and suppression of the Arabidopsis *Pgb1* was associated with enhanced resistance against hemibiotrophic and necrotrophic fungal isolates (Mur et al., 2012b). These responses were mediated by NO and associated to fluctuations in jasmonic acid and ethylene levels (Mur et al., 2013).

The leaves with suppressed *ZmPgb1* had an increased expression of several genes involved in ethylene synthesis, indicative of a higher production of this growth regulator in these leaves, and confirming the effect of Pgbs on ethylene synthesis under abiotic stress conditions (Manac'h-Little et al., 2005). Of interest, ethylene responses to inoculation with *Clavibacter nebraskensis*, estimated by the expression of *EBF1* and *ERF2*, was delayed in WT leaves relative to ZmPgb1:RNAi leaves, an observation consistent with the expression of the PR genes *PI* and

PRI, and suggestive that a reduction in *ZmPgb1* may have accelerated plant responses to the bacterial infection. In fact, a possible link between Pgbs and PR- proteins was also proposed by Seregélyes et al. (2004) who showed alterations in PR1 expression in tobacco cells following altered level of *Pgb1*.

The suppression of *ZmPgb1* causes a rapid increase in the expression of RBHOA and D, with a concomitant elevation in staining patterns of both hydrogen peroxide and superoxide in areas adjacent to the infection site. The more prominent accumulation of ROS observed in the leaves with suppressed *ZmPgb1* is associated with the presence of TUNEL-positive cells, suggesting the induction of PCD, which is a key event of the hypersensitive response, and is in agreement with the observation of Mur et al. (2012). This also suggests that the rapid transcriptional activation of ethylene synthesis and the response observed in leaves with suppressed *ZmPgb1* might be required to induce the activity of NADPH oxidase, the major source of ROS in plant cells (Miller et al., 2010; Suzuki et al., 2011), composed of several sub-units encoded by the Respiratory Burst Oxidase Homolog (*RBOH*) genes (Sagi and Fluhr, 2006).

Our results obtained *in planta* indicate the participation of ethylene, ROS, and PCD in the enhanced tolerance of ZmPgb1:RNAi plants to *C. nebraskenesis*, but do not necessarily provide a cause-effect relationship among the different components. To address this issue, we developed an *in vitro* system more amenable to pharmacological treatments. Culture cells elicited with Cn displayed a rapid accumulation of NO, especially in those suppressing *ZmPgb1*, consistent with the early involvement of NO in plant responses to pathogens (Keller et al., 1998; Delledonne et al., 1998), and the role of Pgbs as NO scavengers (Igamberdiev et al., 2004; Hebelstrup et al., 2006). The higher accumulation of NO following suppression of *ZmPgb1* is required for the transcriptional induction of ethylene synthesis and response, and ultimately the accumulation of

ethylene. The observation that these events are increased by elevating NO (by SNP) in WT cells, and attenuated by scavenging NO (by cPTIO) in the ZmPgb1:RNAi cells, places NO upstream of ethylene, as also observed in Pgb-mediated responses to abiotic stress (Mur et al., 2008).

Suppression of *ZmPgb1* has a promotive effect on the expression of *RBOHs* and production of ROS, in a model requiring the participation of NO as a component up-stream of ethylene. This was demonstrated in WT cells, where a reduction in ethylene by AOA attenuated the inductive effect of SNP on *RBOH* transcription and ROS accumulation, and in ZmPgb1:RNAi cells where an elevation of ethylene by ETH reverted the inhibitory effect of cPTIO on the same responses. The observation that a reduction in *ZmPgb1* elevates the transcript levels of *RBOHA* and *RBOHD in planta* and all *RBOHs in vitro*, might be indicative of differences between the two systems in the mechanisms regulating ROS production.

The rise in ROS in ZmPgb1:RNAi cells relative to their WT counterparts, is needed to induce PCD, a key event of the HR response which delays of the pathogen's progression (Torres and Dangl, 2005). The regulation of cell fate by Pgbs has been recently elucidated. Huang et al. (2014) observed the induction of PCD in maize embryogenic cells suppressing *ZmPgb1* and demonstrated this event to be crucial for shaping the embryo body. Similarly, the induction of PCD as a result of *Pgb* suppression makes tissues and organs more susceptible to abiotic stress responses including drought and hypoxia (Mira et al., 2016; 2017).

Based on the above, a mechanistic model for *ZmPgb1* action during infection with Cn is proposed (Fig. 3.21). Suppression of *ZmPgb1* elevates the level of NO which is needed to induce ethylene synthesis and response. These events are required to activate the expression of pathogenesis-related genes, as well as several *RBOH* genes which promote the accumulation of

ROS in proximity to the infection site. The cells accumulating ROS are destined to die by PCD, thus restricting the advancement of the pathogen. These events, which are compromised in WT, are very similar to those described during hypoxia, thus suggesting a universal function of Pgbs in regulating stress responses.

Our study indicates that low *Pgb* expression in maize correlates with higher tolerance to the highly aggressive *C. nebraskensis* isolate Cmn 14-5-1. This effect is mediated by NO, ethylene and ROS signaling. Hence, future directions for this study would include the use of Pgb as a molecular marker to screen the resistance levels of commercial varieties. Hybrid plants of the most productive lines with low Pgb expression lines could be developed. This would be useful to produce stress-tolerant varieties to improve corn resilience in predominately corn growing regions. The findings from this study could also be extended to other economically important corn bacterial diseases such as Stewart's wilt caused by the pathogen *Pantoea stewartii*. Also, further investigation to ascertain the effects of *Pgb1* response in other host-pathogen interaction such as bacterial blight of soybean by modulating other plants hormones, NO and ROS could be assessed.

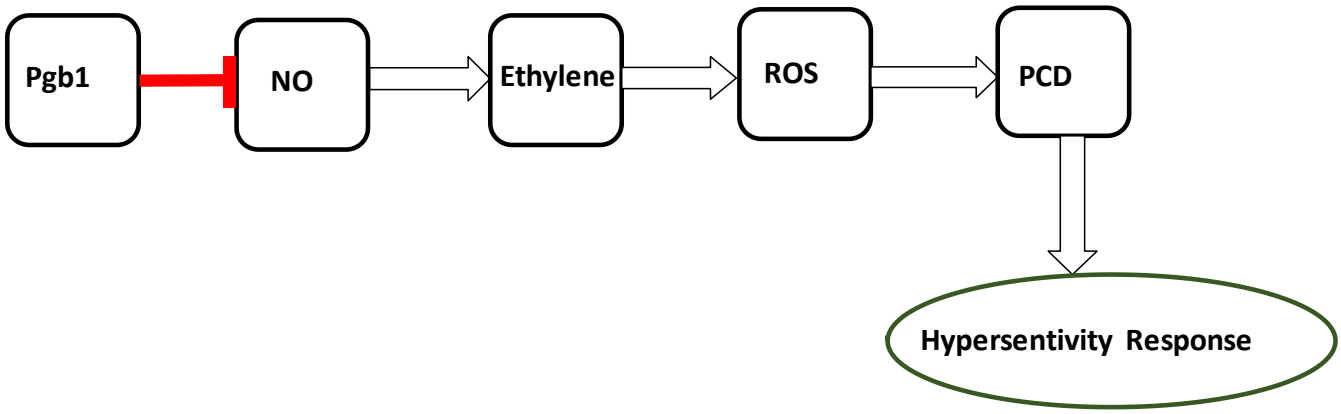


Figure 3.21. Proposed model of the participation of *Pgb1* during corn responses to biotic stress. Overexpression of *ZmPgb1* reduces the level of active NO in the plant cells. Suppression of *ZmPgb1* results in increased NO levels, which induce higher ethylene levels, resulting in increased ROS production, enhancing PCD and eventually HR to reduce the spread of pathogen infection and plant disease development.

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