2013

OVEREXPRESSION/SILENCING OF SELECTED SOYBEAN GENES ALTERS RESISTANCE TO PATHOGENS

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OVEREXPRESSION/SILENCING OF SELECTED SOYBEAN GENES ALTERS RESISTANCE TO PATHOGENS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By

Mohamed El-Habbak

Lexington, Kentucky

Director: Dr. Said Ghabrial, Professor of Plant Pathology

Lexington, Kentucky

2013

ABSTRACT OF DISSERTATION

OVEREXPRESSION/SILENCING OF SELECTED SOYBEAN GENES ALTERS RESISTANCE TO PATHOGENS

Plant diseases remain a major obstruction to meeting the world's increased demand for soybean oil and protein. Reducing the losses caused by diseases in order to improve crop production is a high priority for agricultural research. The need for novel strategies for plant disease control cannot be overstated. In the present study, selected defense-related genes were silenced and/or overexpressed in soybean using a virus-based vector and the resultant plants were tested for their responses to pathogens. The first part of the study focused on *Rps1k* (Resistance to *Phytophthora sojae*) gene. The two conserved domains encoding 'P-Loop NTPase' and 'PLN03210' of *Rps1k* were independently overexpressed. Stem inoculation assays for the overexpressing plants showed significant resistance to virulent races; 90% standing plants compared to 10% in controls. Lesion length was greatly restricted only in case of plants overexpressing 'PLN03210'. Simultaneous silencing of *Rps1k*-1 and *Rps1k*-2 resulted in remarkable susceptibility to avirulent races when tested by a detached-leaf assay. The second part of the study entailed silencing/overexpression of the chlorophyllase genes *GmCLH1* and *GmCLH2* and testing the responses of the silenced/overexpressing plants to the sudden death pathogen *Fusarium virguliforme*. Four weeks post root inoculation, *GmCLH2*-silenced plants showed enhanced resistance while the *GmCLH2*-overexpressing plants exhibited markedly increased susceptibility when compared to empty vector control. RT-PCR assay of *PR* genes revealed elevated expression of *PR2* and *PR4* in *GmCLH2*-silenced plants. In the third part of the study, soybean plants silenced for a leucine-rich repeat receptor-like kinase (*GmRLK3*) gene were examined for their responses to different pathogens. Silencing of *GmRLK3* enhanced susceptibility to infection with *Alternaria tenuissima* or *Sclerotinia sclerotiorum* as revealed by rapid disease progress on treated leaves. Surprisingly, silencing of *GmRLK3* in known susceptible soybean cultivars rendered the silenced plants resistant to *P. sojae*. The ensuing partial resistance to *P. sojae* was consistent with results of RT-PCR assays that showed a significant increase in the transcript level of the osmotin-encoding gene (*PR5a*) in the *GmRLK3*-silenced plants. *PR5a* is considered a marker for systemic acquired resistance.

KEYWORDS: Soybean resistance, Gene overexpression/silencing, Resistance to *Phytohphthora sojae* (*Rps1k*), Chlorophyllase (*GmCLH*), Receptor-like kinase (*GmRLK*)

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February 4, 2013

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February 4, 2013

DEDICATION

Dedicated with my love to

my honorable parents, FATEMA and HAMED

and my precious wife, JIHAN

ACKNOWLEDGMENTS

Praise is due to almighty Allah for gifting me the health and time for the completion of this research.

I would like to express my deepest gratitude to my major advisor, Dr. Said Ghabrial, for his excellent guidance, caring, patience, and providing me with an excellent environment for doing research. His constant encouragement, constructive criticism and great insight were instrumental for accomplishing my work and completing my dissertation. I would like to thank him for letting me experience research in molecular biology and for patiently correcting my writing during his recovery from eye surgery.

I would also like to thank my advisory committee members Dr. Aardra Kachroo, Dr. Lisa Vaillancourt and Dr. Todd Pfeiffer for guiding my research for the past several years. Each member provided great insights in her/his specific area of expertise. I am grateful for Dr. Aardra Kachroo for her helpful discussions and valuable ideas in my research.

Special thanks go to Dr. Dennis Egli, who accepted to serve as the outside examiner in my final exam committee. His comments during the dissertation defense were valuable. I would also like to thank Dr. Nicholas McLetchie who was willing to serve at the same position before Dr. Egli was selected.

I would like to thank the faculty members of the Branch of Plant Pathology, Department of Agricultural Botany, Banha Univeristy, Egypt who taught me and provided with me the knowledge I had prior to enrolling in this Ph.D. program.

My research would not have been possible without the help of Dr. Suryadevara Rao who provided overexpression constructs and dry soybean tissues silenced for the soy chlorophyllase genes, the sequence specific primers of the soybean pathogenesis related genes and some technical guidance and innumerable discussions during the time he shared with me in the lab. Dr. Padmanaban Annamalai provided dried soybean tissues silenced for *GmRLK3* gene. I am also grateful to Ms. Wendy Havens for all her technical assistance and her excellent job in maintaining the lab ready for work. Mrs. Amy Crume for maintaining the greenhouse. Dr. Keshun Yu for his help with the gas chromatography. Mr. Mohamed El-Shetehy for his help in the estimation of hydrogen peroxide. Dr. Paul Vincelli for providing the *Phytophthora sojae* isolates and Dr. Glen Hartman for providing the *Fusarium virguliforme* isolate.

The admission to this Ph.D. program would have been impossible without the academic scholarship granted to me by the Ministry of Higher Education, Egypt. Also the completion of it mainly relied on the assistantship provided by the Plant Pathology Department, University of Kentucky.

Finally, I would like to thank my wife for her continuous support and encouragement during my years of study. She stood by me through the hard times and celebrated the good ones. It would be impossible to describe how much gratitude and appreciation I owe my parents who raised me, showered me with their endless love and sincere prayers and instilled the good values in my character. To my brothers who helped me through the financial difficulties that I and my family faced during the past few years and to my sisters who always supported me and encouraged me, I owe my thanks.

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CHAPTER 1

INTRODUCTION

The world population is in continuous growth that in turn contributes to excessive consumption. The agriculture system plays a significant role to meet the increased demand for food at present and in the future. One of the most important factors to achieve this goal is the wise use of the continuously decreasing cultivated land in the next decades worldwide, which could be attained by following a series of steps including giving priority to high importance crops that could supply the globe with food, feed and fiber such as soybean (*Glycine max* (L.) Merrill).

1.1 Nutritional use and medicinal value of soybean

The main processing of soybean beans starts with extracting its 20% oil content to produce the edible, widely consumed soybean oil. The remaining part forms the soybean meal that contains a high level of protein followed by carbohydrate (40% and 35% of the seed weight, respectively). Close to 85% of the global soybean is crushed for oil and meal. It is worth mentioning that soybean oil is the second largest consumed vegetable oil in the world and the soybean protein meal represents by itself more than two thirds of the world's consumption (Lee *et al.*, 2007; Qiu and Chang, 2010; SoyStats, 2012).

In addition to protein, oil and carbohydrates, soybean seed composition also includes fatty acids, isoflavones, tocopherols, lecithin, saponins, sterols and raffinosacharides that make it ideal nutritional source for both human and animal. Recently, researchers shed more light on the benefits of soybean nutritional elements – especially isoflavones - for human health. Isoflavones are flavonoides (phenolic compounds isolated from plants) that are known as phytoestrogens because of their

structural similarities to estrogens. Consumption of soy foods was found helpful in preventing or controlling some major human diseases. Pharmaceutical benefits of soybean components were reviewed by Isanga and Zhang (2008). There have been studies showing that soy-based meal as a dietary replacement promotes weight loss and reduces blood cholesterols in obese men and women (Mikkelsen *et al.*, 2000; Allison *et al.*, 2003; Deibert *et al.*, 2004; Kwak *et al.*, 2012). Furthermore, there has been much focus during the past two decades on the cancer-preventive effects of soy foods. Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. Population studies showed that consumption of soy foods and soy isolflavones is associated with reduction in breast cancer risk in women (Yamamoto *et al.*, 2003) and reduction in prostate cancer in men (Lee *et al.*, 2003; Kurahashi *et al.*, 2007). Most of these studies were conducted in Japan and China, where the soy consumption is higher than that in the United States.

It has been shown that soy protein can help lower the low-density lipoprotein (LDL) cholesterol by 3% to 5% (Taku *et al.*, 2007). This modest reduction has considerable relevance, as each 1 percent reduction in LDL cholesterol helps to reduce heart disease risk by 2 - 4%. For this reason, the Food and Drug Administration (FDA) allowed in 1999 a health claim on food labels stating that including 25 grams of soy protein in a daily low-saturated-fat diet may reduce the risk of heart diseases (DHHS, 1999).

Maintaining optimal control of glucose and insulin levels is essential for overall good health, especially for obesity and diabetes. Recent studies revealed that soy foods might be valuable in this regard. Soy protein intake reduces fasting serum glucose levels

in postmenopausal women who are moderately high in fasting glucose levels. Soy foods have a low glycemic index (Jenkins *et al.*, 1981; Foster-Powell *et al.*, 2002). The glycemic index refers to the relative blood glucose response to sugar-containing foods. Some evidence indicates that foods with a high glycemic index - which may cause greater rises in serum glucose and insulin levels - increase risk for chronic diseases including cardiovascular diseases (Liu *et al.*, 2000), obesity (Brand-Miller *et al.*, 2002) and diabetes (Salmerón *et al.*, 1997). Thus, the low glycemic index of soy foods suggests that they have a role to play in helping control or protect from these diseases (Washburn *et al.*, 1999; Erdman Jr, 2000; Bhathena and Velasquez, 2002).

1.2 Soybean production

Globally, soybean production has expanded nearly 10-fold between 1961 and 2011, and it has doubled since the mid-1990s which made it a billion dollars' value crop. Currently, soybean ranks number 4 among the top cultivated crops in general and in particular ranks number 1 among oilseed crops as well as number 1 among the leguminous crops. In 2011, the total cultivated area of soybean in the world was 102 million Ha harvested out a yield of 25548.04 Hg/Ha giving a total production of 9.239 billion bushels of which 81% was shared by only 3 countries. USA ranked the first with 3.056 billion bushels (33%), then Brazil with 2.645 billion bushels (29%) and Argentina with 1.764 billion bushels (19%) (FAO, 2012; SoyStats, 2012).

Domestically, the crop value reached its peak in 2010 with 38 billion dollars. On a smaller scale, the soybean production in Kentucky rose to the highest level in 2009 with 68 million bushels harvested from 1.4 million acres with a crop value of 675 million

dollars that comes in the second place after corn (National Agricultural Statistics Service, 2011).

1.3 Soybean diseases alter crop quantity and quality

Diseases represent a major contributor of soybean yield reduction. The total yield loss due to disease in the United States was estimated as 11% in 1994, this increased to 23% between 2001 and 2003. Yield loss was estimated at 12% between 2003 and 2005 and approximately 11.5% in 2007 (Wrather *et al.*, 1997; Wrather *et al.*, 2001a; Wrather and Koenning, 2006; Wrather and Koenning, 2009; Hartman *et al.*, 2011). All parts of the soybean plant are subjected to a number of diseases that reduce the quality and/or quantity of some or all yield components. Diseases of this crop are caused by different types of fungal, oomycete, bacterial, viral and nematode pathogens that recently reached more than 300 pathogens (Hartman *et al.*, 2011), only a few of them cause diseases of high economic importance. The economic significance of diseases is determined based on the pathogen epidemic nature, the damaged plant part, the growing season in which the disease peaks, the availability of effective plant resistance, and the persistence of the pathogen against this resistance.

To reduce yield losses caused by diseases, producers follow different disease control methods, most effectively the use of resistant cultivars, then cultural practices and chemical control (Hartman and Hill, 2010).

1.4 Exploiting biotechnology for enhancing disease resistance

Scientific research has undertaken the endeavor of reducing production costs and minimizing environmental pollution with agricultural chemical residues by developing

soybean plants with enhanced disease resistance using traditional and modern breeding in addition to biotechnology. Both forward and reverse genetics tools have achieved rapid progress to allow a better understanding of how plants defend themselves against plant pathogens including signal perception and transduction and the associated molecular regulatory network. In a general review, the use of forward or reverse genetics in improving soybean traits was discussed by Bilyeu (2008). Reverse genetics approaches are needed to determine the function of an identified gene sequence or to assign an additional function to a gene with already known function, so called functional analysis. The functional genomic approaches for soybean improvement have been reviewed by Vuong *et al.* (2007). In the post-genomic era, functional analysis strategies have gained priority over the identification of genes which requires a forward genetic approach. For functional analysis, reverse genetics approaches have grown to be ever more popular alternatives to phenotypic screens. Several techniques of reverse genetics have been successfully used for functional analysis of plant genes of interest. Virus-induced gene silencing (VIGS) has emerged as an extremely powerful tool of reverse genetics for functional genomics, which enables transient knockdown of the expression of target plant genes, known as loss of function approach. Recently, transient gene overexpression (gain of function approach) has also been demonstrated to be valuable using virus-based vector systems.

In comparison to other gene functional analysis approaches in plants, researchers gain several advantages when they particularly use VIGS. Of these advantages, the ease of generating the construct by direct cloning of a fragment of the gene of interest into the respective viral vector, the ability to do rapid transient silencing/overexpression and rapid

phenotype analysis when there is no need for stable transformation, therefore the ability of conducting a large-scale screening of candidate genes, the relatively significant reduction of costs, lowering the number of plant population since there is no need for selection, the ability to analyze genes in species that are not amenable to Agrobacterium transformation, targeting an entire gene family by cloning a conserved region in this family, the ability of testing genes with embryo-lethal function and the broad use of viral vectors as most of them based on viruses with a wide host range of plants (Unver and Budak, 2009; Bernacki *et al.*, 2010; Huang *et al.*, 2012).

In 2006, Zhang and Ghabrial developed a bean pod mottle virus (BPMV)-based viral vector by engineering its RNA2 molecule (Zhang and Ghabrial, 2006). The resultant vector, designated pGG7R2-V, was proven to be a powerful VIGS system for functional genomics studies in soybean (Zhang and Ghabrial, 2006; Kachroo and Ghabrial, 2012). Since VIGS requires prior knowledge of genome sequences, the completion of the whole genome sequencing of soybean (Schmutz *et al.*, 2010) with homologous data from model plants such as *Arabidopsis* will boost the utilization of the BPMV vector such that it can be widely and effectively used to define the functions of genes of interest in soybean. In light of the serious necessity for rapid evaluation of new traits involving expression of valuable proteins that confer disease resistance in soybean, such a research will lead to the identification of novel resistance quantitative trait loci (QTLs) and/or the recognition of highly complicated crosstalk/interaction between different defense response pathways. Using efficient gene transformation technologies, such achievements might be practically translated to improved soybean lines with enhanced disease resistance.

1.5 The aim of the work

This dissertation comprises three studies of gene silencing or overexpression in soybean, each study investigates a gene that is structurally different than the other two. The well identified gene, resistance to *Phytophthora sojae* (*Rps1k*) gains its high importance from the ability of overcoming most of the pathogen races in a gene for gene resistance manner. Mapping and sequencing this gene opened the door for more functional studies. The first study aims to finding a partial resistance phenotype to *P. sojae* when only conserved regions of the large repetitive sequence-rich *Rps1k* gene are overexpressed .

Chlorophyllase genes of several plants were cloned and their clear role in most studies was demonstrated to be in chlorophyll degradation (Trebitsh *et al.*, 1993; Jacob-Wilk *et al.*, 1999; Tsuchiya *et al.*, 1999; Benedetti and Arruda, 2002; Tang *et al.*, 2004; Arkus *et al.*, 2005; Zhou *et al.*, 2007). However, their demonstrated correlation with the defense-related plant hormones such as jasmonic acid and ethylene suggests a likely interaction with plant defense pathways (Mitchell *et al.*, 1983; Trebitsh *et al.*, 1993; Tsuchiya *et al.*, 1999; Kariola *et al.*, 2005). Therefore, the second study, through silencing and overexpressing each of the chlorophyllase-encoding genes in soybean (*GmCLH1*and *GmCLH2*) aims to find out whether any of these genes has a potential function in defense against soybean pathogens. Overexpressing/silenced soybean plants are tested against two groups of pathogens; hemibiotrophic pathogens, namely *Fusarium virguliforme* and *P. sojae* and necrotrophic pathogens, namely *Alternaria tenuissima* and *Sclerotinia sclerotiorum*. The resultant susceptible/resistant phenotypes are analyzed.

The third study aims to investigate the function of a leucine rich repeat receptor-like kinase (LRR-RLK)-encoding gene (*GmRLK3*), which belongs to a recently identified gene family that comprises three genes. Although other genes coding for proteins of the same structure in other plants were extensively investigated and found to possess defense-related functions, *GmRLK* family was only recently studied (Yamamoto and Knap, 2001). The investigation is conducted by silencing *GmRLK3* gene and investigating the effect of silencing on the plant defensive response to *P. sojae* using different sets of *P. sojae* race-soybean cultivar and to the necrotrophs *A. tenuissima* and *S. sclerotiorum*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant materials and growth conditions

Soybean cultivars, Williams, Sloan and Williams 82 were used in the *Rps1k* study, Essex and Harosoy in the *GmCLH*s study and Harosoy and Harosoy 63 in the *GmRLK3* study. Seeds of each cultivar were grown in 4-inch pots (10 seeds/pot) containing PRO-MIX® BX MYCORRHIZAE™ (Premier Horticulture, Inc., Quakertown, PA) soil mix, and were thinned just before transcript inoculation (see below) to five evenly sized plants/pot in order to reduce the differences that might occur between individual plants. Unless otherwise specified, all plants were grown in a greenhouse with temperature of 27°/20°C under a 16/8-h light/dark regime.

2.2 Shade conditions

To set up a low light growth conditions for plants, a growth cage completely covered with 2 layers of shade cloth was used. The cage was put on the same growing bench that has the normal light-plants in the greenhouse. Using a Quantum Sensor (LI-190S, LI-Cor, Lincoln, NE, USA), photosynthetically active radiation (PAR) was measured in both light and shade conditions during midday time of a sunny day in June. Average PAR in the regular light was (450.43 $\mu mol.s^{-1}.m^{-2}$) while average PAR in shaded cage (34.37 $\mu mol.s^{-1}.m^{-2}$) that equals 7.6% of the intensity outside the cage. Clear, cloudless conditions prevailed over the entire experimental period.

2.3 Plant height and root length measurements

Thirty days postinoculation with the recombinant virus vector, plant height was measured in centimeters from the soil surface to the apical tip before the shoot was excised from the roots at the soil surface. The pots were emptied and roots were washed from soil and measured in centimeter to the tip of the main root.

2.4 Measurement of chlorophyll

Chlorophyll was measured in leaves using atLEAF+ portable chlorophyll meter (FT GREEN LLC, Wilmington, DE, USA). Chlorophyll content was expressed in atLEAF units.

2.5 Jasmonic acid (JA)/Methyl Jasmonate (MeJA) treatment

JA solution was prepared by dissolving JA (Sigma-Aldrrich, MO, USA) in absolute ethanol and diluted in sterile water to 50 μM. MeJA solution was prepared by dissolving MeJA (Sigma-Aldrich, MO, USA) in methanol to 10% then used directly. Using an aerosol pressurized sprayer, all leaves of 2 weeks old plants were sprayed with JA solution until runoff then plants were placed in glass chambers for 48 hours. Before the chamber was closed, an open flask containing the MeJA solution was put beside the plants to evaporate in the phylloplane. Control plants were sprayed with water. All plants were kept in chambers for 48 hours.

2.6 Pathogens

Phytophthora sojae races R1 and R3 were kindly provided by Dr. Paul Vincelli, Plant Pathology Department, University of Kentucky. *P. sojae* cultures were maintained at 10°C on V8 agar (V8A) medium composed of filtered V8 juice 200 mL, $CaCO_3$ 2.0 g, agar (Difco Laboratories, Detroit, MI, USA) 15.0 g and deionized water 800 mL (Miller 1955). All cultures were grown on the same medium in 9 cm Petri dishes at 23°C.

Fusarium virguliforme (isolate 3) was kindly provided by Dr. Glen Hartman, Laboratory for Soybean Disease Recearch, University of Illinois. Slant cultures were maintained on 2% water agar at 4°C and subcultured on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) medium amended with streptomycin (100mg/L) in 9 cm Petri dishes at 23°C.

Phomopsis longicolla isolate was a subculture of the isolate used in previous studies (Koning *et al.*, 2003) and maintained as slant cultures on acidified potato dextrose agar (aPDA; pH 4.5) medium at 4°C and subcultured on the same medium in 9 cm Petri dishes at 23°C.

Alternaria spp. was isolated from soybean plants in Lexington, Kentucky, single spored, identified microscopically and maintained on slant cultures of PDA at 4°C and subcultured on the same medium in 9 cm Petri dishes at 23°C.

Sclerotium scerotiorum isolate was kindly provided by Dr. Paul Vincelli, (Plant Pathology Department, University of Kentucky). The fungus was maintained as dry sclerotia in capped glass vials and was cultured after surface-sterilisation (3% NaOHCl for 5 min, rinsed in sterilized water and dried between sterilized filter papers)

by placing a single sclerotium on a 9 cm Petri dish of potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) which were then incubated at 20 – 22°C for 3 days. Subculturing was carried out by cutting 5 mm-diameter agar plug from the edge of the growing mycelium using a cork borer and transferring it to the centre of a new PDA-Petri dish.

2.7 Identification of *Alternaria* isolate

The preliminary recognition of the Alternaria spot symptoms along with preliminary microscopic examination of the fungal growth has determined that the isolate belongs to the genus *Alternaria*. Morphological features of conidia were examined by digital imaging of spore preparations with Axioskop microscope and attached Axiocam HRc camera (Carl Zeiss AG, Göttingen, Germany) which were used for measurements of conidial body dimensions (length and width) and length of the peak using AxioVision 4.8 software (Carl Zeiss AG, Göttingen, Germany). Other features like conidium fragmentation were also recorded. The numeric data were compared to the table of 'Description of selected *Alternaria* species by various authors' (Rotem, 1994) to designate the species.

The ITS (Internal Transcribed Spacer) sequence of the *Alternaria* isolate was also obtained via direct sequencing using primers ITS1 and ITS4 (White *et al.*, 1990), and species determination was verified via a GenBank BLAST search.

2.8 Plant inoculations

A list of the different methods used for testing the responses of transcript-infected soybean plants to different pathogens is shown below:

2.8.1 Stem inoculation:

In this method, 14-day old V8A cultures of *P. sojae* grown in 9 cm Petri dishes were used to generate agar mycelial plugs. A sterile lid of a 6 cm Petri dish was used to demarcate a central agar circle in the culture. This circle was then divided into 32 similarly-sized sectors of agar plugs so that all plugs are of comparable age. Stem inoculation was carried out by making a 1.5 cm longitudinal incision below the second node on the stem of plants at V1 stage using a sterile scalpel. After removing the major part of the agar from the inoculum plug, the remaining mycelial growth layer was then immediately placed onto the wound surface. A sterilized-water-saturated piece of cheesecloth (~ 3 × 1.5 cm) was wrapped around the inoculation zone, which was finally covered with parafilm in such a way that seals the wrap to the stem at the bottom edge while the upper edge is still loose. A few drops of sterile-water were then added with disposable plastic Pasteur pipette to ensure that the cheesecloth is fully saturated (Fig. 2.1). Inoculated plants were maintained in a controlled-environment growth chamber with 16 h photoperiod at 27°/20°C (day/night) and 90% relative humidity for 10 days. Disease severity was scored by measuring the length of the lesion along the stem.

2.8.2 Root inoculation

For *F. virguliforme* inoculations, four 3-week old cultures grown on 9 cm Petri dishes were used. Mycelia from each culture was harvested in 10 ml sterilized water using a spatula, filtered with sterile cheese cloth and the filtrate was increased to 200 ml with sterilized water and 0.1% Tween 20 was added. Plants were inoculated at V1 growth stage. After watered in the morning, each plant main root was superficially wounded ~ 2 cm under the soil surface by a sterilized scalpel. A 5-ml plastic syringe was used to

deliver 5 ml of the spore suspension at each root wound. The soil was returned back to fill the hole beside each root. Plants were kept without watering for the next 24 hours. All pots are kept in trays and maintained in a controlled-environment growth chamber with 16 h photoperiod at 23°/18°C (day/night) and 75% relative humidity for 6-9 weeks.

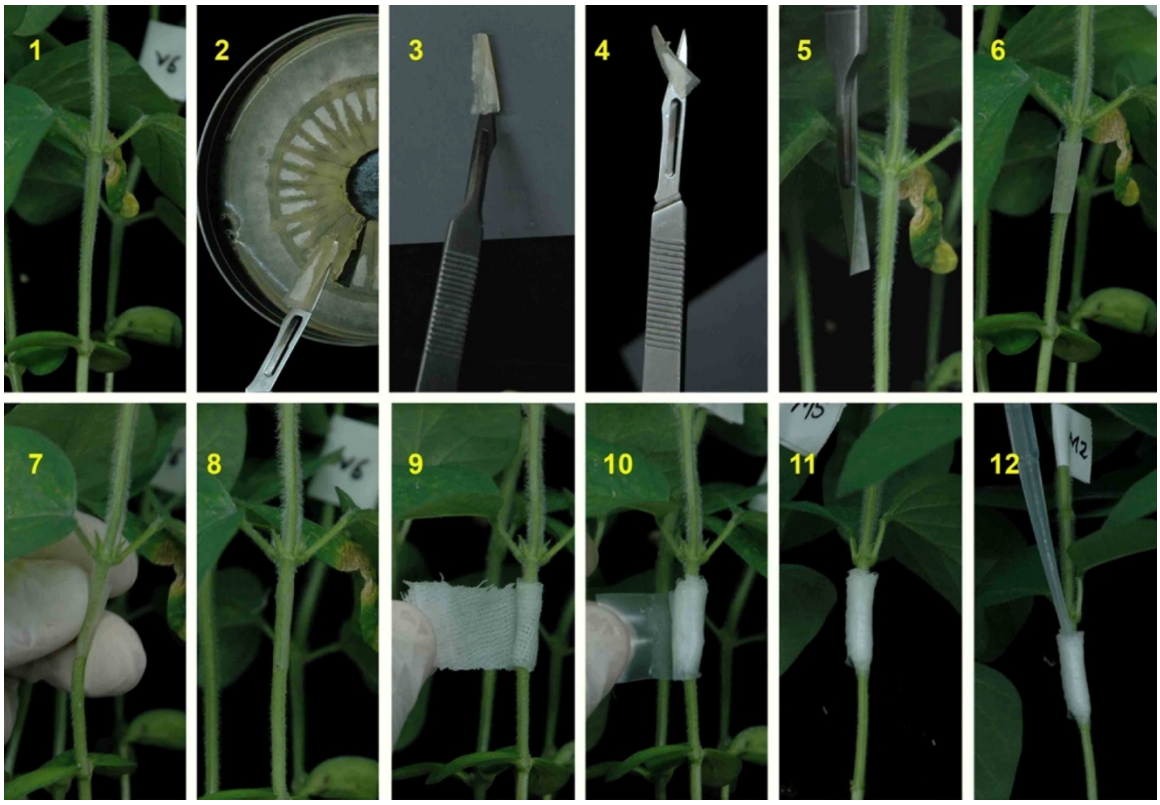


Fig. 2.1 Steps of stem inoculation method. 1, Performing a small longitudinal incision (~15 mm) below the second node on the stem using a sterile scalpel. **2,** *P. sojae* inoculum preparation: a centric agar circle was generated by pressing a sterile lid of a 60 mm Petri dish in the middle of 90 mm Petri culture of *P. sojae*. The circle was then cut into sector-shaped agar plugs of equal size. **3,** An individual agar plug was removed from the culture and a sterile scalpel was used to remove the major basal part of the agar. **4,** The remaining mycelial growth layer which will be used in inoculation. **5-6,** Putting the inoculum face-down on the surface of the wound. **7-8,** Finger-pressing on the back of

inoculum to firm it on the wounded stem surface. **9,** Wrapping a sterilized-water-saturated piece of cheesecloth (~ 3 x 1.5 cm) around the inoculum. **10,** Wrapping a layer of parafilm around the cheese cloth forming a cup shape (sealing the bottom edge of the parafilm to the stem while the upper edge is loose around). **11,** The final shape of the inoculation zone. **12,** Adding a few drops of sterile water inside the wrap using disposable plastic Pasteur pipette

2.8.3 Detached leaf inoculation

A laboratory incubation set was specifically developed for use in the detached leaf assay (Fig. 2.2). It comprises a clean empty box that holds 1.0 ml pipette tips to which 200 ml of milliQ water were added. A rectangular piece of cheesecloth was used to cover the tips' rack except for a small hole in the middle of the front side to allow the petiole to go through into the box. When covering the rack, it was taken in consideration that the front side of the cheesecloth dips into the water so it can irrigate the leaves with sufficient moisture by being in contact with the petiole. The lid was closed and taped by a thermal autoclave tape to indicate autoclaving efficiency. After autoclaving, the set was left to cool down for a day in a laminar air flow hood where the remaining part of the work took place. For the bioassay, soybean trifoliolate leaves were collected fresh from plants, surface-sterilized with 0.05% sodium hypochlorite solution and subsequently washed 3 times with sterile water and dried between sterile paper towels. The petioles were angularly trimmed at their ends with a sterile scalpel then each trifoliolate leaf was placed on the rack with the petiole penetrating the front hole with its cut surface in contact with the cheesecloth. Inoculum preparation varied for different pathogens as follows:

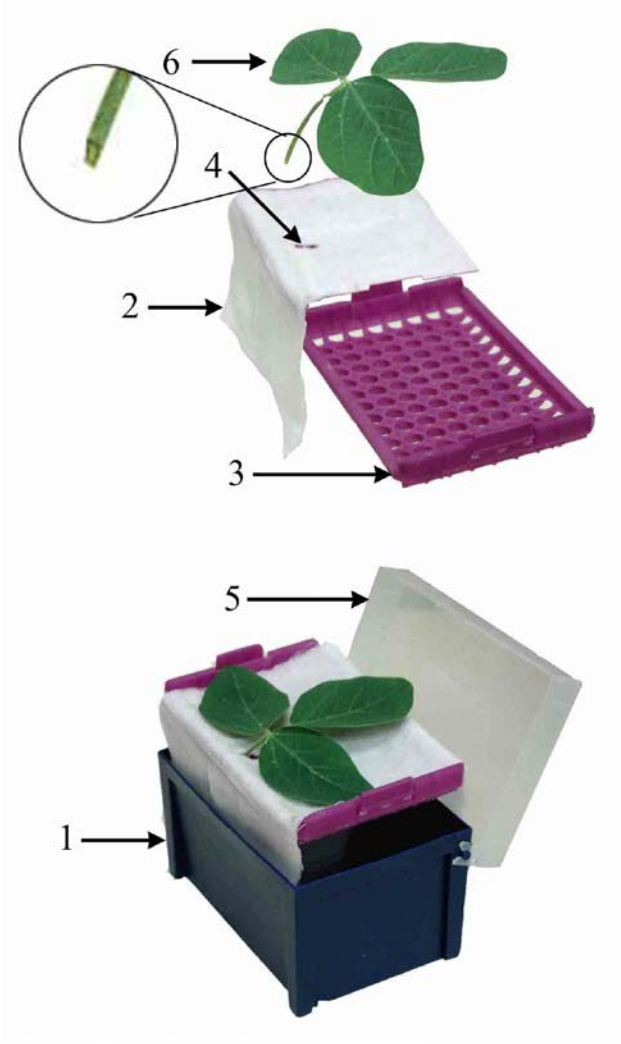


Fig. 2.2 Detached leaves inoculation set: components, assembly and method. The set comprises an empty used 1 ml pipette tips box (1), filled in with a 200 ml of milliQ water. A rectangular piece of cheesecloth (2) was used to cover the tips' rack (3) with a long side to the front. A small hole (4) was punctured in the middle front side of the cheese cloth to allow the petiole to go through into the box. The box lid (5) to be closed before autoclaving and might be open only in the hood then closed back after inoculation to maintain sterilized conditions. Soybean trifoliolate leaf (6) to be rested on the covered rack and its angularly trimmed petiole penetrates the whole to maintain continuous watering to the leaf during the experiment.

***Phytophthora* inoculum** was prepared using a 5-mm cork borer to produce mycelial agar plug discs from a 14-day old culture of *P. sojae* at similar distances from the culture center. A drop of sterile water (5 µl) was placed at the center of each leaflet using a micropipette and then a mycelial agar plug was placed facing down on the water drop. Lids were then closed and sets were incubated in the lab at room temperature (23-25°C).

***Phomopsis* inoculum** was prepared by harvesting mycelia, pycnidia and stroma from a 3-4 week old culture and homogenizing the combined material using sterile mortar and pestle in 3 ml sterilized water. From the homogenate, a 40-µl drop was placed on each leaflet. Lids were closed and sets were incubated at 25°C and the lesions were evaluated 10 days post inoculation.

***Alternaria* inoculum,** a 20-µl drop of spore suspension (3-4 x 10^4 spores/ml) prepared from a 14-day old culture of *A. tenuissima* was placed at the center of each leaflet. Lids were closed and sets were incubated at 28°C for 24 hours, after which they were transferred to 20°C for 6 days. The disease was evaluated visually by comparing lesion size to that of the susceptible mock-inoculated control.

***Scelrotinia* inoculum** was prepared using a 5-mm cork borer to cut mycelial agar plug discs from a 5-day old culture of *S. sclerotiorum* at similar distances from the culture center. A drop of sterile water (5 µl) was placed at the center of each leaflet using a micropipette and then a mycelial agar plug was placed facing down on the water drop. After inoculation was carried out, lids were closed and sets were incubated in the dark at 20°C.

2.9 Recombinant BPMV constructs for overexpression/silencing of target genes

Total RNA was extracted from soybean (cv. Williams 82) leaves. Using a Veriti™ 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA), a reverse transcription-polymerase chain reaction (RT-PCR) was carried out to synthesize first strand cDNA using oligo $(dT)_{17}$ primer and Superscript® Reverse Transcriptase II (SS RT II; Invitrogen™ Life Technologies, Carlsbad, USA). Constructs were made for the following genes:

2.9.1 Resistance to *Phytophthora sojae* (*Rps1k*)

Three constructs were generated to study this gene. Using sequence specific primers (Table 2.1), three fragments within the *Rps1k* gene were amplified using polymerase chain reaction (PCR). For overexpression, two different pairs of specific primers were designed to amplify fragments of 573 bp and 846 bp encoding the highly conserved 'PLN03210' and 'P-Loop NTPase' domains, respectively, of the Rps1k protein (GenBank accession No. AAX89382). For silencing the target gene, a third pair of specific primers was used to amplify a 273 bp-fragment. Each of the three fragments was engineered to contain a *BamH*I at its 5' end and an *Msc*I at its 3' end for cloning in the BPMV vector.

2.9.2 Chlorophyllases (*GmCLHs*)

Two constructs were generated for silencing *GmCLH1* and *GmCLH2* (GenBank accession No. AB181947 and AB181948, respectively). Sequence specific primers (Table 2.1) were used to PCR-amplify 237 bp-fragment in *GmCLH1* and 240 bp-

fragment in *GmCLH2*. The full-length sequences of 981 bp of *GmCLH1* and 951 bp of *GmCLH2* were amplified to generate another 2 constructs for overexpression.

2.9.3 Receptor like kinase 3 (*GmRLK3*)

A nucleotide sequence of 258 bp was PCR-amplified to generate a silencing construct for *GmRLK3* (GenBank accession No. TC219395) in soybean. *BamH*I and *Msc*I restriction sites were added to the fragment during amplification.

All amplified products were cloned first into the pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA) and sequenced. Inserts were excised from the recombinant pGEM-Teasy vector using the appropriate restriction enzymes and purified. Similarly, the BPMV-based vector 'pGG7R2' (Zhang and Ghabrial 2006) was digested using the same endonucleases and subsequently cloned in the BPMV vector and the resulted positive clones were used for further analysis.

2.10 DNA sequencing

Universal M13 primers were used for sequencing pGEM-T easy clones and vector sequence specific reverse primer was used for sequencing BPMV vector clones, meanwhile PCR products were sequenced using gene-specific sequencing primers. Purified DNA by DNA purification kit (Fermentas, Hanover, MD, USA) from PCR products or gel excised fragments was prepared for sequencing in 10 µl reaction volumes in 200 µl PCR tubes. To 2 µl primer (5 µM), 1µl sequencing buffer (5X) and 1 µl of BigDye® Terminator v3.1 Cycle (Applied Biosystems, Foster City, CA, USA) was added 3µl DNA (~ 50-100 ng). Thermocycling was performed on a Veriti™ 96-well Thermal

Cycler (Applied Biosystems, Foster City, CA, USA) as follows: initial activation step of heating to 95°C for 2 min, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 4 minutes and finally 4°C for 5 min. DNA was then precipitated for 30 min at room temperature by addition of 75 µl absolute ethanol and 5 µl of 125 mM EDTA, and centrifugation (13,000 rpm, 25 minutes). Precipitated DNA was washed twice by addition of 100 µl ethanol (70% v/v in sterile water) followed by centrifugation (13,000 rpm, 5 minutes). After discarding the supernatant, the precipitated DNA was dried in Savant Integrated SpeedVac® system ISS110 (ThermoSavant, Holbrook, NY, USA) for 5 min. Samples were submitted to the Advanced Genetic Technologies Center (AGTC; Plant science Building, University of Kentucky) where they were dissolved in 30 µl Hi-Di Formamide (Applied Biosystems, Foster City, CA, USA) then sequencing was performed using ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Resulted sequences were analyzed by BLASTn software to confirm the sequences of the clones or the amplified genes/fragments of the respective genes.

2.11 RNA transcription and transcript inoculation

For *in-vitro* RNA transcription, recombinant RNA2 vectors carrying full-length coding sequences or silencing fragments of the target genes and BPMV RNA1 were linearized with *Sal*I and *Sal*I/*Not*I, respectively to be used as templates for RNA transcription. Empty vector was used instead of the recombinant one to serve as control (Fig. 2.3A; Diaz-Camino et al., 2011; Kachroo and Ghabrial, 2012). Transcription reaction was carried out as previously described (Kachroo and Ghabrial, 2012). After

transcription, 10µl of each reaction mixture was analyzed by electrophoresis on a 1% ethedium bromide-stained agarose gel in TBE buffer (90 mM Tris–borate, 2 mM EDTA) to assess yield and quality of the transcripts (Fig. 2.3B). Transcripts of BPMV RNA1 and recombinant RNA2 were mixed to produce an infectious transcript and immediately rub-inoculated on the fully expanded unifoliolate leaves of 7-day old soybean seedlings (Kachroo and Ghabrial, 2012). Buffer-inoculated plants served as mock. All plants were kept in the greenhouse and were monitored for symptom development and phenotypic changes (Kachroo and Ghabrial 2012).

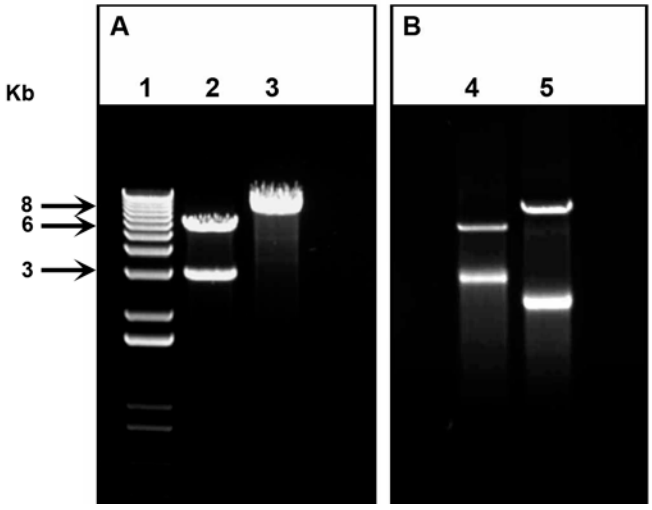


Fig. 2.3 Linearization of the recombinant BPMV plasmids and transcription products. A. Lane 1. 1Kb+ DNA marker, 2. *Sal*I/*Not*I-linearized HopR1 plasmid and 3. *Sal*I linearized- pGG7R2-*GmCLH2*-951 **B.** RNA Transcripts from Hop RNA1 (Lane 4) and pGG7R2-*GmCLH2*-951 (Lane 5). Upper bands are the remaining linearized DNA plasmids (reaction input) and lower bands are RNA transcripts (reaction output) after 3 hr incubation at 37°C.

2.12 RNA extraction and RT-PCR analysis

RNA was extracted from liquid N_2-frozen leaves using TriReagent® (Molecular Research Center, Inc., Cincinnati, OH) following manufacturer instructions. First strand cDNA synthesis was carried out using oligo $(dT)_{17}$ primers and Superscript® Reverse Transcriptase II (SS RT II; Invitrogen™ Life Technologies, Carlsbad, USA). RT-PCR and semi-quantitative RT-PCR assays were performed to assess the levels of the respective target genes' transcripts, the response of the respective target genes to different factors or the induction of pathogenesis related (*PR*) genes due to silencing/overexpression of the respective target genes. All cDNAs syntheses and DNA amplifications were performed on a Veriti™ 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) however, PCR reaction mixture, cycle number and PCR conditions varied according to the genes amplified and the purpose of the assay. Aliquots (10 µl) of the PCR products were analyzed in comparison to DNA molecular weight marker (1Kb Plus DNA Ladder; Invitrogen™ Life Technologies, Carlsbad, USA) by electrophoresis on a 1% ethedium bromide-stained agarose gel in TBE buffer (90 mM Tris–borate, 2 mM EDTA) at 90 V for 50 min. The amplified DNA fragments were UV-visualized and photographed using Molecular Imager®, Gel Doc™ XR system (Bio-Rad Laboratories Inc., Hercules, CA, USA). To compare band intensities, gel images were analyzed using ImageJ software, version 1.46r (Rasband, W.S. 1997-2012. Bethesda, Maryland. U.S. National Institutes of Health). Images were imported into ImageJ and independently analyzed using the Uncalibrated Optical Density (OD) function, whereas the intensity of individual bands was measured as gray values then were converted to uncalibrated OD values (arbitrary units corresponding to gray levels). ImageJ converts

pixel intensities into optical density using the function: Unc. OD = $\log_{10}(^{255}/_{\text{pixel value}})$. For each gel image, the relative values of transcript levels were mathematically calculated as a percentage of empty vector control peak's area, which served as 100% control.

2.13 Protein evaluation and western plot analysis

Total protein extraction from soybean leaves was performed as described by Osherov and May (1998). Protein concentration was estimated by the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was carried out as previously described (Srinivasa *et al.*, 2001) using an antiserum to BPMV-CP.

2.14 Jasmonic acid assay

For jasmonic acid estimations, 1 g of fresh leaves were extracted using a solution containing glacial acetic acid, methanol, chloroform and potassium chloride (0.9%) (1:4:8:8 by vol) and dihydrojasmonic acid (DHJA) as an internal standard. The lower phase was dried under compressed nitrogen, and samples were derivatized using diazomethane and suspended in acetonitrile and analyzed by a gas chromatograph equipped with mass spectrometry. A Varian FAME 0.25 mm × 50 m column was used for this analysis.

2.15 Hydrogen peroxide (H_2O_2) quantification

For H_2O_2 determination, leaves were ground in 40 mM Tris-HCl, pH 7.0 followed by adding 20 mM 2'7'-dichlorofluorescein. The samples were incubated in the dark for 1

hour and the H_2O_2 levels were measured with a spectrofluorimeter at 488 and 583 nm wavelengths. The concentration of H_2O_2 was determined as mmol/mg protein by extrapolating from the standard H_2O_2 curve.

2.16 Determination of chlorophyllase activity

For the enzyme substrate, an acetonic extract of fresh soybean trifoliate leaves was prepared at 4^oC (2 g leaves/ 6 ml acetone) and vacuum-filtered through filter paper, washed with cold acetone and increased to 15 ml filtrate. The extract was stored for 3 days at 4^o C to precipitate carotenoids which were removed by filtration and the substrate was collected in a clean dry dark tube. For chlorophyllase extraction, an acetone-dried powder was prepared from fresh leaves by grinding ~ 1 g of fresh leaves in 5 ml cold acetone at 4^o C. After vacuum-filtering through filter paper, the solid material was washed with cold acetone to remove excess pigments then left at room temperature to dry out. To 600 μl of the enzyme substrate, 30 mg of the acetonic powder, 400 μl of 100 mM sodium citrate was added. The final pH of the mixture should be ≈ 8.0. The mixture was incubated in the dark at 40°C for 24 hours. The supernatant was transferred to clean tube with Pasteur pipette and the retained powder was washed with 500 μl of 80% acetone that was added to the same tube then a 500 μl of 2% NaCl was added to block the enzyme activity. Chlorophyllide *a* absorption in samples was measured at wavelength λ 665 nm on a Beckman DU 640 UV-Visible spectrophotometer (Beckman Instruments, Fullerton, CA, USA) and the quantity of chlorophyllide *a* was calculated based on its molar absorbance coefficient $\alpha_\lambda = 76.79\ mM^{-1}\ cm^{-1}$.

Table (1): Primers

Gene	Name	Sequence (5'-3')	Amplicon size (bp)
Rps1k	PLN-*BamH*I-OE-For	TACTAC GGA TCC ACT TGA GAG TTT TAT C	573
	PLN-*Msc*I-OE-Rev	ATG TCC TGG CCA TAT AGC CTT TTA	
Rps1k	PLoop-*BamH*I-OE-For	GCA GGA TCC AGG GAG AAA GAT AGG GAG GC	846
	PLoop-*Msc*I-OE-Rev	CAG TGG CCA ATC TTC AGC CAT CCA CAA CAA	
Rps1k	*K1+2-BamH*I-SIL-For	TTA GGA TCC GAT GAC TTA CTC GAC CAT	273
	*K1+2-Msc*I-SIL-Rev	TAA ATA TGG CCA CTC CGA CAA CAA CT	
Rps1k	PLN-ExpChk-For	ACT TGA GAG TTT TAT CAT TTT GTG ACT TC	573
	PLN-ExpChk-Rev	TAT AGC CTT TTA TTC TCA ACG ATT CAA TG	
Rps1k-1	*K1*-ExpChk-For	GGA ATC GAA CGA GAA CAC AAC A	434
	K1-ExpChk-Rev	CAC CAT AAG GCC AAC TAC TTC TAT T	
Rps1k-2	*K2*-ExpChk-For	ATG CCT TGT TGG GAG GTG	590
	K2-ExpChk-Rev	AAT GAC TCT GCC CCT GAA AC	
GmCLH1	*CLH1-BamH*I-SIL-For	GGA TCC ACG GAT TAT GGT CAC ATG GAC AT	216
	*CLH1-Bal*I-SIL-Rev	TGG CCA TGG TAA GTA TCG CAC ATC ATC AAG	

Table (1) - continued

GmCLH1	*CLH1-Bgl*II-OE-For	ATT AAG ATC TAT GCA AAA CTT TGC AGA ATC TCA TCA ACT TTC	981
	*CLH1-Sma*I-OE-Rev	ATT ACC CGG GGA TAT CCA GAA AAG AAT CAA ATT TGA TCT CCA CTG GTA	
GmCLH2	*CLH2-Bgl*II-SIL-For	ATA TAG ATC TAT GGC GCA GAG AGC TCA ACC AGC G	243
	*CLH2-Sma*I-SIL-Rev	ATT ACC CGG GTG GCC ATG GTA AGT ATC GCA CAT CAT CAA G	
GmCLH2	*CLH2-Bgl*II-OE-For	ATA TAG ATC TAT GGC GCA GAG AGC TCA ACC AGC G	951
	*CLH2-Sal*I-OE-Rev	ATA TGT CGA CTT ATG GCC ATG GTA AGT ATC GCA CAT CAT CAA G	
GmRLK3 AF244890	*RLK3-BamH*I-SIL-For	CAG GAT AGG ATC CAT TAT CTC TCT G	258
	*RLK3-MSc*I-SIL-Rev	AAC AGC TTG GCC ATA CAC TGA CTG A	
GmRLK3 AF244890	*RLK3*-ExpChk-For	GAG CTC TAC ATC GGC TAC TAC	1200
	RLK3-ExpChk-Rev	TGT AAT TGA AGT AGC TGA ATT GAC	
PR1a AF136636	*PR1a* -ExpChk-For	ATGGGGTACATGTGCATTAAGATTTCGTTTTGTGTG	522
	PR1a -ExpChk-Rev	CAGTTTGTAGGGTCTTTCACCAACAAAGTTGCC	
PR2 M37753	*PR2*-ExpChk-For	ATG GCT AAG TAT CAT TCA AGT GGG AAA AGC TCT TC	400
	PR2-ExpChk-Rev	TGA GTG TTC GGG TTT CAC TTC ATT TCC CAC TG	
PR3 AF202731	*PR3*-ExpChk-For	ATG AAA AAC ATG AAA TTG TGT TCG GTG ATG CTA TGC TTA T	390
	PR3-ExpChk-Rev	ACC GGT TGT GCC AAA GCC ATT GAA AGA	

Table (1) - continued

PR4 BT090788	*PR4*-ExpChk-For	AGG CCT GGT GGT ATT GCT GTG TTT GAT T	550
	PR4-ExpChk-Rev	GTC GAG CTC ATT CCC ACA ATC CAC AAA C	
PR5a CX701785	*PR5*-ExpChk-For	GGC CGG TTT TGG GCC CGA AC	532
	PR5-ExpChk-Rev	GGC AGA ATA TGA TGG CAT AGT CAG GTC CGT T	
GmPAL1	*PAL1*-ExpChk-For	TTG CCT TCA AAT CTC ACT GCC AGC	773
	PAL1-ExpChk-Rev	TTC CCT TGG CAC AAA GCA GTG AAC	
GmICS1	*ICS1*-ExpChk-For	TGC AGG TGC CAA TTG AAG AGC AAG	617
	ICS1-ExpChk-Rev	AGT GCT ACG AGC TAG CAC AAC CTT	
β-Tubulin B3 M21297	*β*-Tubulin-For	TCT CCG CAA CCA TGA GTG GTG TTA	565
	β-Tubulin-Rev	TCG TTC ATG TTG CTC TCT GCC TCT	

CHAPTER 3

3 Overexpression of coding sequences of *Rps1k* conserved domains confers resistance to *P. sojae* in soybean

3.1 LITERATURE REVIEW

Phytophthora root and stem rot of soybean (*Glycine max* (L.) Merrill), caused by numerous physiologic races of the oomycete pathogen *Phytophthora sojae,* Kaufmann and Gerdmann, is one of the most destructive diseases of soybean in the field. It ranked among the top 4 diseases causing the greatest losses of soybean yield in the United States in the years 2003 – 2005 with an estimated annual loss of 300 million dollar in North America (Wrather and Koenning, 2006) and approximately $1 billion to the annual world soybean crop (Nicholls, 2004).

Phytophthora (meaning 'plant destroyer') may attack soybean plants at any stage of development. Early symptoms are seed rot, pre-emergence or post-emergence damping off. On older seedlings, stems may appear water soaked, leaves may turn yellow and the plants wilt and die. On mature plants, leaves gradually turn yellow, wilt but remain attached to the dead plants. Roots show severe rot on the taproot and destruction of the lateral roots. On the stem, symptoms appear as lesions involving cortex and vascular tissue discoloration that progress up the stem before the plants die. The disease is most severe in poorly drained soils with high clay content (Schmitthenner, 1999).

The primary management method for this disease relies on utilizing resistant cultivars. Different types of host resistance were described, i.e. root resistance, partial or complete resistance (Dorrance and Schmitthenner, 2000; Dorrance *et al.*, 2003; Mideros *et al.,* 2007). Commercial soybean cultivars that have the later type of resistance carry

one or a combination of single dominant resistance (*R*) genes - so called Resistance to *Phytophthora sojae* (*Rps*) - that confer specific resistance to one or more races of the pathogen. This monogenic resistance encoded by *Rps* genes has been somewhat durable, effectively lasting for 8 to 15 years so it has been reliably used to control the disease for a few decades (Schmitthenner, 1985; Sandhu *et al.*, 2005). To date, the identified members of the *Rps* gene family have reached 15 alleles at 8 loci (Tyler, 2008). Of these loci, *Rps1k* was reported to provide resistance to a wide range of *P. sojae* races. Therefore, plant breeders designed programs to transfer *Rps1k* to commercial cultivars in order to produce potentially resistant cultivars to the majority of the known races of *P. sojae* (Abney *et al.*, 1997). The best example is introducing the *Rps1k* from its source variety 'Kingwa' to the cultivar 'Williams' to develop the new cultivar 'Williams 82' by the USDA-ARS and the Illinois Agricultural Experiment Station in the early 1980s (Bernard and Cremeens, 1988).

During the last decade, remarkable progress was attained related to the isolation and cloning of *R* genes in soybean despite the difficulties in cloning soybean genes and soybean transformation. The high importance of the *Rps1k* locus attracted most attention and efforts were directed to investigate the genomic content in this *Rps1k* region and how it has evolved in order to understand the mechanism underlying the stable resistance conferred by this locus. A series of studies including mapping, isolation and DNA sequencing identified a large cluster of highly polymorphic paralogous *Rps1k* sequences located adjacent to the *Rps1k* region that might have facilitated the expansion of *Rps1k* gene numbers and the generation of new recognition specificities (Bhattacharyya *et al.*, 1997; Bhattacharyya *et al.*, 2005; Gao *et al.*, 2005; Gao and Bhattacharyya, 2008). The

Rps1k genomic region comprised repetitive sequences involving 16 simple repeats and 63 tandem repeats. Although the majority of the genes in this region are truncated and probably nonfunctional, a sequence of 184 kb was found to include 4 highly similar genes - designated *Rps1k*-1 to *Rps1k*-4 - with a disease resistance gene-like sequence, which were found to be members of the coiled coil-nucleotide binding site-leucine rich repeat (CC-NBS–LRR)-type of disease resistance genes. Based on nucleotide sequence identity, these four genes were grouped into two different classes; class I includes genes 1, 3 and 4 while class II comprises only gene 2 (Gao *et al.*, 2005). Mutational and overexpression studies through stable transformation of soybean showed that 2 of these 4 genes, *Rps1k*-1 and *Rps1k*-2 work in modulating the race-specific interaction. The fact that this *Rps1k* locus is located in a gene-poor region - therefore the frequency of recombination is low - and the presence of two functional genes explained the stable and broad-spectrum resistance conferred by this locus. Functional sequencing identified specific nucleotide sequences that encode variant conserved domains in the predicted proteins (Bhattacharyya *et al.*, 2005; Gao and Bhattacharyya, 2008).

The main objective of this study was to overexpress the sequences encoding two conserved protein domains. The first domain is P-Loop NTPase, belongs to superfamily that contains a phosphate-binding loop (P-Loop) motif that have previously been identified to be associated with pathogen resistance. The other domain is PLN03210, which is defined in NCBI to be associated with resistance function to *Pseudomonas syringae* pv. *glycinea* race 6. The study also aimed to silence the *Rps1k*-1 and *Rps1k*-2 genes using the bean pod mottle virus-based vector (Zhang and Ghabrial 2006). The responses of silenced/overexpressing plants to *P. sojae* infection were also investigated.

3.2 RESULTS

3.2.1 Overexpression of the conserved domains PLN03210 and P-Loop NTPase

For the purpose of overexpressing the conserved domains 'PLN03210' and 'P-Loop NTPase', the full-length nucleotide sequence encoding each of these domains was PCR-amplified and independently cloned in the BPMV vector. For PLN03210, sequence specific primers (Table 2.1) were designed to amplify a 573-nucleotide stretch starting at nt position 19705 and ending at 20277 in *Rps1k*-1 (GenBank Accession No. AY963292) and starting at nt position 44135 and ending at 44707 in *Rps1k*-2 (GenBank Accession No. AY963293; Fig. 3.1). For P-Loop NTPase, specific primers (Table 2.1) were designed to amplify an 846 bp fragment at positions from 18465 to 19310 in *Rps1k*-1 and from 42898 to 43746 in *Rps1k*-2 (Fig. 3.1). The resultant recombinant vectors were designated 'pGG7R-PLN' and 'pGG7R-Ploop', respectively.

Both soybean plants (cv. Williams) inoculated with 'pGG7R-PLN' - hereinafter referred to as 'PLN (OE)' and plants inoculated with 'pGG7R-Ploop' - hereinafter referred to as 'Ploop (OE)' developed distinct phenotypic changes compared to the empty vector control plants. Blistering on leaves, especially of the Ploop (OE) plants, was observed at different stages of the plant life (Fig. 3.2).

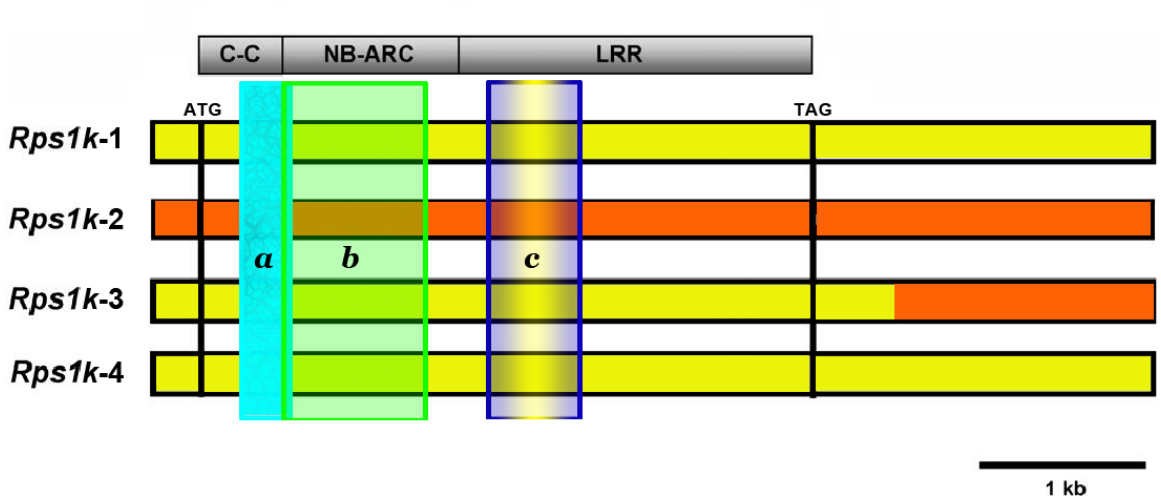


Fig. 3.1 Schematic diagram for the positions of overexpression/silencing fragments in the *Rps1k* gene family. The open reading frames (ORF) of the genes colored in grey share 100% identity at the nucleotide level and therefore grouped together in class I of the family while the gene ORF colored in black (class II) is only 93% identical with the genes of class I at the nucleotide level. *a* indicates the 273 bp amplified for silencing both gene classes. *b* and *c* indicate the 846 bp amplified for the P-Loop NTPase overexpression and the 573 bp amplified for the PLN03210 overexpression, respectively. Modified from Gao *et al.* (2005).

In the absence of antisera specific for Rps1k proteins, I relied on reverse transcriptase polymerase chain reaction (RT-PCR) to assess overexpression of the sequences coding for the *Rps1k* conserved domains in plants infected with recombinant vectors. For this purpose, I used a semi-quantitative RT-PCR assay and gene-specific primers (Table 2.1) to examine transcript levels in the various treatments.

Given that quantitative PCR was not available during this study, the results of the semi-quantitative PCR suggested marked increase in the level of the recombinant

transcript coding for the PLN sequence in PLN (OE) plants, estimated at 12-fold higher than in empty vector control plants (Fig. 3.3A and 3.3B). On the other hand, an estimate of 6-fold increase in the transcript level was achieved in case of Ploop (OE) plants (Fig. 3.3C and 3.3D). Refer to section 2.12 of this dissertation for the fold increase calculation method.

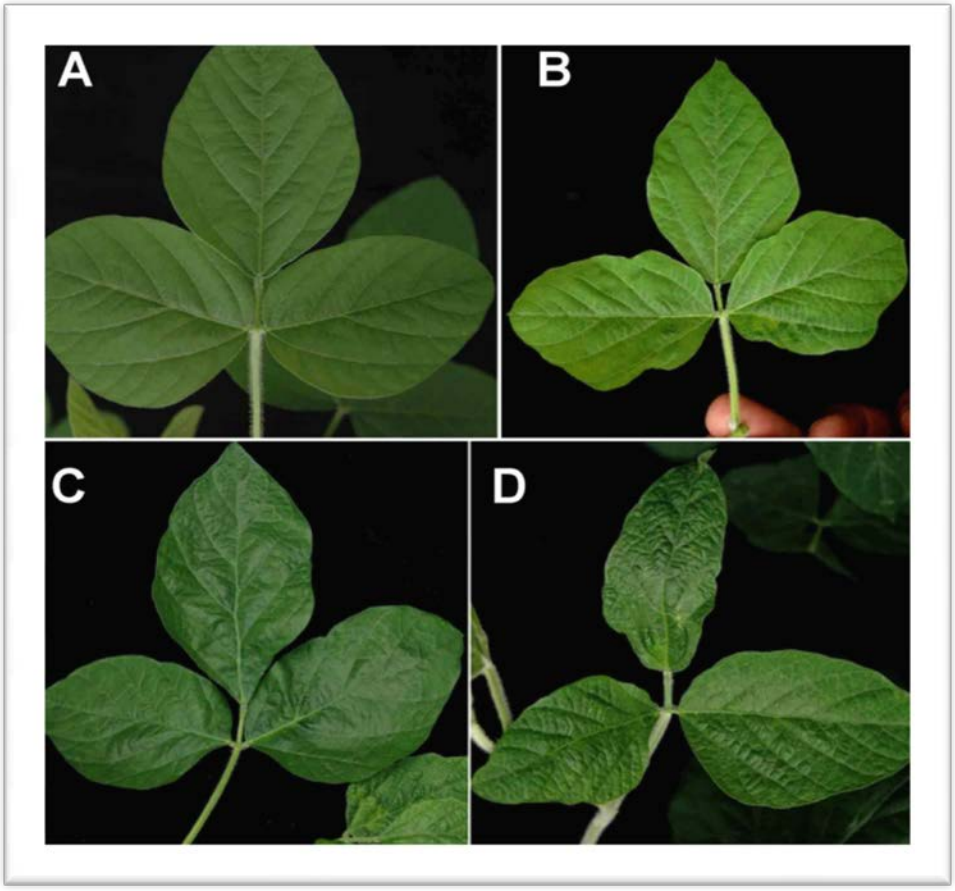
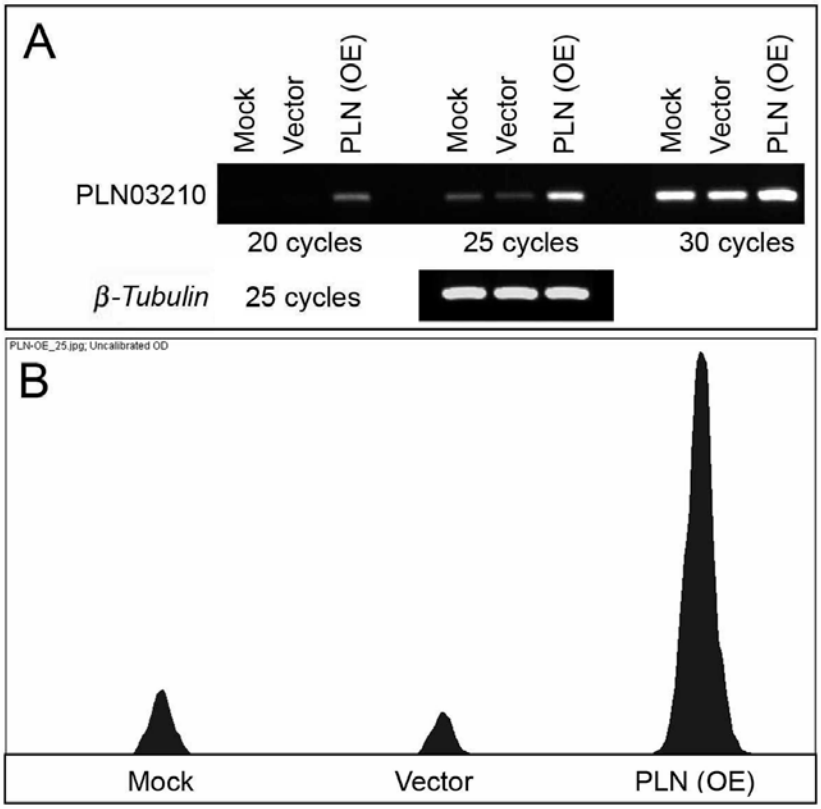


Fig. 3.2 Phenotypic changes of plants overexpressing PLN03210 domain and plants overexpressing P-Loop NTPase domain. Fourth tripholiolate leaves of soybean plants (cv. Williams) show characteristic phenotypic differences between **A,** mock, **B,** empty vector-infected, **C,** plants infected with overexpression recombinant vector- PLN03210 and **D,** plants infected with overexpression recombinant vector-P-loop NTPase.


A
Mock
Vector
PLN (OE)
Mock
Vector
PLN (OE)
Mock
Vector
PLN (OE)
PLN03210
20 cycles
25 cycles
30 cycles
β-Tubulin
25 cycles
PLN-OE_25.jpg; Uncalibrated OD
B
Mock
Vector
PLN (OE)

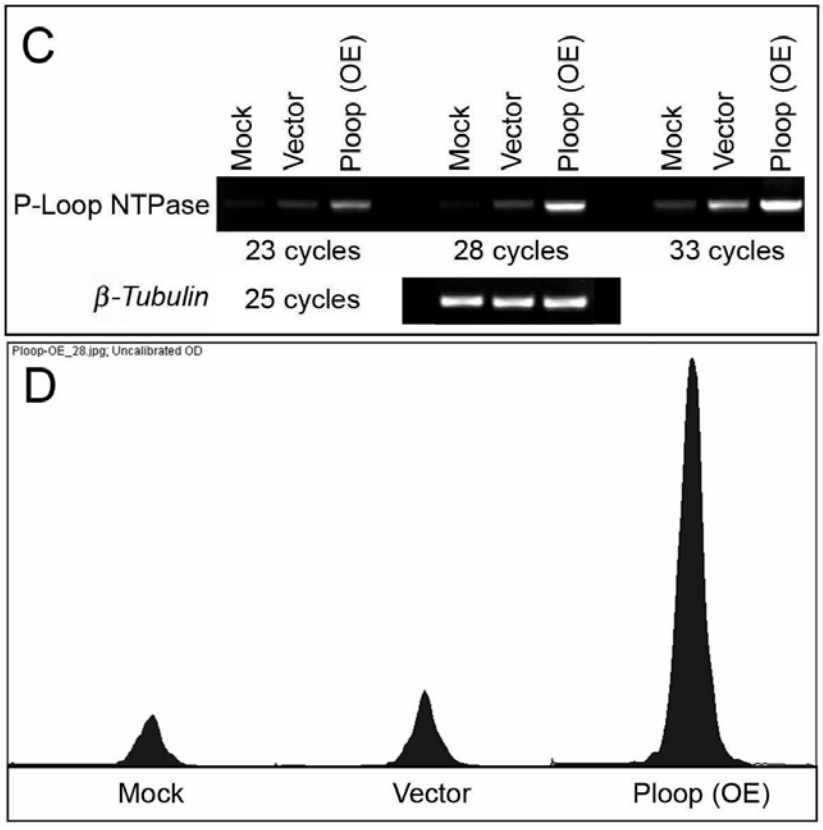

C
Mock
Vector
Ploop (OE)
Mock
Vector
Ploop (OE)
Mock
Vector
Ploop (OE)
P-Loop NTPase
23 cycles
28 cycles
33 cycles
β-Tubulin
25 cycles
Ploop-OE_28.jpg; Uncalibrated OD
D
Mock
Vector
Ploop (OE)

Fig. 3.3 Transcript levels of PLN03210 or P-Loop NTPase domains in the overexpressing soybean plants. Semi-quantitative RT-PCR analysis show transcript levels in soybean plants (cv. Williams) overexpressing coding sequences of two conserved Rps1k domains compared to mock and vector only-infected plants. RT-PCR is based on cDNA synthesized from RNA extracted from soybean plants. *β -tubulin* was used as a loading control. Number of PCR cycles is indicated under the lanes. Sequence-specific primer pairs were used to amplify: **A,** full length sequence PLN03210 and **C**, full length sequence P-Loop NTPase. **B and D,** Profile plots of the RT-PCR gel image displays one-dimensional graph in which each band is converted to a peak based on the calculated gray value of the band. Differences between peak areas represent the differences between band intensities. Values have been calculated and graph has been generated by the software ImageJ. **B**, Peaks for the transcript levels of PLN03210 following 25 cycles and **C,** Peaks for the transcript levels of and P-Loop NTPase following 28 cycles. PLN (OE) = Plants overexpressing PLN03210 and P-Loop (OE) = Plants overexpress P-Loop NTPase.

3.2.2 PLN (OE) and Ploop (OE) plants showed various levels of resistance to *P. sojae*

To evaluate the possible involvement of the conserved domains 'PLN03210' or 'P-Loop NTPase' in conferring soybean resistance to *Phytophthora sojae*, the overexpressing plants were tested for their responses to the pathogen using the stem inoculation assay in a growth chamber. Disease assessment was intended to evaluate two traits; first, upward or downward lesion expansion (upward expansion is more likely to

occur) at the inoculation site by measuring the lesion length for each individual plant in millimeters. This trait represents the ability of the plant to resist superficial colonization by the pathogen. Second, the percentage of plants that remain standing at the end of the experiment; this trait relies on plant resistance to interior colonization of the vascular tissues of the stem by the pathogen causing tissue softness, morbidity and stem disintegration.

The experiment was carried out using the susceptible cultivars Williams or Sloan tested against *P. sojae* races 1 or 3, which are virulent against both cultivars.

'Ploop' overexpression improved only the attribute of stem rigidity, maintaining 90% of inoculated plants standing compared to 10% of empty vector control, while the lesion length attribute was not significantly altered. On the other hand, by measuring the lesion expansion overtime, results showed that PLN (OE)-plants recorded dramatic slow-down after the 6^{th} day post inoculation (dpi) then completely stopped on the 7th day, whereas Ploop (OE)-plants and empty vector control plants acted similarly with 24 hrs average delays, while in mock plants lesion development slowed down on 7 dpi but kept a low increasing rate till the end of the experiment or plants are completely dead (Fig. 3.4A and 3.4B). Overall, PLN (OE)-plants showed considerable advantage in minimizing both the lesion length and the morbidity of the stem, which resulted in an effective suppression of the disease (Fig. 3.5 and 3.6).

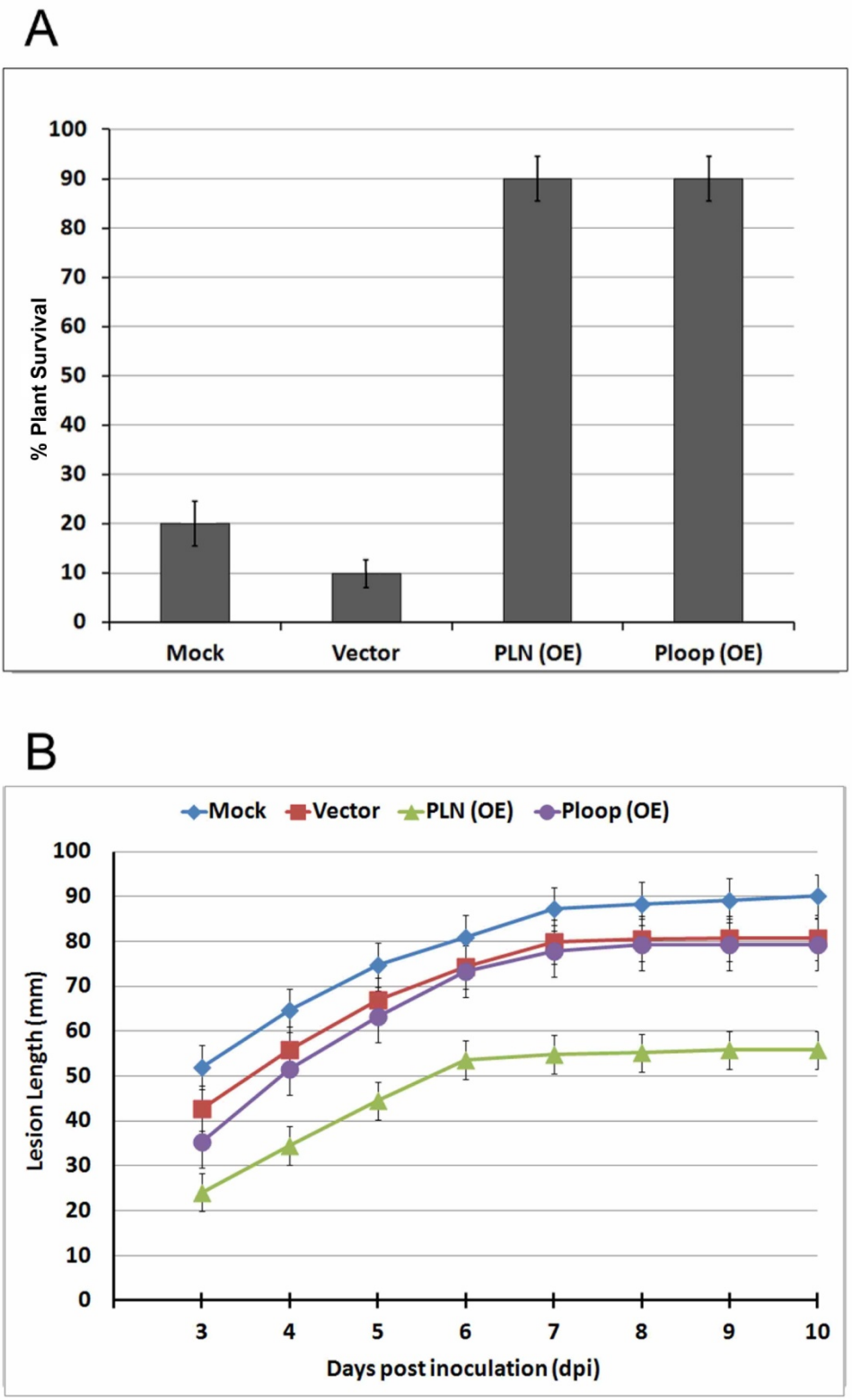


Fig. 3.4 Response of plants overexpressing PLN03210 and plants overexpressing P-Loop NTPase to *Phytophthora sojae* R3: survival percentage and disease progress

curve. A, Percent survival of plants (plants that remain standing) compared to mock and empty vector-infected plants 10 days post inoculation (dpi) describes the ability of overexpressing plants to prevent the pathogen from colonizing the vascular tissue (n = 10 plants, error bars represent standard deviation). **B,** Stem lesion length in millimeter measured everyday starting 3 dpi to 10 dpi describe the ability of the infected plants to suppress the pathogen spread along the cortex of the stem. The curves also show the overtime progression of the lesions (each value is average of lesion length on 10 plants, error bars represent standard deviation).

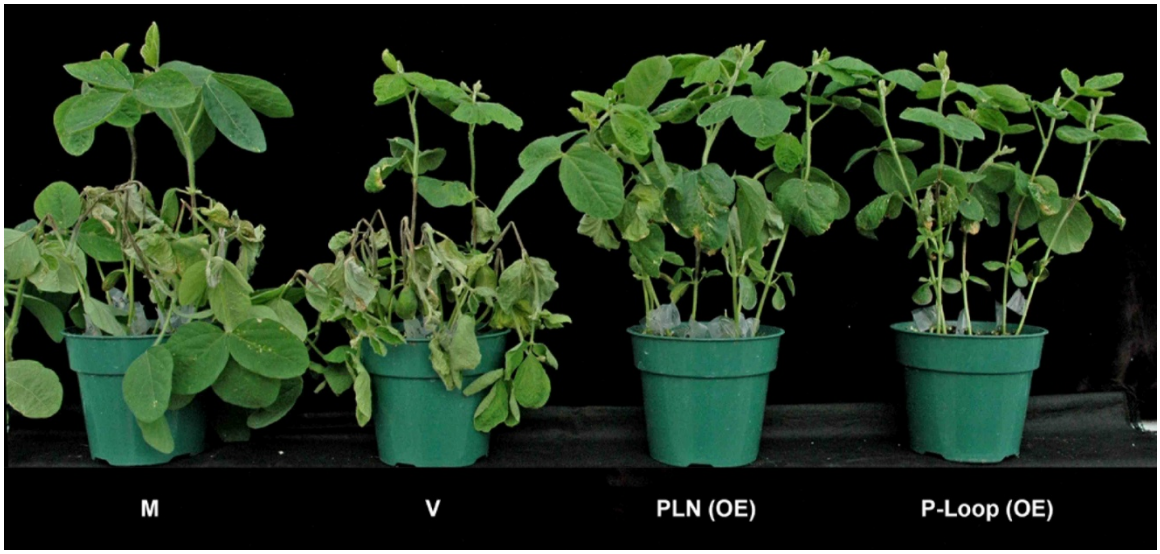


Fig. 3.5 Plants overexpressing PLN03210 and plants overexpressing P-Loop NTPase collectively surviving *P. sojae* infection 5 dpi. Susceptible soybean plants (cv. Williams) used to compare the response of non-infected mock plants, vector only-infected plants, PLN (OE) plants and Ploop (OE) plants against artificial stem inoculation with *P. sojae* R3, 5 dpi. An expected rapid spread of the stem rot clearly seen on both controls, while both overexpression treatments maintained the plants standing. The

experiment was carried out 3 times using the same experimental materials with consistent results.

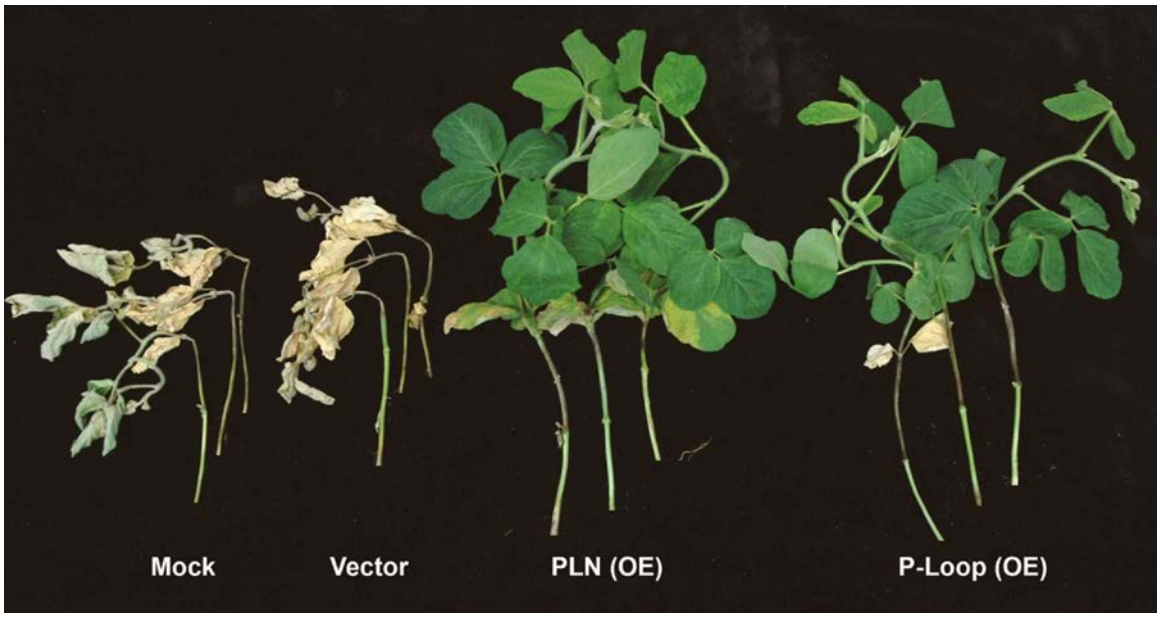


Fig. 3.6 Conferred resistance phenotypes of plants overexpressing PLN03210 and plants overexpressing P-Loop NTPase against *P. sojae.* The susceptible soybean plants (cv. Williams) became resistant to *P. sojae* R3 as a result of overexpressing PLN03210 [PLN (OE)] or P-Loop NTPase [Ploop (OE)] 13 dpi. Non-infected mock plants and empty vector-infected plants show collapse and death resulting from extensive stem rot. Plants in both overexpression treatments remained viable, but the observed variation in the nature of defense response between them is the lesion spread along the stem. While the reduction in lesion length on Ploop (OE) is not remarkable, PLN (OE) shows great limitation to the lesion to only few centimeters around the inoculation site. The experiment was carried out 3 times using the same experimental materials with consistent results.

3.2.3 Silencing of the *Rps1k* gene

The nucleic acid and deduced amino acid sequence identities between ORFs of Class I (*Rps1k*-1, 3 & 4) and class II (*Rps1k*-2) genes are 93% and 89.9%, respectively (Gao *et al.*, 2005). In order to silence the two gene classes simultaneously, specific primers were designed for amplifying a short silencing fragment of 273 bp comprising a 123 bp that is identical between *Rps1k*-1 and *Rps1k*-2. The silencing recombinant vector - pGG7R-*Rps1k*-273 - targeted nucleotides at position 18235 to 18507 of the *Rps1k*-1 ORF as well as nucleotides from position 42668 to 42940 of the *Rps1k*-2 ORF (Fig. 3.1).

The pGG7R-*Rps1k*-273-inoculated plants - referred to hereinafter as '*k* (SI)'- exhibited enhanced mottling and severe blistering on leaves at 2 weeks after rub-inoculation. In addition, malformation and blade reduction of the trifoliolate leaves has also been observed on the silenced plants. All of these phenotypic changes were observed on cultivars Essex and Williams 82 (Fig. 3.7). It is worth mentioning that these symptoms remained visible on silenced plants at the time the experiment was terminated 35 days after rub-inoculation.

Transcript levels of *Rps1k*-1 and *Rps1k*-2 genes were compared between the '*k* (SI)'-silenced, empty vector control and mock plants by semi-quantitative RT-PCR in which specific primers were designed based on sequences flanking the silencing fragments. In silenced plants, a significant reduction, 70.6% and 80.4%, in transcript levels of *Rps1k*-1 and *Rps1k*-2, respectively, were achieved in '*k* (SI)'-silenced plants when compared to that of empty vector control (Fig. 3.8). The higher reduction in transcript levels in '*k* (SI)'-silenced plants indicated more effective down-regulation of *Rps1k*-2 than of *Rps1k*-1.

It is noteworthy that the endogenous transcript levels of *Rps1k*-1 in mock, when amplified using specific primers at 30 cycles, was much lower than that of *Rps1k*-2 even at 20 cycles. These results differ from those of Gao *et al.* (2005) whose RT-PCR results showed low transcript levels of both genes, which could be detected only when these authors used a highly sensitive two-step RT-PCR experiment.

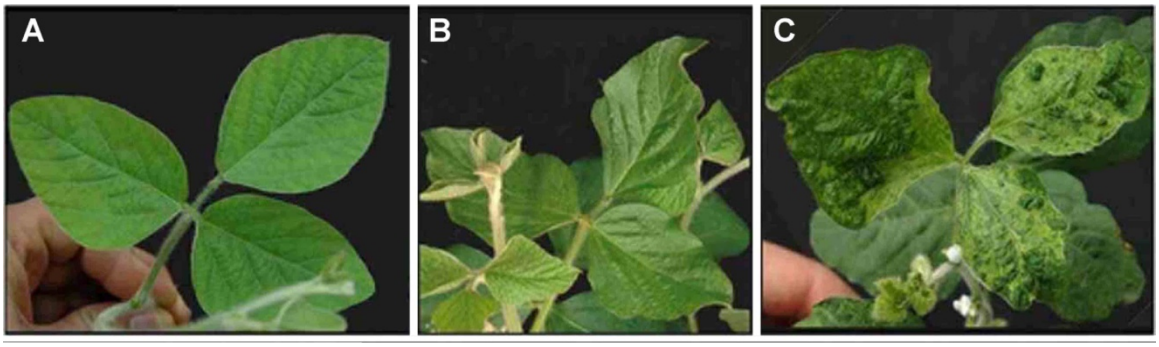


Fig. 3.7 Phenotypic changes of plants silenced for *Rps1k* gene. Photographs show phenotype of 6th trifoliolate (cv. Williams 82) 4 weeks post rub inoculation of different treatments: **A,** mock, **B,** empty vector-infected plants and **C,** *Rps1k*-silenced plants at the same age and leaf position. Silencing of *Rps1k* resulted in enhanced mottling and severe blistering and sometimes malformation and blade reduction on leaves starting 2 weeks after rub-inoculation and symptoms remained visible till the time the experiment was terminated 35 days after rub-inoculation.

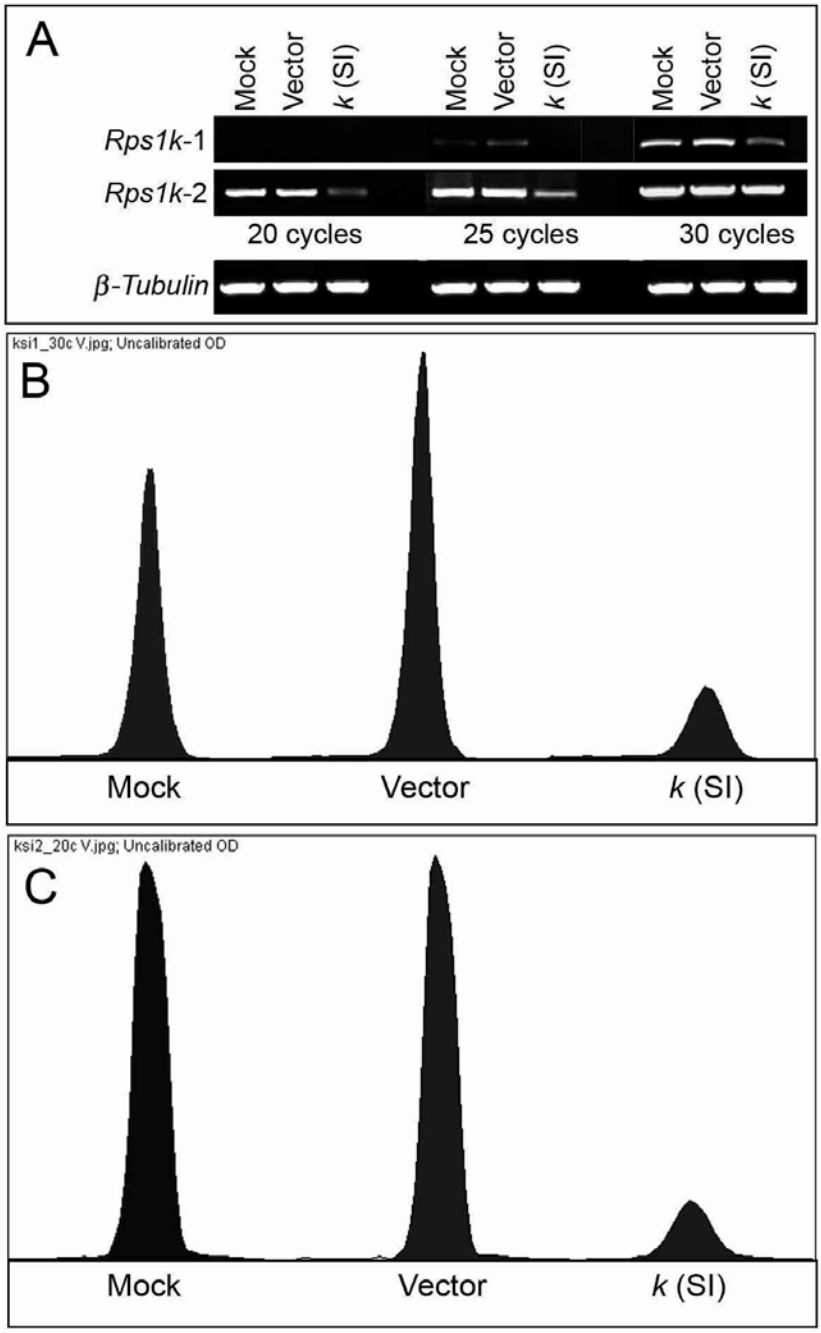


Fig. 3.8 Transcript levels of *Rps1k*-1 and *Rps1k*-2 in a soybean plant silenced for both genes. Semi-quantitative RT-PCR analysis is based on cDNA synthesized from RNA extracted from mock, empty vector-infected and *Rps1k*-silenced soybean plants. β-tubulin was used as a loading control. Number of PCR cycles is indicated under the lanes. **A,** Sequence-specific primer pairs were used to amplify 2 different sequences flanking

the silencing fragment for *Rps1k*-1 and *Rps1k*-2 in the upper and middle panels, respectively. **B and C,** Profile plots of the semi-quantitative RT-PCR gel image displays one-dimensional graph in which each band is converted to a peak based on the calculated gray value of the band. Differences between peak areas represent the differences between band intensities. Values have been calculated and graph has been generated by the software ImageJ. **B**, Peaks for the transcript levels of and *Rps1k*-1 (following 30 cycles of RT-PCR) **C,** Peaks for the transcript levels of and *Rps1k*-2 (following 25 cycles of RT-PCR). *k* (SI) = Plants silenced for *Rps1k*-1 and *Rps1k*-2 loci. Consistent results were obtained in repeated RT-PCR analyses.

3.2.4 Silencing of *Rps1k* inhibits the defense response to avirulent *P. sojae*

To determine if silencing of both genes using the BPMV vector could counteract the *Rps1k* single gene resistance to *P. sojae* races 1 and 3, the resistant cultivar Williams 82 was selected for this purpose using a laboratory detached leaf assay. When assessing the disease, changes from the resistant response were observed and recorded. The race-specific complete resistance endowed by most of the *Rps* gene family is often expressed as hypersensitive response (HR; Dorrance *et al.*, 2008). Detached leaves of both mock and vector control plants showed typical hypersensitive response (a dark brown localized necrosis limited to the site of inoculation) to *P. sojae*. In contrast, leaves of *Rps1k*-silenced plants showed light brown-colored water-soaked tissues that spread rapidly around the inoculation site. The macerated area enlarged faster in case of *P. sojae* race 1 than race 3 (Fig. 3.9A and 3.9B).

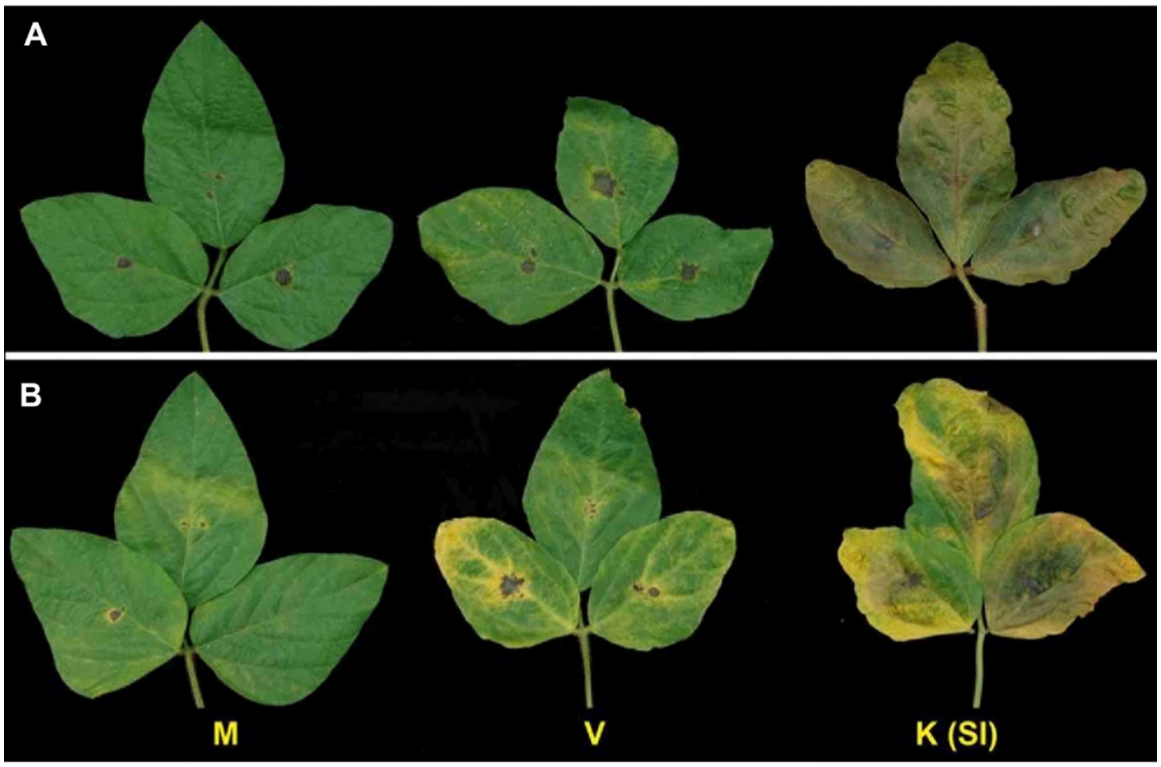


Fig. 3.9 Loss of the race-specific resistance to *P. sojae* in the resistant cultivar Williams 82 due to silencing of *Rps1k*-1 and *Rps1k*-2. Results of a laboratory detached leaf bioassay using the sixth soybean trifoliolate leaves from *Rps1k*-silenced plants (cv. Williams 82) exhibited susceptible response to *P. sojae* marked by developing maceration and discoloration (light brown) of the tissues around the inoculum, while both controls, mock and empty vector-infected plants, showed limited necrotic tissue as an evidence of complete resistance to **A,** *P. sojae* R1 (5 dpi) and **B,** *P. sojae* R3 (10 dpi)**.** The bioassay was carried out 3 times with consistent results.

3.3 DISCUSSION

The *Rps1k* gene has been widely utilized in commercial soybean cultivars for successful and effective control of most physiological races of *P. sojae* over the last few decades (Sandhu *et al.*, 2005). However, as a natural response of the pathogen, new races have developed to overcome this complete (i.e. gene-for-gene) resistance, hence it is very important to search for new sources of resistance. In addition to attempts of identifying new *Rps* genes, identification of partial disease resistance is highly encouraged (Gijzen and Qutob, 2009). Partial resistance is independent of the pathogen's virulence genes specificity; therefore it is more durable against the rapid evolution of these genes and most likely, it works through reducing the lesion expansion and disease severity (Mideros *et al.*, 2007; Tyler *et al.*, 2008).

The common genetic redundancy of soybean makes it difficult to target genes for studies of their molecular functions in order to improve soybean for certain traits required by producers or consumers (Schmutz *et al.*, 2010). One of the best examples is the *Rps* gene family, which comprises 8 loci with 15 alleles identified to date (Sandhu *et al.*, 2005). Furthermore, mapping the *Rps1k* locus showed the presence of 4 highly similar genes, and that the race-specific resistance is conditioned by dual expression of *Rps1k*-1 and *Rps1k*-2 (Bhattacharyya *et al.*, 2005; Gao and Bhattacharyya, 2008). A previous study on different *Rps* loci (1, 4 and 6) suggests that the copy number variants of *Rps* may play a role in their degree of pathogen race-specificity (Gijzen and Qutob, 2009).

Virus-induced gene silencing (VIGS) is an exceptional reverse-genetic strategy that allows gene functional analysis in species not amenable to stable genetic transformation, e.g. soybean. VIGS is rapid, does not require development of stable

transformants, allows characterization of phenotypes that might be lethal in stable lines, and offers the potential to silence either individual or multiple members of a gene family. Thus targeting a highly conserved nucleotide sequence in a gene family for silencing (Scofield *et al.*, 2005) would potentially allow for silencing all members of the family. The BPMV-based vector (Zhang and Ghabrial, 2006) has been used successfully in the last few years to silence soybean genes and became one of the most powerful tools in functional genomic studies of soybean (Fu *et al.*, 2009; Selote and Kachroo, 2010; Diaz-Camino *et al.*, 2011; Singh *et al.*, 2011; Kachroo and Ghabrial, 2012).

In light of the above-mentioned information, my careful selection of the silencing fragment was based upon the high sequence similarity between *Rps1k*-1 and *Rps1k*-2 genes to gain the best possible silencing efficiency of these two genes.

Gao *et al.* (2005) were unable to observe detectable transcript levels of any of the *Rps1k* genes except when they used a two-step highly sensitive RT-PCR assay. These authors reasoned that possible deleterious effect of their increased protein levels in the plant cell. My RT-PCR assays (Fig 3.4), on the other hand, showed considerably high levels of *Rps1k*-2 transcript in control plants after 20 PCR cycles; this was almost equivalent to that observed for *Rps1k*-1 after 30 PCR cycles.

In silenced plants, RT-PCR showed an estimated reduction of 70.6% and 80.4% in transcript levels compared to empty vector level for *Rps1k*-1 and *Rps1k*-2, respectively. Effective silencing for various soybean genes has also been reported using the BPMV vector as confirmed by RT-PCR analysis (Fu *et al.*, 2009; Selote and Kachroo, 2010; Singh *et al.*, 2011).

It has been stated that the predicted 1229 amino acid-protein (Q2YE88) encoded by *Rps1k* is a defense response protein that functions through programmed cell death (i.e. apoptosis) (www.uniprot.org). Silencing of *Rps1k*-1 and *Rps1k*-2 in Williams 82 broke its resistance and converted the plants to a susceptible phenotype against *P. sojae* avirulent races (R1 and R3). I interpret the resultant susceptibility to be as a consequence of the absence of rapid and limited necrosis due to hypersensitive reaction (HR) at the site of inoculation (Fig. 3.9).

In the current study, I was interested in investigating whether overexpression of a partial sequence of a single dominant *R* gene is able to confer partial resistance to plants. In the *Rps1k* gene, my search in NCBI protein database identified two conserved domains (Geer *et al.*, 2010); the first is P-Loop NTPase, which belongs to domain superfamily that contains a phosphate-binding loop (P-Loop) motif and the other domain is PLN03210, which is defined in that database to be associated with resistance function to *Pseudomonas syringae* pv. *glycinea* race 6. P-Loop NTPase lies in the NBS domain while PLN03210 lies in the LRR domain. I used the BPMV vector to overexpress each domain in susceptible soybean plants. In a recent study, the BPMV vector was successfully used for overexpressing the full-length gene encoding soybean calmodulin (*SCaM4*; S. Rao *et al. unpublished data*).

The overexpression of each domain in soybean plants of the universally susceptible cultivar Williams conferred resistance against the virulent race *P. sojae* (R3). In both cases, the overexpressing plants exhibited partial resistance manifest as limitation of the stem rot and reduction in stem breaking and plant collapse. In the assessment of the

resistance, I relied on the description of Vega-Sánchez *et al.* (2005) for partial resistance to *P. sojae* where the authors reported it as "reduced colonization following inoculation".

Nevertheless, overexpressing plants in both cases did not develop remarkable phenotypic changes different from empty vector-inoculated plants. On the other hand, the RT-PCR analysis for P-Loop NTPase in overexpressing plants showed an estimated transcript level increase of 6-fold over empty vector control. This is compared to an estimated 12-fold increase in PLN overexpressing plants. This may be interpreted by a possible suppression of multiplication of P-loop recombinant vector due the increase in transcript level. The role of P-loop motif in viral resistance has been previously demonstrated. The tobacco mosaic virus (TMV) resistance gene '*N*' is a member of the Toll interleukin 1 receptor-nucleotide binding site-leucine rich repeat (TIR-NBS-LRR) class of plant resistance (*R*) genes and its NBS domain contains a P-Loop motif (Traut, 1994). In a mutational study in NBS region of '*N*', it was found that any amino acid substitution in P-Loop broke resistance to TMV (Dinesh-Kumar *et al.*, 2000).

The P-loop motif - originally named as 'motif A' or 'Walker A' - is the most common conserved motif in the nucleotide-binding (NB) proteins and can be found in both ATP- and GTP-binding proteins (Walker *et al.*, 1982; Saraste *et al.*, 1990). In 1990s, NB region was a common factor among the discovered plant *R* genes' sequences. The presence of P-loop domain as one of the most five conserved domains in NB region was an indicator for their general ATP- and GTP-binding structure. In addition, another structural study revealed that a hydroxyl group of Ser or Thr (S/T) in the P-loop is involved in binding of Mg^{2+} associated with bound nucleotides, but till that time, their biochemical functions had not been demonstrated (Hammond-Kosack and Jones, 1997;

Meyers *et al.*, 1999; Dinesh-Kumar *et al.*, 2000). In a model explaining NBS-LRR proteins' mechanism during defense responses, the NBS has been proposed to be responsible for ATP hydrolysis and release of a signal to be received by the N-terminal of the LRR domain that modulates activation of the C-terminal of the same domain to recognize and interact with the pathogen effectors (Belkhadir *et al.*, 2004). A loss-of-function mutational study revealed that a mutation of the P-loop lysine (K) resulted in ATP-binding reduction (Takken *et al.*, 2006; Lukasik and Takken, 2009). Other investigators predicted that P-loop side by side with other NB motifs are playing a role in nucleotide binding but not ATP hydrolysis (Collier and Moffett, 2009). Based on the sequence and structural features of the NBS region, P-loop motif is the first in order of the domains comprising this region, besides it is characterized with ATPase activity, it is concluded that the P-loop is responsible for signaling. On the other hand, overexpressing P-loop NTPase did not cause a HR reaction and conferred only partial resistance to the plants, which indicates that this protein by itself possesses a function that differs from that of the whole Rps1k protein. Moreover, it is predicted that the NB region has some more motifs with unidentified functions (Meyers *et al.*, 1999; Rairdan and Moffett, 2007). The results from this study support the proposal of Collier and Moffett (2009) that such motifs will be candidate sites for interpreting signal initiation controlled by NB proteins.

The results demonstrated that overexpression of PLN03210 enhanced defense activation in soybean plants when artificially inoculated with *P. sojae*. My search through conserved domain database (NCBI) revealed that the PLN03210 domain function is linked to plant resistance to *P. syringae* pv. *glycinea* race 6 (Geer *et al.*, 2010). Previous

studies have shown that similar proteins have been capable of conferring resistance to oomycete pathogens. In *Arabidopsis*, the RPS6 (resistance to *Pseudomonas syringae* pv. *glycinea* race 6) protein - encoded by the *R* gene *RPS6* - was found to share high amino acid sequence similarity with the *Arabidopsis* protein RAC1. The later determines resistance to the oomycete pathogen *Albugo candida* (Kim *et al.*, 2009b). On the nucleic acid level, the PLN03210 shares 94% sequence identity with '*Glycine max* putative disease resistance RPP13-like'. The *Arabidopsis RPP13* is a member of the 'Recognition for *Peronospora parasitica*' *R* gene family that confers resistance to the biotrophic oomycete, *Peronospora parasitica* (Bittner-Eddy *et al.*, 1999; Bittner-Eddy *et al.*, 2000). The PLN03210 has not been subjected to biochemical and functional investigation, therefore pertinent literature is rare.

In summary, overexpression of any of the two conserved domains, P-Loop NTPase or PLN03210, conferred partial resistance to the specific pathogen *P. sojae* with consideration of a difference in the resistance attributes between both cases.

CHAPTER 4

4 Investigation of the roles of soybean chlorophyllase genes in plant resistance to fungal diseases

4.1 LITERATURE REVIEW

Chlorophyll (Chl) is a major widespread pigment on earth. It absorbs sunlight to initiate the energy required for photosynthesis; a biosynthetic pathway that utilizes water and carbon to produce carbohydrates that sustain plant life. Therefore, together with the blood pigment, they were described as the most important pigments on earth (Rothemund, 1956). At a variety of occasions in plant life, whether associated with natural development or environmental responses, whether in viable or in dead cells, chlorophyll gets to degrade. A few examples are - but not limited to - fruit color change at ripening stage, leaves color change of deciduous trees in autumn, individual leaves or entire plant senescence at maturation stages. In addition, other incidents or premature death of plant tissues, which might occur due to the influence of biotic or abiotic factors, cause chlorophyll degradation. Moreover, the chlorophyll molecule turnover due to reaching steady state in the cell is one of the reasons for degradation (Hendry *et al.*, 1987; Matile *et al.*, 1999). A few estimates of the amount of degraded chlorophyll were given in different studies. Of all, Hendry *et al.* (1987), based on their calculation methods, reported the most acceptable amount of degraded chlorophyll from the planet as one billion tons annually. Chlorophyll breakdown is a complex biodegradative pathway in which the first step has to be done with the activity of chlorophyllase (Chlase; chlorophyll-chlorophyllido hydrolase, EC 3.1.1.14) to produce chlorophyllide (Chlide) after removing the phytol tail from chlorophyll *a* (Chl *a*) molecule (dephytylation),

meanwhile chlorophyll *b* (Chl *b*) has to be initially converted to chlorophyll *a* (Chl *a*) by chlorophyll *b* reductase then be subjected to the activity of Chlase (Tsuchiya *et al.*, 1997; Matile *et al.*, 1999; Scheumann *et al.*, 1999; Kräutler and Hörtensteiner, 2006). It has also been reported that Pheide *a* Oxygenase, the key enzyme in the breakdown pathway, exclusively accepts Pheide *a* but not Pheide *b* (Hörtensteiner *et al.*, 1995). The final products of this multi-step degradation process known as nonfluorescent chlorophyll catabolites (NCCs) are being directed to the vacuole which counts as their terminal storage in the cell (Kräutler and Hörtensteiner, 2006; Hörtensteiner, 2012).

Since its discovery in 1910 by Willstatter and Stoll (Holden, 1961), the Chlase enzyme has been isolated and characterized from many plant species (reviewed by (Tsuchiya *et al.*, 1997). On the molecular level, genes encoding Chlase have been cloned consecutively from higher plants, namely *Chenopdium album* (Tsuchiya *et al.*, 1999), *Arabidopsis thaliana* (Benedetti *et al.*, 1998; Tsuchiya *et al.*, 1999), *Citrus unshiu* (Trebitsh *et al.*, 1993), *Citrus sinensis* cv. Valencia (Jacob-Wilk *et al.*, 1999), *Triticum aestivum* (Arkus *et al.*, 2005), *Brassica olearacea* cv. Italica (Zhou *et al.*, 2007), *Ginkgo biloba* (Tang *et al.*, 2004), *Citrus limon*, *Ricinus communis*, , *Vitis vinifera*, *Oryza sativa*, *Nicotiana tabacum* and *Glycine max*. Identification of new CLH genes in vegetable, ornamental or field crop plants may prove valuable in controlling age-associated senescence in plants of economic importance. This is because senescence causes less productivity and undesired market value of the product due to short life span and postharvest yellowing (Cahoon, 2007; Guo and Gan, 2005).

In the model plant *Arabidopsis*, the chlorophyllase coding gene *AtCLH1* was extensively investigated regarding its potential signaling role in plant defense pathways

against necrotrophic pathogens. The knowledge gained from the investigation carried out on model systems are cornerstones for building upon in the scientific research.

As chlorophyll degradation is associated with senescence in plants, it is believed that *CLH* gene regulation is primarily controlled by endogenous plant growth regulators (phytohormones) (Kräutler and Hörtensteiner, 2006). Misra and Biswal (1980) reported that retardation of chlorophyll loss in detached wheat leaves by kinetin, indole acetic acid (IAA) and gibberellins (GA) *in vitro* was as a result of direct action of the hormones on aging chloroplasts, thus preventing yellowing of senescing leaves.

Foliar application of gibberellic acid (GA_3) improved the chlorophyll levels in salinity-stressed maize (*Zea mays* L.) plants which may be due to down-regulation of chlorophyll degrading enzymes such as chlorophyllase (Tuna *et al.*, 2008). In a similar study, the cholorphyllase activity showed reduction in strawberry fruits treated with GA_3 during ripening (Martínez *et al.*, 1996). Application of abscisic acid (ABA; senescence enhancer) on *Piper betle* L. showed significant decrease in chlorophyll content (85%) 6 days post treatment and led to a rise in Chlase activity (Gupta *et al.*, 2012).

Likewise, ethylene was found to influence the regulation of chlorophyllase activity. Trebitsh *et al.* (1993) determined a role for ethylene in chlorophyll degradation in fruit peel. Exogenous ethylene treatments showed up to 4-fold increase in Chlase activity in citrus fruits which showed immediate drop in chlorophyll content (Amir-Shapira *et al.*, 1987) and elevated the steady state level of *CLH* mRNA in citrus fruit peel (Jacob-Wilk *et al.*, 1999). Although most studies showed that ethylene is involved in Chlase degradation in fruits, it was not clear whether ethylene has a role in inducing chlorophyllase activity, as some investigations were not in agreement with the conclusion

of a positive correlation between ethylene exposure and chlorophyllase activity (Purvis and Barmore, 1981). On the other hand, using inhibitors of ethylene (i.e 1-methylecyclopropene; 1-MCP) reduced postharvest chlorophyll degradation and delayed ripening of avocado fruits (Schnabel *et al.*, 2005) and retained the green pigment in the fruit peel for longer time (Schnabel *et al.*, 2005). Porat *et al.* (1999) found that exogenous 1-MCP treatment altered the regulation of fruit senescence of 'Shamouti' (*Citrus sinensi*s L. Osbeck) oranges by deactivating the degreening process as a result of ethylene effects inhibition.

Weidhase *et al.* (1987) observed a rapid loss of chlorophyll in barley leaf segments treated with methyl jasmonate (MeJA; an endogenous plant signaling molecule). They suggested that MeJA causes degradation of chloroplast constituents, besides playing indirect role by being a cause for enhanced synthesis of cytoplasmic polypeptides involved in the senescence syndrome. Later enzymic and molecular studies by Mitchell *et al.* (1983) showed that *Arabidopsis thaliana* plants treated with wounding, MeJA or coronatine (COR; a bacterial phytotoxin produced by pathovars of *Pseudomonas syringae* and believed to act as a homologue to MeJA causing chlorosis in plant tissues) showed induction of *AtCLH1* (*ATHCOR1*) but not *AtCLH2* mRNA which indicated that transcription of some of the *CLH* genes is not hormonally activated (Benedetti *et al.*, 1998; Tsuchiya *et al.*, 1999). In another study, coronataine-insensetive 1 (*coi1*) mutants in *Arabidopsis* has shown induction in *AtCLH1* when treated with coronataine, MeJA or ethylene (Benedetti *et al.*, 1998). The well studied role of ethylene, MeJA or coronataine in plant defense signaling pathways besides their confirmed induction of *CLH* expression has shed some light on the possible role of *CLH* in plant

defense. In the most recent report, a link between chlorophyll degradation and plant response to pathogen infection was concluded (Hörtensteiner, 2012). Kariola *et al.* (2005) found that due to the tissue damage resulting from infection of *Arabidopsis thaliana* plants with the necrotrophic pathogen *Alternaria brassicicola*, the expression of *AtCLH1* was rapidly induced. On the other hand, free chlorophyll was released from the thylakoid membranes. They concluded that *AtCLH1* degraded (detoxified) the free Chl in the cell; hence the cell has been protected from any accumulation of the reactive oxygen species (ROS), such as hydrogen peroxide H_2O_2 and superoxide radical $^{\cdot}O_2^-$ - due to possible phototoxicity (Takamiya *et al.*, 2000) - and promoted a JA-dependent pathway that led to resistance response. ROS is generated in the cell in response to exposure to various environmental factors, stresses or pathogen attack (Scandalios, 1993) and its accumulation in the cell is proposed to cause natural (age-induced) senescence (Munné-Bosch and Alegre, 2002).

The high incidence of sudden death syndrome (SDS) of soybean in the United States has made this disease a serious constraint to soybean production and has placed this crop at a risk of devastating yield reductions. In 2005, this reduction resulted in a loss estimated at $118.9 million for the whole country (Wrather and Koenning, 2006). The disease is caused by 4 distinct species of the fungal pathogen *Fusarium*; i.e. *F. virguliforme* in both North and South America and *Fusarium brasiliense* sp. *nov*., *F. cuneirostrum* sp. *nov*., *F. tucumaniae* in South America only (Aoki *et al.*, 2003; Aoki *et al.*, 2005). Symptoms include root rot associated with vascular discoloration and reduction of the total root system. Foliar symptoms characterized by interveinal chlorosis often turns into necrosis on the leaves that defoliate prematurely in case of severe

infection while the petioles remain attached to the stem (Melgar *et al.*, 1994; Schmitthenner, 1999). The most effective management of SDS relies on planting cultivars possessing moderate to high levels of quantitative resistance. In addition, applying some cultural practices such as improving soil physical characteristics and drainage, delaying planting and tillage should help in reducing the disease incidence and/or severity (Tsuchiya *et al.*, 1997; Schmitthenner, 1999).

The goal of the current study was to examine the potential role of soybean chlorophyllases in amending the plant defense to *F. virguliforme* through gene silencing/overexpression of the target genes. Here, by using BPMV-based vector (Zhang and Ghabrial, 2006), a role of *GmCLH2* in plant susceptibility to SDS has been identified. In addition, a few functional differences between *GmCLH1* and *GmCLH2* have been demonstrated.

4.2 RESULTS

4.2.1 Silencing/overexpression of *GmCLH1* or *GmCLH2*

Based on database search results, the nucleotide sequence information available from GenBank showed that *Glycine max* has a family of Chlase genes; they are those encoding Chlorophyllase 1 (*GmCLH1*; AB181947), Chlorophyllase 2 (*GmCLH2*; AB181948) and Chlorophyllase 3 (*GmCLH3*; AB181949) where *GmCLH1* and *GmCLH3* share 100% identity at the nucleotide sequence level and 98% identity at the amino acid sequence level.

In order to investigate the role of the *GmCLH*s in host defense, a reverse genetics approach using the BPMV-based silencing vector was used. Two cDNA fragments (216 and 243 bp based on *GmCLH1* and *GmCLH2* sequences, respectively) were cloned independently in the BPMV vector that resulted in generating two silencing recombinant vectors, pGG7R-*GmCLH1*-216 (referred to hereinafter as *CLH1* (SI) and pGG7R-*GmCLH2*-243 (referred to hereinafter as *CLH2*-SI).

Since the BPMV vector was recently demonstrated to be a useful tool for overexpression of endogenous genes in soybean (S. Rao *et al., unpublished data*), full-length cDNA sequences of both genes were cloned independently in the BPMV vector. As a result, two new recombinant vectors for overexpression were developed; pGG7R-*GmCLH1*-981 (referred to hereinafter as *CLH1* (OE) and pGG7R-*GmCLH2*-951 (referred to hereinafter as *CLH2*-OE).

The phenotypes of the recombinant vector-infected plants were observed and recorded. The *CLH1* (SI)-inoculated plants showed the mildest phenotypic changes among plants infected with transcripts from the four recombinant vectors; they exhibited

more distinct leaf mottling than empty vector-inoculated plants. More distinct mottling was still shown by the *CLH2* (OE) plants (Fig. 4.1). Based on severity of the blistering phenotype, plants could be arranged ascendingly in the following order: vector only, *CLH1* (SI), *CLH2* (OE), *CLH2* (SI) and *CLH1* (OE) (Fig. 4.1). As this study was repeated and the plants were grown in the greenhouse during an extended period of the year, some other phenotypic differences between treatments could be observed due to light intensity change in the greenhouse. During the highest intensity and longest period /day of sunlight, chlorotic and necrotic lesions have been developed on plants as follows: *CLH2* (SI) plants showed tiny necrotic lesions covering most of the leaf area basically on the lower part and occasionally on the middle part of the plant (Fig. 4.2A). Large chlorotic areas were developed on leaves at the lower through the middle plant parts of *CLH1* (OE) which in most cases turned into well developed necrosis on the old leaves to such an extent that scorches were recorded on some leaves (Fig. 4.2B). Empty-vector, *CLH1* (SI) or *CLH2* (OE) grown in the same conditions did not show any similar disorders. In addition to the above phenotypic changes, a significant increase in both stem and root lengths due to silencing of *GmCLH1* was noted (Fig.4.3).

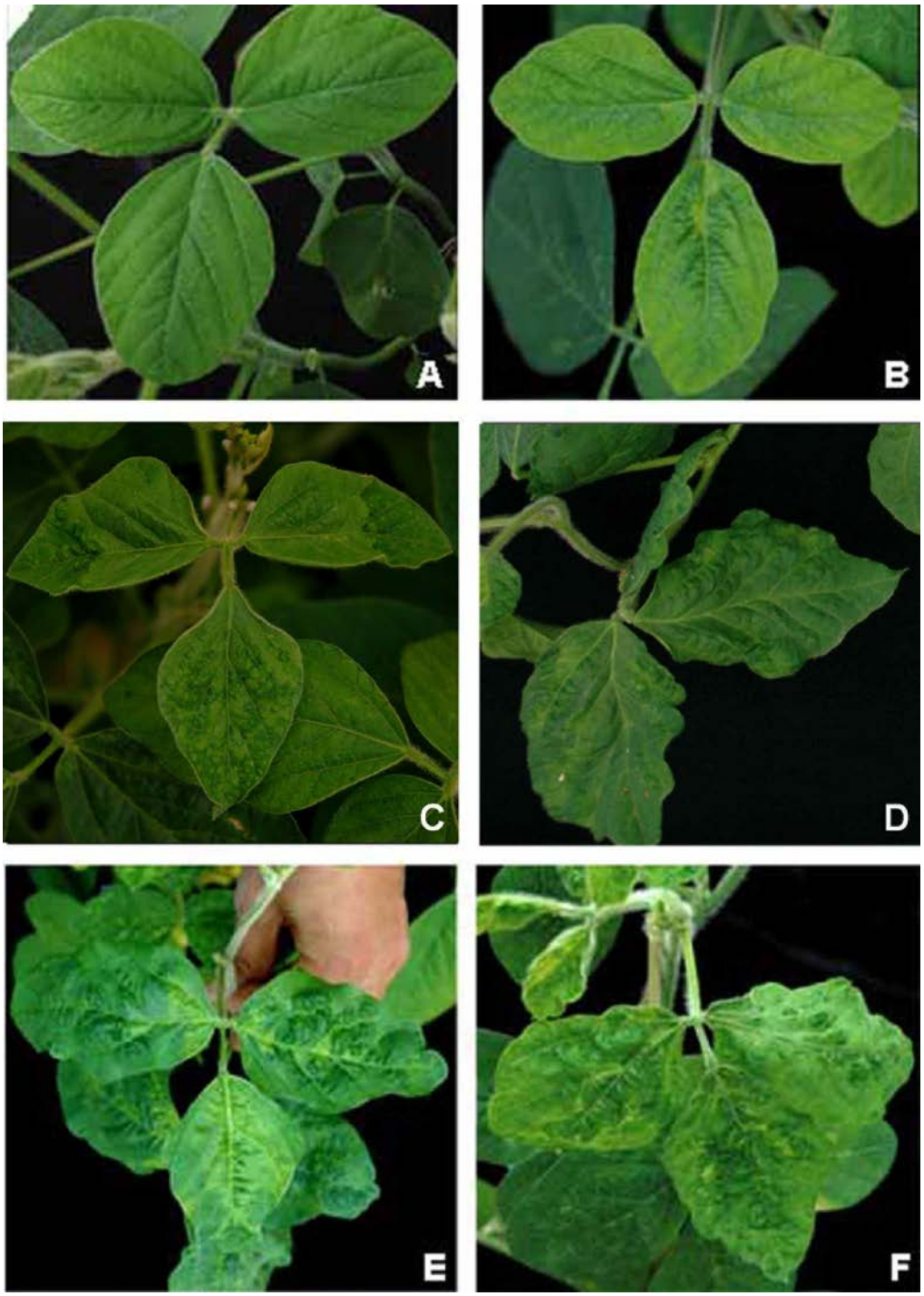


Fig.4.1 Phenotypic changes exhibited on leaves of soybean plants due to infection with different BPMV-CLH recombinant vectors. A, mock, **B,** empty vector-inoculated, **C,** *GmCLH1* (SI), **D,** *GmCLH2* (SI), **E,** *GmCLH1* (OE) and **F,** *GmCLH2* (OE). The *CLH1* (SI)-inoculated plants showed the mildest phenotypic changes among

plants infected with transcripts from the four recombinant vectors; they exhibited more distinct leaf mottling than empty vector-inoculated plants. More distinct mottling was still shown by the *CLH2* (OE) plants. Based on severity of the blistering phenotype, plants could be arranged ascendingly in the following order: V, *CLH1* (SI), *CLH2* (OE), *CLH2* (SI) and *CLH1* (OE). Leaves at the same position were compared.

A.

B.

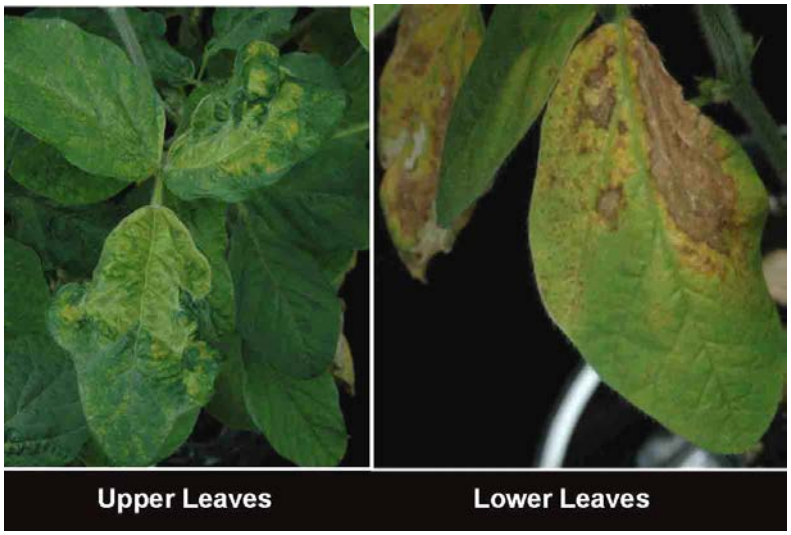


Fig.4.2 Chlorosis/necrosis phenotypes developed on plants silenced for *GmCLH2* or plants overexpressing *GmCLH1*. Leaves at various positions of soybean plants (cv. Essex) developed additional symptoms. **A,** Silencing of *GmCLH2* resulted in the development of tiny necrotic lesions covering most of the leaf area basically on the lower part and occasionally on the middle part of the plant. **B,** Overexpssion of *GmCLH1*resulted in the development of large chlorotic areas on leaves at the lower through the middle plant parts which in most cases turned into well developed necrosis on the old leaves. V, *CLH1* (SI) or *CLH2* (OE) grown in the same conditions did not show any similar disorders. Photographs were taken at 27 dpi.

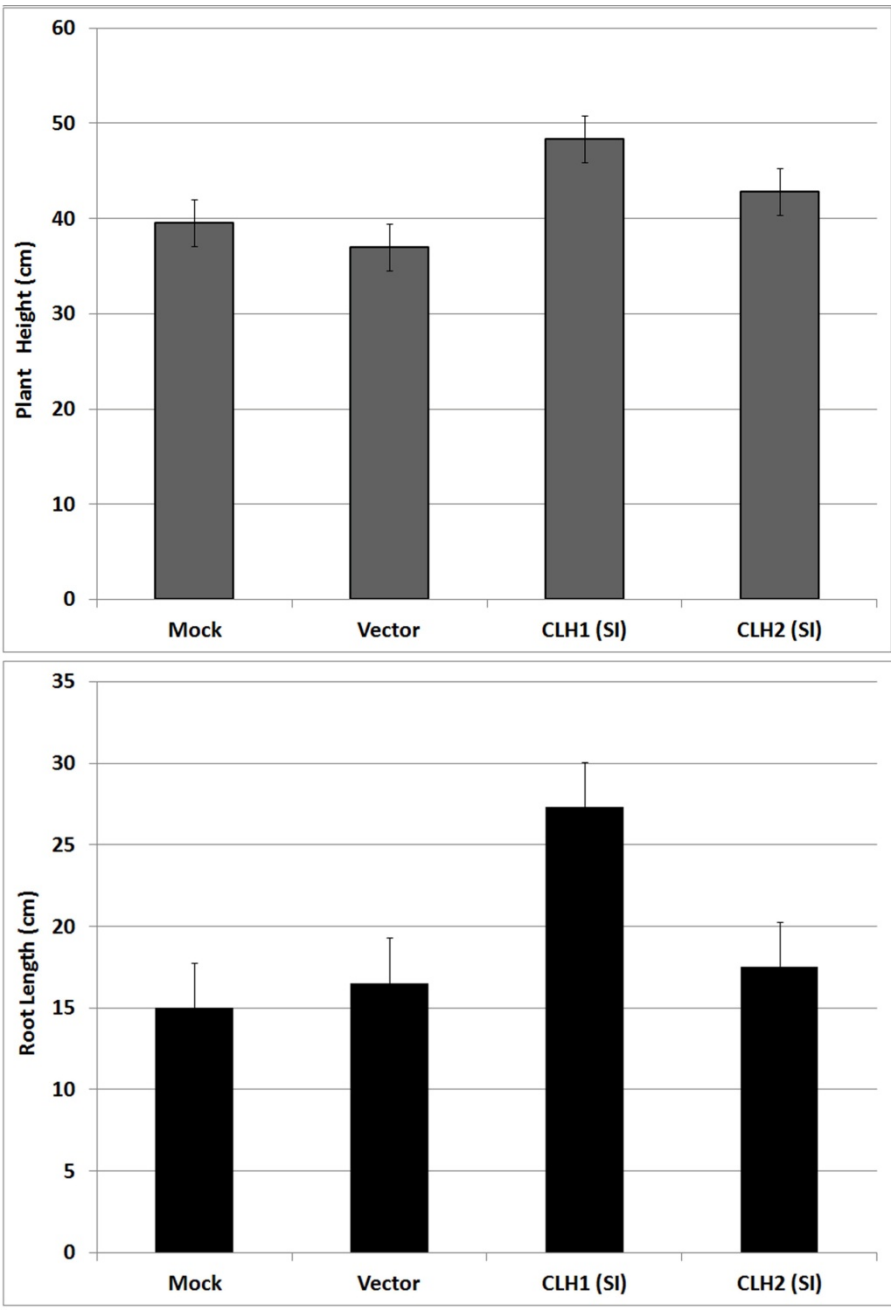


Fig. 4.3 Shoot and root length increase in plants silenced for *GmCLH1*. A significant increase was recorded of plant height (upper chart) and root length (lower chart) of soybean plants as a result of silencing of *GmCLH1*. Measurements were conducted on soybean plants (cv. Essex) at 4 weeks post inoculation (n=10 plants).

4.2.2 Silencing of *GmCLH1* reduces BPMV virus titer

BPMV titers in leaves of V, *CLH1* (SI) and *CLH2* (SI) plants were determined by ELISA and the results confirmed that the observed phenotypic differences correlated well with virus titer. A significant reduction in BPMV accumulation in *CLH1* (SI) plants compared to empty vector-inoculated plants was observed, whereas a significant increase in virus accumulation was recorded in *CLH2* (SI) plants (Fig. 4.4).

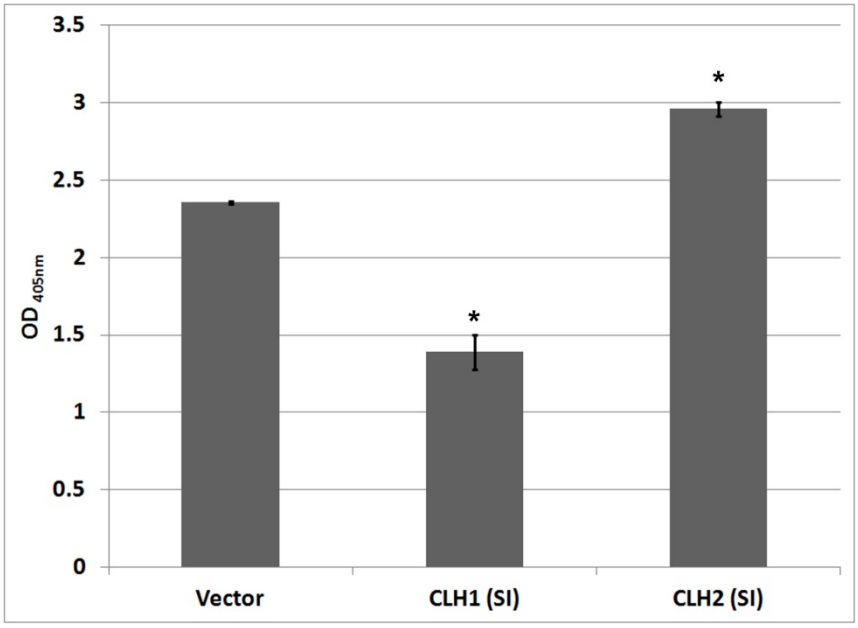


Fig. 4.4 Influence of BPMV-mediated silencing of *GmCLH1* or *GmCLH2* on BPMV virus titer in silenced plants. Reduction or increase of the BPMV levels in plants silenced for *GmCLH1* or plants silenced for *GmCLH2*, respectively. The virus titer was assessed as optical density (OD) units at wavelength 405 nm using enzyme-linked immunosorbent assay (ELISA). Samples were collected from soybean plants (cv. Essex) at 14 days post inoculation (n=2 plants). Asterisks denote data significantly different from Vector ($P < 0.005$).

4.2.3 Delayed senescence due to silencing of *GmCLH2* or overexpression of *GmCLH1*

Interestingly, different treatments showed variation in their senescence time. Ten weeks after recombinant vectors inoculation, *CLH2* (SI) plants and *CLH1* (OE) plants were still green while all other treatments were already dead of senescence (Fig. 4.5).

Mock Vector

CLH1 (SI) *CLH2* (SI)

CLH1 (OE) *CLH2* (OE)

Fig. 4.5 Delayed senescence phenotype exhibited by plants overexpressing *GmCLH1* and plants silenced for *GmCLH2*. *CLH1* (OE) and *CLH2* (SI) plants remained green while other treatments including controls showed age-associated senescence. Photographs were taken 9 weeks after inoculation of soybean plants (cv. Williams 82).

4.2.4 RT-PCR analysis of *GmCLH*-silenced/overexpressing plants

A reverse transcriptase polymerase chain reaction (RT-PCR) assay was used to check transcript levels of both genes in each of the four treatments of the study compared to mock and empty vector control plants. An effective silencing has been attained in both plants infected with pGG7R-*GmCLH1*-216; *CLH1* (SI) (Fig. 4.6A) or pGG7R-*GmCLH2*-243; *CLH2* (SI) (Fig. 4.6B). Although about 10-fold of the transcript level of *GmCLH1* was attained in case of *CLH1* (OE) plants (Fig. 4.6C), transcript level of *GmCLH2* from plants infected with pGG7R-*GmCLH2*-951 reached at least 20-fold than vector plants (Fig. 4.6D).

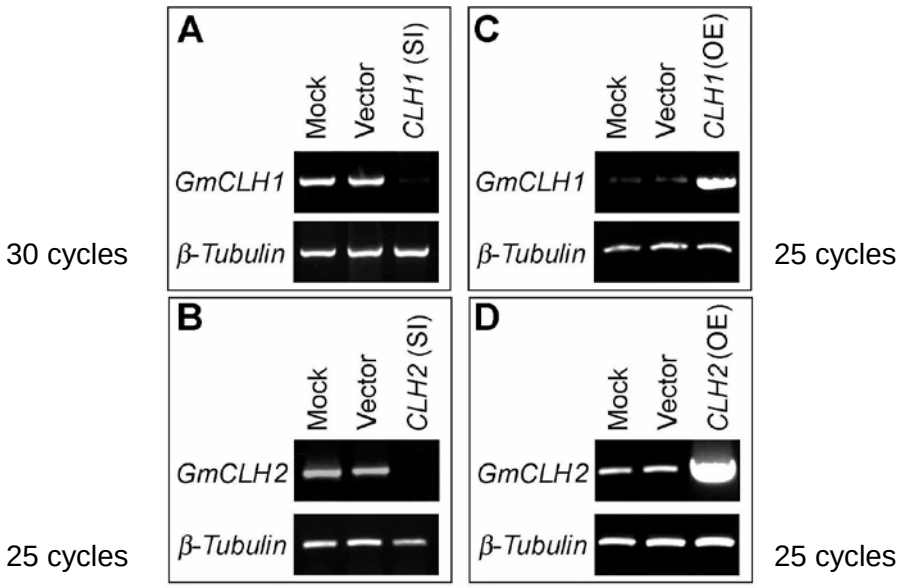


Fig. 4.6 RT-PCR analysis showing effect of BPMV-mediated overexpression/ silencing on transcript level of *GmCLH1* and *GmCLH2*. In both *CLH1* (SI) and *CLH2* (SI) leaves, no detectable transcript was visualized whereas transcripts were clearly detected in mock as well as empty vector-inoculated plants (**A** and **B**). Significant increase in the accumulation of the recombinant transcript encoding *GmCLH1* and *GmCLH2* sequences were detected in *CLH1* (OE) and *CLH2* (OE), respectively (**C** and **D**). *β-tubulin* was amplified as an internal cDNA control.

4.2.5 Response of silenced/overexpressing plants to *Fusraium virguliforme*

Soybean plants (cv. Essex) were used to test the effect of silencing/overexpression of *GmCLH1* or *GmCLH2* on plant response to *F. virguliforme*, the causal agent of SDS. A disease scale from 0 to 5 for foliar symptoms was used to assess disease severity on infected plants where 0 is resistant plant and 5 is dead plant. Empty vector-inoculated plants were used as a control. Seven weeks after root inoculation with the pathogen, *CLH1* (OE) plants showed no difference from the empty vector-inoculated plants as they both showed leaf interveinal necrosis on the lower half of the plant and interveinal chlorosis on some leaves of the upper half and leaf defoliation was rare so they have been rated in category 3. Because the interveinal leaf necrosis reached the upper parts of the plants besides a frequent leaf defoliation in *CLH1* (SI) plants, they have been considered more susceptible than the later treatments and were ranked in category 4. Given that *CLH2* (OE) plants lost their leaves and showed necrosis and a cup-shaped leaf on few remaining leaves on the upper part of the plant, they recorded the highest severity among the treatments having a No. 5 category. in contrast, *CLH2* (SI) were given a rating of 1 as

they lacked most of the foliar symptoms except for some mild chlorosis on a few older leaves on the plants expressing the most resistance on the foliage (Fig. 4.7).

4.2.6 Effect of *GmCLH* silencing/overexpression on jasmonic acid levels in leaves

To determine whether the enhanced resistance to SDS foliar symptoms of *CLH2* (SI) plants and/or its enhanced susceptibility by *CLH2* (OE) plants due to increase or decrease - respectively - in the phytohormone jasmonic acid (JA), JA analysis was conducted in the different treatments after the artificial inoculation of the roots with *F. virguliforme*. The analysis showed that *CLH2* (SI) plants accumulated almost double the quantity of empty vector-inoculated plants while *CLH1* (SI) showed 57% reduction relative to the empty vector-inoculated plants. *CLH1* (OE) reduction was not significant compared to *CLH2* (OE) that showed 38% reduction than the control (Fig.4.8).

4.2.7 Effect of *GmCLH* silencing/overexpression on H_2O_2 levels in plants

In plants, levels of reactive oxygen species (ROS) correlate negatively with successful resistance to necrotrophic pathogens (Torres *et al.*, 2006; Mengiste, 2012). As a measure of ROS, hydrogen peroxide (H_2O_2) levels were evaluated in the leaves of *F. virguliforme*-inoculated plants that were subjected to the different treatments. Compared to empty vector-inoculated plants, a significant increase in H_2O_2 levels was achieved in *CLH2* (OE) and highly significant in *CLH1* (OE). For silenced plants, *CLH1* (SI) did not show significant change and the only significant reduction was in the *CLH2* (SI) treatment (Fig.4.9).

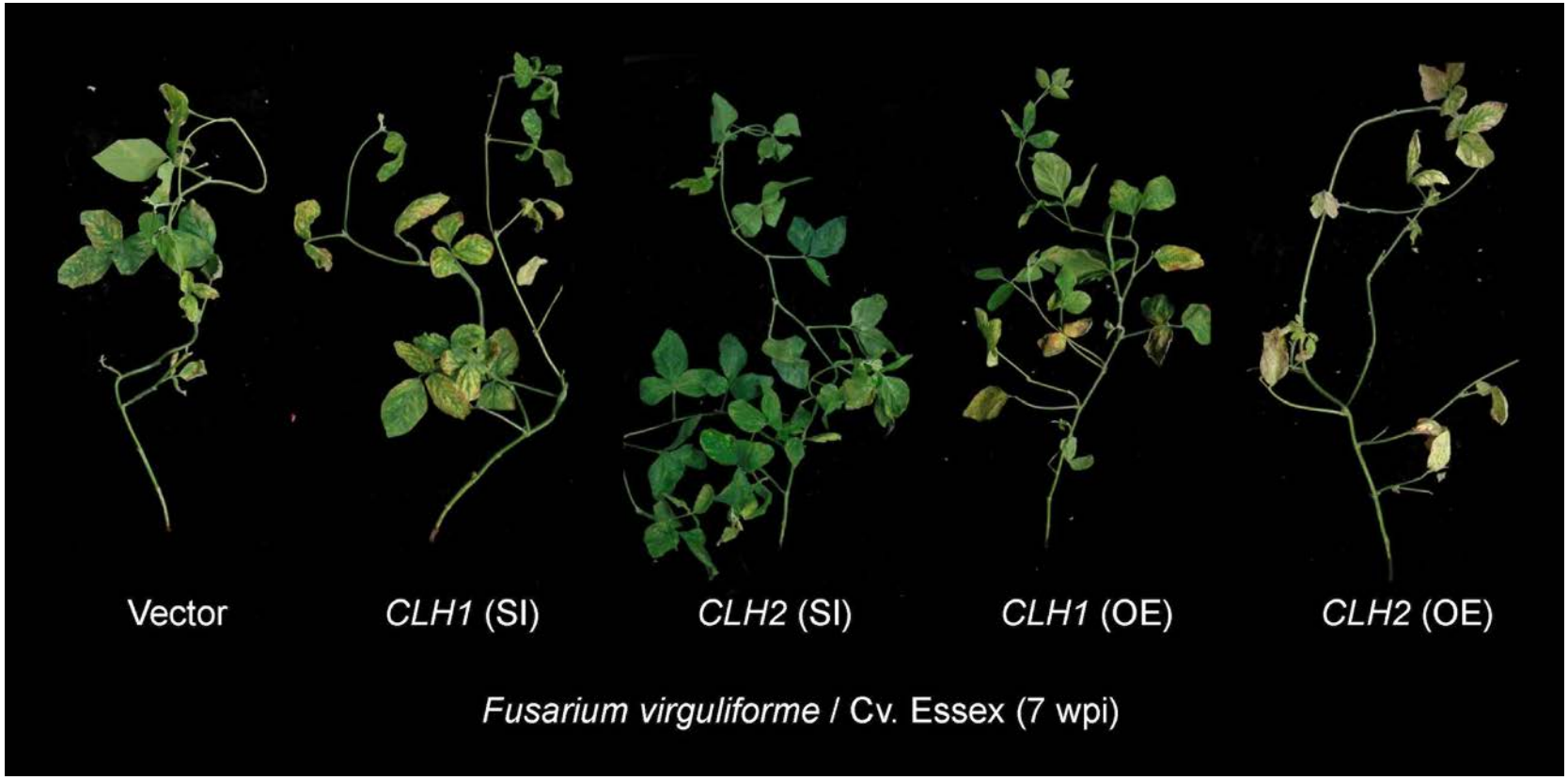


Fig. 4.7 Silencing of *GmCLH2* enhances resistance to *Fusarium virguliforme* and overexpression increases susceptibility when compared to empty vector-inoculated plants. cv. Essex generally shows moderate susceptibility to *F. virguliforme*. Disease assessment was conducted seven weeks post pathogen inoculation.

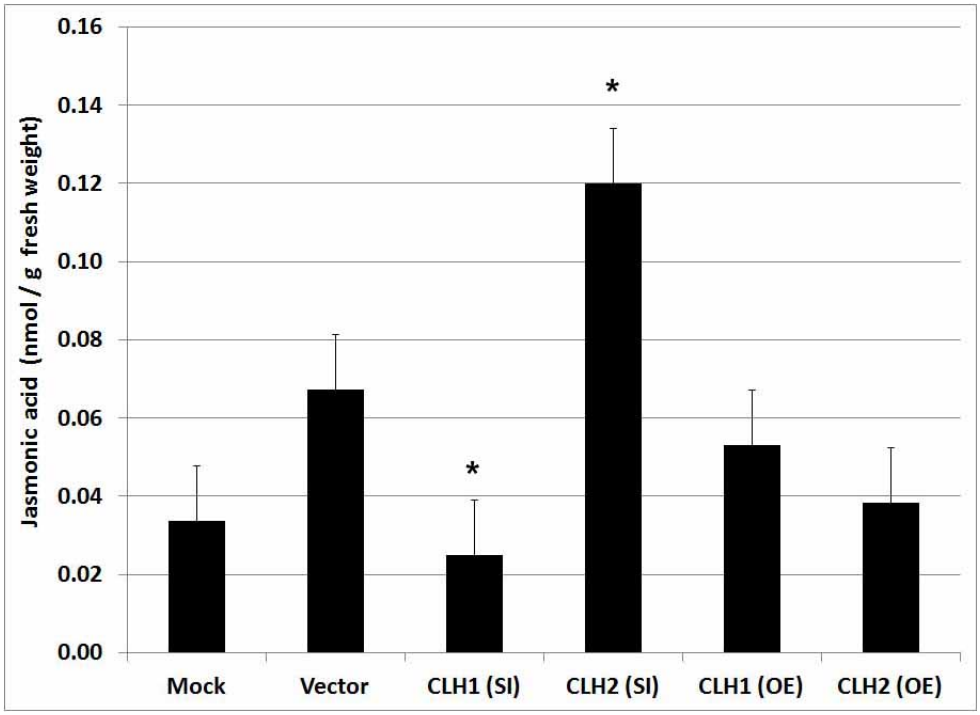


Fig. 4.8 Plants silenced for *GmCLH2* accumulate high level of jasmonic acid (JA). Quantification of JA was done using 1 g of fresh leaves with adding internal standard of Dihydro-JA. Samples were collected 24 hours after inoculating all treatments with *F. virguliforme*. Results are representative of two independent analyses, each comprised samples of 3 plants/treatment. Asterisks denote data significantly different from Vector treatment ($P < 0.005$).

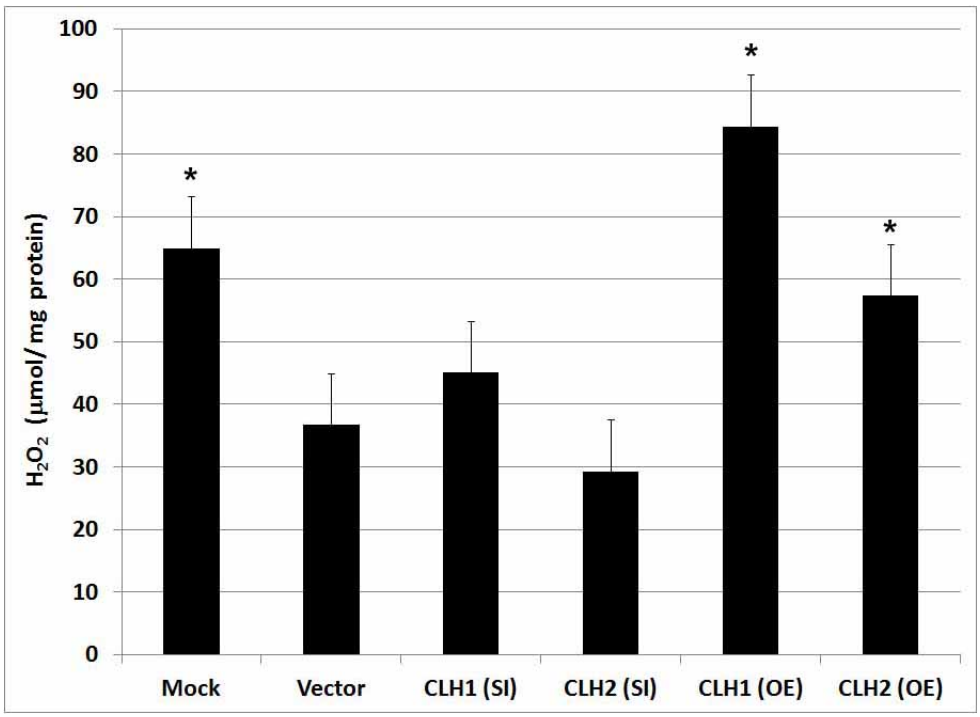


Fig. 4.9 Evaluation of H_2O_2 in plants overexpressing/silenced for *GmCLH1* or *GmCLH2*. The H_2O_2 levels were measured with a spectrofluorimeter at 488 and 583 nm wavelengths. The concentration of H_2O_2 was determined as mmol/mg protein by extrapolating from the standard H_2O_2 curve. Asterisks denote data significantly different from Vector treatment ($P < 0.005$).

4.2.8 Enhanced expression of some *PR* genes due to *GmCLH* silencing/ overexpression

Expression of *PR* genes increase in resistant plants. RT-PCR assay was used to check the expression of selected *PR* genes in silenced plants and controls. Results (Fig 4.10) show enhanced expression of *PR1a* and *PR4* in both *CLH1* (SI) and *CLH2* (SI) 3 days post root inoculation of with *F. virguliforme*. Meanwhile, the level of *PR2* increased

only in *CLH2* (SI) plants. No bands were visualized in *PR2* or *PR4* when amplified from plants 15 dpi or 30 dpi. Although *PR1a* transcripts were null in Mock and empty vector-inoculated plants 3 dpi, it has been amplified efficiently at 15 and 30 dpi from all treatments.

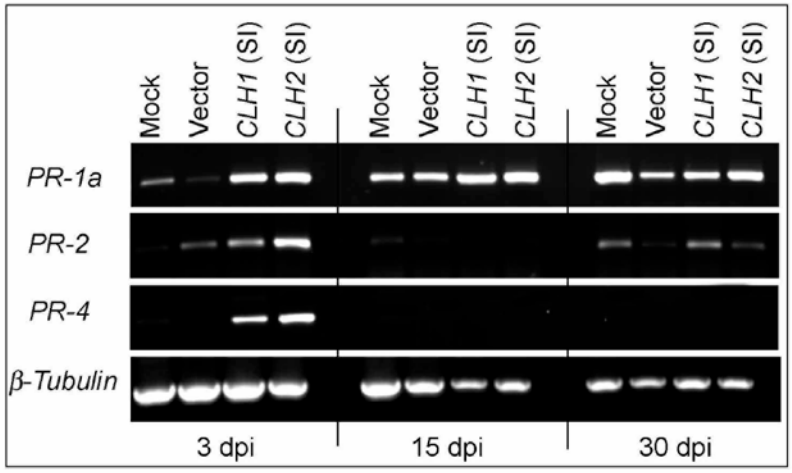


Fig. 4.10 Temporal expression pattern of selected *PR* genes in plants silenced for *GmCLH1*; *CLH1* (SI) and plants silenced for *GmCLH2*; *CLH2* (SI). Transcript profiles of *PR1a*, *PR2* and *PR4* in *F. virguliforme*-inoculated plants were analyzed using RT-PCR. At 3dpi, *CLH2* (SI) showed induction in *PR1a*, *PR2* and *PR4* transcripts while *PR2* was not induced in *CLH1* (SI). RT-PCR of the constitutively expressed *β-tubulin* was performed to ensure equal cDNA amount in each reaction.

4.2.9 Response of *GmCLH*-silenced plants to *Phytophthora sojae*

Soybean plants (cv. Harosoy) were used to test the effect of silencing of *GmCLH1* or *GmCLH2* on plant response to *Phytophthora sojae*. The soybean cultivar Harosoy is susceptible to *P. sojae* R3. Silencing of *GmCLH2* significantly altered this susceptibility

as *CLH2* (SI) plants showed reduction of 40% in lesion length compared to empty vector -inoculated plants. On the other hand, plants silenced for *GmCLH1* did not show significant change in lesion length compared empty-inoculated plants (Fig. 4.11A and 4.11B).

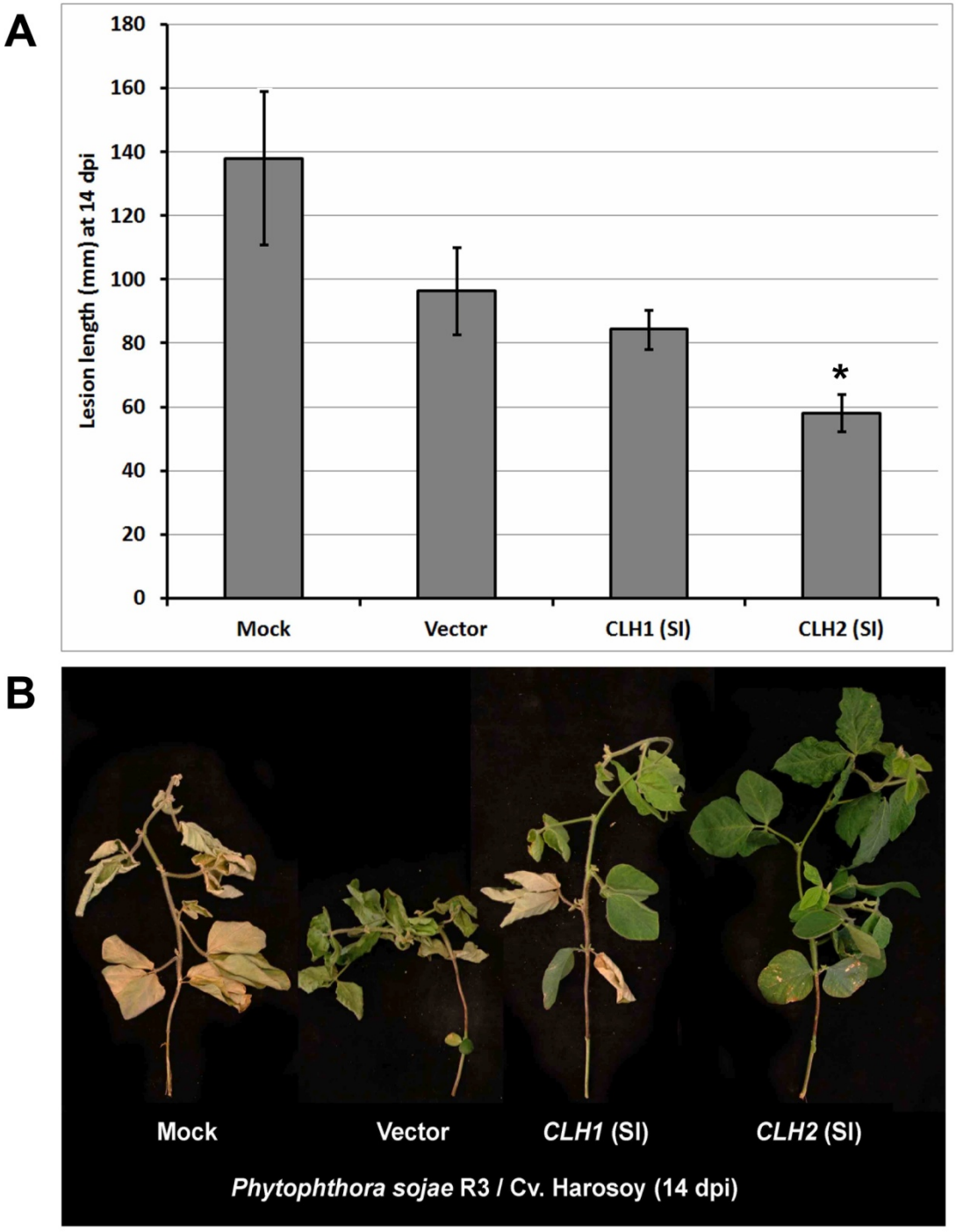


Fig. 4.11 Response of *GmCLH1*- or *GmCLH2*-silenced plants to *P. sojae* inoculation: lesion length and phenotype. Soybean plants (of the susceptible cultivar Harosoy) infected with *GmCLH1* or *GmCLH2*-silencing constructs were inoculated with *P. sojae* R3. **A,** Average lesion length on plant stem measured longitudinally in millimeters. The data represent 3 independent experiments with 4-5plants/treatment. Asterisks denote lesion length significantly different from Vector treatment ($P < 0.005$). **B,** Response phenotypes exhibited by different treatments to *P. sojae* inoculation. Photographs were taken 14 days post pathogen inoculation.

4.2.10 Induced susceptibility of *CLH1* (SI) to necrotrophic fungal pathogens

Preliminary observations from detached leaf assays showed that *CLH1* (SI) exhibited high susceptibility to all the tested fungal pathogens while *CLH2* (SI) did not show significant changes than controls. Hence, this part focused on *CLH1* (SI) to shed some light on its role in plant resistance. *CLH1* (SI)-infected plants were tested against the following fungal pathogens: *Phomopsis longicolla*, *Alternaria tenuissima* and *Sclerotinia sclerotiorum* and their responses were compared to those inoculated with empty-inoculated plants. Preliminary comparative virulence assays showed that Cv. Harosoy is the best of my cultivar collection to do these experiments due to its moderate susceptibility to *P. longicolla* and *A. tenuissima*. When tested to *P. longicolla*, *CLH1* (SI) showed an increase of about 2-fold of the macerated area on the leaf more than mock or empty vector-inoculated treatment (Fig. 4.12A). In case of *A. tenuissima*, the response was more dramatic as the leaf tissue of *CLH1* (SI) completely turned water-soaked when

the progress of the spot on the vector only inoculated plant leaf had not exceed 30% of the blade and the mock was even less (Fig. 4.12B). The response of *CLH1* (SI) to *S. sclerotiorum* was interestingly characterized by a very rapid progress of the maceration to cover the whole blade area in 4 days while none of the controls recorded any disease (Fig. 4.12C); a phenomenon that was confusing at the time of the experiment but later on, the used isolate of *S. sclerotiorum* was proven in a separate investigation in my lab to be hypovirulent due to a dsRNA infection (Xie and Ghabrial, 2012).

In an attempt to find out the reason behind the dissimilar act of *GmCLH1* and *GmCLH2* in plant response to pathogens, the following experiments were carried out:

4.2.11 Phylogenetic relationship of soybean chlorophyllases to other known chlorophyllases:

The response of silenced/overexpressing plants to various pathogens showed a difference in all cases between *GmCLH1* and *GmCLH2* genes. In an attempt to shed some light on what causes the differences between their effects, I carried out a phylogenetic analysis of 34 known plant CLHs including *Glycine max* CLHs and some other well studied CLHs. The analysis was conducted by MEGA5 software (Tamura *et al.*, 2011) and a neighbor-joining tree was generated (Saitou and Nei, 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. This analysis was done with the expectation of learning more about relationships among the three soybean CLHs and the selected known CLH proteins. The resultant phylogenetic tree showed the CLHs could grouped in two main clades, which is predicted

based on their amino acid sequences (Fig. 4.13). As shown in Fig. 4.13, soybean chlorophyllases were distributed between the two groups with GmCLH1 and GmCLH3 - which are almost identical - in one group, while GmCLH2 is placed with the other group.

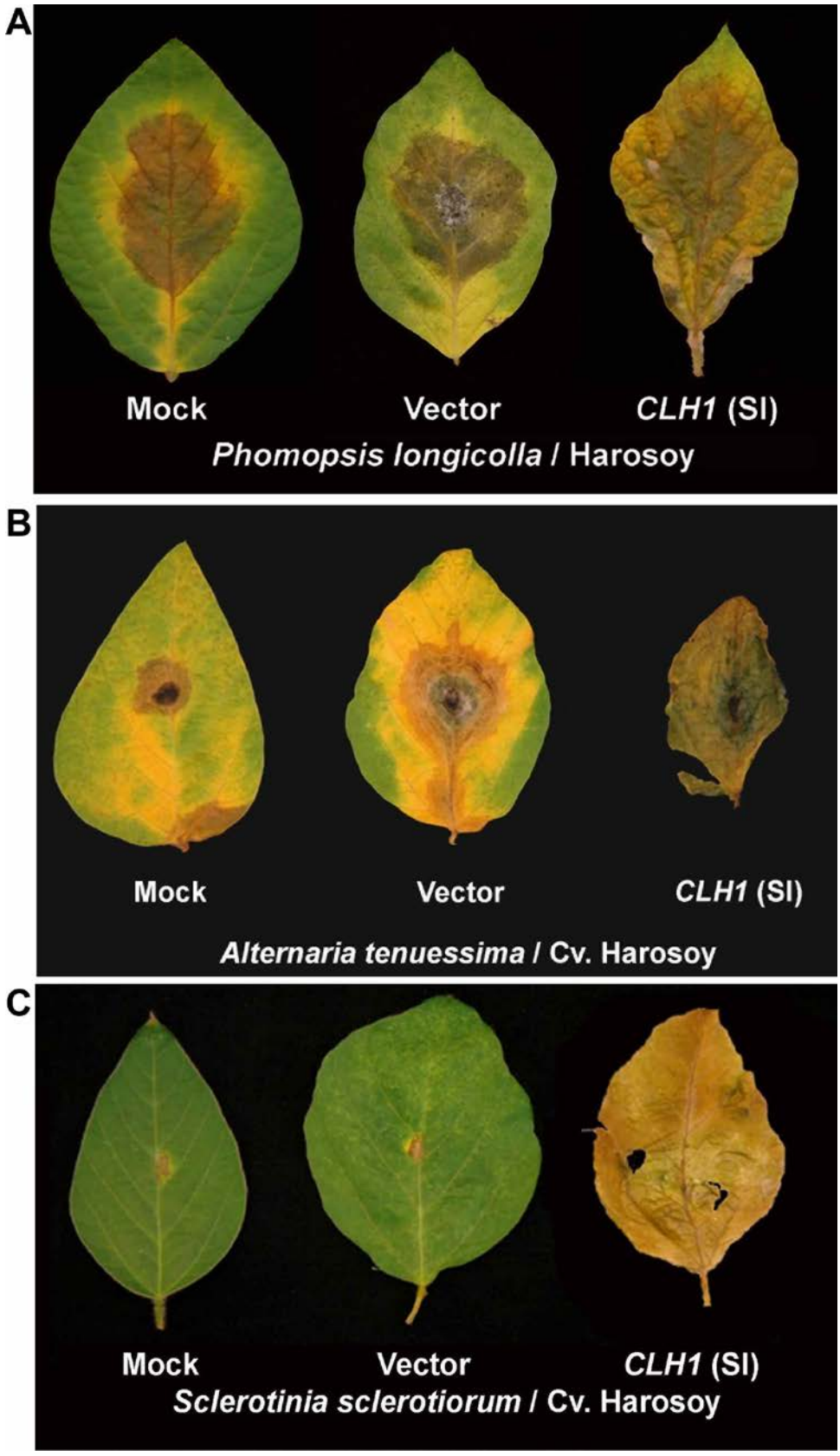

A
Mock
Vector
CLH1 (SI)
Phomopsis longicolla / Harosoy
B
Mock
Vector
CLH1 (SI)
Alternaria tenuessima / Cv. Harosoy
C
Mock
Vector
CLH1 (SI)
Sclerotinia sclerotiorum / Cv. Harosoy

Fig. 4.12 Enhanced susceptibility of *GmCLH1*-silenced plants to necrotrophic fungal pathogens. Leaves of soybean plants (cv. Harosoy) were tested in a laboratory bioassay to **A,** *Phomopsis longicolla* **B,** *Alternaria tenuissima* and **C,** *Sclerotinia sclerotiorum*. Assessment of the disease in all cases was done by the visual contrast of the *CLH1* (SI) treatment to the controls. Each experiment was repeated at least 3 times using 3 leaves from 3 independent plants per each treatment.

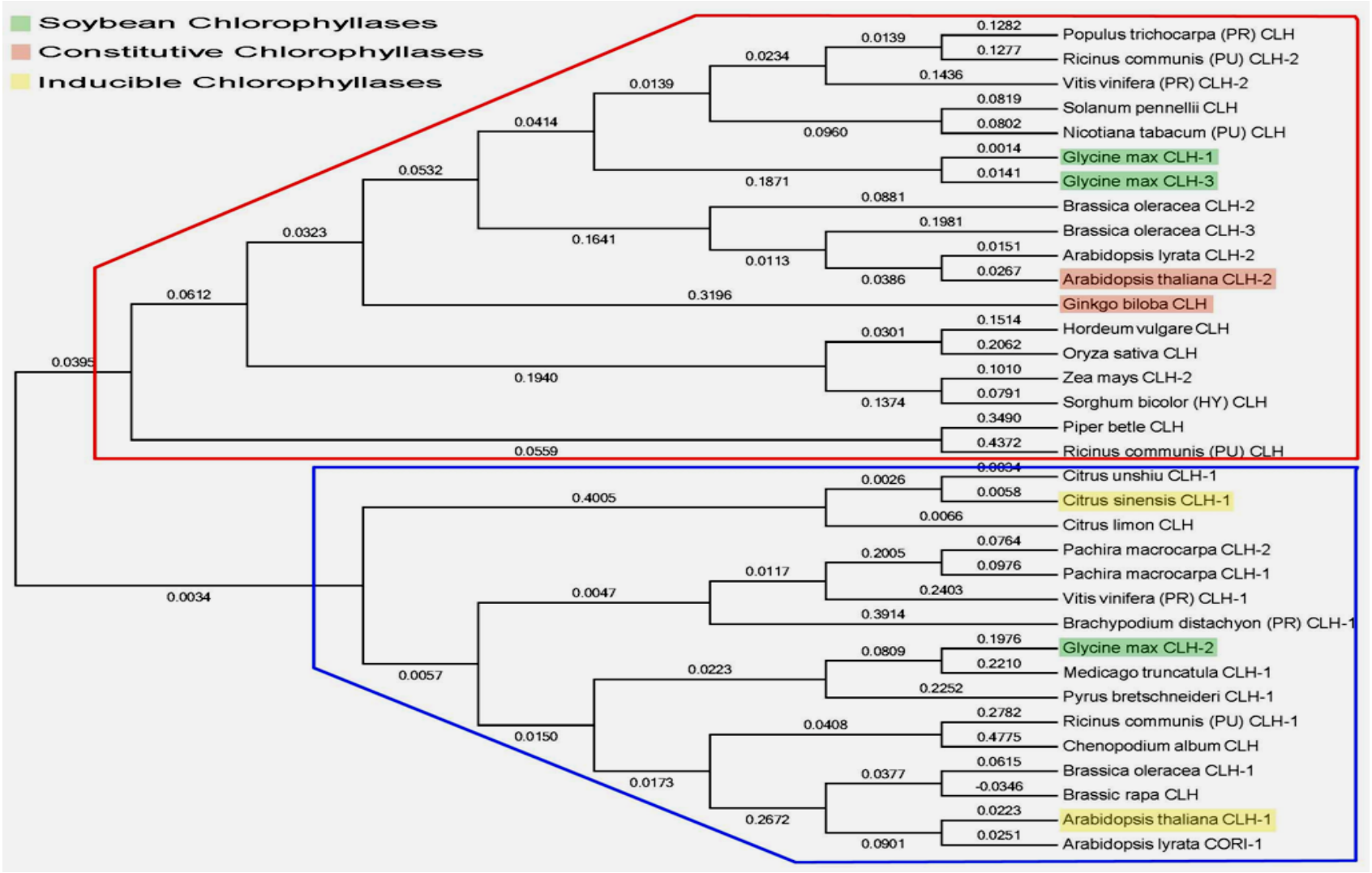

Soybean Chlorophyllases
Constitutive Chlorophyllases
Inducible Chlorophyllases
Populus trichocarpa (PR) CLH
Ricinus communis (PU) CLH-2
Vitis vinifera (PR) CLH-2
Solanum pennellii CLH
Nicotiana tabacum (PU) CLH
Glycine max CLH-1
Glycine max CLH-3
Brassica oleracea CLH-2
Brassica oleracea CLH-3
Arabidopsis lyrata CLH-2
Arabidopsis thaliana CLH-2
Ginkgo biloba CLH
Hordeum vulgare CLH
Oryza sativa CLH
Zea mays CLH-2
Sorghum bicolor (HY) CLH
Piper betle CLH
Ricinus communis (PU) CLH
Citrus unshiu CLH-1
Citrus sinensis CLH-1
Citrus limon CLH
Pachira macrocarpa CLH-2
Pachira macrocarpa CLH-1
Vitis vinifera (PR) CLH-1
Brachypodium distachyon (PR) CLH-1
Glycine max CLH-2
Medicago truncatula CLH-1
Pyrus bretschneideri CLH-1
Ricinus communis (PU) CLH-1
Chenopodium album CLH
Brassica oleracea CLH-1
Brassic rapa CLH
Arabidopsis thaliana CLH-1
Arabidopsis lyrata CORI-1

Fig. 4.13 Phylogenetic analysis of GmCLH1, GmCLH2, GmCLH3 and other chlorophyllases from flowering plants. The analysis involved the amino acid sequences of 34 proteins, which were obtained from GenBank. The neighbor-joining tree was constructed using MEGA5, with branch lengths (next to the branches) written in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis revealed 2 clusters encircled with red and blue lines. The three soybean chlorophyllase proteins are highlighted with green. Chlorophyllases that were previously reported as phytohormone-inducible are highlighted with yellow. Proteins highlighted with pink are chlorophyllases that were previously reported as constitutive.

4.2.12 Effect of exogenous jasmonic acid treatment on *GmCLH* expression in systemic leaves

Previous studies showed that chlorophyllases are classified according to their response to phytohormones -such as MeJA, ethylene, coronatine - into two groups; responsive and unresponsive *CLH*s. For example, the expression level of *AtCLH2*, *Arabidopsis thaliana* clorophyllase 2, has been found unresponsive to MeJA (Tsuchiya *et al.*, 1999). Similarly, the *Ginkgo biloba* chlorophyllase *GbCLH* was constitutive and did not respond to MeJA either (Tang *et al.*, 2004). On the other hand, *AtCLH1* mRNA level was enhanced due to application of MeJA or coronatine (Benedetti *et al.*, 1998) and chlorophyllase of *Citrus sinensis* was induced due to ethylene treatment (Jacob-Wilk *et al.*, 1999). In the current study exogenous application of JA induced the expression of

GmCLH2 but did not alter the expression of *GmCLH1* as concluded from RT-PCR assay genes (Fig. 4.14). This shows a second difference between the two genes.

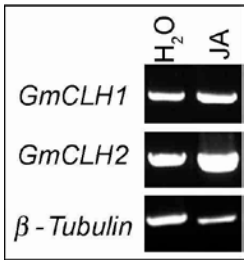


Fig. 4.14 Induction of endogenous transcript of *GmCLH2* by exogenous jasmonic acid application. RT-PCR analysis of *GmCLH1* and *GmCLH2* transcripts in plants treated with jasmonic acid. Plants treated with water served as control. RT-PCR of the constitutively expressed *β-tubulin* was performed to ensure equal cDNA amount.

4.2.13 Chlorophyllase activity in *GmCLH*-silenced plants

This experiment was carried out to determine the direct effects of silencing both *GmCLH1 and GmCLH2* genes on loss of chlorophyllase enzyme activity and whether both genes equally regulate the enzyme activity. Results showed that silencing of *GmCLH1* was significantly more effective in lowering the enzyme activity than *GmCLH2* (Fig. 4.15).

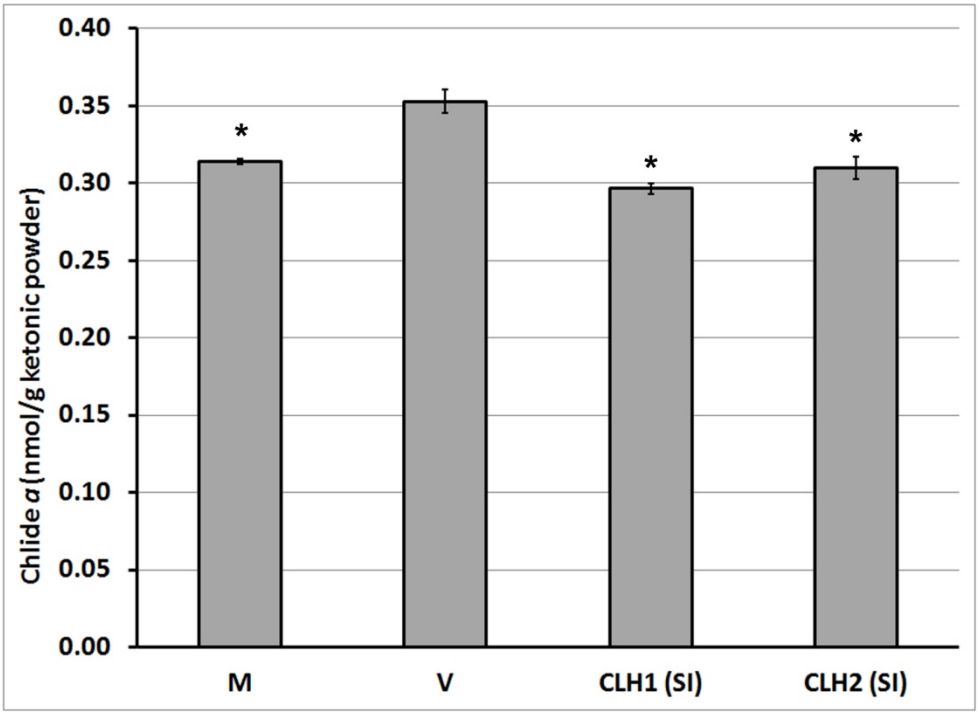


Fig. 4.15 Reduction of chlorophyllase activity in soybean plants silenced for *GmCLH1* or *GmCLH2*. Chlorophyllase activity was evaluated in non-senescent silenced and control plants. The amount of Chlide *a*, released as a result of chlorophyll degradation, was measured spectrophotometrically and expressed as nmol/gram of acetone powder. More reduction of the enzyme activity was characterized in *GmCLH1*-silenced plants. The data represent 2 independent experiments with 3 plants/treatment. Asterisks denote measurements significantly different from Vector treatment.

4.2.14 Influence of *GmCLH1* and *GmCLH2* silencing on plant tolerance to low light intensity

Under growth conditions of low light intensity, it is expected that less chlorophyll will accumulate in the tissues. When *GmCLH1* was silenced, the case remained the same, meanwhile when *GmCLH2* was silenced, I could record a chlorophyll amount that is

higher than mock or empty vector-inoculated plants. It is known that abiotic stress factors induce early senescence of plant. When all treatments subjected to low light, treatments that have normal regulation of *GmCLH2* (mock, empty vector-inoculated, *CLH1* (SI) showed significant drop in total chlorophyll content, while the only treatment that tolerated that stress was the plants silenced for this gene. Taken together with the observed delayed senescence phenotype of *CLH2* (SI), it is suggested that *GmCLH2* plays a role in senescing signaling (Fig. 4.16).

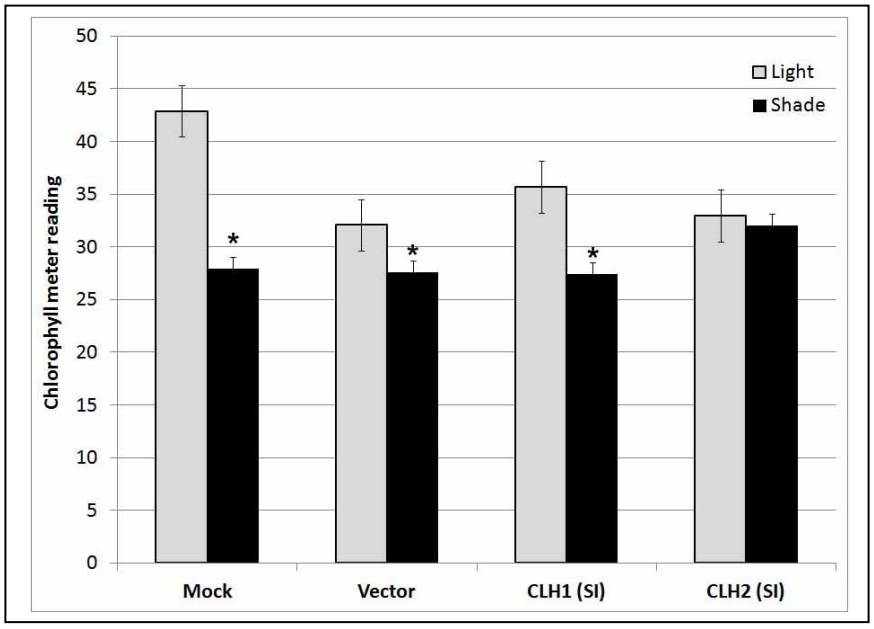


Fig. 4.16 Tolerance of *GmCLH2*-silenced plants to the decrease of chlorophyll concentration when subjected to low light intensity. A set of plants grown under normal light conditions served as controls. Chlorophyll was measured in leaves using atLEAF+ portable chlorophyll meter and the chlorophyll content was expressed in atLEAF units. Asterisks denote decrease of chlorophyll content significant from normal light conditions ($P < 0.005$).

4.3 DISCUSSION

Infection of soybean with recombinant BPMV-*CLH* silencing/overexpressing vectors resulted in distinct phenotypes. *CLH1* (SI) plants showed the mildest phenotypic changes (mottling and blistering) compared to other treatments and showed a significant reduction of BPMV titer. This may be a result of the suppression or the induction of JA synthesis in *CLH1* (SI) and *CLH2* (SI), respectively. This in turn, due to the antagonistic effect of JA on SA, thus promoting an SA defense pathway to BPMV in the first case or interfering with it in the second case. SA is known to be essential for local and systemic resistance of plants to viruses through different mechanisms (Chivasa *et al.*, 1997; Murphy *et al.*, 1999; Murphy and Carr, 2002; Kachroo, 2006). Although no data was obtained on SA accumulation in the present study, previous studies showed that JA and SA pathways in the plant are antagonistic (Takahashi *et al.*, 2004).

Moreover, *CLH1* (SI) plants were characterized by increased shoot and root lengths. Obviously, silencing of *GmCLH1* resulted in reduction in the chlorophyllase activity and allowed higher accumulation of chlorophyll in the leaves. Therefore, light absorption may be enhanced and the photosythestic rate increased. Photosynthesis rate in soybean leaves was found to be highly correlated with chlorophyll content (Buttery and Buzzell, 1977). Furthermore, increased growth rates of different plant species were attributed to high amounts of chlorophyll in their leaves (Brougham, 1960). Another explanation is that repression of JA in the *CLH1* (SI) treatment eliminated the growth inhibitory effect of JA, so the plants of this treatment have an increase in height and root length. Studies on jasmonates demonstrated that they have a few physiological roles in plants including mature leaves' senescence, inhibition of growth and germination and leaf

abscission (Ueda and Kato, 1980; Dathe *et al.*, 1981; Curtis, 1984; Meyer *et al.*, 1984; Corbineau *et al.*, 1988; Creelman *et al.*, 1992).

The characterisitic necrosis/chlorosis developed on leaves of *CLH1* (OE) plants and the chlorosis spread on *CLH2* (SI) plant leaves is considered as evidence of the formation of harmful types of ROS in the leaves. Sharma *et al.* (2012) explained that singlet oxygen ($^1O_2$) - a highly reactive and destructive form of ROS - may be produced in the light from the triplet chlorophyll (^{3}Chl) state in the reaction center of photosystem II as well as in the antenna system. Plants suffer from high phototoxicity of free chlorophyll that reacts rapidly with oxygen (O_2) in the presence of light to produce highly reactive $^1O_2$ (Feild *et al.*, 2001). This suggests that *GmCLH2*, but not *GmCLH1*, is the responsible chlorophyllase for degrading free chlorophyll in the cell. This result is in line with the finding of and model created by Kariola *et al.* (2005) who suggested that the free chlorophyll radicals generated by light in *Arabidopsis* plants are being degraded under normal conditions by the regulation of *AtCLH1*, so plants silenced for this gene accumulated ROS due to the phototoxicity of free chlorophyll.

Legumes appear to be particularly tolerant of mutations influencing Chl degradation, possibly because nitrogen fixation compensates for the lower accessibility of internal nitrogen from which stay-greens tend to suffer. The stay-green phenotype and other senescence traits in soybean are under the control of at least nine separate genetic loci (Thomas and Smart, 1993; Matile *et al.*, 1999).

RT-PCR analyses (Fig 4.16), carried out in this study to compare the expression levels of transcripts of *GmCLH1* or *GmCLH2* in all silenced/overexpressing treatments, have clearly confirmed silencing or overexpression in the respective plants. Virus-

induced gene silencing (VIGS) is an excellent reverse-genetic strategy that allows gene functional analysis in species not amenable to stable genetic transformation, like soybean. VIGS is quick, does not necessitate development of stable transformants, and allows characterization of phenotypes that might be lethal in stable lines. Furthermore, VIGS offers the ability to silence either individual or multiple members of a gene family. Thus targeting for silencing a highly conserved sequence in a gene family (Scofield *et al.*, 2005) would potentially allow for silencing all members of the family. The BPMV-based vector (Zuo *et al.*, 2007) has been used successfully in the past 6 years to silence soybean genes and became one of the most effective tools in functional genomics of soybean (Tsuda *et al.*, 2009; Diaz-Camino *et al.*, 2011; Singh *et al.*, 2011).

To examine the silenced/overexpressing plants for their responses to *F. virguliforme*, a preliminary experiment was conducted to test fungal virulence on four different cultivars. On a 0-5 disease scale where 0 is most resistant, the rating of those cultivars was Harosoy (1), Essex (2), Clark or Williams 82 (5). Essex was selected for use in these experiments because of its moderate susceptibility. Essex susceptibility to SDS has been previously reported (Iqbal *et al.*, 2001).

CLH2 (SI) plants showed higher resistance to SDS while *CLH1 (SI)* plants did not differ from the vector only inoculated-plants. On the other hand, *CLH2* (OE) plants exhibited the highest level of disease severity among all treatments. In *A. thaliana*, of the two genes encoding chlorophyllases *AtCLH1* and *AtCLH2*, only the former was rapidly induced in response to wounding, methyl jasmonate (MeJA) and the bacterial jasmonate-mimicking toxin coronatine (COR). Further, the expression of *AtCLH1* was reduced in the JA-insensetive *coi1* mutant plants (Benedetti and Arruda, 2002). By using RNA

interference (RNAi), the specific silencing of *AtCLH1* led to accumulation of ROS. In addition, leaf inoculation of silenced plants with *Erwinia carotovoa* subsp. *carotovora* resulted in no visible symptoms. In contrast, plants overexpressing the same gene exhibited disease symptoms such as tissue maceration after only 24 hours post inoculation (Kariola *et al.*, 2005).

Furthermore, detached leaves bioassays of *CLH1* (SI) showed enhanced susceptibility to three necrotrophic fungal pathogens (i.e. *Alternaria tenuissima*, *Phompopsis longicolla* and *Sclerotinia sclerotiorum*) - characterized by severe chlorosis and maceration - when compared to mock and empty vector-inoculated plants. This may be directly correlated with the reduction of JA level in the same treatment that reached 57% of the empty vector-inoculated plants. Typically, JA was previously demonstrated to be an essential element in triggering defense signaling against necrotrophic fungi (Antico *et al.*, 2012). These results are consistent with the enhanced susceptibility to *Alternaria brassicola* in *A. thaliana* plants silenced for *AtCLH1*. This was explained by the activation of SA-dependent defense pathway due to the accumulation of ROS in the cells as a result of photooxicity of the increased level of free chlorophyll, the latter could not be degraded as a direct result of silencing the chlorophyllase encoding gene (Kariola *et al.*, 2005).

The questions that need to be addressed now are: What is the difference between *F. virguliforme* and the other three necrotrophs? And why is *CLH2* (SI) did not enhance resistance response to these necrotrophs although it did for *F. virguliforme*?

One of two different explanations may apply. The first explanation indicates the possibility of the foliage susceptibility of *CLH2* (SI) plants to the direct effect of the toxin

produced by SDS pathogen. Plant resistance to SDS is divided into two major parts; resistance to the root necrosis caused by the fungus and resistance to the toxin effect produced by the fungus in the root and transferred to the foliage (Lightfoot, 2008; Ding *et al.*, 2011). The RT-PCR (Fig. 4.10) for pathogenesis related (*PR*) genes in *F. virguliforme*-inoculated plants 72 hours post inoculation (hpi) showed that transcript levels of *PR1a* has been induced in both *CLH1* (SI) and *CLH2* (SI) to similar levels, *PR4* was induced in *CLH2* (SI) as 2-fold as the induction in *CLH1* (SI) while *PR2* has been induced only in *CLH2* (SI). The induced level of *PR2* might inhibit the fungal colonization of the root due to the direct effect of β-1,3-glucanase, encoded by *PR2*, through degrading the hyphal cell wall components which certainly leads to a reduction of the toxin accumulation in the plant. Hevein-like protein (HEL) is the gene product of *PR4* in *Arabidopsis*, which marks the activation of JA signaling. The hevein and wheat win 1 and 2 proteins have typical antifungal activity. Repression of fungal growth of *Fusarium culmorum* has been reported *in vitro* due to exposure to the wheat win 1 or 2 proteins extracted from wheat (Caruso *et al.*, 2001; Bertini *et al.*, 2003; Roberti *et al.*, 2008; Bertini *et al.*, 2009). The resistance to and/or necrotic symptoms delay of *Fusarium pseudograminearum* - the causal agent of crown rot of wheat - was specifically correlated with JA defense pathway and JA-induced resistance genes (Desmond *et al.*, 2005). The formerly-mentioned 2-fold increase of accumulated JA in *CLH2* (SI) soybean plants 72 hpi with *F. virguliforme* disagree with the findings of Kariola *et al.* (2005) who determined JA repression in *AtCLH1*-RNAi silenced *Arabidopsis* plants.

The second explanation depends on phytotoxins mode of action in the plant cell which classically comprises DNA breakdown, shrinking cells and activation of proteases.

Most of those mechanisms are similar to the physiological reaction of plant cells during senescence and abiotic stresses. Although *CLH2* (SI) plants exhibited high level of JA that works as a senescence factor in the plant cell, it is predicted that FvTOX1, the toxin purified recently from *F. virguliforme* (Ding *et al.*, 2011), in one way or another depends on *GmCLH2* to degrade the chlorophyll and express the symptoms of SDS. When *GmCLH2* is silenced, the toxin could not exert its effect. The evidence for this is that the *CLH2* (SI) plants have a 10 -14 days delayed senescence than other treatments in this study. In addition, a low light stress experiment was conducted for *CLH1* (SI) and *CLH2* (SI) showed that the later has tolerated this stress conditions more than the former or the controls. Given the facts that *CLH2* (SI) plants are senescence-delayed and tolerant to senescence enhancers (i.e. stress/phytotoxin) suggests that the *GmCLH2* mediates senescence in the plant. In contrast, the traits of delayed senescence and moderate resistance to SDS exhibited by *CLH1* (OE) proposes the possibility for *GmCLH1* in mediating a process other than senescence such as chlorophyll homeostasis.

Phytophthora sojae R3 inoculations on Harosoy plants showed significant reduction in the length of stem lesions of *CLH2* (SI) when compared to controls. No dead plants recorded in *CLH1* (SI) or *CLH2* (SI) - in contrast to mock or empty vector-inoculated plants. Nevertheless, the stem lesion length reduction was not significant in *CLH1* (SI). Hemibiotrophic defense pathways are generally different than those of necrotrophic. It is still accepted that the increased JA level in *CLH2* (SI) is the reason for the resistance in this case. Singh *et al.* (2011) reported effective resistance of soybean to the same *P. sojae* race in *GmFAD3*-silenced plants that are exhibiting high SA and JA.

It is clear at this point of the current study that both genes act completely contradictory to each other. In an attempt to discover more about the reason for this difference, I have generated a phylogenetic analysisbased on amino acid sequences of *Glycine max* chlorophyllase 1, 2 and 3 as well as additional 32 chlorophyllases from higher plants. The phylogenetic tree (Fig. 4.13) predicted the classification of the selected proteins into two main clades. One of them included *Gm*CLH1 and *Gm*CLH3 which share a 98% identity, while *Gm*CLH2 belonged to the other group and shares only 48% amino acid identity sequence with the former two. This confirms that *Gm*CLH1 and *Gm*CLH2 are only distantly related. Tang *et al.* (2004); Schnabel *et al.* (2005) carried out a phylogenetic analysis of 9 chlorophyllases from higher plants and explained their distribution in two groups based on their response to the phytohormones MeJA, ethylene (ET) or coronatine (COR). The first group had individuals characterized by their active response to phytohormones while the second included constitutive genes that are expressed at low level (Jacob-Wilk *et al.*, 1999; Tsuchiya *et al.*, 1999; Tang *et al.*, 2004; Schnabel *et al.*, 2005). In order to examine the responsiveness of the soybean chlorophyllase 1 and 2 to the same phytohormones, exogenous JA was applied to non-inoculated seedlings while a second group of plants was sprayed with water as a control. Transcript levels of *GmCLH1* or *GmCLH2* were determined by RT-PCR. Only *GmCLH2* transcript level was elevated due to JA application while that of *GmCLH1* did not show change over the control. Interestingly, it is clear from the phylogentic grouping, that *Gm*CLH1 (as well as 3) is in the cluster that includes the *A. thaliana At*CLH2 and the *Ginkgo biloba Gb*CLH which are both phytohormone-unresponsive (constitutive) (Tsuchiya *et al.*, 1999; Tang *et al.*, 2004; Schnabel *et al.*, 2005). On the other hand, JA-

responsive *Gm*CLH2 has been positioned in the second group which included the inducible chlorophyllases *At*CLH1 and *Cs*CLH1 (from *Citrus sinensis*) (Jacob-Wilk *et al.*, 1999; Tsuchiya *et al.*, 1999). Due to the high correlation between senescence and jasmonates/ethylene, the inferred results from this phylogentic analysis confirm also the functional difference between the two groups of chlorophyllases. The first group individuals including *Gm*CLH1 are phytohormone unresponsive, so they are predicted to be not required for senescence but have a role in chlorophyll degradation for homeostasis. Meanwhile, the second group individuals including *Gm*CLH2 are phytohormone-inducible which makes them functional during senescence. Tang *et al.* (2004); Schnabel *et al.* (2005) ended up to a similar conclusion in his investigations on the *Ginkgo biloba* CLH.

Chlorophyllase activity determination indicated that silencing of *GmCLH1* is repressing the enzyme activity more than silencing *GmCLH2* suggesting that both genes do not contribute equally to the enzyme activity at least at a specific time point. My suggestion is that the *GmCLH2* is upregulated during senescence time, while during the regular growth and development time, the *GmCLH1* upregulated more. I also predict that they may vary in the localization of their encoded proteins in the cell.

When chlorophyll content was measured in the *CLH1* (SI) and *CLH2* (SI) plants, the results showed significant increase of leaves' chlorophyll content for both treatments in comparison with empty vector-inoculated plants. Furthermore, higher chlorophyll content was found in *CLH1* (SI) plants in comparison with *CLH2* (SI), which indicates that in the former treatment chlorophyll degradation has been altered further when compared to the later which means that the chlorophyll degradation mediated by either

gene is positively correlated with the chlorophyllase enzyme activity. Previous studies showed that chlorophyll content may or may not be correlated with CLH activity (Fang *et al.*, 1998; Ben-Yaakov *et al.*, 2006). When all grown in the shade, all treatments showed reduction in chlorophyll content than the concentrations determined in the light. It was demonstrated that leaves grown in the sun on the average contain more chlorophyll per leaf area unit than leaves grown in the shade of the same species (Lichtenthaler *et al.*, 1981). Interestingly, the least reduction in chlorophyll content was found in *CLH2* (SI) plants which indicates less suffering from or higher tolerance to low light conditions than the *CLH1* (SI) plants.

CHAPTER 5

5 *GmRLK3* plays different roles in response to necrotrophs or hemibiotrophs attacks in soybean

5.1 LITERATURE REVIEW

Plant Receptor-like kinases (*RLK*s) comprise a large gene family encoding a superfamily of proteins that share a common domain organization consisting of a ligand-binding N-terminal extracellular domain (ECD), a single-pass transmembrane (TM) domain, and a C-terminal intracellular serine/threonine kinase domain (KD) (Shpak *et al.*, 2004). The first plant RLK protein (ZmPK1) was identified in maize in 1990 followed by successful identification and cloning of novel RLKs, most of them were from *Arabidopsis thaliana* (Walker and Zhang, 1990; Moran and Walker, 1993; Walker, 1993). To date, nearly 610 RLKs have been identified in *Arabidopsis* alone, a number that represents about 2.5% of its protein coding genes and which forms a vast monophyletic protein superfamily (Shpak *et al.*, 2004). Moreover, many RLKs have been identified from other plant species such as rice (1131 members) (Lehti-Shiu *et al.*, 2009). This large number accompanied with diversity of plant RLKs suggest that they may be involved in the perception of a wide range of stimuli and in their speculated role in plant development. Based on the structure of their extracellular domain motifs - e.g. leucine-rich-repeats (LRRs), lysine-motif (LysM), lectin-domain or without any signal peptide (known as cytoplasmic kinases) - they are classified into 44 subfamilies (Shpak *et al.*, 2004; Lehti-Shiu *et al.*, 2009). Considering their large numbers, only a few RLKs have been functionally characterized and they seem to play roles in development, growth, plant

defense and symbiosis. This variation in roles is another feature that enabled scientists to classify RLKs based on function into two main groups. The first group regulates plant growth and development, represented by ERECTA (ER), which regulates the overall plant shape (Torii *et al.*, 1996), CLAVATA1 (CLV1) which regulates the development of the shoot apical meristem (SAM) (Schnabel *et al.*, 2005) and Brassinosteroid-Insensitive 1 (BRI1), which senses the plant steroid hormone brassinosteroid (BR) (Wrather *et al.*, 2001b). The second group comprises RLKs that mediate plant–microbe interactions such as symbiosis or defense reaction where they work as surface-exposed pattern-recognition receptors (PRRs) that mediate the recognition of highly conserved microbial/pathogen molecules termed microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) and recently termed DAMPs for damage-associated molecular patterns (Medzhitov and Janeway, 1997; Chisholm *et al.*, 2006). Examples of these RLKs are flagellin-sensing 2 (FLS2; Kim *et al.*, 2009a), the bacterial EF-Tu receptor (EFR) - EF-Tu are peptides derived from the bacterial flagellin and elongation factor Tu - (Zipfel *et al.*, 2006), and the chitin elicitor receptor kinase 1 (CERK1) that recognizes flagellin, EF-Tu or fungal chitin (Miya *et al.*, 2007), as well as several conserved proteins secreted from bacteria, fungi and oomycetes, and the polysaccharides chitin and beta-glucans (Postel *et al.*, 2010). The previously mentioned examples have concluded that, like other eukaryotic perception systems, RLKs at the plasma membrane confer onto the cell the ability to perceive external chemical signals and direct the translated information to the appropriate signaling pathway in the cell. The LRR-receptor kinase FLS2 protein is the earliest characterized PRR in *Arabidopsis* and the first receptor-like kinase involved in PAMP perception in plants. Almost all known plant RLKs are believed to bind their

corresponding ligands or perceive uncharacterized developmental or environmental signals on the plasma membrane (Torii *et al.*). The largest subfamily of RLK possesses LRRs as extracellular domain, there are over 230 of them in *Arabidopsis* only (Zhou *et al.*, 2007), and all of the plant LRR-RLKs analyzed to date possess Ser/Thr kinase activity. Loss of function mutation studies showed that some LRR-RLKs have roles in diverse physiological processes during plant growth. Specifically, the LRR domain is implicated in protein-protein interactions, therefore it is able to bind proteinaceous ligand or ligand complex (Kobe and Deisenhofer, 1994; Schnabel *et al.*, 2005). This is also true in animals where LRRs are found in various membrane proteins involved in pathogen recognition such as the Drosophila toll and mammalian toll-like receptor proteins (TLRs). In addition, LRR structural studies demonstrated that this domain is composed of tandem repeats consisting of 20 - 30 amino acids rich in leucine. Structure analysis of the LRR domain revealed a curved solenoid structure. The solenoid concave is particularly suitable for protein-protein interactions as PAMP molecule insertion within this loop - regardless of the mechanism - is critical for the recognition process (Bell *et al.*, 2003; Bella *et al.*, 2008). Furthermore, the majority of the plant resistance (*R*) genes, that confer resistance against pathogens through specific interactions with corresponding avirulence *(Avr)* genes of the pathogen, encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins which are localized in the cytosol or may be associated with the plasma membrane, which are similar in having the LRR domain but can be further divided into subgroups according to the domain at their amino terminus (Nimchuk *et al.*, 2003; Klink *et al.*, 2007). In the *Arabidopsis* (ecotype Columbia) genomic sequence, 149 NB-LRR–encoding genes have been identified (Meyers *et al.*, 2003). According to the gene-for-

gene hypothesis, R proteins play a role in the recognition of diverse pathogens (McHale *et al.,* 2006) but in contrast to mammals, no intracellular NB-LRR protein recognizing a PAMP has yet been identified in plants (Monaghan and Zipfel, 2012). A well-characterized member of the LRR-type of *R* genes is the rice (*Oryza sativa* L.) *Xa21* that confers resistance to *Xanthomonas oryzae* pv. *oryzae* secreting the corresponding effector avrXa2 (recently renamed Ax21; activator of XA21-mediated immunity) (Song *et al.,* 1995; Lee *et al.,* 2009). Based on the information that the gene encoding the brassinosteroid receptor BRI1 exhibits a domain organization comparable with XA21, the extracellular LRR plus the TM domain of *BRI1* were fused with the intracellular kinase domain of *Xa21*. The resultant chimeric protein activated defense responses in cultured rice cells after treatment with brassinosteroids (Yamamoto *et al.,* 2000; Shpak *et al.,* 2004). From these results, it has been concluded that different LRR domains mediate the recognition specificity of their distinct ligands while the protein kinase domains play a significant role in setting up the signal-transduction pathway and the subsequent activation of plant defense mechanisms (Ellis *et al.,* 1999; Yamamoto *et al.,* 2000; Dodds *et al.,* 2001; Romeis, 2001; Shen *et al.,* 2003). On the other hand, LRRs are predicted to play another important role as negative regulators that block inappropriate NB activation, but very little is known about its mechanism or the signaling events required for it, reviewed by (Zipfel *et al.,* 2006).

Other than preformed resistance barriers, it is now clear that there are two different types of plant defense mechanisms determined by two groups of proteins. The first group including RLKs, i.e. PRRs, transduces a defense signal pathway due to the direct extracellular detection of their ligands (i.e. PAMPs), therefore called PAMP-

triggered immunity (PTI), which constitutes a front-line defense against pathogens. It makes sense that PTI is most likely induced by necrotrophic pathogens, pathogens that obtain their nutrition from host dead cells. The enhanced biosynthesis of signaling molecules such as jasmonates (JA) or ethylene (ET) is a PTI indicator as well as means for activating other defense pathways and diverse factors collectively leading to suppression of the pathogen capability of causing more extensive cell death in the plant. On the other hand, the second group; R proteins, directly/indirectly succeed in detecting the corresponding effector molecules that are intracellularly secreted/injected by the pathogen hence initiate a signal transduction pathway leading to targeted programmed cell death as part of the hypersensitive response (HR), which restricts pathogen growth and/or deprives it of nutrition. The later mechanism of resistance so called effector-triggered immunity (ETI) is a second line of defense in plants which is, in contrast to the first, often induced by biotrophic pathogens, pathogens that obtain their nutrition from living host tissues, in a specific interaction and at the same time deactivates any PTI response (Hein *et al.*, 2009; Dodds and Rathjen, 2010; Eckardt, 2011). Taken all together, the common occurrence of the LRR domain in all plant PRRs and in the vast majority of the R proteins leaves no doubt about the essential role of this domain in pathogen detection.

Moreover, the recently identified ability of LRR-RLKs to bind more than one ligand (e.g., BRI can bind brassinolide; BL and systemin) and of a single LRR-RLK to interact with multiple receptors (e.g., BRI1-associated kinase 1; BAK1 with BRI1, FLS2 and other PRRs) suggests that the plant cell might utilize some inter-PRR (between PRR)

combinations to recognize numerous ligands and that more than one signal transduction pathway could be regulated by a single receptor (Zhou *et al.*, 2007).

In spite of the large number of *RLK*s in *Arabidopsis* and rice and their diverse vital roles discussed earlier, a few of them have been identified in soybean (*Glycine max L.*). In 2000, two soybean homologs of *the Arabidopsis CLAVATA1 (CLV1),* designated *GmCLV1A* and *GmCLV1B* were isolated. Functional analyses were not done for the genes at that point (Yamamoto *et al.*, 2000). In 2001, Yamamoto and Knap (2001) isolated three LRR-RLK genes, namely *GmRLK1, GmRLK2* and *GmRLK3. GmRLK2*, and *GmRLK3* share 98% of nucleotide sequence identity. The genes are homologs of *Arabidopsis thaliana AtRLK* and the deduced amino acid sequence of their encoded proteins have the same characteristics of *CLV1* protein. The nodule autoregulation receptor kinase *GmNARK (GmCLV1B)* was cloned and identified for the first time and its similarity to *Arabidopsis* CLAVATA1 was also characterized. Contrary to CLV1, Shpak *et al.* (2004) found that when GmNARK expressed, it plays a significant role in long-distance communication with nodules and lateral root primordial tissues. On the other hand, the cloning and functional analysis of three different LRR receptor-like protein kinases *rlpk1*, *2* and *3* revealed a role for *rplk1* and *rplk2* in regulating leaf senescence of soybean. A phylogentic analysis of their proteins showed that RLPK1 and RLPK2 share an independent branch while RLPK3 share common nodes with several RLKs known to have stress response functions (Zipfel *et al.*, 2006). Further studies using RNAi silencing of *rlpk2* suggested a potential role as a negative regulator of leaf functions (e.g. photosynthesis) and/or chloroplast structure (Zuo *et al.*, 2007). Another *LRR-RLK GmSARK* (*Glycine max* senescence-associated receptor-like kinase) was isolated and

identified as a regulator of leaf senescence in soybean (Zuo *et al.*, 2007). A cytoplasmic *RLK* was cloned from soybean and designated *GmRLCK* and phylogentic analysis suggests that it shares common ancestors with senescence-associated RLKs so it may also be involved in senescence (Zuo *et al.*, 2007).

Considering that previous studies on soybean *RLK*s demonstrated or infrequently predicted that they possess growth or development functions and rarely any of them dealt with responses to fungal pathogens, this work was designed to analyze the role of *GmRLK3* in the defense response of soybean plants to different oomycete/fungal pathogens aiming to provide, at least, clues about the signal pathway utilized/activated by *RLK3* in the plant.

5.2 RESULTS

5.2.1 Silencing of *GmRLK3* gene and phenotypic changes in silenced plants

For silencing of *GmRLK3* gene, a cDNA fragment of 258 bp based on the open reading frame (ORF) sequence was PCR-amplified and inserted into the *BamHI*/*MscI* sites of the BPMV vector. Plants infected with the VIGS recombinant vector pGG7R2-*GmRLK3*, referred to herein as *RLK3* (SI), were observed for any changes in their phenotypes. Vastly different than empty vector-inoculated plants, the leaves of *RLK3* (SI) plants were characterized by severe blistering and intense chlorotic mottling appearance (Figures 5.1 and 5.2). These severe symptoms were visulalized on all soybean cultivars tested including Essex, Williams, Harosoy and Harosoy 63. Only cv. Jack showed slghtly milder response when infected with *RLK3*(SI). Moreover, the leaves on the lower part of *RLK3* (SI) plants showed variably sized necrostic spots, which appeared starting at the V2 to V4 growth stage (Fig. 5.1). Except for the necrotic spots, this severe version of phenotypic changes is reminiscent of the phenotype of soybean plants silenced for omega-3 fatty acid desaturase (*GmFAD3*) gene using the BPMV VIGS system (Singh *et al.*, 2011).

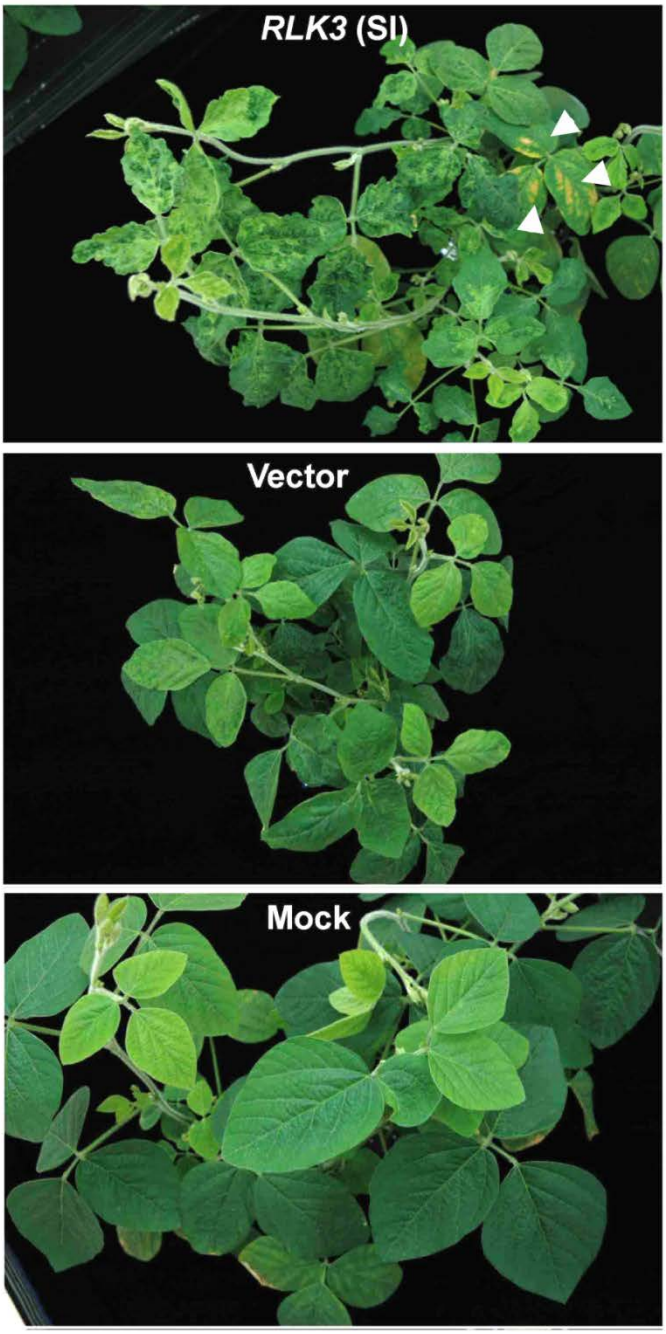


Fig. 5.1 Phenotypic changes in soybean plants silenced for *GmRLK3.* Soybean plants (cv. Essex) infected with the BPMV-*GmRLK3* silencing construct at V6 growth stage, showing severe mottling and blistering of leaves (upper panel) compared with empty vector-inoculated plants (middle pannel) and mock plants (lower pannel). White arrows in the upper pannel indicate necrotic areas on the lower leaves of the plant (see text).

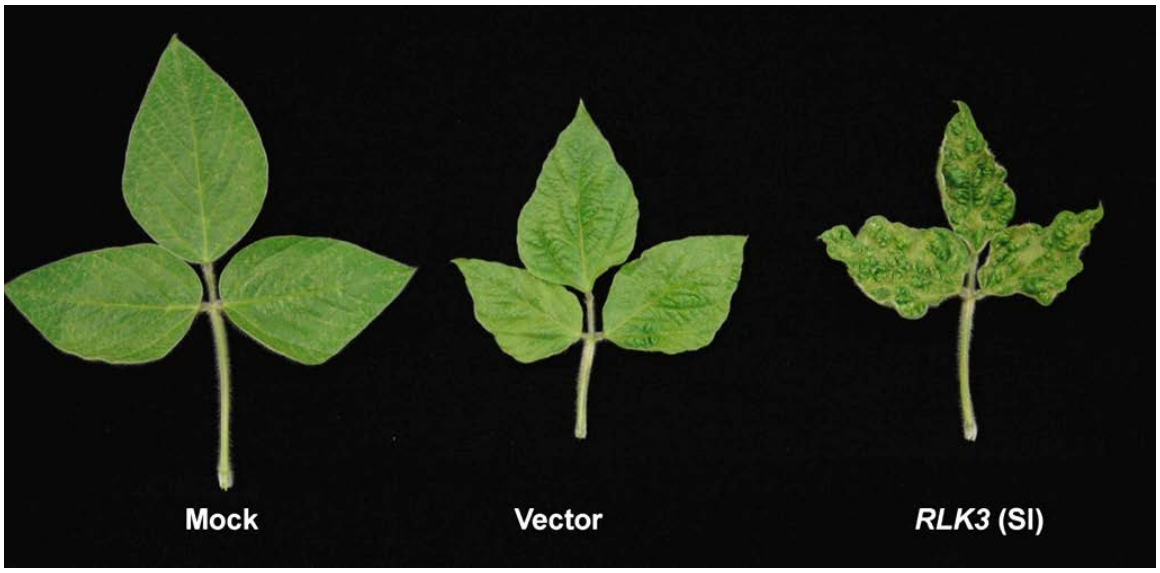


Fig. 5.2 Phenotypic changes in soybean trifoliolates of plants silenced for *GmRLK3.* Close up of detached 7th trifoliolates of soybean plants (cv. Harosoy 63) infected with the BPMV-*GmRLK3* silencing construct showing severe phenotypic changes on *RLK3* (SI) leaf compared to leaves of mock and empty vector-inoculated plants.

5.2.2 *GmRLK3* expression analysis in silenced plants

RT-PCR assay was used to examine the level of *GmRLK3* transcript in the *RLK3* (SI) plants compared to mock and empty vector control plants. Although bands of the predicted size were visualized clearly in mock and empty vector-treated plants after 30 RT-PCR cycles, no band corresponding to the *RLK3* fragment was detected in *RLK3* (SI) plants. This suggests highly effective silencing of the target gene (Fig. 5.3). The BPMV-VIGS vector was previously demonstrated to be highly efficient in silencing of diverse genes in soybean (Zhang and Ghabrial, 2006; Fu *et al.*, 2009; Selote and Kachroo, 2010; Diaz-Camino *et al.*, 2011; Singh *et al.*, 2011).

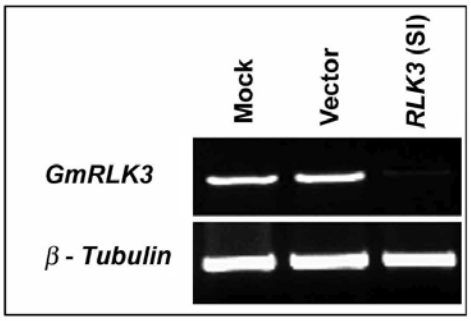


Fig. 5.3 Transcript level of *GmRLK3* in *RLK3* (SI) plants. RT-PCR analysis showing effect of VIGS-mediated silencing on transcript level of *GmRLK3.* In *RLK3* (SI) leaves, no detectable transcript was visualized following 30 cycles, whereas transcripts were clearly detected in mock as well as empty vector-inoculated plants. *β-tubulin* was amplified as an internal cDNA control.

5.2.3 Silencing of *GmRLK3* enhances soybean susceptibility to necrotrophic fungal pathogens

In this experiment, 2 similar sets of soybean (cv. Harosoy) detached leaves from *GmRLK3*-silenced plants, empty vector-inoculated plants and mock plants were used. Each set was subjected to artificial inoculation with the necrotrophic fungal pathogens *Alternaria tenuissima* or *Sclerotinia sclerotiorum*, the causal agents of Alternaria leaf spot and Sclerotinia stem rot of soybean, respectively. Leaves of *RLK3* (SI) responded similarly to both pathogens. In a short period of time (6 dpi), *A. tenuissima* was able to cause distinct symptoms on *RLK3* (SI) treatment before any symptoms started to appear on the controls (Fig. 5.4). The disease progress was characterized by extended maceration of the leaf tisuues and mycelial growth around the inoculation site. In case of *S.*

sclerotiorum, the entire leaf of *RLK3* (SI) turned into water soaked tissue by the end of the experiment whereas empty vector-inoculated and mock treatments were resistant (Fig. 5.5).

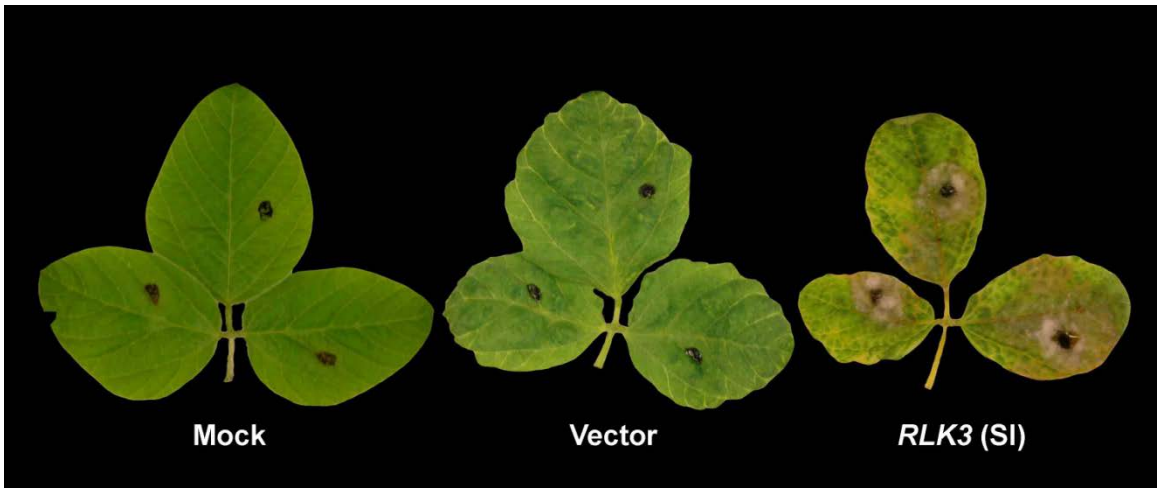


Fig. 5.4 Enhanced susceptibility to the necrotroph *Alternaria tenuissima* of plants silenced to *GmRLK3*, 6 dpi. Detached leaf bioassay showing representative *RLK3* (SI) trifoliolate leaf with enhanced susceptibility to the necrotroph *Alternaria tenuissima* 6 dpi.

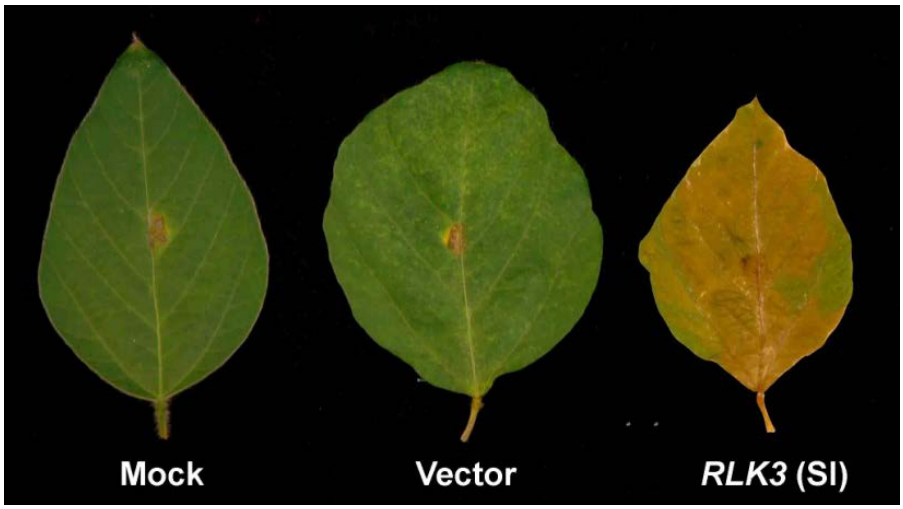


Fig. 5.5 Enhanced susceptibility to the necrotroph *Sclerotinia sclerotiorum* of plants silenced for *GmRLK3*, 14 dpi. Detached leaf bioassay showing complete maceration of a leaflet from an *RLK3* (SI) plant 14 days post *Sclerotinia sclerotiorum* inoculation. Leaflets from mock and empty vector-inoculated control are resistant to *S. sclerotiorum*.

5.2.4 Silencing of *GmRLK3* confers resistance against virulent *P. sojae* R3

In a separate experiment, intact soybean plants were used in a growth chamber to evaluate the response of *RLK3* (SI) plants to the hemibiotroph *P. sojae*, the specific oomycete causing root and stem rot of soybean. Due to the specificity of the pathogen races, it was essential to select the race and the corresponding cultivar according to the purpose of the experiment. To examine any possibility of resistance breaking, the incompatible cultivar-race; Williams 82-R3 combination was used. Negative results were obtained in this experiment as all treatments showed complete resistance to the pathogen. A new experiment was carried out using the compatible system Harosoy 63-R3. Interestingly, even at 10 days post inoculation, the *RLK3* (SI) plants showed a high resistance response comparable to the specific race resistance obtained in the first case. This resistance was characterized by restriction of the lesion to the inoculation site and maintaining complete rigidity of the stem even at the inoculation site (Fig. 5.6A). In contrast, mock and empty vector plants showed the typical symptoms of the compatible reaction, which was characterized by fast enlargment of the lesion along and surrounding the stem, gradual wilting then death of trifoliolate leaves and finally reached the apical shoot tip (Fig. 5.6A). To the end of the experiment, 100% of plants silenced for *GmRLK3*

survived, while low percentage of survivals was recorded for mock and empty vector-inoculated treatments (Fig. 5.6B).

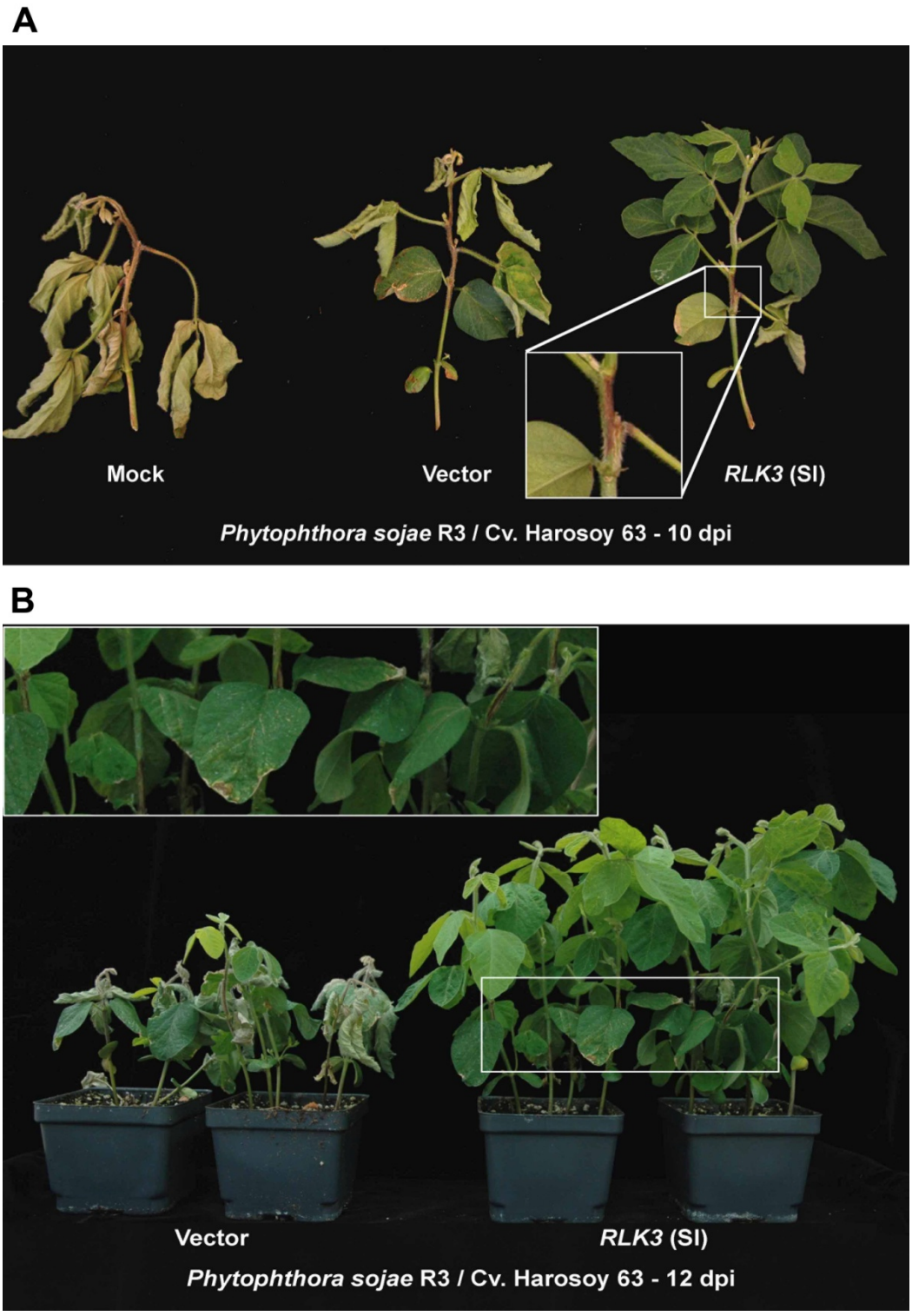


Fig. 5.6 Enhanced partial resistance to *P. sojae* in plants silenced to *GmRLK3*: Resistance phenotype and plant surviving 12 dpi. Results of stem inoculation with *P. sojae* R3 on soybean plants of cv. Harosoy 63. **A,** At 10 dpi, both mock and empty vector-inoculated plants showed susceptibility leading to wilt and death due to the expected compatible interaction between the cultivar and the pathogen race. The lesions on *RLK3* (SI) plants were restricted as shown in a close up photograph (see insert). **B,** Comparative survival rate of *P. sojae* R3-stem inoculated plants at 12 dpi. Two groups of soybean plants (cv. Harosoy 63), the group to the left side are empty vector-inoculated that show typical susceptibility to the pathogen race, while the plants silenced for *GmRLK3* (to the right) shows high resistance. The upper pannel is a close up of the plants encircled with a white rectangle on the *RLK3* (SI) plants to show appearance of the resistant phenotype.

5.2.5 Silencing *GmRLK3* induces *PR5a* and *GmICS* expression

In an attempt to determine if any of the well known signaling pathways is involved in the resistance response of *RLK3* (SI) plants to *P. sojae*, an RT-PCR assay was conducted for the expression of some *PR* genes that are previously known to be characteristic of the salycilic acid (SA)-triggered defense pathway or the jasmonic acid (JA)-triggered defense pathway. Transcript levels of *PR2*, *PR3*, *PR4* and *PR5a* were evaluated in leaves of *P. sojae* R3 infected plant leaves of all treatments using specific gene primers for 30 cycles. Visualization of bands clearly showed that *PR5a* was highly induced in *RLK3* (SI) whearas the transcript was barely detected in mock or empty

vector-inoculated plants (Fig. 5.8A). To learn more about the induced defense signaling pathways, *GmPAL1* (for phenylalanine ammonia lyase) and *GmICS* (for isochorismate synthase) were also examined for their expression in silenced plants and controls. The activity of each gene is known to be essential in two different pathways of SA biosynthesis. Although *PAL* was constitutively expressed in high amounts in all treatments *ICS* was expressed in relatively lower amounts in mock and vector compared to *RLK3* (SI) plants (Fig 5.8B).

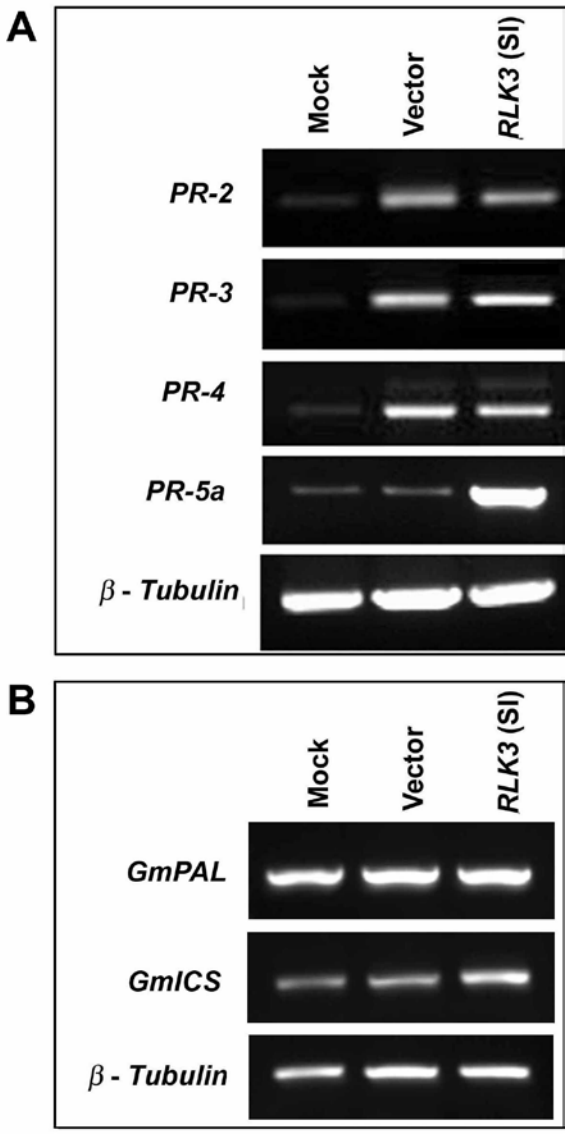


Fig. 5.7 Induction of *PR5a* and *GmICS* transcripts in *P.sojae*-inoculated plants silenced for *GmRLK3.* RT-PCR analysis of selected soybean genes. **A.** RT-PCR results showed *PR5a* transcript level in *RLK3* (SI) plants to be significantly higher than mock or empty vector-inoculated plants. RT-PCR assays were done using specific gene primers for 30 cycles. **B.** Following 35 RT-PCR cycles, transcripts of *GmPAL1* was constitutive in all treatments while *GmICS* was moderately induced in *RLK3* (SI). *β-tubulin* was amplified as an internal cDNA control. The analyses were done at least 3 times with reproducible results.

5.3 DISCUSSION

The frequent cloning and characteriztion of novel plant receptor like kinases (RLKs) helps to add new members to their large superfamily. Simple or complex functional studies of most of these genes of unknown functions eventually allow their proper classification either in the growth and development regulators group or in the plant-microbe interaction regulators group. In this study, generating a *GmRLK3*-BPMV VIGS construct was a useful tool towards understanding the mechanism of potential defense-related functions of this gene in soybean plants. Efficient silencing of the gene was confirmed using RT-PCR. Yamamoto and Knap (2001) identified an extracellular region consisting of an LRR domain in the cloned *GmRLK3*. Based on evolutionary perspective, LRR domains were predicted to be involved more than the kinases in pathogen recognition (Ruben *et al.*, 2006). Hence, in the current study, it was hypothesized that silencing *GmRLK3* will negatively affect plant resistance to pathogens. Results showed that silenced plants exhibited enhanced susceptibility to two fungal necrotrophs, namely *Alternaria tenuissima* and *Sclerotinia sclerotiorum*. In contrast, *RLK3*-silenced plants acquired resistance to the specific hemibiotrophic oomycete *Phytophthora sojae*.

Necrotrophic pathogens employ very destructive approaches in their parasitism causing cell death in their hosts revealing general symptoms varying from necrotic spots to rots (macerated tissues; Thomma *et al.*, 2001). In order for necrotrophs to obtain their nutrition from the host, they possess a machinery to produce cell wall degrading enzymes (CWDEs) and sometimes phytotoxins during pre-infection, infection and colonization stages (Zhang *et al.*, 2010). *Alternaria* spp. and *Sclerotinia* spp. are nectrophic fungal

pathogens. *A. tenuissima* produces β-1,3 glucanases, β-1,6 glucanses, proteases and mannanases (Jirků *et al.*, 1980) and *S. sclerotiorum* produces cellulases, pectinases, glucanases, glycosidases, xylanases, cutinases and proteases (Bolton *et al.*, 2005). In addition, production of nonhost-specific toxins was reported for both *Alternaria* and *Scelrotinia* genera (Scheffer, 1991). The released plant cell wall components owing to the act of CWDEs work as PAMPs that alert the immune system of the plant through the PRRs at the plasma membrane to induce a defense signaling pathway. The enhanced susceptibility shown by *RLK3* (SI) to *A. tenuissima* or *S. sclerotiorum* is suggested to be a direct result of disruption of the corresponding perception system as a result of the absence of *GmRLK3* expression; this implies that *GmRLK3* plays some role in signaling for PTI. Likewise, other RLKs were previously demonstrated to be responsible for the immune response to necrotrophic pathogens. In *Arabidopsis*, BAK1 works with other RLKs (e.g., FLS2 and EFR) in a PRR complex for PAMPs recognition to initiate a signal for PTI response (Chinchilla *et al.*, 2007). Therefore, loss of BAK1 results in increased susceptibility to the necrotrophic fungus *Alternaria brassicola* (Kemmerling *et al.*, 2007), the hemibiotrophic bacterium *Pseudomonas syringae* and the biotrophic oomycete *Hyaloperonospora arabidopsidis* (Kemmerling *et al.*, 2007; Roux *et al.*, 2011). Another well known RLKs such as WAK1 (for wall-associated kinase 1) that perceives the oligogalacturonides-confered resistance in transgenic plants overexpressing the same gene against the necrotroph *Botrytis cinerea* (Brutus *et al.*, 2010) and the LysM-RLK CERK1 mutation was found responsible for the attenuation of resistance of the *Arabidopsis* plants to *A. brassicicola* (Miya *et al.*, 2007). When the LRR-RLK ERECTA, a regulator for the development of aerial plant organs, was used to transform the

Arabidopsis plant accession L*er* which is susceptible to the bacterium *Ralstonia solanacearum*, ERECTA-transgenic L*er* managed to show resistance characterized by wilt reduction and bacterial growth inhibition (Godiard *et al.*, 2003). Furthermore, both LRR and kinase domains of ERECTA were found to be specifically required for the *Arabidopsis* resistance to the necrotrophic fungus *Plectosphaerella cucumerina*, a type of resistance that relies on three quantitative trait loci (QTLs) (Llorente *et al.*, 2005).

RLK3 (SI) plants were included in more experiments for testing plant response to pathogens. Different sets of *P. sojae* race-soybean cultivar were used to perform the experiments. These sets included the R3-Williams 82 incompatible set (produces resistance) and the R3-Harosoy 63 compatible set (produces susceptibility). Williams 82 carries the single resistance gene *Rps1k* that overcomes most of the identified *P. sojae* races including R3 (*Avr1a* and *Avr7* but lacks *Avr1k*), meanwhile R3 is cabable to overcome Harosy 63 resistance because this cultivar carries only *Rps1a* and *Rps7* (Roy *et al.*, 1997; Schmitthenner, 1999; Dorrance *et al.*, 2004).

Silencing *GmRLK3* in Williams 82 did not reverse or even attenuate the race-specific resistance response to R3. Surprisingly, silencing this particular gene in Harosoy 63 converted the absolute susceptibility to R3 to resistance. Based on a review of some studies on nucleotide binding (NB)-LRR proteins, Zipfel *et al.* (2006) predicted that the LRR domains might play as negative regulators of their protein-mediated resistance as they block any inappropriate activation of NB domains and in turn the induction of ETI. However, they could not explain the utilized mechanism or identify the signaling pathway. Even by considering the fact that *Gm*RLK3 comprises LRR domain, the previous hypothesis cannot explain the herein resistance of *RLK3* (SI) plants to *P. sojae*

because the *Gm*RLK3, in contrast to NB-LRR proteins, is a transmembrane RLK protein with an extracellular LRR. On the other hand, the Pto protein kinase was found to positively regulate the Prf (NB-LRR protein)-mediated resistance to *Pseudomonas syringae* pv. *tomato* in tomato by direct recognition of and interaction with the pathogen effector AvrPto. Therefore, AvrPto effector is able to activate the Prf only when the later is in a protein complex of Prf-Pto (Tang *et al.*, 1996; Mucyn *et al.*, 2006). Although that case is compared to this study in dealing with specific resistance and the same *R* gene class (NB-LRR), but the case here is indeed different because silencing the RLK gene positively regulated the defense response.

Close examination of this resistance confirms that it is a partial resistance type to *P. sojae*, leads to considering the idea that *RLK3* silencing might induce other different host genes that are cabable of triggering defense pathways leading to partial resistance response. Zhang *et al.* (2011) reported the induction of a number of candidate genes with potential functions in regulating the expression of defense-related pathways for resistance to *P. sojae* based on a microarray study from both the pathogen and the host during *P. sojae*-soybean interaction.

Based on the *PR* gene expression analysis, the large increase in the transcript level of *PR5a* that appeared only in the *GmRLK3*-silenced plants infected with *P. sojae* R3 might explain the conferred resistance in this treatment. *PR* genes generally are thought to be molecular markers for the systemic acquired resistance (SAR) (Durrant and Dong, 2004). *PR1, PR2* and *PR5* genes are known to be induced by SA, while *PR3, PR4* and *PR12* genes are usually being upregulated by JA or ET (Thomma *et al.*, 2001). In a previous study, *PR1, PR2* and *PR5* expression have shown intensive induction of their

transcript levels after heat shock treatment as a SAR inducer and the heat-challenged *Arabidopsis* plants showed resistance (90% reduction of bacterial growth) to the virulent strain DC3000 of the hemobiotroph *Pseudomonas syringae* pv. *tomato* (Kusajima *et al.*, 2012). PR5 protein family comprise thaumatins and thaumatin-like proteins (osmotins) have been demonstrated to inhibit the growth of fungi and the fungi/oomycetes were found to be either osmotin-susceptible or osmotin-resistant (Monteiro *et al.*, 2003). *In vitro*, their mechanism was demonstrated in causing hyphal rupture or increasing plasma membrane permeability which leads to leakage of cytoplasmic material (Vigers *et al.*, 1992; Niderman *et al.*, 1995; Anžlovar and Dermastia, 2003; Klink *et al.*, 2010) and their antagonistic activity against oomycetes has been reviewed (Van Loon *et al.*, 2006). Another mechanism of the PR5 is based on their previously demonstrated ability to bind some fungal cell surface components such as phosphomannoproteins of yeast and β-1,3 glucans (Salzman *et al.*, 2004). This attribute might set them up as PRRs, exactly like RLKs, that sense and interact with fungal PAMPs.

Extensive plant molecular and biochemical studies are in agreement that SA defense pathways are commonly associated with resistance to biotrophic pathogens while necrotrophic pathogens generally enhance JA/ET defense pathways, and that SA and JA/ET defense pathways are mutually antagonistic (Thomma *et al.*, 2001; Kunkel and Brooks, 2002; Turner *et al.*, 2002; Rojo *et al.*, 2003; Zipfel *et al.*, 2006; Adie *et al.*, 2007; Kliebenstein and Rowe, 2008; Klink *et al.*, 2010). SA can be synthesized through two different main synthesis pathways in plants, one is from phenylalanine catalyzed by PAL, while the other utilizes ICS in catalyzing chorismate. Recently, it has been demonstrated that during plant response to pathogen infection, the majority of accumulated SA is

synthesized from chorismate (Wildermuth *et al.*, 2001). The induction of *GmICS* gene expression supports the same explanation. In contrast, overexpression of CRK13 in *Arabidopsis* caused induction for *PR1*, *PR5*, *ICS1*, SA accumulation and regulation of defense (Acharya *et al.*, 2007). This inconsistency in results may be due to structure of CRK13 which is a cysteine-rich receptor like kinase and, in addition, contains DUF26 motif in the extracellular domain. Nawrath and Métraux (1999) found that the *Arabidopsis* SA induction-deficient (*sid*) mutant which do not accumulate SA as a result of pathogen infection, either *Pseudomonas syringae* or *Hyaloperonospora parasitica*, this mutant still show induction of *PR2* and *PR5*, According to theses results, it is predicted that *ICS* induction in the current study might be a secondary result (working downstream and not upstream) in the defense signaling pathway. *PAL* was constitutively expressed at high levels in all treatments. The high level recorded even in mock plants is consistent with results of Moy *et al.* (2004) who found that *PAL* is one of the induced genes in the host upon *P. sojae* infection.

In agreement with the current results, *bak1* null mutation in *Arabidopsis* that shows susceptibility to necrotrophic pathogens was found to be extremely resistant to biotrophic pathogens, a phenomenon that was explained by the absence of cell death phenotype (Dodds and Rathjen, 2010). The role of BAK1 was discussed above in perceiving pathogen PAMPs individually or in protein complexes to trigger PTI. Taken altogether, with the highly induced susceptibility in the silenced plants to necrotrophs, it is suggested that silencing of *GmRLK3* confers partial resistance against the virulent race of *P. sojae* possibly as consequence of the induction of *PR5*; this in turn predicts a negative regulatory role of *GmRLK3* for SAR. Correspondingly, the mitogen-activated

protein kinase (MAPK) in *Arabidopsis* was known from biochemical investigations to regulate different functions in the plant including immunity as a response to diverse stimuli, but the *mpk4* mutant line showed enhanced resistance to a virulent race of the *P. syringae* bacterium as well as to the *P. parasitica* fungus. The resistance was characterized by induction of some genes' expression known to be markers of SAR and repression of JA-dependent gene expression. Therefore, the results were broadly interpreted that MPK4 plays as a negative regulator in the plant defense (reviewed by Romeis (2001).

It is also concluded from the current investigation that *GmRLK3*, in contrast to the formerly mentioned function, works regularly as expected for the majority of LRR-RLKs, as PRR that senses and interacts with PAMPs/DAMPs to enhance the plant immunity to different pathogens through the PTI mechanism. Shpak *et al.* (2004) concluded that it is likely for a single pattern-recognition receptor (PRR) to mediate more than one signal transduction pathway. As many other plant genes, it is concluded that *GmRLK3* has been assigned two different functions in signaling network.

APPENDIX I

ABBREVIATIONS

ABA	Abscisic acid
aPDA	Acidified potato dextrose agar
ATP	Adenosine triphosphate
Avr	Avirulence gene
Ax21	*Xanthomonas oryzae* pv. *oryzae* effector
BAK1	BRI1-associated kinase 1
BPMV	Bean pod mottle virus
BR	Brassinosteroid
BRI1	Brassinosteroid-insensitive 1
CC-NBS–LRR	Coiled coil-nucleotide binding site-leucine rich repeat
cDNA	Complimentary DNA
CERK1	Chitin elicitor receptor kinase 1
Chl	Chlorophyll
Chlase	Chlorophyllase
Chlide	Chlorophyllide
CLH	Chlorophyllase
CLV1	CLAVATA1
coi1	Coronataine-insensetive 1 mutant
COR	Coronatine
CRK13	Cysteine-rich receptor like kinase 13
cv.	Cultivar
CWDE	Cell wall degrading enzymes
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
ECD	Extracellular domain
EF-Tu	Elongation factor Tu
ELISA	enzyme-linked immunosorbent assay
ET	Ethylene
ETI	Effector-triggered immunity
FAD3	Omega-3 fatty acid desaturase gene
FAO	The Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FLS2	Flagellin-sensing 2
FvTOX1	*Fusarium virguliforme* phytotoxin
GA_3	Gibberellic acid
Gm	*Glycine max*
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HEL	Hevein-like protein
HR	Hypersensitive response
IAA	Indole acetic acid

ICS	Isochorismate synthase gene
ITS	Internal transcribed spacer
JA	Jasmonic acid
KD	Kinase domain
LDL	low-density lipoprotein
LRR	Leucine rich repeat
LysM	Lysine motif
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MCP	Methylecyclopropene
MeJA	Methyl jasmonate
N	Tobacco resistance gene to tobacco mosaic virus
NARK	Nodule autoregulation receptor kinase gene
NB	Nucleotide binding
NBS	Nucleotide binding site
NCCs	Nonfluorescent chlorophyll catabolites
OD	Optical density
OE	Overexpressing
ORF	Open reading frame
PAL	Phenylalanine ammonia lyase gene
PAMP	Pathogen-associated molecular pattern
PAR	Photosynthetically active radiation
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
P-Loop NTPase	Phosphate-binding loop Nucleoside Triphosphatase
PR	Pathogenesis related gene
PRR	Pattern-recognition receptor
PTI	PAMP-triggered immunity
Pto	Tomato resistance protein to *Pseudomonas syringae* pv. *tomato*
pv.	Pathovar
QTL	Quantitative trait loci
R	Resistance gene
RLK	Receptor-like-kinase
RLPK	Receptor-like protein kinase
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RPP13	Recognition for *Peronospora parasitica*
Rps	Resistance to *Phytophthora sojae*
RPS	Resistance to *Pseudomonas syringae*
RT-PCR	Reverse transcription-polymerase chain reaction
S/T	Serine or Threonine
SA	Salicylic acid
SAM	Shoot apical meristem
SAR	systemic acquired resistance
SARK	Senescence-associated receptor-like kinase

SDS	Sudden death syndrome
SI	Silenced
sid	SA induction-deficient mutant
TLR	Toll-like receptor
TM	Transmembrane domain
TMV	Tobacco mosaic virus
V plant	Empty BPMV vector-inoculated plant
V8A	V8 agar
VIGS	Virus-induced gene silencing
WAK1	Wall-associated kinase 1
XA21	Rice resistance protein for *Xanthomonas oryzae* pv. *oryzae*

APPENDIX II

Molecular Identification of an Isolate of *Alternaria* sp.

- **Nucleotide sequence of the internal transcribed spacer (ITS) of *Alternaria* sp. isolated from soybean seedlings in Lexington, KY**

  ACAATTTGGAGGCGGGCTGGACCTCTCGGGGTTACAGCCTTGCTG
  AATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGT
  TCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCA
  GCGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCTC
  TTGGTTCTGGCATCGATGAAGAACGCAGC

- **BLAST matching result:**

  gb|HM467832.1| *Alternaria tenuissima* strain MG3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence Length=544.

  Score = 377 bits (204), Expect = 5e-101

  Identities = 209/211 (**99%**), Gaps = 1/211 (0%)

  Strand=Plus/Plus

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