8-12-2016

The Defense Response of Glycine Max to its Major Parasitic Nematode Pathogen Heterodera Glycines

Shankar R. Pant

The defense response of *Glycine max* to its major parasitic nematode pathogen

Heterodera glycines

By

Shankar Raj Pant

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

August 2016

The defense response of *Glycine max* to its major parasitic nematode pathogen

Heterodera glycines

By

Shankar Raj Pant

Approved:

_____

Vincent Klink
(Major Professor)

_____

Gary W. Lawrence
(Co-Major Professor)

_____

Donna M. Gordon
(Committee Member)

_____

Shien Lu
(Committee Member)

_____

Mark E. Welch
(Graduate Coordinator)

_____

Rick Travis
Interim Dean
College of Arts & Sciences

Name: Shankar Raj Pant

Date of Degree: August 12, 2016

Institution: Mississippi State University

Major Field: Biological Sciences

Major Professor: Dr. Vincent Klink

Title of Study: The defense response of *Glycine max* to its major parasitic nematode pathogen *Heterodera glycines*

Pages in Study 162

Candidate for Degree of Doctor of Philosophy

Heterodera glycines, soybean cyst nematode (SCN) causes more than one billion dollars soyben production loss in the U.S. annually. SCN is an obligate parasite of specialized feeding cells within the host root known as syncytium. The SCN resistance genes and signaling pathways in soybean have not been fully characterized. Gene expression analysis in syncytium from compatible and incompatible interactions identified candidate genes that might involve conferring resistance to SCN. This dissertation aimed to investigate the biological functions of the candidate resistance genes to confirm the roles of these genes in resistance to SCN. The study demonstrated a role of syntaxin 31-like genes (Gm-SYP38) in resistance to SCN. Overexpression of Gm-SYP38 induced the transcriptional activity of the cytoplasmic receptor-like kinase *BOTRYTIS INDUCED KINASE 1* (Gm-BIK1-6). Overexpression of Gm-BIK1-6 rescued the resistant phenotype. In contrast, Gm-BIK1-6 RNAi increased parasitism. In another experiment, the expression of a *Glycine max* homolog of *LESION SIMULATING DISEASE1* (*LSD1*) resulted in the transcriptional activation of *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) and *NONEXPRESSOR OF PR1* (*NPR1*), that function in salicylic acid (SA)

signaling, implicating the involvement of the antiapoptotic, environmental response gene *LESION SIMULATING DISEASE1 (LSD1)* in defense that is demonstrated here. The study also investigated the role of SNARE components (genes functioning in membrane fusion) in resistance to SCN. Experiments showed that SNARE functions in concert with a beta-glucosidase having homology to *PEN2* and an ATP binding cassette transporter having homology to *PEN3.* This study provides novel information for the genetic improvement of soybean for enhanced disease resistance.

DEDICATION

This dissertation dedicated to my grandfather Moti Ram Pant for his support, love and encouragement to continue my education.

ACKNOWLEDGEMENTS

I would like to express my deep respect and gratitude to my advisor, Dr. Vincent Klink, for his meticulous guidance and constant encouragement to be an independent researcher with an attitude toward strenuous research efforts. I am much thankful to Dr. Klink for always being there for me when I needed help, and for making this a challenging but a rewarding Ph. D.

I would like to express my sincere gratitude to my co-advisor Dr. Gary Lawrence for graciously giving his precious time and guidance to carry out this research throughout my graduate career. I thank him wholeheartedly for giving insight of nematology and truly encouraging me to develop into a good researcher. I find myself indebted to my advisory committee Dr. Donna Gordon and Dr. Shien Lu for giving constructive advices and encouragement at all the stages of my work which helped in the successful completion of my work. I am especially grateful Dr. Gary Ervin and Dr. March Welch for being immensely helpful to carry out this study.

My doctoral research has been carried out under the auspices of many individuals. I would like to acknowledge my former and present lab members Dr. Aparna Krishnavajhala, Bisho Ram Lawaju, Brant McNeece Jillian Harris, Jian Jiang, Keshav Sharma, Prakash M. Niruala, Dr. Prachi Matsye, Tineka Burkhead and Weasam Adnan Radhi Aljaafri for supporting me to achieve my academic and research goals as well as for enriching my life with indelible memories. I am also thankful to Yi Xiu Pinnix (Jan)

for her help to carry out this research in green house. I am especially thankful to undergraduate student workers and volunteers for their invaluable assistance. I am deeply indebted to my parents and family for all that you have done. I have no words to express my gratitude for my mother for her unconditional love who stood by me in thick and thin. No acknowledgement would be complete without giving thanks to my loving wife Binita for giving the strength and reassurance to tackle each and every challenge.

I would also like to thank all my teachers for making me capable of pursuing higher studies. My sincere gratitude is extended to the Department of Biological Sciences, Mississippi Agricultural and Forestry Experimental Station and Mississippi Soybean Promotion Board for providing financial resources for this research. Finally, thank you to all of my friends, faculty and staffs in Department of Biological Sciences, MSU.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Soybean Cyst Nematode (SCN)

The SCN is the most devastating pathogen of soybean, causing 7-10 % production loss annually worldwide (Sinclair and Backman, 1989; Pratt and Wrather, 1998; Wrather and Koenning, 2006). SCN was first identified in Japan in 1881 (Schmitt and Noel, 1984). It was first reported in 1954 in U.S. in North Carolina (Winstead *et al.* 1955). In a few years, SCN had spread to several other states, including Mississippi. It was first reported in Mississippi in 1957 (Spears, 1957). Recent data shows that SCN has infected almost all the soybean production areas in the U.S. (Wrather *et al.* 2001). SCN is considered an invasive species in the U.S. and it causes more losses than rest of the soybean pathogens combined (Wrather *et al.* 2001; Wrather and Koenning, 2006).

SCN has complex genetic diversity which contributes to its ability to infect several legume and non-legume species (Golden *et al.* 1970; Riggs and Schmitt, 1988, 1991; Niblack *et al.* 2002). In order to separate major genetic groups of SCN population for host compatibility within species, nematologists had performed race test for SCN populations based on relative development of SCN females on four soybean plant introduction (PI) lines *G. max*[Pickett/PI 548988], *G. max*[Peking/PI 548402], *G. max*[PI 88788] and *G. max*[PI 90763] which classified SCN into four races (Golden *et al.* 1970). Immediately, the four race scheme was found to be inadequate to describe existing complex SCN genetic

diversity in various soybean production areas (Miller, 1970; Epps and Duclos 1970; Niblack *et al.* 2002). To address this problem, the four race scheme of SCN classification was expanded into a 16 race scheme by Riggs and Schmitt (1988). This classification was further modified into 'HG (*Heterodera glycines*) Type' scheme which describes SCN population variation based on their ability to reproduce on a set of seven soybean indicator lines (Niblack *et al.* 2002; Niblack and Riggs 2004).

Life cycle of SCN

Heterodera glycines is an obligate endoparasite of *G. max* with a life cycle of approximately 30 days, depending on temperature, soil, nutrition, and geographical location (Lauritis *et al.* 1983; Koenning, 2004). Eggs are encased in a cyst. The cyst is a hardened structure composed of the carcass of the female that encloses the eggs. The first hatching takes place inside the cyst as a result the second stage pre-infective juveniles (pi-J2s) emerge from the cyst and migrate toward and burrow into the root. The infective juveniles (i-J2s) then burrow through the cortex toward the root stele (Noel, 2004). Migration is accomplished by using a tubular mouthpiece called a stylet, to slice through the cells. When *H. glycines* reaches a targeted cell (typically a pericycle cell or neighboring root cell), secretory proteins released from the stylet are secreted into the targeted cell. At this point, the nematodes are parasitic juveniles (p-J2). The secretory proteins are synthesized in esophageal and/or sub-ventral gland cells with each gland cell providing certain substances at specific times during parasitism. The secretory proteins alter the physiology and metabolism of invaded and surrounding host cells, to remodel the cells for syncytium formation (Davis *et al.*, 2000). Shortly after, the infected root cell fuses with neighboring cells by breakdown of cell wall material at or near the

plasmodesmata. The fusion of the cell wall results in the free flow of cytoplasm, organelles and even nuclei in and out of former cellular boundaries. The repeated cell fusion events produce a multinucleate giant cell known as a syncytium. The mature syncytium, which acts as a nurse cell contains approximately 200 cells sharing a common cytoplasm (Jones and Northcote 1972; Jones, 1981). The p-J2 nematodes which develop into males then feed for several days. During feeding, the males become sedentary until the end of their J3 stage. The males then stop feeding and subsequently molt into vermiform J4 males. The J4 males remain encased within the J2 and J3 cuticles until they burrow out of the cuticles and root in preparation for mating. In contrast to the males, the p-J2s that are destined to develop into females become and remain sedentary during and after the establishment of their nurse cell. The female nematodes increase in size and become pear-shaped. The process is followed by J3 and J4 molts. After J4 molts, the posterior of the female will erupt out the root epidermis. By having the female posterior outside of the root boundary, the males that are living in the soil gain access for copulation. After copulation, the adult females will keep growing while it lays eggs internally. However, the female will also secrete some of her eggs in a gelatinous matrix outside her body. As the life cycle ends, the color of the female changes from a creamy white to yellow-tan, indicating sign of mortality of the female. However, the eggs within its carcass remain viable. The SCN can complete several life cycles during the soybean production season and rapidly infest the soybean field with cysts. The cyst can remain dormant in the field for up to 9 years (Inagaki and Tsutsumi 1971).

Cytological reactions during resistance

Several cytological changes have been observed after the initiation of infection by SCN. The celluar reaction to SCN infection can be divided into 2 phases (Ross, 1958; Endo, 1964, 1965, 1991; Riggs *et al.* 1973; Kim *et al.* 1987; Mahalingam and Skorpska 1996). Phase 1 is an early cellular response of SCN infection and is similar in both resistant and susceptible genotypes. The early events of SCN infection leading to syncytium formation include hypertrophy, dissolution of cell walls, dense cytoplasm, and enlargement of nuclei, ER and ribosome (Endo, 1964, 1965; Riggs *et al.* 1973; Kim *et al.* 1987; Kim and Riggs 1992; Mahalingam and Skorpska 1996). The enlargement of nuclei and dense ER and ribosome content indicates an increase in gene expression and protein synthesis, as a result of manipulation of host cell physiology by the nematode to obtain nutrition.

The susceptible and resistant reaction varies in phase 2 of SCN parasitism. The major events of susceptible reaction in phase 2 are hypertrophy of nuclei and nucleolus, reduction and dissolution of vacuole and cell wall fusion (Endo and Veech 1970; Gipson *et al.* 1971; Jones and Northcote 1972; Riggs *et al.* 1973; Jones 1981). The reduction and dissolution of vacuole indicates the events of membrane fusion and maintenance in parasitized cells. In contrast, resistant reaction in phase 2 varies depending upon the soybean genotype being tested. A number of studies have been done to characterize defense response in several resistant genotypes. Based on the similarity and difference in cellular defense response against SCN parasitism, soybean genotypes have been categorized into *G. max*$_{[\text{Peking/PI 548402}]}$ and *G. max*$_{[\text{PI 88788}]}$ -types of defense responses (Colgrove and Niblack 2008). The *G. max*$_{[\text{Peking/PI 548402}]}$ type of defense response is

characterized by the development of a necrotic layer that surrounds the head of the nematode, followed by necrosis of initial parasitized cells, separating the syncytium from its surrounding cells (Kim *et al.* 1987; Endo, 1991). In contrast, the *G. max*[PI 88788] -type of defense response involves necrosis of initial parasitized cells. However, the necrotic layer that surrounds the head of the nematode is absent. The *G. max*[Peking/PI 548402] -type of defense response is potent and rapid in which most of the nematodes die at the parasitic second juvenile (p-J2) stage (Colgrove and Niblack 2008). In contrast, the *G. max*[PI 88788] -type of defense response is potent but prolonged in which nematodes die at the J3 or J4 stage (Kim *et al.* 1987; Colgrove and Niblack 2008). In both types of defense response, the syncytium eventually collapses to prevent the SCN from completing its life cycle (Endo, 1965; Riggs *et al.* 1972; Kim *et al.* 1987).

Another difference in mode of defense response between *G. max*[PI 88788] and *G. max*[Peking/PI 548402] is cell wall apposition (CWA). CWA is a physical and chemical barrier to cell penetration by pathogen (Aist *et al.* 1976, Schmelzer, 2002; An *et al.* 2006a, 2006b; Hardham *et al.* 2008). The CWA type of defense is found in *G. max*[Peking/PI 548402] and *G. max*[PI 437654] genotypes but is lacking in *G. max*[PI 88788] during pathogen invasion (Mahalingam and Skorpska 1996). The molecular mechanism of CWA formation and its role in defense response is not fully known. The major constituents of CWA are lignin, suberin, chitin, and pectin which are synthesized via the phenylpropanoid pathway (Bhuiyan *et al.* 2007). Klink *et al.* (2007b, 2009) found elevated transcript level of genes of monolignol biosynthesis (phenylpropanoid pathway) including phenylalanine ammonia lyase (PAL), caffeic acid O-methyltransferase (CAOMT), caffeoyl-CoA methyltransferase (CCoAMT), and cinnamyl alcohol dehydrogenase (CAD) during

defense against SCN for the cells in the syncytium. The CWA formation involves the aggregation of subcellular components at the site of infection. The process is further evident by the finding of polarization of actin at the site of infection (Klink *et al.* 2007b, 2009). The CWA formation was observed in several plant pathogen interaction studies. CWA formation was reported in plant infected with fungi (Aist 1976; Hückelhoven and Panstruga 2011), in *G. max*[Peking/PI 548402] and *G. max*[PI 437654] by SCN (Kim *et al.* 1987; Mahalingham and Skorupska 1996) and in barley by *Blumeria graminis* (Bohlenius *et al.* 2010). A number of studies in the cereal-powdery mildew patho-system found the formation of CWA which was a dome-shaped cell wall apposition established by epidermal cell between the cell wall and cell membrane during fungal invasion (Hückelhoven and Panstruga 2011; Nielsen *et al.* 2012).

Genetics of soybean resistance to SCN

Since 1898, the USDA National Plant Germplasm System has been collecting soybean accessions from all over the world. Out of 20,000 publicly available PIs, screening of 5,800 soybean accessions has led to the identification of three major genetic sources for SCN resistance genes in the *G. max* accessions; *G. max*[Peking/PI 548402], *G. max*[PI 88788], and *G. max*[PI 437654] (Ross and Brim 1957; Concibido *et al.* 2004). Currently, *G. max*[Peking/PI 548402] and *G. max*[PI 88788] germplasm are present in more than 97% of all commercial cultivars in the U.S. (Concibido *et al.* 2004). Efforts have been made to map SCN resistance genes and SCN resistance quantitative trait loci (QTLs) have been identified from a soybean germplasm. Studies have identified QTLs that map to 17 linkage groups. *G. max*[Peking/PI 548402] has at least nine and *G. max*[PI 88788] has at least five QTLs (Concibido *et al.* 2004). Genetic analyses identified three recessive resistance loci

rhg1, *rhg2,* and *rhg3* (Caldwell *et al.* 1960) and two dominant resistance loci *Rhg4* (Matson and Williams 1965) and *Rhg5* (Rao-Arelli, 1994). *rhg1*, *rhg2,* and *Rhg4* loci are found in genotype *G. max*[Peking/PI 548402], *G. max*[PI 88788], and *G. max*[PI 437654]. The *rhg3* locus found in *G. max*[Peking/PI 548402] while *Rhg5* found in *G. max*[PI 437654] and *G. max*[PI 88788]. The *rhg1* is the most widely studied locus. *Rhg1* is found on linkage group 4 on chromosome 18 and is an important locus that confers the resistance to SCN. The dominant *Rhg4* locus is in linkage group A2 and is located on chromosome 8. The genes present in the *rhg1* and *Rhg4* loci have recently been identified (Matsye *et al.* 2012; Cook *et al.* 2012; Liu *et al.* 2012). The metabolic networks of these defense genes and role in resistance are not understood (Liu *et al.* 2012) and therefore, warrant further investigation.

Gene expression in soybean during SCN parasitism

Several gene expression studies have been carried out in both compatible and non-compatible interactions to understand how soybean responds to the SCN infection (Klink *et al.* 2005; Alkharouf *et al.* 2006; Ithal *et al.* 2007; Klink *et al.* 2007b). Alkharouf *et al.* (2006) identified the defense related genes from whole infected soybean root collected 6 days post infection (prior to feeding site selection) during susceptible reaction. These gene included Kunitz trypsin inhibitor (KTI), germin, peroxidase, phospholipase D, 12-oxyphytodienoate reductase (OPR), pathogenesis related-1 (PR1), phospholipase C, lipoxygenase, WRKY6 transcription factor and calmodulin. Ithal *et al. (*2007) also reported similar lists of defense genes expressed in other susceptible soybean genotypes.

The identification of gene expression within syncytium is challenging, as it requires physical isolation of syncytia undergoing parasitism during SCN infection. The physical isolation of syncytia was first described by Klink *et al.* (2005). In this experiment the syncytia were precisely isolated using laser capture microdissection (LCM) technique at different time points. The isolated syncytia were used to construct cDNA libraries, cloning and sequencing full length genes, making probes for *in situ* hybridization, quantitative PCR (qPCR) and immunocytochemistry (Klink *et al.* 2005). This experiment provides the basis for genome wide gene expression analysis.

Klink *et al.* (2007b) examined gene expression profiles of both susceptible and resistance reaction in same genotype *G. max*[Peking/PI 548402], to avoid error due to difference in genotype or complication caused by mutant during analysis. The experiment used *H. glycines*[NL1-RHg/HG-type 7] (also called race 3 [*H. glycines*[NL1-RHg/HG-type 7/race 3]]) to study resistant reaction (*G. max*[Peking/PI 548402] is resistant to *H. glycines*[NL1-RHg/HG-type 7/race 3]) and *H. glycines*[TN8/HG-type 1.3.6.7] (also called race 14 [*H. glycines*[TN8/HG-type 1.3.6.7/race 14]]) to study susceptible reaction (Peking is susceptible *H. glycines*[TN8/HG-type 1.3.6.7/race 14]). The experiment provided a unique opportunity for direct comparison of genes expression in resistant reaction with those expressed during susceptible reactions in syncytium of same genotype. The study showed some genes are highly expressed during susceptible reaction compared to resistance reaction. The highly expressed genes during early stage of infections in susceptible reactions included: expansin, peroxidase, plasma membrane intrinsic protein 1C (PIP1C), germin-like protein (GER) 1, β-Ig-H3 domain containing protein, chorismate mutase, 4-coumarate CoA ligase family protein, trans-ketolase, cytochrome P450, peroxidase, metallo-proteinase, matrixin family protein, and a lipid

transfer protein (LTP). Interestingly, the expression of these genes was supressed during resistance reaction. The genes which expression was highly induced during resistance reaction are lipoxygenase-9, lipoxygenase-4, the *EARLY-RESPONSIVE TO DEHYDRATION* 2 gene and the *GENERAL REGULATORY FACTOR* 2. The study also found induced expression of genes related to phenylpropanoid pathway, the phenylalanine ammonia lyase, chalcone isomerase, isoflavone reductase, cinnamoyl-CoA reductase, and caffeic acid O-methyltransferase.

A number of studies demostratred that vesicular transport machinery protein component known as syntaxin was involve in the formation of CWA during SCN infection (Collins *et al.* 2003; Assaad *et al.* 2004; Kalde *et al.* 2007). No information existed on how syntaxin interacts with other vesicular transport proteins to accomplish plant defense to pathogens until Matsye *et al.* (2012) reported the role of syntaxin in defense in SCN infected roots, and further indicated the direct and indirect interaction with other vesicular transport protein components. The role of other vesicular transport protein components in pathogen defense in plant is unknown. Genetic studies in *Arabidopsis thaliana* showed the *PENETRATION1* (*PEN1*) gene (syntaxin 121 (SYP121) homolog in Arabidopsis) is involved in defense response against *Blumeria graminis* f. sp. *hordei* (Sanderfoot *et al.* 2000; Collins *et al.* 2003). SYP121 protein forms a complex on the plasmamembrane with two vesicle-associated membrane proteins (VAMPs) (VAMP721 and VAMP722), and is important for transporting vesicles between the Golgi apparatus and the plasma membrane (Collins *et al.* 2003). The SYP121 protein plays important role in CWA assembly by delivering the cargo recquired for cell wall maintainace. Others studies showed involvement of *PENETRATION2* (*PEN2*) gene, (a

secreted signal peptide-containing β-thioglucoside glucohydrolase gene) and *PENETRATION3* (*PEN3*) (encodes an ATP binding cassette (ABC) G-type transporter) in pathogen defense in *A. thaliana* (Lipka *et al.* 2005; Stein *et al.* 2006). Other components of vesicular transport machinery that directly interact with syntaxin are N-ethylmaleimide-sensitive factor attachment protein (NSF) (Malhotra *et al.* 1988), the soluble N-ethylmaleimidesensitive factor attachment receptor protein (SNARE) complex, and synaptosomal-associated protein 25 (SNAP25) (Oyler *et al.* 1989), the soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) (Weidman *et al.* 1989; Clary *et al.* 1990; Collins *et al.* 2003; Assaad *et al.* 2004; Kalde *et al.* 2007). The homolog of α-SNAP was first identified in *Saccharomyces cerevisiae* as a temperature sensitive secretion (*Sec*) mutant known as Sec17p (Novick *et al.* 1980). Later work determined Sec17p is required for vesicle transport from the endoplasmic reticulum (ER) to the Golgi apparatus with mutations resulting in the accumulation of 50 nm vesicles (Novick *et al.* 1981). In humans, α-SNAP binds to syntaxin through its N-terminal syntaxin binding domain and a C-terminal coiled-coil domain that binds both syntaxin and NSF leading to its general role in membrane fusion (Glick and Rothman 1987; Clary *et al.* 1990; Morgan *et al.* 1995; Barszczewski *et al.* 2008; Wickner and Schekman 2008; Jahn and Fasshauer 2012). Homologs of α-SNAP and syntaxin physically interact in other biological systems (Hardwick and Pelham 1992; Lupashin *et al.* 1997). However, a role in plant defense to pathogens was not known.

The identification of α-SNAP in resistance shed some light as to how the defense response may be functioning in *G. max* upon infection by *H. glycines*. Matsye *et al.* (2011) studied expression mapping analysis at the *rhg1* locus. The study found induced

expression of amino acid transporter and α-snap throughout the defense response (3, 6, and 9 days post infection [dpi]). A number of studies demostratred that α-SNAP plays important role in defense through the vesicular transport pathway (Liu *et al.* 2005; Hofius *et al.* 2009). The position of *G. max* (Gm) Gm-α-SNAP in the vesicle transport pathway may explain how its overexpression very potently and negatively affects *H. glycines* parasitism since it would be in place to mediate the fusion of different types of vesicles that may be transporting and delivering different types of contents simultaneously in the cell (Figure 1.2). This prediction would be consistent with the observations of CWAs at the cell periphery during the resistant reaction (Endo, 1965, 1991; Endo and Veech 1970; Kim *et al.* 1987; Kim and Riggs 1992; Mahalingham and Skorupska 1996). Furthermore, other specialized transport vesicles may also be involved in resistance which is known to exist (An *et al.* 2006a, 2006b). Regardless, vesicles are becoming more appreciated for their role(s) in defense. However, a role of vesicle transport proteins in plant defense to pathogens is not fully known.

Identification of candidate resistance genes in soybean

The availability of full genome sequence, expressed sequence tag (EST) dataset and construction of whole genome Affimatrix DNA chip make it possible to identify candidate genes in soybean root during SCN parasitism. Matsye *et al.* (2011) identified a pool of 1,787 genes that are expressed specifically in the cells undergoing the resistant reaction, using detection call methodology (DCM). The expression of about 1,000 transcripts was further confirmed by Illumina RNA sequence analysis. RNA sequence analyses has allowed for the 1,787 genes to be further narrowed down to ~100 that are expressed to higher absolute levels (Matsye *et al.* 2011). Furthermore, experiments have

used the gene expression data to map the activity of genes in the *rhg1* locus which ultimately resulted in the identification of the resistance gene, α soluble NSF attachment protein (α-SNAP) whose engineered expression suppressed infection (Matsye *et al.* 2011, 2012). The premise of the planned experiments is to examine these cell type specific transcripts that are found in cells undergoing the resistant reaction and identify their functional role during SCN infection.

Aim of the research project

The main goal of this study was to investigate and elucidate the soybean defense mechanisms using soybean-SCN patho-system. The proposed work aims to determine at cellular resolution why plants with normally functional resistance genes accommodate the success of the pathogen and vice versa. Collecting and analyzing the cells directly involved in infection with validation, prioritization and functional studies permits unprecedented resolution in determining the genetics and biochemistry of the process. Prior work has demonstrated the efficacy of the approach in identifying genes whose activity culminates in suppressing the ability of the plant parasitic nematode to infect *Glycine max* (Matsye *et al.* 2011, 2012). The proposed experiments expand on that work to more fully understand the process.

The specific objectives of this study were to clone the genes identified as expressed in *H. glycines*-induced feeding sites undergoing a resistant reaction (Matsye *et al.* 2011) into overexpression and RNAi plasmid vectors, and evaluate their biological function. In addition, the candidate genes were explored further by studying transcriptional expressional analysis on susceptible *G. max*[Williams 82/PI 518671] roots that obtain the engineered defense response in *G. max*[Williams 82/PI 518671]. This has identified the

genes whose expression the candidate gene is activating or identifing additional gene members that function in the same biochemical pathway or biological process.

References

Aist, J. R. (1976) Papillae and related wound plugs of plant cells. Annu Review of Phytopathology 14: 145-163.

Alvarez, J.P., Pekker, I., Goldshmidt, A., Blum, E., Amsellem, Z., Eshed, Y. (2006) Endogenous and synthetic microRNAs stimulate simultaneous, efficient, and localized regulation of multiple targets in diverse species. Plant Cell 18: 1134-1151.

An, Q., Ehlers, K., Kogel, K.H., VanBel, A.J., Hückelhoven, R. (2006a) Multivesicular compartments proliferate in susceptible and resistant MLA12-barley leaves in response to infection by the biotrophic powdery mildew fungus. New Phytologist 172: 563-57.

An, Q., Hückelhoven, R., Kogel, K.H., VanBel, A. J. (2006b) Multivesicular bodies participate in a cell wall-associated defence response in barley leaves attacked by the pathogenic powdery mildew fungus. Cellular Microbiology 8: 1009-1019.

Assaad, F.F., Qiu J.L., Youngs, H., Ehrhardt, D., Zimmerli, L., Kalde, M., Wanner, G., Peck, S.C., Edwards, H., Ramonell, K., Somerville, C.R., Thordal-Christensen, H. (2004) The *PEN1* syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Molecular Biology of the Cell 15: 5118-5129.

Barszczewski, M., Chua, J.J., Stein, A., Winter, U., Heintzmann, R., Zilly, F.E., Fasshauer, D., Lang, T. and Jahn R. (2008) A novel site of action for α-SNAP in the SNARE conformational cycle controlling membrane fusion. Molecular Biology of the Cell 19: 776-784.

Bhuiyan, N.H., Liu, W., Liu, G., Selvaraj, G., Wei, Y. and King, J. (2007) Transcriptional regulation of genes involved in the pathways of biosynthesis and supply of methyl units in response to powdery mildew attack and abiotic stresses in wheat. Plant Molecular Biology 64: 305-318.

Bohlenius, H., Mørch, S.M., Godfrey, D., Nielsen, M.E., Thordal-Christensen, H. (2010) The multivesicular body-localized GTPase ARFA1b/1c is important for callose deposition and ROR2 syntaxin-dependent preinvasive basal defense in barley. Plant Cell 22: 3831-3844.

Caldwell, B.E., Brim, C.A., Ross, J.P. (1960) Inheritance of resistance of soybeans to the soybean cyst nematode, *Heterodera glycines*. Agronomy Journal 52: 635-636.

Castagnone-Sereno, P. (2002) Genetic variability of nematodes: a threat to the durability of plant resistance genes Euphytica 124: 193-199.

Chen, S.Y., Dickson, D.W. (1996) Pathogenicity of fungi to eggs of *Heterodera glycines*. Journal of Nematology 28: 148-158.

Chilton, M.D., Tepfer, D.A., Petit, A., David, C., Casse-Delbart, F., Tempé, J. (1982) Agrobacterium rhizogenes inserts T-DNA into the genomes of the host plant root cells. Nature 295: 432-434.

Clary, D.O., Griff, I.C., Rothman, J. E. (1990) SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. Cell 61: 709-721.

Colgrove, A.L., Niblack, T.L. (2008) Correlation of female indices from virulence assays on inbred lines and field populations of *Heterodera glycines*. Journal of Nematology 40: 39-45.

Collins, N.C., Thordal-Christensen, H, Lipka. V., Bau, S., Kombrink, E., Qiu, J.L., Hückelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S.C., Schulze-Lefert, P. (2003) SNARE protein mediated disease resistance at the plant cell wall. Nature 425: 973-977.

Concibido, V.C., Diers, B.W., Arelli, P.R. (2004) A decade of QTL mapping for cyst nematode resistance in soybean. Crop Science 44: 1121-1131.

Cook, D.E., Lee, T.G., Guo, X., Melito, S., Wang, K., Bayless, A.M., Wang, J., Hughes, T.J., Willis, D.K., Clemente, TE., Diers B. W. (2012). Copy number variation of multiple genes at *Rhg1* mediates nematode resistance in soybean. Science 338: 1206-1209.

Cooper, J.L., Till, B.J., Laport, R.G., Darlow, M.C., Kleffner, J.M., Jamai, A., EI-Mellouki, T., Liu, S., Ritchie, R., Nielsen, N., Bilyeu, KD., Meksem, K., Comai, L., Henikoff, S. (2008) TILLING to detect induced mutations in soybean. BMC Plant Biology 8: 123-132.

Davis, E.L., Hussey, R.S., Baum, T.J., Bakker, J., Schots, A., Rosso, M. N., Abad, P. (2000) Nematode parasitism genes. Annual Review in Phytopathology 38: 365-396.

Davis, E.L., Hussey, R.S., Baum, T.J. (2004) Getting to the roots of parasitism by nematodes. Trends in Parasitology 20: 134-141.

Endo, B.Y. (1964) Penetration and development of *Heterodera glycines* in soybean roots and related and related anatomical changes. Phytopathology 54: 79-88.

Endo, B.Y. (1965) Histological responses of resistant and susceptible soybean varieties, and backcross progeny to entry development of *Heterodera glycines*. Phytopathology 55: 375-381.

Endo, B.Y. (1991) Ultrastructure of initial responses of susceptible and resistant soybean roots to infection by *Heterodera glycines*. Revue of Nématology 14: 73-84.

Endo, B.Y., Veech, J.A. (1970) Morphology and histochemistry of soybean roots infected with *Heterodera glycines*. Phytopathology 60: 1493-1498.

Epps, J.M., Duclos, L.A. (1970) Races of the soybean cyst nematode in Missouri and Tennessee. Plant Disease Reports 54: 319-320.

Francl, L.J., Dropkin, V.H. (1986) *Heterodera glycines* population dynamics and relation of initial population density to soybean yield. Plant Disease 70: 791-795.

Gao, B., Allen, R., Maier, T., Davis, E.L., Baum, T.J. and Hussey, R.S. (2003) The parasitome of the phytonematode *Heterodera glycines*. Molecular Plant Microbe Interactions 16: 720-726.

Gipson, I., Kim, K.S., Riggs, R.D. (1971) An ultrastructural study of syncytium development in soybean roots infected with *Heterodera glycines*. Phytopathology 61: 347-353.

Golden. A.M., Epps, J.M., Riggs, R.D., Duclos LA, Fox JA, Bernard RL. (1970) Terminology and identity of infraspecific forms of the soybean cyst nematode (*Heterodera glycines*). Plant Disease Reports 54: 544-546.

Hardham. A.R., Takemoto, D., White, R.G. (2008) Rapid and dynamic subcellular reorganization following mechanical stimulation of Arabidopsis epidermal cells mimics responses to fungal and oomycete attack. BMC Plant Biolology 8: 63.

Hardwick, K.G., Pelham, H.R. (1992) SED5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. The Journal of Cell Biology 119: 513-521.

Hewezi, T., Howe, P., Maier, T.R., Hussey, R.S., Mitchum, M.G., Davis, E.L., Baum, T.J. (2008) Cellulose binding protein from the parasitic nematode Heterodera schachtii interacts with Arabidopsis pectin methylesterase: cooperative cell wall modification during parasitism. Plant Cell 20: 3080-3093.

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakanomyo, I., Ogawa, M., Sawa, S., Ohashi-Ito, K., Matsubayashi, Y., Fukuda, H. (2008) Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. Proceedings of the National Academy of Sciences USA 105: 15208-15213.

Hofius, D., Schultz-Larsen, T., Joensen, J., Tsitsigiannis, D.I., Petersen, N.H., Mattsson, O., Jørgensen, L.B., Jones, J.D., Mundy, J., Petersen. M. (2009) Autophagic components contribute to hypersensitive cell death in Arabidopsis. Cell 137: 773-783.

Huang, G., Gao, B., Maier, T., Allen, R., Davis, E.L., Baum, T.J., Hussey. R.S. (2003) A profile of putative parasitism genes expressed in the esophageal gland cells of the root-knot nematode Meloidogyne incognita. Molecular Plant Microbe Interactions 16: 376-381.

Hückelhoven, R., Panstruga, R. (2011) Cell biology of the plant-powdery mildew interaction. Current Opinion in Plant Biology 14:738-746.

Inagaki, H., Tsutsumi, M. (1971) Survival of the soybean cyst nematode, *Heterodera glycines* Ichinohe (Tylenchida: Heteroderidae) under certain storage conditions. Applied Entomology and Zoology 8: 53-63.

Ithal, N., Recknor, J., Nettleston, D., Hearne, L., Maier, T., Baum, T.J., Mitchum, M.G. (2007) Developmental transcript profiling of cyst nematode feeding cells in soybean roots. Molecular Plant Microbe Interactions 20: 293-305.

Jahn, R., Fasshauer, D. (2012) Molecular machines governing exocytosis of synaptic vesicles. Nature 490: 201-207.

Veech, J.A. Dickson, D.W. (1987) VISTAS on nematology: a commemoration of the Twenty-fifth Anniversary of the Society of Nematologists. Vistas on Nematology: A Commemoration of the Twenty-fifth Anniversary of the Society of Nematologists.

Jones, M.G., Northcote, D.H. (1972) Nematode-induced syncytium-a multinucleate transfer cell. Journal of Cell Science 10: 789–809.

Jones M.G. (1981) The development and function of plant cells modified by endoparasitic nematodes. In: Zuckerman BM, Rohde RA (eds) Plant parasitic nematodes, vol 3. Academic Press, New York, pp 255-279.

Jones, J.T., Kumar, A., Pylypenko, L.A., Thirugnanasambandam, A., Castelli, L., Chapman, S., Cock, P.J., Grenier, E., Lilley, C.J., Phillips, M.S., Blok, V.C. (2009) Identification and functional characterization of effectors in expressed sequence tags from various life cycle stages of the potato cyst nematode Globodera pallida. Molecular Plant Pathology 10: 815-828.

Kalde, M., Nühse, T.S., Findlay, K., Peck, S.C. (2007) The syntaxin SYP132 contributes to plant resistance against bacteria and secretion of pathogenesis-related protein 1. Proceedings of the National Academy of Sciences USA 104: 11850-11855.

Kandoth, P.K., Ithal, N., Recknor, J., Maier, T., Nettleton, D., Baum, T.J., Mitchum, M.G. (2011) The Soybean *Rhg1* locus for resistance to the soybean cyst nematode *Heterodera glycines* regulates the expression of a large number of stress- and defense-related genes in degenerating feeding cells. Plant Physiology 155: 1960-1975.

Kim, Y.H., Riggs, R.D., Kim, K.S. (1987) Structural changes associated with resistance of soybean to *Heterodera glycines*. Journal of Nematology 19: 177-187.

Kim, K.S., Riggs, R.D. (1992) Cytopathological reactions of resistant soybean plants to nematode invasion. Pp. 157–168 in J. A. Wrather and R. D. Riggs, eds. Biology and Management of the Soybean Cyst Nematode. St. Paul: APS Press.

Klink, V.P., MacDonald, M., Alkharouf, N., Matthews, B.F. (2005) Laser capture microdissection (LCM) and expression analyses of *Glycine max*(soybean) syncytium containing root regions formed by the plant pathogen *Heterodera glycines* (soybean cyst nematode). Plant Molecular Biology 59: 969-983.

Klink, V.P., Overall, C.C., Alkharouf, N., MacDonald, M.H., Matthews, B.F. (2007a) Laser capture microdissection (LCM) and comparative microarray expression analysis of syncytial cells isolated from incompatible and compatible soybean roots infected by soybean cyst nematode (*Heterodera glycines*). Planta 226: 1389-1409.

Klink, V.P., Overall, C.C., Alkharouf, N., MacDonald, M.H., Matthews, B.F. (2007b) A comparative microarray analysis of an incompatible and compatible disease response by soybean (Glycine max) to soybean cyst nematode (*Heterodera glycines*) infection. Planta 226: 1423-1447.

Klink, V.P., MacDonald, M.H., Martins, V.E., Park, S-C., Kim, K-H., Baek, S-H., Matthews, B.F. (2008) MiniMax, a new diminutive *Glycine max* variety, with a rapid life cycle, embryogenic potential and transformation capabilities. Plant Cell, Tissue and Organ Culture 92: 183-195.

Klink, V.P., Kim, K.H., Martins, V.E., MacDonald, M.H., Beard, H.S., Alkharouf, N.W., Lee, S.K., Park, S-C., Matthews, B.F. (2009a) A correlation between host-mediated expression of Engineered Soybean Cyst Nematode Resistance parasite genes as tandem inverted repeats and abrogation of the formation of female *Heterodera glycines* cysts during infection of Glycine max. Planta 230: 53-71.

Klink, V.P., Hosseini, P., Matsye, P., Alkharouf, N., Matthews, B.F. (2009b) A gene expression analysis of syncytia laser microdissected from the roots of the *Glycine max*(soybean) genotype PI 548402 (Peking) undergoing a resistant reaction after infection by *Heterodera glycines* (soybean cyst nematode) Plant Molecular Biology 71: 525-567.

Klink, V.P., Hosseini, P., MacDonald, M.H., Alkharouf, N., Matthews, B.F. (2009c) Population-specific gene expression in the plant pathogenic nematode *Heterodera glycines* exists prior to infection and during the onset of a resistant or susceptible reaction in the roots of the *Glycine max* genotype Peking. BMC Genomics 10: 111.

Klink, V.P., Matsye, P.D., Lawrence, G.W. (2010a) Developmental Genomics of the Resistant Reaction of Soybean to the Soybean Cyst nematode, Pp. 249-270, In Plant Tissue Culture and Applied Biotechnology. Eds. Kumar A., Roy S. Aavishkar Publishers, Distributors, India.

Klink, V.P., Hosseini, P., Matsye, P., Alkharouf, N., Matthews, B.F. (2010b) Syncytium gene expression in Glycine max[PI 88788] roots undergoing a resistant reaction to the parasitic nematode *Heterodera glycines* Plant Physiology and Biochemistry 48: 176-193.

Klink, V.P., Overall, C.C., Alkharouf, N., MacDonald, M.H., Matthews, B.F. (2010c) Microarray detection calls as a means to compare transcripts expressed within syncytial cells isolated from incompatible and compatible soybean (Glycine max) roots infected by the soybean cyst nematode (*Heterodera glycines*). Journal of Biomedicine and Biotechnology 1-30.

Klink, V.P., Hosseini, P., Matsye, P.D., Alkharouf, N., Matthews, B.F. (2011a) Differences in gene expression amplitude overlie a conserved transcriptomic program occurring between the rapid and potent localized resistant reaction at the syncytium of the *Glycine max*genotype Peking (PI 548402) as compared to the prolonged and potent resistant reaction of PI 88788. Plant Molecular Biology 75: 141-165.

Klink, V.P., Matsye, P.D., Lawrence, G.W. (2011b) Cell-specific studies of soybean resistance to its major pathogen, the soybean cyst nematode as revealed by laser capture microdissection, gene pathway analyses and functional studies. in Soybean – Molecular Aspects of Breeding pp. 397-428. Ed. Aleksandra Sudaric. Intech Publishers.

Lauritis, J.A., Rebois, R.V., Graney, L.S. (1983) Development of *Heterodera glycines* Ichinohe on soybean, *Glycine max*(L.) Merr. under gnotobiotic conditions. Journal of Nematology 15: 272.

Lee, C., Chronis, D., Kenning, C., Peret, B., Hewezi, T., Davis, E.L., Baum, T.J., Hussey, R., Bennett, M., Mitchum, M.G. (2011) The novel cyst nematode effector protein 19C07 interacts with the Arabidopsis auxin influx transporter LAX3 to control feeding site development. Plant Physiology 155: 866-880.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D., Llorente, F., Molina, A., Parker, J., Somerville, S., Schulze-Lefert, P. (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science 310: 1180-1183.

Liu, Y., Schiff, M., Czymmek, K., Tallo´czy, Z., Levine, B., Dinesh-Kumar, S.P. (2005) Autophagy regulates programmed cell death during the plant innate immune response. Cell 121: 567-577.

Liu, S., Kandoth, P.K., Warren, S.D., Yeckel, G., Heinz, R., Alden, J., Yang, C., Jamai, A., El-Mellouki, T., Juvale, P.S., Hill, J. (2012) A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens. Nature 492: 256-260.

Lupashin, V.V., Pokrovskaya, I.D., McNew, J.A. and Waters, M.G. (1997) Characterization of a novel yeast SNARE protein implicated in Golgi retrograde traffic. Molecular Biology of the Cell 8: 2659-2676.

Mahalingham, R., Skorupska, H.T. (1996) Cytological expression of early response to infection by *Heterodera glycines* Ichinohe in resistant PI 437654 soybean. Genome 39: 986-998.

Malhotra, V., Orci, L., Glick, B.S., Block, M.R., Rothman, J.E. (1988) Role of an N-ethylmaleimide-sensitive transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack. Cell 54: 221-227.

Matsye, P.D., Kumar, R., Hosseini, P., Jones, C.M., Alkharouf, N., Matthews, B.F., Klink, V.P. (2011) Mapping cell fate decisions that occur during soybean defense responses. Plant Molecular Biology 77: 513-528.

Matsye, P.D., Lawrence, G.W., Youssef, R.M., Kim, K.H., Lawrence, K.S., Matthews, B.F., Klink, V.P. (2012) The expression of a naturally occurring, truncated allele of an α-SNAP gene suppresses plant parasitic nematode infection. Plant Molecular Biology 80: 131-155.

McLean, M.D., Hoover, G.J., Bancroft, B., Makhmoudova, A., Clark, S.M., Welacky, T., Simmonds, D.H., Shelp, B.J. (2007) Identification of the full-length Hs1pro-1 coding sequence and preliminary evaluation of soybean cyst nematode resistance in soybean transformed with Hs1pro-1 cDNA. Canadian Journal of Botany 85: 437-441.

Meksem, K., Liu, S., Liu, X., Jamai, A., Mitchum, M., Bendahmane, A., El-Mellouki, T. (2008) TILLING: A reverse genetics and a functional genomics tool in soybean. In: The handbook of Plant Functional Genomics: Concepts and Protocols pp. 251-265.

Miller, L.I. (1970) Differentiation of eleven isolates as races of the soybean cyst nematode. Phytopathology 60: 1016.

Morgan, J.A.T., Blair, D. (1998) Relative merits of nuclear ribosomal internal transcribed spacers and mitochondrial CO1 and ND1 genes for distinguishing among Echinostoma species (Trematoda). Parasitology 116: 289-297.

Niblack, T.L., Heinz, R.D., Smith, G.S., Donald, P.A. (1993) Distribution, density, and diversity of *Heterodera glycines* in Missouri. Journal of Nematology 25: 880-886.

Niblack, T.L., Arelli, P.R., Noel, G.R., Opperman, C.H., Orf, J.H., Schmitt, D.P., Shannon, J.G., Tylka, G.L. (2002) A revised classification scheme for genetically diverse populations of *Heterodera glycines*. Journal of Nematology 34: 279-288.

Niblack, T.L. and Riggs, R.D. (2004) Variation in virulence phenotypes. Biology and Management of Soybean Cyst Nematode, Ed. 2: 57-71.

Nielsen, M.E., Feechan, A., Böhlenius, H., Ueda, T., Thordal-Christensen, H. (2012) Arabidopsis ARF-GTP exchange factor, GNOM, mediates transport required for innate immunity and focal accumulation of syntaxin *PEN1*. Proceedings of the National Academy of Sciences USA 109: 11443-11448.

Noel, G. (2004) Resistance in soybean to soybean cyst nematode, *Heterodera glycines*. Nematology 2: 1-9.

Novick, P., Field, C., Schekma, R. (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 21: 205-215.

Novick, P., Ferro, S., Schekman, R. (1981) Order of events in the yeast secretory pathway. Cell 25: 461-469.

Nyczepir, A.P., Thomas, S.H. (2009) 18 Current and Future Management Strategies in Intensive Crop Production Systems. Root-knot Nematodes p.412.

Opperman, C.H., Chang, S. (1990) Plant-parasitic nematode acetylcholinesterase inhibition by carbamate and organophosphate nematicides. Journal of Nematology 22: 481.

Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E., Wilson, M.C. (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. Journal of Cell Biology 109: 3039-3052.

Pratt, P.W., Wrather, J.A. (1998) Soybean disease loss estimates for the southern United States, 1994-1996. Plant Dis 82: 114-116 Winstead NN, Skotland CB Sasser JN. (1955) Soybean cyst nematodes in North Carolina. Plant Disease Reports 39: 9-11.

Rao-Arelli, AP. (1994) Inheritance of resistance to *Heterodera glycines* race 3 in soybean accessions. Plant Disease 78: 898-900.

Riggs, R.D., Kim, K.S., Gipson, I. (1973) Ultrastructural changes in Peking soybeans infected with *Heterodera glycines*. Phytopathology 63: 76-84.

Riggs, R.D., Schmitt, D.P. (1988) Complete characterization of the race scheme for *Heterodera glycines*. Journal of Nematology 20: 392-395.

Riggs, R.D., Schmitt, D.P. (1991) Optimization of the *Heterodera glycines* race test procedure. Journal of Nematology 23: 149-154.

Ross, J.P., Brim, C.A. (1957) Resistance of soybeans to the soybean cyst nematode as determined by a double-row method. Plant Disease Reports 41: 923-924.

Ross, J.P. (1962) Crop rotation effects on the soybean cyst nematode population and soybean yields. Phytopathology 52: 815-818.

Rosskopf, E.N., Chellemi, D.O., Kokalis-Burelle, N., Church, G.T. (2005) Alternatives to methyl bromide: A Florida perspective. APSnet Feature, June.

Sanderfoot, A.A., Assaad, F.F., Raikhel, N.V. (2000) The Arabidopsis genome. An abundance of soluble N-ethylmaleimide-sensitive factor adaptor protein receptors. Plant Physiology 124: 1558-1569.

Sayre, R.M., Starr, M.P. (1985) Pasteuria penetrans (ex Thome, 1940) nom. rev., comb, n., sp. n., a mycelial and endospore-forming bacterium parasitic in plant-parasitic nematodes. Proceedings of the Helminthological Society of Washington 52: 149-165.

Schmelzer, E. (2002) Cell polarization, a crucial process in fungal defence. Trends in Plant Science 7: 411-415.

Schmitt, D.P., Noel, G.R. (1984) Nematode parasites of soybeans. Plant and Insect Nematodes. New York: Marcel Dekker. 13-59.

Siddiqui, Z.A., Mahmood, I. (1996) Biological control of plant parasitic nematodes by fungi: a review. Bioresource Technology 58: 229-239.

Sinclair, J.B., Backman, P.A. (1989) Frogeye leaf spot. Compendium of soybean diseases. 3rd ed., American Phytopathology Society, St. Paul. 19-21.

Smant, G., Stokkermans, J.P., Yan, Y., De Boer, J.M., Baum, T.J., Wang, X., Hussey, R.S., Gommers, F.J., Henrissat, B., Davis, E.L., Helder, J. (1998) Endogenous cellulases in animals: isolation of β-1, 4-endoglucanase genes from two species of plant-parasitic cyst nematodes. Proceedings of the National Academy of Sciences USA 95: 4906-4911.

Spears, J.F. (1957) Review of soybean cyst nematode situation for presentation at public hearing on the need for Federal Domestic Plant Quarantine, July 24.

Stacey, G., Vodkin, L., Parrott, W.A., Shoemaker, R.C. (2004) National Science Foundation-sponsored workshop report. Draft plan for soybean genomics. Plant Physiology 135: 59-70.

Steeves, R.M., Todd, T.C., Essig, J.S., Trick, H.N. (2006) Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress *Heterodera glycines* reproduction. Functional Plant Biology 33: 991-999.

Stein, M., Dittgen, J., Sánchez-Rodríguez, C., Hou, B.H., Molina, A., Schulze-Lefert, P., Lipka, V., Somerville, S. (2006) Arabidopsis *PEN3*/PDR8, an ATP binding cassette transporter,contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18: 731-746.

Weidman, P.J., Melançon, P., Block, M.R., Rothman, J.E. (1989) Binding of an N-ethylmaleimide-sensitive fusion protein to Golgi membranes requires both a soluble protein(s) and an integral membrane receptor. Journal of Cell Biology 108: 1589-1596.

Wickner, W., and Randy, S. (2008) Membrane fusion. Nature Structural and Molecular Biology 15: 658-664.

Wrather, J.A. and Koenning, S.R. (2006) Estimates of disease effects on soybean yields in the United States 2003 to 2005. Journal of Nematology 38: 173-180.

Wrather, J.A., Steinstra, W.C., Koenning, S.R. (2001) Soybean disease loss estimates for the United States from 1996-1998. Canadian Journal of Plant Pathology 23: 122-131.

Zhang, C. and Ghabrial, S.A. (2006) Development of bean pod mottle virus-based vectors for stable protein expression and sequence-specific virus-induced gene silencing in soybean. Virology 344: 401-411.

Zhang, C., Yang, C., Whitham, S.A., Hill, J.H. (2009) Development and use of an efficient DNA-based viral gene silencing vector for soybean. Molecular Plant Microbe Interactions 22: 123-131.

CHAPTER II

SYNTAXIN 31 FUNCTIONS IN *GLYCINE MAX* RESISTANCE TO THE PLANT PARASITIC NEMATODE *HETERODERA GLYCINES*[1]

Abstract

A *Glycine max* syntaxin 31 homolog (Gm-SYP38) was identified as being expressed in nematode- induced feeding structures known as syncytia undergoing an incompatible interaction with the plant parasitic nematode *Heterodera glycines*. The observed Gm-SYP38 expression was consistent with prior gene expression analyses that identified the alpha soluble NSF attachment protein (Gm-α-SNAP) resistance gene because homologs of these genes physically interact and function together in other genetic systems. Syntaxin 31 is a protein that resides on the *cis* face of the Golgi apparatus and binds Gm-α-SNAP-like proteins, but has no known role in resistance. Experiments presented here show Gm-α-SNAP overexpression induces Gm-SYP38 transcription. Overexpression of Gm-SYP38 rescues *G. max*[Williams 82/PI 518671], genetically *rhg1*$^{-/-}$, by suppressing *H. glycines* parasitism. In contrast, Gm-SYP38 RNAi in the *rhg1*$^{+/+}$ genotype *G. max*[Peking/PI 548402] increases susceptibility. Gm-α-SNAP and Gm-

[1] Most of the content of this chapter has been adapted from the journal article: Pant,S.R.,Matsye, P.D., McNeece, B.T., Sharma, K., Krishnavajhala, A., Lawrence, G.W., Klink, V.P. (2014) Syntaxin 31 functions in *Glycine max* resistance to the plant parasitic nematode *Heterodera glycines* Plant Molecular Biology 85: 107-121.

SYP38 overexpression induce the transcriptional activity of the cytoplasmic receptor-like kinase *BOTRYTIS INDUCED KINASE 1* (Gm-BIK1-6) which is a family of defense proteins known to anchor to membranes through a 5' MGXXXS/T(R) *N*-myristoylation sequence. Gm-BIK1-6 had been identified previously by RNA-seq experiments as expressed in syncytia undergoing an incompatible reaction. Gm-BIK1-6 overexpression rescues the resistant phenotype. In contrast, Gm-BIK1-6 RNAi increases parasitism. The analysis demonstrates a role for syntaxin 31-like genes in resistance that until now was not known.

Introduction

The genetic study of secretion led to the identification of highly conserved vesicle-associated proteins involved in essential cellular processes including signaling, cell growth, mitosis, the endocytic cycle, exocytosis, hormonal release, neurotransmission, fertilization, embryogenesis, development, sporulation and cell death (Novick and Schekman 1979; Novick *et al.* 1980; Clary *et al.* 1990; Bennett *et al.* 1992; Lukowitz *et al.* 1995; Boyd *et al.* 1995; Lauber *et al.* 1995, 1997; Burgess *et al.* 1997; Schulz *et al.* 1997; Neiman *et al.* 1998; Peter *et al.* 1998; Ramalho-Santos *et al.* 2000; Waizenegger *et al.* 2000; Sanderfoot *et al.* 2001a ,b, c; Babcock *et al.* 2004; Hong *et al.* 2004; Perrotta *et al.* 2010; Cotrufo *et al.* 2011; Rodrı´guez *et al.* 2011). From these studies, a core set of vesicle-associated proteins involved in membrane fusion has been identified (Gerber *et al.* 2008; Jahn and Fasshauer 2012). Some of the proteins also play important roles in plant resistance, as well as different types of resistance (Ishihara *et al.* 2001; Collins *et al.* 2003; Kalde *et al.* 2007; Kwon *et al.* 2008; Pajonk *et al.* 2008; Meyer *et al.* 2009; Lai *et al.* 2011; Matsye *et al.* 2012). In *A. thaliana*, resistance to the fungal

pathogen *Blumeria graminis* f. sp. *hordei* involves syntaxin 121 (SYP121) known as *PENETRATION* 1 (*PEN1*) that forms a complex on the plasma membrane with the vesicle-associated membrane protein (VAMP) 721/VAMP722 and the soluble N-ethylmaleimide-sensitive factor (NSF) adaptor protein (SNAP33) (Collins *et al.* 2003; Assaad *et al.* 2004; Kalde *et al.* 2007; Kwon *et al.* 2008; Pajonk *et al.* 2008). These observations established vesicular transport and membrane fusion in the plant resistance. However, the observations did not take into account that membrane fusion occurs at various points in the vesicular transport pathway and utilizes specific gene family members at these different points (Kaiser and Schekman 1990; Sanderfoot *et al.* 2001a, b, c).

The vesicle-associated protein alpha soluble NSF attachment protein (α-SNAP) is involved in the resistance process of *G. max* to the plant parasitic nematode *H. glycines* (Matsye *et al.* 2012; Cook *et al.* 2012). *H. glycines* induce the formation of a well-defined nurse cell called a syncytium (Figure 2.1) that develops through cell wall degradation, merging the cytoplasm of 200-250 root cells (Endo, 1964; Gipson and Riggs 1971; Jones and Northcote 1972; Jones, 1981). The role of α–SNAP in countering parasitism was determined through studies that identified the involved genes composing the major resistance locus, *rhg1* (Caldwell *et al.* 1960; Cregan *et al.* 1999; Kim *et al.* 2010; Matsye *et al.* 2011, 2012; Cook *et al.* 2012). How *rhg1* functioned or was regulated was unclear.

α-SNAP was first identified in *S. cerevisiae* as a temperature sensitive secretion (*sec*) mutant of Sec17p (Novick *et al.* 1980). Sec17p is required for vesicle transport from the ER to the Golgi with mutations resulting in the accumulation of 50 nm vesicles (Novick *et al.* 1980, 1981; Esmon *et al.* 1981). In humans, α-SNAP binds to syntaxin

which leads to its general role in membrane fusion (Glick and Rothman 1987; Clary *et al.* 1990; Morgan *et al.* 1995; DeBollo *et al.* 1995; Barszczewski *et al.* 2008; Wickner and Schekman 2008; Jahn and Fasshauer 2012). Homologs of α-SNAP and syntaxin physically interact in other biological systems (Hardwick and Pelham 1992; Lupashin *et al.* 1997). For example, in *S. cerevisae*, Sec17p binds to Sed5p (suppressors of the erd2-deletion 5) (Hardwick and Pelham 1992; Lupashin *et al.* 1997). Sed5p is homologous to the *A. thaliana* syntaxin 31 (SYP31) and has an essential function, localizing to *cis*-Golgi as it mediates anterograde trafficking (Hardwick and Pelham 1992; Banfield *et al.* 1995; Peng *et al.* 2004). The Sed5p homolog in *Nicotiana tabacum* (tobacco) (Nt-SYP31) is also localized to the *cis*-Golgi, but its exact role is not clear and no role in resistance has been identified (Rancour *et al.* 2002; Bubeck *et al.* 2008; Melser *et al.* 2009; Chatre *et al.* 2009). The location of SYP31 at the *cis* face of the Golgi stack would place it upstream in the vesicular transport pathway in relation to SYP121 in a central position with regard to metabolism, consistent with its observed essential role in *S. cerevisiae* (Hardwick and Pelham 1992; Banfield *et al.* 1995; Lupashin *et al.* 1997; Peng *et al.* 2004).

In addition to the function of α-SNAP in resistance, Matsye *et al.* (2012) also found its overexpression leads to high transcript levels of the pathogenesis related gene, *PR1* (Antoniw and Pierpoint 1978). *PR1* encodes a cysteine-rich secretory protein which indicates it cycles through the vesicular transport pathway and its expression is salicylic acid (SA)-inducible. These observations indicated part of α-SNAP's function during the suppression of *H. glycines* parasitism includes SA signaling (Wubben *et al.* 2008; Youssef *et al.* 2013). However, neither study linked the activity to other cellular functions. SA induces the expression of leucine rich repeat receptor like kinase resistance

(R) genes (Cao *et al.* 1994; Delaney *et al.* 1995; Glazebrook *et al.* 1996; Shah *et al.* 1997; Falk *et al.* 1999; Kachroo *et al.* 2000; Wildermuth *et al.* 2001; Feys *et al.* 2001; Shah *et al.* 2001; Van der Biezen *et al.* 2002 Rairidan and Delaney 2002; Takahashi *et al.* 2002; Shirano *et al.* 2002). Furthermore, Xiao *et al.* (2003) demonstrated the existence of a self-amplifying pathway that involves SA signaling and R genes. However, downstream aspects occurring prior to the hypersensitive response including vesicle dynamics were not examined. In the analysis presented here, a framework is presented that explores the role of Gm-SYP31 in the resistance of *G. max* to *H. glycines*.

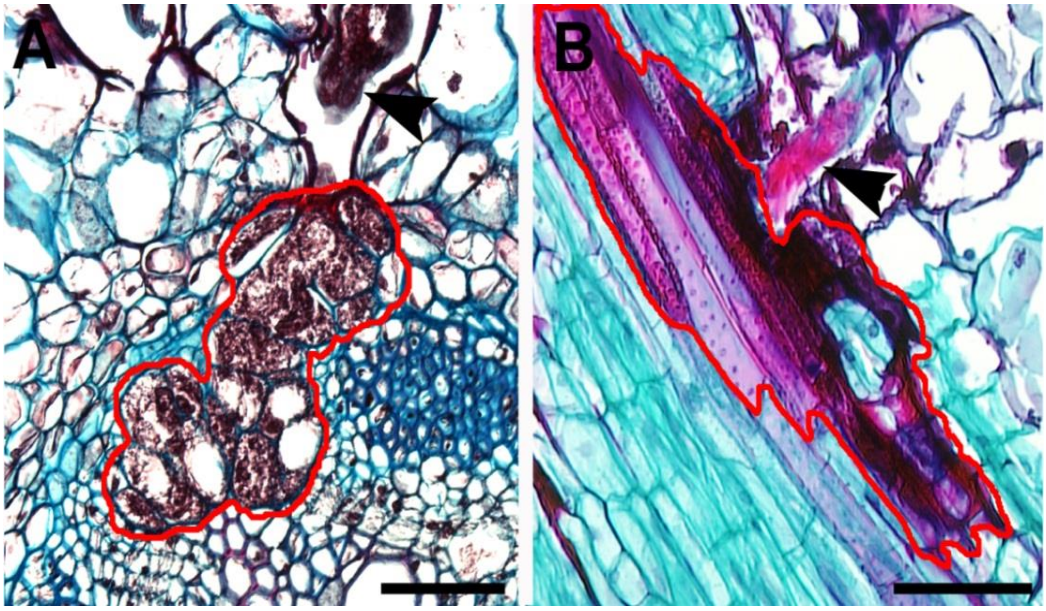


Figure 2.1 Soybean cyst nematode parasitized in soybean root

A. Transverse section of a compatible (susceptible) reaction between the *G. max*$_{[\text{William 82/PI 518671}]}$ and *H. glycines* (black arrowhead) at 6 dpi. The red line demarcates the boundary of the developing syncytium. Bar = 50 µm. B. Longitudinal section of an incompatible (resistant) reaction between *G. max*$_{[\text{Peking/PI 548402}]}$ and *H. glycines* (black arrowhead) at 6 days post infection. The red line demarcates the boundary of the syncytium undergoing the resistant reaction. Bar = 25 µm

Materials and Methods

Gene cloning and genetic transformation

Amplicons generated by PCR (Appendix Table A.1) were gel purified in 1.0% agarose using the Qiagen® gel purification kit, ligated into the directional pENTR/D-TOPO® vector and transformed into chemically competent *E. coli* strain One Shot TOP10. Chemical selection was on LB-kanamycin (50 μg/ml) according to protocol (Invitrogen®). Amplicons were confirmed by sequencing and matching it to their original Genbank accession. The *G. max* amplicon was shuttled into the pRAP15 destination vector using LR clonase (Invitrogen®). The engineered pRAP15 vector was transformed into chemically competent *A. rhizogenes* strain K599 (K599) (Haas *et al.* 1995) using the freeze-thaw method (Hofgen and Willmitzer 1988) on LB-tetracycline (5 μg/ml) according to Klink *et al.* (2008). Genetic transformation experiments were performed according to Matsye *et al.* (2012) in the *rhg1*$^{-/-}$ genetic background of *G. max*[Williams 82/PI 518671], proven by genome sequencing to lack a functional defense response to *H. glycines* parasitism (Bernard and Cremeens 1988; Atkinson and Harris 1989; Schmutz *et al.* 2010; Cook *et al.* 2012).

RNA isolation and quantitative real-time PCR (qPCR)

G. max root RNA was isolated according to Matsye *et al.* (2012). RNA isolation was done using the UltraClean® Plant RNA Isolation Kit (Mo Bio Laboratories®, Inc.; Carlsbad, CA). The RNA was treated with DNase I to remove genomic DNA. The cDNA was reversed transcribed from RNA. This procedure was done using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen®) with oligo d(T) as the primer according to protocol (Invitrogen®). Genomic DNA contamination was assessed by PCR

by using β-conglycinin primer pair (Appendix Table A.1) that amplify across an intron, thus yielding different sized DNA fragments based on the presence/absence of that intron (contaminating DNA). No contaminating genomic DNA existed in the cDNA as demonstrated in PCR reactions containing no template and reactions using RNA processed in parallel but with no Superscript® reverse transcriptase that also served as controls, producing no amplicon.

Primers used in qPCR gene expression experiments are provided (Appendix Table A.1). The experiments used the ribosomal protein gene S21 as a control (Klink *et al.* 2005; Matsye *et al.* 2012). The expression of the candidate genes was tested in relation to several different classes of pathogenesis related (PR) genes. These experiments included the salicylic acid regulated gene *PR1* (Antoniw and Pierpoint 1978), the ethylene responsive *PR2* (Kauffmann *et al.* 1987), the ethylene and jasmonic acid responsive gene *PR3* (Legrand *et al.* 1987) and the SA-responsive gene *PR5* (Kauffmann *et al.* 1990). The qPCR experiments used Taqman® 6-carboxyfluorescein (6-FAM) probes and Black Hole Quencher (BHQ1) (MWG Operon; Birmingham, AL). The qPCR differential expression tests were performed using RNA samples isolated from three independent replicates. The qPCR reaction conditions included a 20 µl Taqman Gene Expression Master Mix (Applied Biosystems; Foster City, CA), 0.9 µl of µM forward primer, 0.9 µl of 100 µM reverse primer, 2 µl of 2.5 µM 6-FAM (MWG Operon®) probe and 9.0 µl of (100 ng/µl) template DNA. The qPCR reactions were performed on an ABI 7300 (Applied Biosystems®). The qPCR conditions included a preincubation of 50° C for 2 min, followed by 95° C for 10 min. This step was followed by alternating 95° C for 15 sec followed by 60° C for 1 min for 40 cycles. The statistical analysis using $2^{-\Delta\Delta CT}$ to calculate

fold change was followed according to the derived formula presented in Livak and Schmittgen (2001).

The infection of *G. max* by *H. glycines*

Female *H. glycines*[NL1-Rhg/HG-type 7/race 3] were purified by sucrose flotation (Jenkins, 1964; Matthews *et al.* 2003, 2013; Klink *et al.* 2007; Matsye *et al.* 2012). Each root was inoculated with one ml of nematodes at a concentration of 2,000 second stage juveniles (J2s)/ml per root system (per plant), infected for 30 days and finally confirmed by acid fuchsin staining (Byrd *et al.* 1983). At the end of the experiment, the cysts (fully matured females) were collected over nested 20 and 100-mesh sieves (Matsye *et al.* 2012). Furthermore, the soil was washed several times and the rinse water sieved to assure collection of all cysts (Matsye *et al.* 2012). The accepted assay to accurately reflect if a condition exerts an influence on *H. glycines* development is the female index (FI) (Golden *et al.* 1970). The FI was calculated as FI = (Nx/Ns) x 100, where Nx is the average number of females on the test cultivar and Ns is the average number of females on the standard susceptible cultivar (Golden *et al.* 1970; Riggs and Schmitt 1988, 1991; Niblack *et al.* 2002; Klink *et al.* 2009; Matthews *et al.* 2013). Nx is the pRAP15-transformed line that had the engineered gene of interest. Ns is the pRAP15 control in their *G. max*[Williams 82/PI 518671]. Because the pRAP15 control has the *ccd*B gene, it also controls for non-specific effects caused by gene expression (Klink *et al.* 2009; Matsye *et al.* 2012). This FI assay is used by other labs using genetically engineered constructs in *G. max* to examine *H. glycines* biology (Steeves *et al.* 2007; McLean *et al.* 2007; Mazarei *et al.* 2007; Li *et al.* 2010; Melito *et al.* 2011; Liu *et al.* 2011; 2012; Cook *et al.* 2012; Matthews *et al.* 2013; Youssef *et al.* 2013). In the experiments of Golden *et al.*

(1970), Riggs and Schmidtt (1988, 1991), Kim *et al.* (1998) and Niblack *et al.* (2002), originally developed and modified the FI, the FI is typically calculated from a total of 3-10 experimental and 3-10 control plants. In those studies, each individual plant serves as a replicate and experimental replicates may or may not be performed. All of the experiments presented here exceed these studies in that regard. The FI was calculated as a function of root mass, tested statistically using the Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, $p < 0.05$ (Matsye *et al.* 2012). The effect that the overexpressed gene and RNAi had on root growth from a representative experiment was determined as a function of root mass, tested statistically using the Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, $p < 0.05$ (Matsye *et al.* 2012).

Microscopy

Histological observation was according to Klink et al. (2005). Briefly, tissue was fixed in Farmer's solution (FS) composed of 75% ethanol, 25% acetic acid (Sass 1958; Klink et al. 2005). *G. max* root tissue was harvested and cut into 0.5 cm pieces. Those pieces were vacuum infiltrated with FS for one hour (h) at 4°C. Fresh FS fixative was then added to their respective samples. Tissue was subjected to an incubation step of 12 h at 4° C. Dehydration of FS-fixed tissue proceeded through a graded ethanol series (75%, 85%, 100%, 100%, 100%), 30 min each. Ethanol was replaced with 1:1 Hemo DE® (Scientific Safety Solvents; Keller, TX, U.S.A.):ethanol for 30 min. Subsequently, three, 100% Hemo DE® incubations (30 min each) were done. The specimens in Hemo DE® were moved from 4°C to into a 58° C oven. Hemo DE® was replaced by paraffin. It is essential that exposure of the tissue to molten paraffin is minimized. The roots were infiltrated sequentially in 3:1, 1:1, 1:3 Hemo DE®:Paraplast+® tissue embedding

medium (Tyco Healthcare Group LP®; Mansfield, MA, U.S.A.) in each step for three h. Three changes of 100% Paraplast+® in each step for three h followed. Tissue was cast and subsequently mounted for sectioning. Serial sections of roots were made on an American Optical 820® microtome (American Optical Co®.; Buffalo, NY, U.S.A.) at a section thickness of 10 μm. Sections were stained in Safranin O (Fisher Scientific Co.; Fair Lawn, NJ, U.S.A.) in 50% ETOH and counter-stained in Fast Green FCF (Fisher Scientific Co.) (Klink *et al*., 2005). For histological analyses, the tissue was permanently mounted in Permount® (Fisher Scientific Co.). Stereoscope images of GOI::*uid*A reporter constructs were obtained on a Wild Heerbrugg stereoscope with Wild Heerbrugg Makrozoom 1:5 lenses having a 6.3-32x scale. GUS-stained images were captured according to Klink *et al.* (2013). Analyses were done using the IMT i-solution computer package (IMT i-solution Inc., Ho Chi Minh City, Vietnam).

Results

Framework

The identification of Gm-α-SNAP as a resistance gene and demonstration that its overexpression specifically induces *PR1* expression led to the development of a testable framework connecting α-SNAP to genes involved in vesicle transport, membrane fusion, SA signaling, R-gene mediated resistance and cell wall modification (Figure 2.2). Gene expression data was mined, resulting in the identification of a *G. max* syntaxin 31 homolog, Gm-SYP38 (Glyma14g06610), that is expressed in syncytia undergoing an incompatible interaction with *H. glycines* (Matsye *et al.* 2011). The known association of Sec17p (α-SNAP) and Sed5p indicated their gene expression may be co-regulated in *G.*

max. RNA isolated from roots overexpressing the *rhg1* gene Gm-α-SNAP, collected prior to *H. glycines* infection, have a 4.86 fold elevation in Gm-SYP38 expression as compared to controls. At this point, it was determined that it was reasonable to functionally test Gm-SYP38 in experiments examining its expression in relation to *H. glycines* parasitism.

Determination of gene expression in transgenic lines

In the functional tests presented here, no statistically significant effect on root growth was observed in the overexpressing roots. As expected, the expression of gene of interest was found induced in respective overexpression lines (Table 2.1) is shown to occur. RNAi experiments resulted in suppressed gene activity and had no statistically significant effect on root growth (Appendix Figure A.1). All overexpression and RNAi experiments were performed in three independent biological replicates. The number of independent transgenic lines used in each biological replicate is presented (Table 2.2). RNA was isolated from all of the tested overexpression and RNAi lines for subsequent quantitative gene expression studies presented later in the study.

Gm-SYP38

While α-SNAP and syntaxin physically interact in other experimental systems, no functional role for Gm-SYP38-like genes in resistance has been identified in plants. The analysis of Gm-SYP38 overexpression in relation to *H. glycines* parasitism examined a total of 69 independent transgenic lines (Table 2.2). Shown here, the overexpression of Gm-SYP38 rescues the ability of the *rhg1*$^{-/-}$ *G. max*$_{[\text{Williams 82/PI 518671}]}$ in suppressing *H. glycines* parasitism (Figure 2.3). In contrast, the analysis of Gm-SYP38 RNAi examined

a total of 64 independent transgenic lines (Table 2.2). Gm-SYP38 RNAi roots decreased its cognate RNA levels by 1.97 fold. Parasitism was increased as compared to controls (Appendix Figure A.2).

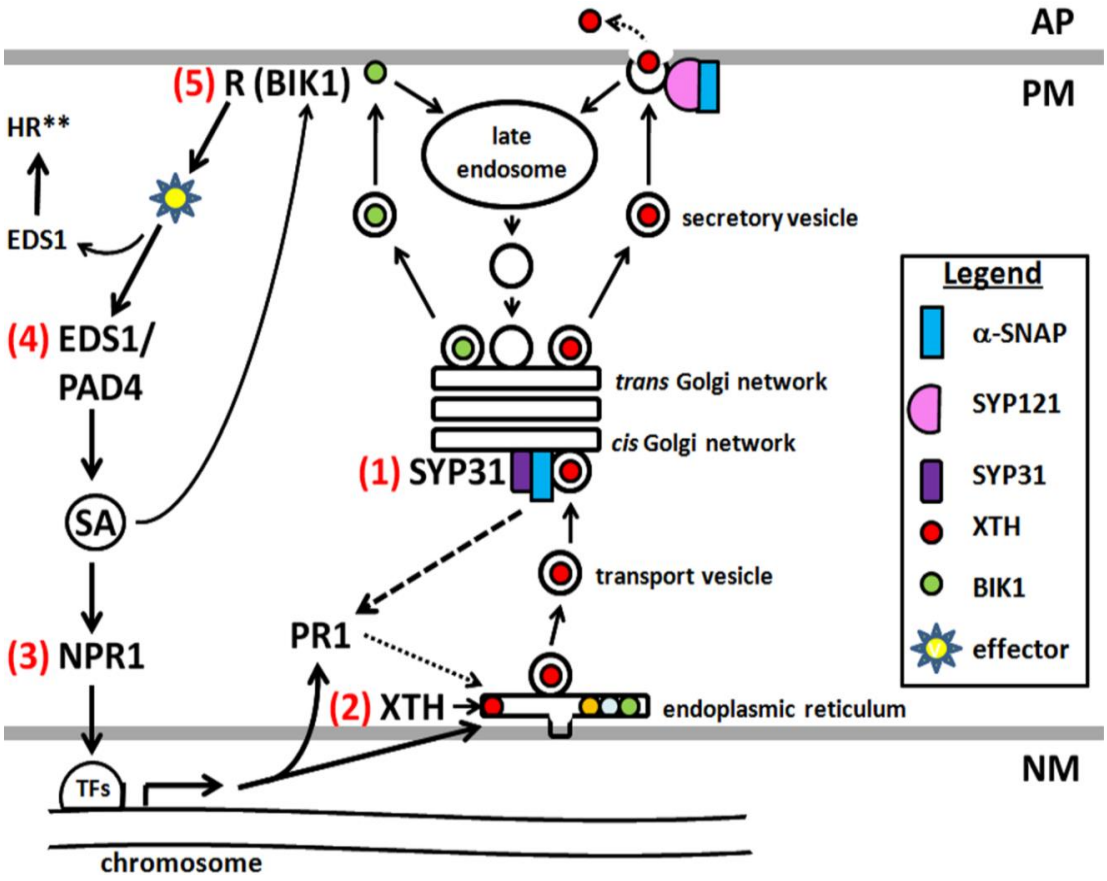


Figure 2.2 The cellular framework regarding the tested genes.

(1) SYP31; (2) XTH, (3) *NPR1*, (4) *EDS1* and (5) BIK1. The heavier dashed line indicates α-SNAP overexpression induces PR1 expression. PR1, a secreted protein, would enter the ER for processing (smaller dashed line). The ER is shown to have different resident proteins (colored circles) each processed and delivered to the cell periphery. α-SNAP (blue box) would likely interact with SYP121 at the cell membrane and SYP31 (purple box) at the cis face of the Golgi apparatus. (1) SYP31 binds to vesicles during membrane fusion. (2) XTH (red circle) metabolizes hemicellulose. (3) *NPR1* functions upstream of PR1 to synthesize it. PR1 enters the Golgi for secretion (4) *EDS1* heterodimerizes with PAD4. (5) BIK1 (green circle) is an R gene that binds to the pathogen effector while activating SA signaling. Endosomes shift from α-SNAP-dependent recycling between the cytoplasm and the inactive membrane bound R protein to degrading material between the active membrane bound R protein and the cytoplasm, delivering cargo. R protein-deactivating effectors directly cleave BIK1, deactivating it. SA signaling also activates R gene expression. AP-apoplastic space, PM-plasma membrane, NM-nuclear membrane, HR-hypersensitive response, TFs-transcription factors. **hypersensitive response (Xiao *et al*. 2003).

Gm-XTH43

RNA-seq analyses identified high expression levels of the xyloglucan endotransglycosylase/hydrolase (XTH) homolog Gm-XTH43 (Glyma17g07250) in syncytia undergoing an incompatible interaction (Fry *et al.* 1992; Klink *et al.* 2010; Matsye *et al.* 2011). This observation indicated that hemicellulose metabolism was actively involved in resistance. XTHs have a signal peptide, allowing targeting to the ER and can be *N*-glycosylated, indicating processing through the secretory pathway (Campbell and Braam 1998; Yokoyama and Nishitani 2001; Henriksson *et al.* 2003; Kallas *et al.* 2005; Genovesi *et al.* 2008; Maris *et al.* 2009). Gm-XTH43 has a signal peptide (Appendix Figure A.3) and is predicted to be *N*-glycosylated (Appendix Figure A.4). Furthermore, XTHs associate with vesicles, indicating regulated trafficking as it is transported to its site of activity (Yokoyama and Nishitani 2001; Albert *et al.* 2004). This observation supports an involvement with Gm-α-SNAP and Gm-SYP38 at some level. Furthermore, Gm-SYP38 overexpressing roots have elevated levels of Gm-XTH43 (presented later in Table 2.3). The analysis of Gm-XTH43 overexpression examined a total of 98 independent transgenic lines (Table 2.2). Roots overexpressing Gm-XTH43 in the *rhg1*$^{-/-}$ *G. max*[Williams 82/PI 518671] (Table 2.1) rescue the ability to suppress *H. glycines* parasitism (Figure 2.4).

Gm-NPR1

Induced gene expression of the secreted protein PR1 occurs in roots overexpressing Gm-α-SNAP (Matsye *et al.* 2012). These observations implicate SA signaling in the process of resistance (Figure 2.2). Furthermore, Gm-SYP38 overexpressing roots have elevated levels of *PR1* and *NONEXPRESSOR of PR1* RNA

(*NPR1*; Gm-NPR1-2 [Glyma09g02430]) (Cao *et al.* 1994) (presented later in Table 2.3). The analysis of Gm-NPR1-2overexpression examined a total of 50 independent transgenic lines (Table 2.2). Gm-NPR1-2 was overexpressed in the *rhg1*$^{-/-}$ *G. max*[Williams 82/PI 518671] where it rescues the ability of *G. max* to suppress *H. glycines* parasitism (Figure 2.4).

Table 2.1 qPCR demonstrating the studied genes are overexpressed in their respective transgenic roots

0 dpi	
Gene	**mRNA Expression (fold change)**
SYP38	132.25
XTH43	25.97
NPR1-1	2.07
EDS1-2	3.15
BIK1-6	27.65

RNA was isolated from transgenic roots genetically engineered to overexpress the candidate genes at the 0 dpi time point. Shown is the relative mRNA fold change expression compared to the pRAP15 vector lines lacking the candidate gene. Shown is the relative mRNA fold change expression of Gm-SYP38 in transgenic roots engineered to overexpress Gm-SYP38; relative mRNA fold change expression of Gm-XTH43 in transgenic roots engineered to overexpress Gm-XTH43; relative mRNA fold change expression of Gm-NPR1-2 in transgenic roots engineered to overexpress Gm-NPR1-2; relative mRNA fold change expression of Gm-EDS1-2 in transgenic roots engineered to overexpress Gm-EDS1-2; relative mRNA fold change expression of Gm-BIK1-6 in transgenic roots engineered to overexpress Gm-BIK1-6. An arbitrary cutoff of +/- 1.5 fold is considered differential expression.

Gm-EDS1

SA is known to influence the expression of upstream R genes and its associated upstream genes like *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) (Falk *et al.* 1999) (Figure 2.2). Matsye *et al.* (2011) identified high absolute levels of Gm-EDS1-2 (Glyma06g19890) in syncytia undergoing resistance. This observation indicated EDS1-2 may play a role in the process that leads to the suppression of *H. glycines* parasitism in *G. max*. Furthermore, Gm-SYP38 overexpressing roots have elevated levels of EDS1-2 (Table 2.3). The analysis of Gm-EDS1-2 overexpression examined a total of 67 independent transgenic lines (Table 2.2). The overexpression of Gm-EDS1-2 in the *rhg1*$^{-}$

$^{/-}$ *G. max*$_{[\text{Williams 82/PI 518671}]}$ (Table 2.1) rescues the ability to suppress *H. glycines* parasitism (Figure 2.4).

Table 2.2 The number of independent transgenic lines used in each replicate experiment for each gene under study

	Independent transgenic lines			
Gene construct	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Total**
SYP38-OE	20	24	25	69
SYP38-RNAi	19	15	11	64
XTH43-OE	40	24	34	98
NPR1-2-OE	17	17	16	50
EDS1-2-OE	25	23	19	67
BIK1-6-OE	26	25	20	71
BIK1-6-RNAi	19	7	12	38

Gm-BIK1

In other pathosystems, R genes activate SA signaling leading to the suppression of pathogen infection (Falk et al. 1999; Feys et al. 2001; Shirano et al. 2002; Xiao et al. 2003). In A. thaliana, this process involves *EDS1* (Falk et al. 1999). Gene expression studies of syncytia undergoing resistance identified a *G. max* homolog of the *A. thaliana* R gene *BOTRYTIS INDUCED KINASE1* (*BIK1*), referred to here as Gm-BIK1-6 (Glyma14g07460), was 10% of the studied transcripts (Veronese *et al.* 2006; Klink *et al.* 2010; Matsye *et al.* 2011). Vesicular transport and membrane fusion are processes that cycle R genes like *BIK1* and involve α-SNAP (Itin *et al.* 1997; Veronese *et al.* 2006; Lu *et al.* 2010; Laluk *et al.* 2011; Wu *et al.* 2011). Prior analyses of *A. thaliana BIK1* have shown that it localizes to cell membranes through a 5' MGXXXS/T(R) *N*-myristoylation membrane-anchoring consensus sequence (Thompson and Okuyama 2000; Veronese *et al.* 2006; Abuqamar *et al.* 2008). Gm-BIK1-6, like its 8 other related paralogs, have an

MGXXXS/T(R) *N*-myristoylation consensus sequence (Appendix Figure A.5). Gm-BIK1-6 overexpression analyses examined a total of 71 independent transgenic lines (Table 2.2). The overexpression of Gm-BIK1-6 in the *rhg1*$^{-/-}$ *G. max* [Williams 82/PI 518671] (Table 2.1) rescues the ability to suppress *H. glycines* parasitism (Figure 2.3). The effect resembles a resistant reaction, cytologically (Figures 2.1 and 2.4). In contrast, the analysis of Gm-BIK1-6 RNAi examined a total of 38 independent transgenic lines (Table 2.2). RNAi of Gm-BIK1-6 decreased its RNA levels by 2.21 fold and increased parasitism as compared to controls (Appendix Figure A.2).

Table 2.3 Gene expression analysis using qPCR of selected genes at 0 dp

	Transgenic lines					
Genes tested	**α-SNAP**	**SYP38**	**XTH**	***NPR1***	***EDS1***	***BIK1***
α-SNAP	11.08	-1.5	-1.23	-1.51	1.41	1.01
SYP38	4.86	132.25	-1.27	-1.19	-2.93	-1.12
XTH	-12.41	1.67	25.97	10.26	1.77	3.85
NPR1	2	6.9	2.22	2.07	-1.4	3.81
EDS1	1.02	5.8	-3.76	-1.6	3.15	-1.22
BIK1	3.5	1.99	-2.25	3.21	3.83	27.65
PR1	11.64	52.02	3.35	2.47	1.17	-2.42
PR2	15.69	-37.68	-1.53	-4.77	-43.47	-2.15
PR3	-4.95	195.07	5.81	38.63	125.27	-1.06
PR5	6.78	-2.09	1.22	-1.53	-3	1.03
WIP	-1.97	-3.07	-2.63	5.48	3.74	-1.92
AAT	-6.82	-1.4	-1.76	5.5	3.26	-4.72
SHMT	-6.89	-1.88	1.37	5	1.67	-3.39

RNA was isolated from roots of the overexpressing candidate genes (top) at the 0 dpi time point; Gm-α-SNAP; Gm-SYP38; Gm-XTH43; Gm-NPR1-2; Gm-EDS1-2; Gm-BIK1-6. The left column represents the same genes and additional pathogenesis related (PR) genes and genes composing the *rhg1* and *Rhg4* loci. * represents expression presented in Matsye et al. (2012). Dark gray boxes, gene activity in its overexpressing line. Light gray boxes, overexpressed genes under study. White boxes, PR genes and additional genetically identified *rhg1* and *Rhg4* resistance genes. Red, induced; green, suppressed; black, not significant. An arbitrary cutoff of +/- 1.5 fold, $p < 0.05$ was used for differential expression.

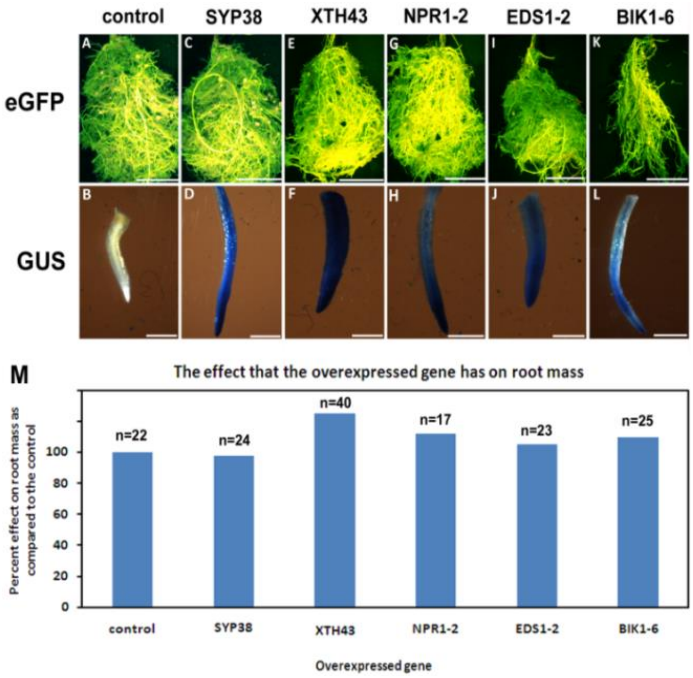


Figure 2.3 Representative roots genetically engineered to overexpress *G. max* XTH43, SYP38, NPR1-2, EDS1-2 or BIK1-6 in the *rhg1*$^{-/-}$ *G. max*[Williams 82/PI 518671].

The analysis procedure averaged the mass of the root and presented the data as a percentage difference in the mass between the lined genetically engineered for each target candidate gene and the pRAP15-engineered lines lacking the target gene. (A) pRAP15 control; (B) pRAP15 control (no *uid*A); (C) XTH43-OE; (D) XTH43::*uid*A-OE; (E) SYP38-OE; (F) SYP38::*uid*A-OE; (G) NPR1-2-OE; (H) NPR1-2::*uid*A-OE; (I) EDS1-2-OE; (J) EDS1-2::*uid*A-OE; (K) BIK1-6-OE; (L) BIK1::*uid*A-OE. The roots in A, C, E, G, I and K are from those used to show statistically that the overexpression of the candidate gene had no effect on root development. A, C, E, G, I and K bars = 10 cm; B, D, F, H, J and L bars = 1 cm. (M). The effect that the overexpressed gene has on root mass is shown as compared to the control. n = number of roots examined in the analysis. Control (pRAP15) (n = 22), SYP38 (n = 24), p = 0.05; XTH43 (n = 40), p = 0.112417; NPR1-2 (n = 17), p = 0.197757; EDS1-2 (n = 23), p = 0.05; BIK1-6 (n = 25), 0.346635. None of the experiments had statistically significant differences in root growth between the overexpressing lines and the controls ($p < 0.05$).

Comparative analyses through gene expression

The observations demonstrate *G. max* homologs of α-SNAP, SYP38, XTH43, EDS1-2, NPR1-2 and BIK1-6 rescue the *rhg1*$^{-/-}$ *G. max*[Williams 82/PI 518671] by suppressing H. glycines infection in comparison to controls. Expression experiments using qPCR was then performed to the observations demonstrate *G. max* homologs of α-SNAP, SYP38, XTH43, EDS1-2, NPR1-2 and BIK1-6 rescue the *rhg1*$^{-/-}$ *G. max*[Williams 82/PI 518671] by

suppressing H. glycines infection in comparison to controls. Expression experiments using qPCR was then performed to determine whether the expression of these individual genes was in some way influencing each other. The gene expression studies also examined PR1, PR2, PR3 and PR5 (defined in Materials section). Furthermore, to understand relationship with genetically identified resistance genes, qPCR experiments were performed on the *G. max rhg1* associated genes AAT and WIP and the *Rhg4* gene, serine hydroxymethyltransferase (SHMT) that is well known to function in biotic and abiotic stress (Woo 1979; Moreno et al. 2005; Cook et al. 2012; Liu et al. 2012) (Table 2.3).

Table 2.4 Gene expression analysis using qPCR of selected genes at 3 dpi

	Transgenic lines			
Genes tested	**Syntaxin**	**XTH**	***EDS1***	***BIK1***
Syntaxin	86.8	-1.33	-1.57	-1.34
XTH	1.72	27.86	2.99	1.8
EDS1	1.33	4.13	3.14	-18.55
BIK1	-2.33	1.41	1.99	48.93
PR1	19.24	-1.29	-1.01	-1.79
PR2	-13.1	-5.13	-3.13	-3.35
PR3	105.52	-1.17	4.86	-1.15
PR5	-1.1	-3.02	1.72	2.01
α-SNAP	-1.39	-1.03	-4.45	-1.67
WIP	-1.08	1.5	-2.28	-17.53
AAT	-3.91	-1	2.04	-3.46
SHMT	1.17	-1.42	-1.05	-24.1

RNA was isolated from roots of the overexpressing candidate genes (top) at the 3 dpi time point; Gm-α-SNAP; Gm-SYP38; Gm-XTH43; Gm-EDS1-2; Gm-BIK1-6. The left column represents the same genes and additional pathogenesis related (PR) genes and genes composing the *rhg1* and *Rhg4* loci. * represents expression presented in Matsye et al. (2012). Dark gray boxes, gene activity in its overexpressing line. Light gray boxes, overexpressed genes under study. White boxes, PR genes and additional genetically identified *rhg1* and *Rhg4* resistance genes. Red, induced; green, suppressed; black, not significant. An arbitrary cutoff of +/- 1.5 fold, $p < 0.05$ was used for differential expression.

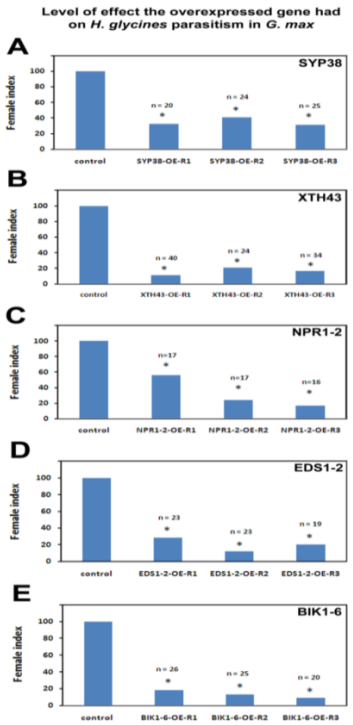


Figure 2.4 Rescue experiments in the genetically hypomorphic *rhg1−/− G. max*[Williams 82/PI 518671]

The female index (FI) was calculated for *H. glycines* infected roots in all experiments. Control is presented graphically as a function of it being compared to itself (FI = 100 %). n = number of independent transformants examined, also presented in Table 2. For all experiments, * = statistically significant $p < 0.05$. **a** SYP38 overexpression (SYP38-OE) analysis. SYP38-OE-R1 (replicate 1) (n = 20); SYP38-OE-R1 females/ gram = 14.55; control (n = 17), females/gram = 44.78; FI = 32.49; *p* value = 0.0000787183*. SYP38-OE-R2 (n = 24); SYP38-OE-R2 females/gram = 8.65; control (n = 22); females/gram = 30.33; FI = 41.14; *p* value = 4.20178e−07*. SYP38-OE-R3 (n = 25); SYP38-OE-R3 females/gram = 7.80; control (n = 25); females/ gram = 24.90; FI = 31.33; *p* value = 4.8109e−07*. **b** XTH43 overexpression (XTH43-OE) analysis. XTH43-OE-R1 (n = 40 plants); XTH43-OE-R1 females/gram = 4.97; control (n = 36); females/gram = 44.80; FI = 11.10; *p* value = 0.00032207*. XTH43-OE-R2 (n = 24 plants); XTH43-OE-R2 females/ gram = 6.33; control (n = 22); females/gram = 30.34; FI = 20.86; *p* value = 1.05354e−07*. XTH43-OE-R3 (n = 34 plants); XTH43-OE-R3 females/gram = 6.05; control (n = 22); females/ gram = 36.50; FI = 16.60; *p* value = 0.000000000043*. **c** NPR1-2 overexpression (NPR1-2-OE) analysis. NPR1-2-OE-R1 (n = 17); NPR1-2-OE-R1 females/gram = 16.94; control (n = 22); females/ gram = 30.34; FI = 55.82; *p* value = 0.00803383*. NPR1-2-OE-R2 (n = 17); NPR1-2-OE-R2 females/gram = 8.54; control (n = 22); females/gram = 35.31; FI = 24.18; *p* value = 5.54068e−07*. NPR1-2-OE-R3 (n = 16); NPR1-2-OE-R3 females/gram = 5.55; control (n = 20); females/gram = 32.88; FI = 16.82; *p* value = 1.75556e−07*. **d** EDS1-2 overexpression (EDS1-2-OE) analysis. EDS1-2-OE-R1 (n = 23); EDS1-2-OE-R1 females/ gram = 10.06; control (n = 22); females/gram = 28.49; FI = 24.18; *p* value = 4.65287e−07*. EDS1-2-OE-R2 (n = 23); EDS1-2-OE-R2 females/gram = 3.61; control (n = 22); females/gram = 30.34; FI = 11.89; *p* value = 2.26135e−08*. EDS1-2-OE-R3 (n = 19); EDS1-2-OE-R3 females/gram = 11.59; control (n = 20); females/ gram = 57.72 (n = 20); FI = 20.60; *p* value = 1.57906e−07*. **e** BIK1-6 overexpression (BIK1-6-OE) analysis. BIK1-6-OE-R1 (n = 26); BIK1-6-OE-R1 females/gram = 6.55; control (n = 22); females/gram = 35.31; FI = 18.55; *p* value = 0.00000000716054*. BIK1-6-OE-R2 (n = 25); BIK1-6-OE-R2 females/gram = 4.03; control (n = 22); females/gram = 30.34; FI = 13.28; *p* value = 0.0000000287057*. BIK1-6-OE-R3 (n = 20); BIK1-6-OE-R3 females/gram = 5.44; control (n = 20); females/gram = 57.72; FI = 9.42; *p* value = 4.68492e−08*

Discussion

Genetic experiments in *G. max* have shown the vesicle-associated and membrane fusion gene α-SNAP is at least part of the *rhg1* locus that is responsible for a resistance phenotype (Matsye *et al.* 2012; Cook *et al.* 2012). However, some crucial remaining problems still remained. Firstly, it was unclear how α-SNAP was involved in the process of resistance. Secondly, and a more overarching problem from all the genetic studies, was the identity of an R gene functioning in resistance. Both of those problems were addressed here.

Table 2.5 Gene expression analysis using qPCR of selected genes at 6 dpi

	syntaxin	XTH	EDS1	BIK1
syntaxin	183.75	-1.37	2.16	-2.87
XTH	7.93	13.58	-1.85	-2.27
EDS1	1.64	-2.33	4.1	-65.09
BIK1	1.72	-1.95	1.02	7.58
PR1	27.68	2.32	-2.33	-5.15
PR2	-12.35	-5.54	3.84	-25.64
PR3	23.53	2.21	-7.95	-9.64
PR5	1.32	-1.16	1.36	1.17
α-SNAP	68.83	-1.14	-5.77	-1.08
WIP	1.42	-2.46	-1.71	-40.38
AAT	1.08	-3.86	-1.04	-12.29
SHMT	1.57	-1.67	-1.4	-60.26

RNA was isolated from roots of the overexpressing candidate genes (top) at the 6 dpi time point; Gm-α-SNAP; Gm-SYP38; Gm-XTH43; Gm-EDS1-2; Gm-BIK1-6. The left column represents the same genes and additional pathogenesis related (PR) genes and genes composing the *rhg1* and *Rhg4* loci. * represents expression presented in Matsye *et al*. (2012). Dark gray boxes, gene activity in its overexpressing line. Light gray boxes, overexpressed genes under study. White boxes, PR genes and additional genetically identified *rhg1* and *Rhg4* resistance genes. Red, induced; green, suppressed; black, not significant. An arbitrary cutoff of +/- 1.5 fold, $p < 0.05$ was used for differential expression.

The regulation of *G. max* α-SNAP and SYP38 transcription

The position of SYP31-like proteins at the *cis* face of the Golgi apparatus places it at the base of the vesicular transport machinery (Banfield *et al.* 1995; Lupashin *et al.* 1997; Leyman *et al.* 1999; Collins *et al.* 2003; Peng *et al.* 2004; Bubeck *et al.* 2008). Its location at this position may explain how its overexpression very potently and negatively affects *H. glycines* parasitism since it would mediate the import of numerous proteins from the ER into the Golgi apparatus. In yeast, Sed5p binds directly to Sec17p (it's only SNAP) (Lupashin *et al.* 1997). Therefore, the involvement of Gm-SYP38 in the resistant reaction of *G. max to H. glycines*, as shown here, links its function directly to the *rhg1* locus gene, α-SNAP. Furthermore, Gm-SYP38 has a very strong influence on α-SNAP gene expression by 6 dpi when the resistant reaction is fully engaged. The nature of the strong positive influence that Gm-SYP38 expression has on Gm-α-SNAP transcription is unknown and requires further study. Since Gm-α-SNAP gene expression is not constitutively induced in roots overexpressing Gm-SYP38, the effect may not be direct. Gm-α-SNAP may require additional prerequisite activities for its transcription to become activated. However, the high level of α-SNAP expression indicates that amount of transcriptional activity is important to the potent resistant reaction as already demonstrated (Matsye *et al.* 2012). This result is consistent with the localized high levels of transcription observed for the other genes tested here as being important for resistance (Matsye *et al.* 2011, 2012). Furthermore, the very high relative levels of Gm-α-SNAP found at 6 dpi indicate that different types of vesicles may be transporting and delivering different types of contents simultaneously in the cell. In *A. thaliana*, a genetic pathway involving the β-thioglucoside glucohydrolase, *PEN2*, and the ABC transporter, *PEN3*,

bring glucosinolates to the cell periphery for defense (Lipka *et al.* 2005; Stein *et al.* 2006). β-thioglucoside glucohydrolase is a protein found in a specialized transport vesicle involved in defense called ER bodies and other types of vesicles involved in defense such as multivesicular bodies are known (Matsushima *et al.* 2003a, b; An *et al.* 2006a, b; Ogasawara *et al.* 2009).

The R gene, Gm-BIK1-6, functions in resistance

Genetic studies in *G. max*, in relation to *H. glycines* resistance, determined *rhg1* and *Rhg4* did not contain the expected R genes (Kim *et al.* 2010; Melito *et al.* 2010; Liu *et al.* 2011, 2012; Cook *et al.* 2012). Thus, a remaining problem was determining whether an R gene was involved in the process. R genes have been long known to be involved in resistance to plant parasitic nematodes (Milligan *et al.* 1998). A good candidate *G. max* R gene for acting in suppressing *H. glycines* parasitism was Gm-BIK1-6 which we originally identified as being expressed only in syncytia undergoing an incompatible reaction (Klink *et al.* 2010; Matsye *et al.* 2011). Gm-BIK1-6 overexpression was shown here to suppress *H. glycines* parasitism in *G. max* to levels greater than 90% in some replicates with the cellular response resembling a resistant reaction. In contrast, the suppression of Gm-BIK1-6 expression by RNAi resulted in an increase in infection in the *rhg1*$^{+/+}$ *G. max*[Peking/PI 548402]. This result indicated that the high absolute levels of Gm-BIK1-6 found originally in Matsye *et al.* (2011) were relevant to their biological function. In *A. thaliana*, pathogens have the capacity to inhibit the function of *BIK1* through an effector called HopF2 (Wu *et al.* 2011). This result indicated *H. glycines* may have effectors that target Gm-BIK1-like proteins, but their activity is overcome by high levels of localized expression of plant defense genes. Matsye *et al.* (2011) identified a

second highly expressed Gm-BIK1 homolog (Gm-BIK1-2, Glyma02g41490) whose effect on nematode parasitism was not presented here. This observation indicated a diverse repertoire of *BIK1*-like genes may be important for resistance. Consistent with this hypothesis, we identified 9 closely related *BIK1*-like genes having the MGXXXS/T(R) *N*-myristoylation membrane binding consensus sequence in the genome of *G. max* (Veronese *et al.* 2006; Abuqamar *et al.* 2008; Zhang *et al.* 2010; Lu *et al.* 2010; Laluk *et al.* 2011). The identification of Gm-BIK1-like genes having the *N*-myristoylation consensus sequence also indicated an association with vesicles or vesicle-related structures, linking it to the *rhg1* gene, α-SNAP (Branch *et al.* 2006; Laluk *et al.* 2011). Furthermore, Gm-α-SNAP and Gm-SYP38 overexpressing roots induce Gm-BIK1 gene expression at 0 dpi. These observations indicate the vesicle transport system may somehow function upstream of Gm-BIK1-mediated resistance.

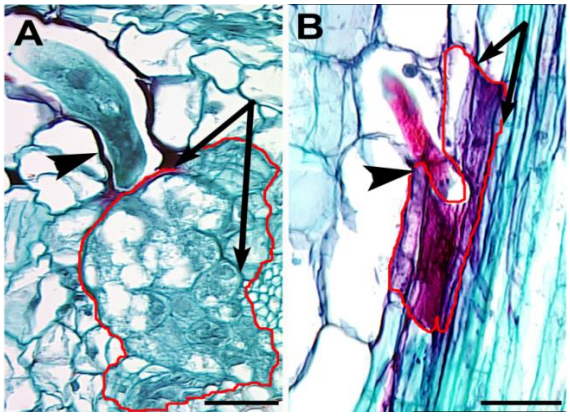


Figure 2.5 Gm-BIK1-6 overexpression in the *rhg1*−/− *G. max*[Williams 82/PI 518671], results in an outcome resembling a resistant reaction to parasitism by *H. glycines* by 6 dpi.

A control, **B** Gm-BIK1-6-OE. *Bars* = 50 μm. *Red line* is the boundary of the syncytium. *Black arrowhead*, *H. glycines*. *Red stain* is safranin which is known to label cells undergoing a resistant reaction (Ross 1958). In the control (**A**), *H. glycines* is exhibiting growth as compared to (**B**).

High levels of localized expression are important to the defense response

In *A. thaliana*, the cycling of *BIK1* through endosomes is associated with the regulation of SA levels (Branch *et al.* 2006; Laluk *et al.* 2011). If these observations are true in *G. max,* the results would link Gm-BIK1-6 to SA signaling. In reciprocal experiments, Gm-EDS1-2 and Gm-NPR1-2 overexpressing lines each exhibit induced Gm-BIK1-6 transcription. Thus, the ability of pathogens to inhibit this activity would be important to their success. Heidrich *et al.* (2011) demonstrated in *A. thaliana* that *EDS1*, like *BIK1*, is also a target of pathogen effectors. The results show that multiple proteins examined here could be the targets of *H. glycines* effectors, but high localized expression of these genes as presented in RNA-seq experiments by Matsye *et al.* (2011) indicate a plant mechanism to circumvent parasitism. The mechanism is probably conserved (Humphry *et al.* 2010).

Components of a conserved signaling circuit exist in *G. max*

Xiao *et al.* (2003) showed the existence of an R-gene-involved, SA-dependent amplification circuit functioning in resistance in *A. thaliana*. From the analysis presented here, it appears a related framework including vesicle-associated and membrane fusion proteins exists in *G. max* as it suppresses parasitism by *H. glycines*. The functional data presented here in *G. max* indicates that SYP38 may reinforce this circuit by activating the expression of α-SNAP (*rhg1*), BIK1-6, EDS1-2, NPR1-2 and XTH43. Notably, greater transcriptional activation of Gm-α-SNAP and Gm-XTH43 happens later during the resistant reaction as the cytoplasm is undergoing reorganization and the cell walls of cells surrounding the syncytium are undergoing structural modification. The work presented here clearly support vesicular transport and the delivery of their contents and/or

membrane anchored proteins as major components in the ability of *G. max* to defend itself from *H. glycines* parasitism. The nature of these proteins, in particular Gm-SYP38, indicates that they probably function in a related manner in different organisms. However, the complexity of vesicle transport with its diverse, but essential roles, make it a fertile area of future study.

References

Abel, S., Theologis, A. (1994) Transient transformation of Arabidopsis leaf protoplasts: a versatile experimental system to study gene expression. Plant Journal 5: 421-427.

Abuqamar, S., Chai, M.F., Luo, H., Song, F., Mengiste, T. (2008) Tomato protein kinase 1b mediates signaling of plant responses to necrotrophic fungi and insect herbivory. Plant Cell 20: 1964-1983.

Albert, M., Werner, M., Proksch, P., Fry, S.C., Kaldenhoff, R. (2004) The cell wall-modifying xyloglucan endotransglycosylase/hydrolase LeXTH1 is expressed during the defence reaction of tomato against the plant parasite Cuscuta reflexa. Plant Biology (Stuttg) 6: 402-407.

An, Q., Ehlers, K., Kogel, K.H., Van Bel, A.J., Hückelhoven, R. (2006a) Multivesicular compartments proliferate in susceptible and resistant MLA12-barley leaves in response to infection by the biotrophic powdery mildew fungus. New Phytologist 172: 563-57.

An, Q., Hückelhoven, R., Kogel K.H., Van Bel, A.J. (2006b) Multivesicular bodies participate in a cell wall-associated defence response in barley leaves attacked by the pathogenic powdery mildew fungus. Cellular Microbiology 8: 1009-1019.

Antoniw, J.F., Pierpoint, W.S. (1978) The purification and properties of one of the 'b" proteins from virus-infected tobacco plants. Journal of General Virology 39: 343-350.

Assaad, F.F., Qiu, J.L., Youngs, H., Ehrhardt, D., Zimmerli, L., Kalde, M., Wanner, G., Peck, S.C., Edwards, H., Ramonell, K., Somerville, C.R., Thordal-Christensen, H. (2004) The *PEN1* syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Molecular Biology of the Cell 15: 5118-5129.

Atkinson, H.J., Harris, P.D. (1989) Changes in nematode antigens recognized by monoclonal antibodies during early infections of soya bean with cyst nematode *Heterodera glycines*. Parasitology 98: 479-487.

Babcock, M., Macleod, G.T., Leither, J., Pallanck, L. (2004) Genetic analysis of soluble N ethylmaleimide-sensitive factor attachment protein function in Drosophila reveals positive and negative secretory roles. Journal of Neuroscience 24: 3964-3973.

Banfield, D.K., Lewis, M.J., Pelham, H.R. (1995) A SNARE-like protein required for traffic through the Golgi complex. Nature 375:806-809.

Barszczewski, M., Chua, J.J., Stein, A., Winter, U., Heintzmann, R., Zilly F.E., Fasshauer, D., Lang, T., Jahn R. (2008) A novel site of action for α-SNAP in the SNARE conformational cycle controlling membrane fusion. Molecular Biology of the Cell 19: 776-784.

Bennett, M.K., Calakos, N., Scheller, R.H. (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257: 255-259.

Bernard, R.L., Cremeens, C.R. (1988) Registration of 'Williams 82' Soybean. Crop Science 28: 1027.

Boyd, R.S., Duggan, M.J., Shone, C.C., Foster K.A. (1995) The effect of botulinum neurotoxins on the release of insulin from the insulinoma cell lines HIT-15 and RINm5F. Journal of Biological Chemistry 270: 18216-18218.

Branch, C., Hwang, C.F., Navarre, D.A., Williamson, V.M. (2004) Salicylic acid is part of the Mi-1-mediated defense response to root-knot nematode in tomato. Molecular Plant Microbe Interactions 17: 351-356.

Bubeck, J., Scheuring, D., Hummel, E., Langhans, M., Viotti, C., Foresti, O., Denecke, J., Banfield, D.K., Robinson, D.G. (2008) The syntaxins SYP31 and SYP81 control ER-Golgi trafficking in the plant secretory pathway. Traffic 9: 1629-1652.

Burgess, R.W., Deitcher, D.L., Schwarz, T.L. (1997) The synaptic protein syntaxin1 is required for cellularization of Drosophila embryos. Journal of Cell Biology 138: 861-875.

Byrd, D.W. Jr., Kirkpatrick, T., Barker, K.R. (1983) An improved technique for clearing and staining plant tissue for detection of nematodes. Journal of Nematology 15: 142-143.

Caldwell, B.E., Brim, C.A., Ross, J.P. (1960) Inheritance of resistance of soybeans to the soybean cyst nematode, *Heterodera glycines*. Agronomy Journal 52:635-636.

Campbell, P., Braam, J. (1998) Co- and/or post-translational modifications are critical for TCH4 XET activity. Plant Journal 15: 553-561.

Cao, H., Bowling, S.A., Gordon, A.S., Dong, X. (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6: 1583-1592.

Chatre, L., Wattelet-Boyer, V., Melser, S., Maneta-Peyret, L., Brandizzi, F., Moreau, P. (2009) A novel di-acidic motif facilitates ER export of the syntaxin SYP31. Journal of Experimental Botany 60: 3157-3165.

Chitwood, D.J. (2003) Nematicides. Pages 1104-1115 in J. R. Plimmer, ed., Encyclopedia of Agrochemicals, Vol. 3. New York: John Wiley, Sons.

Clary, D.O., Griff, I.C., Rothman, J.E. (1990) SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. Cell 61: 709-721.

Collins, N.C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu J.L., Hückelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S.C., Schulze-Lefert, P. (2003) SNARE-protein mediated disease resistance at the plant cell wall. Nature 425**:** 973-977.

Cook, D.E., Lee, T.G., Guo, X., Melito, S., Wang, K., Bayless, A., Wang, J., Hughes, T.J., Willis, D.K., Clemente, T., Diers, B.W., Jiang, J., Hudson, M.E., Bent, A.F. (2012) Copy Number Variation of Multiple Genes at *Rhg1* Mediates Nematode Resistance in Soybean. Science 338: 1206-1209.

Cotrufo, T., Pérez-Brangulí, F., Muhaisen, A., Ros O., Andrés, R., Baeriswyl, T., Fuschini, G., Tarrago, T., Pascual, M., Ureña, J., Blasi, J., Giralt, E., Stoeckli, E.T., Soriano, E. (2011) A signaling mechanism coupling netrin-1/deleted in colorectal cancer chemoattraction to SNARE-mediated exocytosis in axonal growth cones. Journal of Neuroscience 31: 14463-14480.

Cregan, P.B., Mudge, J., Fickus, E.W., Danesh, D., Denny, R.,, Young, N.D. (1999) Two simple sequence repeat markers to select for soybean cyst nematode resistance conditioned by the *rhg1* locus. Theoretical and Applied Genetics 99: 811-818.

DeBello, W.M., O'Connor, V., Dresbach T., Whiteheart, S.W., Wang, S.S., Schweizer, F.E., Betz, H., Rothman, J.E., Augustine, G.J. (1995) SNAP-mediated protein-protein interactions essential for neurotransmitter release. Nature 373: 626-630.

Delaney, T.P., Friedrich, L., Ryals, J.A. (1995) *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. Proceedings of the National Academy of Sciences USA 92: 6602-6606.

Endo, B.Y. (1964) Penetration and development of *Heterodera glycines* in soybean roots and related and related anatomical changes. Phytopathology 54: 79-88.

Endo, B.Y. (1965) Histological responses of resistant and susceptible soybean varieties, and backcross progeny to entry development of *Heterodera glycines*. Phytopathology 55: 375-381.

Endo, B.Y. (1991) Ultrastructure of initial responses of susceptible and resistant soybean roots to infection by *Heterodera glycines*. Revue of Nématology 14: 73-84.

Esmon, B., Novick, P., Schekman, R. (1981) Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. Cell 25: 451-460.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J., Parker, J.E. (1999) *EDS1*, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proceedings of the National Academy of Sciences USA 96: 3292-3297.

Feys, B.J., Moisan, L.J., Newman, M.A., Parker J.E. (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, *EDS1* and PAD4. EMBO Journal 20: 5400-5411.

Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K., Matthews, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. Biochemical Journal 282: 821-828.

Genovesi, V., Fornalé, S., Fry, S.C., Ruel, K., Ferrer, P., Encina, A., Sonbol, F.M., Bosch, J., Puigdomènech, P., Rigau, J., Caparrós-Ruiz, D. (2008) ZmXTH1, a new xyloglucan endotransglucosylase/hydrolase in maize, affects cell wall structure and composition in *Arabidopsis thaliana*. Journal of Experimental Botany 59: 875-889.

Gerber, S.H., Rah, J.C., Min, S.W., Liu, X., de Wit, H., Dulubova, I., Meyer, A.C., Rizo, J., Arancillo, M., Hammer, R.E., Verhage, M., Rosenmund, C., Südhof, T.C. (2008) Conformational switch of syntaxin-1 controls synaptic vesicle fusion. Science 321: 1507-1510.

Gipson, I., Kim, K.S., Riggs, R.D. (1971) An ultrastructural study of syncytium development in soybean roots infected with *Heterodera glycines*. Phytopathology 61: 347-353.

Glazebrook, J., Rogers, E.E., Ausubel, F.M. (1996) Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics 143: 973-998.

Glick, B.S., Rothman, J.E. (1987) Possible role for fatty acyl-coenzyme A in intracellular protein transport. Nature 326: 309-312.

Golden, A.M., Epps J.M., Riggs, R.D., Duclos, L.A., Fox, J.A., Bernard R.L. (1970) Terminology and identity of infraspecific forms of the soybean cyst nematode *(Heterodera glycines).* Plant Disease Reports 54: 544-546.

Haas, J.H., Moore, L.W., Ream, W., Manulis, S. (1995) Universal PCR primers for detection of phytopathogenic Agrobacterium strains. Applied Environmental Microbiology 61: 2879-2884.

Hardwick, K.G., Pelham, H.R. (1992) SED5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. Journal of Cell Biology 119: 513-521.

Heidrich, K., Wirthmueller, L., Tasset, C., Pouzet, C., Deslandes, L., Parker J.E. (2011) Arabidopsis *EDS1* Connects PathogenEffector Recognition to Cell Compartment–Specific Immune Responses Science 334: 1401-1404.

Henriksson, H., Denman, S.E., Campuzano, I.D., Ademark, P., Master, E.R., Teeri, T.T., Brumer, H. 3rd. (2003) N-linked glycosylation of native and recombinant cauliflower xyloglucan endotransglycosylase 16A. Biochemical Journal 375: 61-73.

Hofgen, R., Willmitzer, L. (1988) Storage of competent cells for Agrobacterium transformation. Nucleic Acids Research 16: 9877.

Hong, K–K., Chakravarti, A., Takahashi, J.S. (2004) The gene for soluble N-ethylmaleimide sensitive factor attachment protein a is mutated in hydrocephaly with hop gait (hyh) mice. Proceedings of the National Academy of Sciences USA 101: 1748-1753.

Humphry, M, Bednarek, P, Kemmerling, B, Koh, S, Stein, M, Göbel, U, Stüber, K, Pislewska-Bednarek, M, Loraine, A, Schulze-Lefert, P, Somerville, S, Panstruga, R. (2010) A regulon conserved in monocot and dicot plants defines a functional module in antifungal plant immunity. Proceedings of the National Academy of Sciences USA 107: 21896-21901.

Ishihara, N., Hamasaki, M., Yokota, S., Suzuki, K., Kamada, Y., Kihara, A., Yoshimori, T., Noda, T., Ohsumi, Y. (2001) Autophagosome requires specific early Sec proteins for its formation and NSF/SNARE for vacuolar fusion. Molecular Biology of the Cell 12: 3690-3702.

Itin, C., Rancaño, C., Nakajima, Y., Pfeffer, S.R. (1997) A novel assay reveals a role for soluble N-ethylmaleimide-sensitive fusion attachment protein in mannose 6-phosphate receptor transport from endosomes to the trans Golgi network. Journal of Biological Chemistry 272: 27737-27744.

Jahn, R., Fasshauer, D. (2012) Molecular machines governing exocytosis of synaptic vesicles. Nature 490: 201-207.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO Journal 6: 3901-3907.

Jenkins, W.R. (1964) A rapid centrifugal flotation technique for separating nematodes from soil. Plant Disease Reports 48: 692.

Jones, M.G.K., Northcote D.H. (1972) Nematode-induced syncytium-a multinucleate transfer cell. Journal of Cell Science 10: 789-809.

Jones, M.G.K. (1981) The development and function of plant cells modified by endoparasitic nematodes. Pages 255-279 in: Plant Parasitic Nematodes, Vol. III. B. M. Zuckerman and R. A. Rohde, eds. Academic Press, New York, U.S.A.

Kachroo, P., Yoshioka, K., Shah, J., Dooner, H., Klessig, D.F. (2000) Resistance to turnip crinkle virus in Arabidopsis requires two host genes and is salicylic acid dependent but *NPR1*, ethylene and jasmonate independent. Plant Cell 12: 677-690.

Kaiser, C.A., Schekman, R. (1990) Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. Cell 61: 723-733.

Kalde, M., Nühse, T.S., Findlay, K., Peck, S.C. (2007) The syntaxin SYP132 contributes to plant resistance against bacteria and secretion of pathogenesis-related protein 1. Proceedings of the National Academy of Sciences USA 104: 11850-11855.

Kallas, A.M., Piens, K., Denman, S.E., Henriksson, H., Fäldt, J., Johansson, P., Brumer, H., Teeri, T.T. (2005) Enzymatic properties of native and deglycosylated hybrid aspen (Populus tremulaxtremuloides) xyloglucan endotransglycosylase 16A expressed in Pichia pastoris. Biochemical Journal 390: 105-113.

Kauffmann, S., Legrand, M., Geoffroy, P., Fritig, B. (1987) Biological function of 'pathogenesis-related" proteins: four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO Journal 6: 3209-3212.

Kauffmann, S., Legrand, M., Fritig, B. (1990) Isolation and characterization of six pathogenesis-related (PR) proteins of Samsun NN tobacco. Plant Molecular Biology 14: 381-390.

Kim, D.G., Riggs, R.D., Mauromoustakos, A. (1998) Variation in Resistance of Soybean Lines to Races of *Heterodera glycines*. Journal of Nematology 30: 184-191.

Kim, M., Hyten, D.L., Bent, A.F., Diers, B.W. (2010) Fine mapping of the SCN resistance locus *rhg1-b* from PI 88788. The Plant Genome 3: 81-89.

Klink, V.P., Alkharouf, N., MacDonald, M., Matthews, B.F. (2005) Laser capture microdissection (LCM) and analysis of *Glycine max* (soybean) syncytial cells formed by the soybean cyst nematode *Heterodera glycines*. Plant Molecular Biology 59: 969-983

Klink, V.P., Overall, C.C., Alkharouf, N., MacDonald, M.H., Matthews, B.F. (2007) Laser capture microdissection (LCM) and comparative microarray expression analysis of syncytial cells isolated from incompatible and compatible soybean roots infected by soybean cyst nematode (*Heterodera glycines*). Planta 226: 1389-1409.

Klink, V.P., MacDonald, M.H., Martins, V.E., Park, S.C., Kim, K.H., Baek, S.H., Matthews, B.F. (2008) MiniMax, a new diminutive *Glycine max*variety, with a rapid life cycle, embryogenic potential and transformation capabilities. Plant Cell, Tissue and Organ Culture 92: 183-195.

Klink, V.P., Kim, K-H., Martins, V.E., MacDonald, M.H., Beard, H.S., Alkharouf, N.W., Lee S-K., Park, S-C., Matthews, B.F. (2009) A correlation between host-mediated expression of parasite genes as tandem inverted repeats and abrogation of the formation of female *Heterodera glycines* cysts during infection of Glycine max. Planta 230: 53-71.

Klink, V.P., Overall, C.C., Alkharouf, N., MacDonald, M.H., Matthews, B.F. (2010) Microarray detection calls as a means to compare transcripts expressed within syncytial cells isolated from incompatible and compatible soybean (Glycine max) roots infected by the soybean cyst nematode (*Heterodera glycines*). Journal of Biomedicine and Biotechnology 2010: 1-30.

Klink, V.P., Thibaudeau, G., Altig, R.G. (2013) A novel sample preparation method that enables nucleic acid analysis from ultrathin sections. Microscopy and Microanalysis 19: 1-7.

Kwon, C., Neu, C., Pajonk, S., Yun, H.S., Lipka, U., Humphry, M., Bau, S., Straus, M., Kwaaitaal, M., Rampelt, H., El Kasmi, F., Jürgens, G., Parker, J., Panstruga, R., Lipka, V., Schulze-Lefert, P. (2008) Co-option of a default secretory pathway for plant immune responses. Nature 451: 835-840.

Lai, Z., Wang, F., Zheng, Z., Fan, B., Chen, Z. (2011) A critical role of autophagy in plant resistance to necrotrophic fungal pathogens. Plant Journal 66: 953-968.

Laluk, K., Luo, H., Chai, M., Dhawan, R., Lai, Z., Mengiste, T. (2011) Biochemical and Genetic Requirements for Function of the Immune Response Regulator BOTRYTIS-INDUCED KINASE1 in Plant Growth, Ethylene Signaling, and PAMP-Triggered Immunity in Arabidopsis. Plant Cell 23: 2831-2849.

Lauber, M.H., Waizenegger, I., Steinmann, T., Schwarz, H., Mayer, U., Hwang, I., Lukowitz, W., Jürgens, G. (1997) The Arabidopsis KNOLLE protein is a cytokinesis-specific syntaxin. Journal of Cell Biology 139: 1485-1493.

Legrand, M., Kauffman, S., Geoffroy, P., Fritig, B. (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis related proteins are chitinases. Proceedings of the National Academy of Sciences USA 84: 6750-6754.

Leyman, B., Geelen, D., Quintero, F.J., Blatt, M.R. (1999) A tobacco syntaxin with a role in hormonal control of guard cell ion channels. Science 283: 537-540.

Li, J., Todd, T.C., Oakley, T.R., Lee, J., Trick, H.N. (2010) Host derived suppression of nematode reproductive and fitness genes decreases fecundity of *Heterodera glycines*. Planta 232: 775-785.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D., Llorente, F., Molina, A., Parker, J., Somerville, S., Schulze-Lefert, P. (2005) Pre-and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science 310: 1180-1183.

Liu, X., Liu, S., Jamai, A., Bendahmane, A., Lightfoot, D.A., Mitchum, M.G., Meksem, K. (2011) Soybean cyst nematode resistance in soybean is independent of the *Rhg4* locus LRR-RLK gene Functional and Integrative Genomics 11: 539-549.

Liu, S., Kandoth, P.K., Warren S.D., Yeckel, G., Heinz, R., Alden, J., Yang, C., Jamai, A., El-Mellouki, T., Juvale, P.S., Hill, J., Baum, T.J., Cianzio, S., Whitham, S.A., Korkin, D., Mitchum, M.G., Meksem, K. (2012) A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens. Nature 492: 256-260.

Livak, K.J., Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., He, P. (2010) A receptor-like cytoplasmic kinase, *BIK1*, associates with a flagellin receptor complex to initiate plant innate immunity. Proceedings of the National Academy of Sciences USA 107: 496-501.

Lukowitz, W., Mayer, U., Jürgens, G. (1996) Cytokinesis in the Arabidopsis embryo involves the syntaxin-related KNOLLE gene product. Cell 84: 61-71.

Lupashin, V.V., Pokrovskaya, I.D., McNew, J.A., Waters, M.G. (1997) Characterization of a novel yeast SNARE protein implicated in Golgi retrograde traffic. Molecular Biology of the Cell 8: 2659-2676.

Maris, A., Suslov, D., Fry, S.C., Verbelen, J.P., Vissenberg, K. (2009) Enzymic characterization of two recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of Arabidopsis and their effect on root growth and cell wall extension. Journal of Experimental Botany 60: 3959-3972.

Matsushima, R., Hayashi, Y., Yamada, K., Shimada, T., Nishimura, M., Hara-Nishimura, I. (2003a) The ER body, a novel endoplasmic reticulum derived structure in Arabidopsis. Plant Cell Physiology 44: 661-666.

Matsushima, R., Kondo, M., Nishimura, M., Hara-Nishimura, I. (2003b) A novel ER-derived compartment, the ER body, selectively accumulates a β- glucosidase with an ER-retention signal in Arabidopsis. Plant Journal 33: 493-502.

Matsye, P.D., Kumar, R., Hosseini, P., Jones, C.M., Tremblay, A., Alkharouf, N.W., Matthews, B.F., Klink, V.P. (2011) Mapping cell fate decisions that occur during soybean defense responses. Plant Molecular Biology 77: 513-528.

Matsye, P.D., Lawrence, G.W., Youssef, R.M., Kim K-H., Matthews, B.F., Lawrence, K.S., Klink, V.P. (2012) The expression of a naturally occurring, truncated allele of an α-SNAP gene suppresses plant parasitic nematode infection. Plant Molecular Biology 80: 131-155.

Matthews, B., MacDonald, M.H., Thai, V.K., Tucker, M.L. (2003) Molecular characterization of argenine kinase in the soybean cyst nematode (*Heterodera glycines*). Journal of Nematology 35: 252-258.

Matthews, B.F., Beard, H., MacDonald, M.H., Kabir, S., Youssef, R.M., Hosseini, P., Brewer, E. (2013) Engineered resistance and hypersusceptibility through functional metabolic studies of 100 genes in soybean to its major pathogen, the soybean cyst nematode. Planta 237: 1337-1357.

Mazarei, M., Elling, A.A., Maier, T.R., Puthoff, D.P., Baum, T.J. (2007) Gm-EREBP1 is a transcription factor activating defense genes in soybean and Arabidopsis. Molecular Plant Microbe Interactions 20: 107-119.

McLean, M.D., Hoover, G.J., Bancroft, B., Makhmoudova, A., Clark, S.M., Welacky T., Simmonds, D.H., Shelp, B.J. (2007) Identification of the full-length *Hs1*$^{pro-1}$ coding sequence and preliminary evaluation of soybean cyst nematode resistance in soybean transformed with *Hs1*$^{pro-1}$ cDNA. Canadian Journal of Botany 85: 437-441.

Melito, S., Heuberger, A., Cook, D., Diers, B., MacGuidwin, A., Bent, A. (2010) A nematode demographics assay in transgenic roots reveals no significant impacts of the *Rhg1* locus LRR-Kinase on soybean cyst nematode resistance. BMC Plant Biology 10: 104.

Melser, S., Wattelet-Boyer, V., Brandizzi, F., Moreau, P. (2009) Blocking ER export of the Golgi SNARE SYP31 affects plant growth. Plant Signaling and Behavior 4: 962-964.

Meyer, D., Pajonk, S., Micali, C., O'Connell, R., Schulze-Lefert, P. (2009) Extracellular transport and integration of plant secretory proteins into pathogen-induced cell wall compartments. Plant Journal 57: 986-999.

Milligan, S.B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., Williamson, V.M. (1998) The root knot nematode resistance gene Mi from tomato is a member of the leucine zipper, nucleotide binding, leucine- rich repeat family of plant genes. Plant Cell 10: 1307-1319.

Moreno, J.I., Martín, R., Castresana, C. (2005) Arabidopsis SHMT1, a serine hydroxymethyltransferase that functions in the photorespiratory pathway influences resistance to biotic and abiotic stress. Plant Journal 41: 451-463.

Morgan, A., Burgoyne, R.D. (1995) A role for soluble NSF attachment proteins (SNAPs) in regulated exocytosis in adrenal chromaffin cells. EMBO Journal 14: 232-239.

Neiman, A.M. (1998) Prospore membrane formation defines a developmentally regulated branch of the secretory pathway in yeast. Journal of Cell Biology 140: 29-37.

Niblack, T.L., Arelli, P.R., Noel, G.R., Opperman, C.H., Orf, J.H., Schmitt, D.P., Shannon, J.G., Tylka, G.L. (2002) A revised classification scheme for genetically diverse populations of *Heterodera glycines*. Journal of Nematology 34: 279-288.

Novick, P., Schekman, R. (1979) Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences USA 76: 1858-1862.

Novick, P., Field, C., Schekman, R. (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 21: 205-215.

Novick, P., Ferro, S., Schekman, R. (1981) Order of events in the yeast secretory pathway. Cell 25: 461-469.

Ogasawara, K., Yamada, K., Christeller, J.T., Kondo, M., Hatsugai, N., Hara-Nishimura, I., Nishimura, M. (2009) Constitutive and inducible ER bodies of *Arabidopsis thaliana* accumulate distinct β-glucosidases Plant Cell Physiology 50: 480-488.

Pajonk, S., Kwon, C., Clemens, N., Panstruga, R., Schulze-Lefert, P. (2008) Activity determinants and functional specialization of Arabidopsis *PEN1* syntaxin in innate immunity. Journal of Biological Chemistry 283: 26974-26984.

Peng, R., Gallwitz, D. (2004) Multiple SNARE interactions of an SM protein: Sed5p/Sly1p binding is dispensable for transport. EMBO Journal 23: 3939-3949.

Perrotta, C., Bizzozero, L., Cazzato, D., Morlacchi, S., Assi, E., Simbari, F., Zhang, Y., Gulbins, E., Bassi, M.T., Rosa, P., Clementi, E. (2010) Syntaxin 4 is required for acid sphingomyelinase activity and apoptotic function. Journal of Biological Chemistry 285: 40240-40251.

Peter, F., Wong, S.H., Subramaniam, V.N., Tang, B.L., Hong, W. (1998) α -SNAP but not gamma-SNAP is required for ER-Golgi transport after vesicle budding and the Rab1-requiring step but before the EGTA-sensitive step. Journal of Cell Science 111: 2625-2633.

Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature Methods 8: 785-786.

Ramalho-Santos, J., Moreno, R.D., Sutovsky, P., Chan, A.W., Hewitson, L., Wessel, G.M., Simerly C.R., Schatten G. (2000) SNAREs in mammalian sperm: possible implications for fertilization. Developmental Biology 223: 54-69.

Rancour, D.M., Dickey, C.E., Park, S., Bednarek, S.Y. (2002) Characterization of AtCDC48. Evidence for multiple membrane fusion mechanisms at the plane of cell division in plants. Plant Physiology 130: 1241-1253.

Raridan, G.J., Delaney, T.P. (2002) Role of salicylic acid and NIM1/*NPR1* in race-specific resistance in Arabidopsis. Genetics 161: 803-811.

Riggs, R.D., Schmitt, D.P. (1988) Complete characterization of the race scheme for *Heterodera glycines*. Journal of Nematology 20: 392-395.

Riggs, R.D., Schmitt, D.P. (1991) Optimization of the *Heterodera glycines* race test procedure. Journal of Nematology 23: 149-154.

Rodrı´guez, F., Bustos, M.A., Zanetti, M.N., Ruete, M.C., Mayorga, L.S., Tomes, C.N. (2011) α–SNAP prevents docking of the acrosome during sperm exocytosis because it sequesters monomeric syntaxin. PLoS ONE 6: e21925.

Ross, J.P., Brim, C.A. (1957) Resistance of soybeans to the soybean cyst nematode as determined by a double-row method. Plant Disease Reports 41: 923-924.

Ross, J.P. (1958) Host-Parasite relationship of the soybean cyst nematode in resistant soybean roots. Phytopathology 48: 578-579.

Sasser, J.N., Freckman, D.W. (1987) A world perspective on nematology: the role of the society. In: Veech JA, Dickerson DW. (eds) Vistas on nematology. Society of Nematologists, Hyattsville, pp 7-14.

Sakamoto, A.N., Lan, V.T., Puripunyavanich, V., Hase, Y., Yokota, Y., Shikazono, N., Nakagawa, M., Narumi, I., Tanaka, A. (2009) A UVB hypersensitive mutant in *Arabidopsis thaliana* is defective in the DNA damage response. Plant Journal 60: 509-517.

Sanderfoot, A.A., Pilgrim, M., Adam, L., Raikhel, N.V. (2001a) Disruption of individual members of Arabidopsis syntaxin gene families indicates each has essential functions. Plant Cell 13: 659-666.

Sanderfoot, A.A., Farhah, F., Assaad, F.F., Natasha, V., Raikhel, N.V. (2001b) The Arabidopsis genome. An abundance of soluble N-ethylmaleimide-sensitive factor adaptor protein receptors. Plant Physiology 124: 1558-1569.

Sanderfoot, A.A., Kovaleva, V., Bassham, D.C., Raikhel, N.V. (2001c) Interactions between Syntaxins Identify at Least Five SNARE Complexes within the Golgi/Prevacuolar System of the Arabidopsis Cell. Molecular Biology of the Cell 12: 3733-3743.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma J., Mitros, T., Nelson, W., Hyten, D.L., Song, Q., Thelen, J.J., Cheng, J., Xu D., Hellsten, U., May, G.D., Yu Y., Sakurai, T., Umezawa, T., Bhattacharyya, M.K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto M., Grant D., Shu S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X.C., Shinozaki, K., Nguyen, H.T., Wing, R.A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R.C., Jackson, S.A. (2010) Genome sequence of the palaeopolyploid soybean. Nature 463: 178-183.

Schulz, J.R., Wessel, G.M., Vacquier, V.D (1997) The exocytosis regulatory proteins syntaxin and VAMP are shed from sea urchin sperm during the acrosome reaction. Developmental Biology 191: 80-87.

Shah, J., Tsui, F., Klessig, D.F. (1997) Characterization of a salicylic acid insensitive mutant (sai1) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. Molecular Plant Microbe Interactions 10: 69-78.

Shah, J., Kachroo, P.K., Nandi, A., Klessig, D.F. (2001) A recessive mutation in the Arabidopsis SSI2 gene confers SA and *NPR1*-independent expression of PR genes and resistance against bacterial and oomycete pathogens. Plant Journal 25: 563-574.

Shirano, Y., Kachroo, P., Shah, J., Klessig, D.F. (2002) A gain-of-function mutation in an Arabidopsis toll interleukin 1 receptor—nucleotide binding site—leucine-rich repeat type R gene triggers defense responses and results in enhanced disease resistance. Plant Cell 14: 3149-3162.

Steeves, R.M., Todd, T.C., Essig, J.S., Trick, H.N. (2006) Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress *Heterodera glycines* reproduction. Functional Plant Biology 33: 991-999.

Stein, M., Dittgen, J., Sánchez-Rodríguez, C., Hou, B.H., Molina, A., Schulze-Lefert, P., Lipka, V., Somerville S. (2006) Arabidopsis *PEN3*/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18: 731-746.

Takahashi, H., Miller, J., Nozaki, Y., Takeda, M., Shah, J., Hase, S., Ikegami, M., Ehara, Y., Dinesh-Kumar, S.P. (2002) RCY1, an *Arabidopsis thaliana* RPP8/HRT family resistance gene, conferring resistance to cucumber mosaic virus requires salicylic acid, ethylene and a novel signal transduction mechanism. Plant Journal 32: 655-667.

Thompson, G.A., Jr., Okuyama, H. (2000) Lipid-linked proteins of plants. Progress in Lipid Research 39: 19-39.

Van der Biezen E.A., Freddie C.T., Kahn K., Parker J.E., Jones J.D.G. (2002) Arabidopsis RPP4 is a member of the RPP5 multigene family of TIR-NB-LRR genes and confers downy mildew resistance through multiple signalling components. Plant Journal 29: 439-451.

Veronese, P., Nakagami, H., Bluhm, B., Abuqamar, S., Chen, X., Salmeron, J., Dietrich, R.A., Hirt, H., Mengiste, T. (2006) The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in Arabidopsis resistance to necrotrophic and biotrophic pathogens. Plant Cell 18: 257-273.

Waizenegger, I., Lukowitz, W., Assaad, F., Schwarz, H., Jürgens, G., Mayer, U. (2000) The Arabidopsis KNOLLE and KEULE genes interact to promote vesicle fusion during cytokinesis. Current Biology 2: 1371-1374.

Wildermuth, M.C., Dewdney, J., Wu G., Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defense. Nature 414: 562-565.

Woo, K.C. (1979) Properties and intramitochondrial localization of serine hydroxymethyltransferase in leaves of higher plants. Plant Physiology 63: 783-787.

Wu, S., Lu, D., Kabbage, M., Wei, H.L., Swingle, B., Records, A.R., Dickman, M., He P., Shan, L. (2011) Bacterial effector HopF2 suppresses Arabidopsis innate immunity at the plasma membrane. Molecular Plant Microbe Interactions 24: 585-593.

Wubben, M.J., Jin, J., Baum, T.J. (2008) Cyst nematode parasitism of *Arabidopsis thaliana* is inhibited by salicylic Acid [SA] and elicits uncoupled SA-independent pathogenesis-related gene expression in roots. Molecular Plant Microbe Interactions 21: 424-443.

Xiao, S., Brown, S., Patrick, E., Brearley, C., Turner, J.G. (2003) Enhanced transcription of the Arabidopsis disease resistance genes RPW8.1 and RPW8.2 via a salicylic acid-dependent amplification circuit is required for hypersensitive cell death. Plant Cell 15: 33-45.

Yokoyama, R., Nishitani, K. (2001) Endoxyloglucan transferase is localized both in the cell plate and in the secretory pathway destined for the apoplast in tobacco cells. Plant Cell Physiology 42: 292-300.

Youssef, R.M., MacDonald, M.H., Brewer, E.P., Bauchan, G.R., Kim, K-H., Matthews, B.F. (2013) Ectopic expression of AtPAD4 broadens resistance of soybean to soybean cyst and root-knot nematodes. BMC Plant Biology 13: 67.

Zhang, J., Li W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S., Mengiste, T., Zhang, Y., Zhou, J.M. (2010) Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a Pseudomonas syringae effector. Cell Host Microbe 7: 290-301.

CHAPTER III

THE SYNTAXIN 31-INDUCED GENE, *LESION SIMULATING DISEASE1* (*LSD1*), FUNCTIONS IN *GLYCINE MAX* DEFENSE TO THE ROOT PARASITE *HETERODERA GLYCINES*[2]

Abstract

Experiments show the membrane fusion genes α-soluble NSF attachment protein (α-SNAP) and syntaxin 31 (Gm-SYP38) contribute to the ability of *Glycine max* to defend itself from infection by the plant parasitic nematode *Heterodera glycines*. Accompanying their expression is the transcriptional activation of the defense genes *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) and *NONEXPRESSOR OF PR1* (*NPR1*) that function in salicylic acid (SA) signaling. These results implicate the added involvement of the antiapoptotic, environmental response gene *LESION SIMULATING DISEASE1* (*LSD1*) in defense. Roots engineered to overexpress the *G. max* defense genes Gm-α-SNAP, SYP38, *EDS1*, *NPR1*, BOTRYTIS INDUCED KINASE1 (*BIK1*) and xyloglucan endotransglycosylase/hydrolase (XTH) in the susceptible genotype *G. max* [Williams 82/PI 518671] have induced Gm-LSD1 (Gm-LSD1-2) transcriptional activity. In

[2] "This is an unofficial translation of a [Taylor & Francis and Routledge Open article / Taylor & Francis and Routledge Open Select article] that appeared in a Taylor & Francis publication. Taylor & Francis and / or the rightsholder has not endorsed this translation." Most of the content of this chapter has been adapted from the journal article: Pant, S.R., Krishnavajhala, A., McNeece, B.T., Lawrence, G.W. and Klink, V.P. (2015) The syntaxin 31-induced gene, LESION SIMULATING DISEASE1 (*LSD1*), functions in *Glycine max* defense to the root parasite *Heterodera glycines*. Plant Signaling and Behaviour 10: 1, e977737

reciprocal experiments, roots engineered to overexpress Gm-LSD1-2 in the susceptible genotype *G. max*[Williams 82/PI 518671] have induced levels of SYP38, *EDS1*, *NPR1*, *BIK1* and XTH, but not α-SNAP prior to infection. In tests examining the role of Gm-LSD1-2 in defense, its overexpression resulted in 52 to 68% reduction in nematode parasitism. In contrast, RNA interference (RNAi) of Gm-LSD1-2 in the resistant genotype *G. max* [Peking/PI 548402] results in an 3.24-10.42 fold increased ability of *H. glycines* to parasitize. The results identify that Gm-LSD1-2 functions in the defense response of *G. max* to *H. glycines* parasitism. It is proposed that *LSD1*, as an antiapoptotic protein, may establish an environment whereby the protected, living plant cell could secrete materials in the vicinity of the parasitizing nematode to disarm it. After the targeted incapacitation of the nematode the parasitized cell succumbs to its targeted demise as the infected root region is becoming fortified.

Introduction

Knowledge of the ability of biological membranes to fuse, resulting in the delivery of vesicle contents to different cellular destinations, is longstanding (Palade, 1975). Genetic experiments and screens in model organisms have identified the proteins that function in the process and ordered the events that lead to material delivery in the form of secretion (Novick and Schekman 1979; Novick *et al.* 1980; Novick *et al.* 1980). Subsequent work in other systems has demonstrated that the core protein machinery involved in membrane fusion is highly conserved, found in all eukaryotes (Reviewed in Jahn and Fasshauer 2012). The process of membrane fusion requires fidelity and protective measures are taken by the cell to ensure it happens properly (Lobingier *et al.* 2014).

Through recent studies, a link between membrane fusion at the cell membrane and also the *cis* face of the Golgi apparatus with SA signaling has been made in plants (Zhang *et al.* 2007; Matsye *et al.* 2012; Pant *et al.* 2014). Genetic work in the plant genetic model, *Arabidopsis thaliana* has also identified essential roles for proteins involved in membrane fusion (Mayer *et al.* 1991). The essential nature of these membrane fusion proteins makes them difficult to study since their mutants are lethal or cause highly detrimental developmental anomalies (Novick and Schekman 1979; Novick *et al.* 1980; Mayer *et al.* 1991, Kwon *et al.* 2008). However, it is possible to study these proteins under certain circumstances. For example, a genetic screen employed by Mayer *et al.* (1991) has determined the role of vesicles in embryo cytokinesis. This approach has succeeded because the biosynthesis of the phragmoplast which relies on vesicles occurs early during embryo development. Subsequent identification of one of the *A. thaliana* genes involved in cytokinesis (*KNOLLE* [At-SYP111]) has determined it to be related to a *Saccharomyces cerevisiae* membrane associated protein known as suppressors of the erd2-deletion 5 (Sed5p) which is structurally homologous to syntaxin (Hardwick and Pelham 1992; Lukowitz *et al.* 1996; Sanderfoot *et al.* 2001a). Syntaxin is a protein involved in secretion, functioning in the fusion of membranes (Hardwick and Pelham 1992; Lukowitz *et al.* 1996). Syntaxins perform membrane fusion through their interaction with a number of other proteins (Reviewed in Jahn and Fasshauer 2012). One of these proteins is α-SNAP whose relation to plant defense has been demonstrated (Matsye *et al.* 2012; Hardwick and Pelham 1992; Clary *et al.* 1990*;* Lupashin *et al.* 1997). Since these discoveries, membrane fusion and vesicle transport have been well

documented in plants, with many of the related genes having orthologs in yeast and other systems (Sanderfoot *et al.* 2001a, b, c).

The roles that these core membrane fusion proteins perform in eukaryotes is extensive, ranging from signaling, cell growth, mitosis, the endocytic cycle, exocytosis, hormonal release, neurotransmission, fertilization, embryogenesis, development, sporulation and cell death (Novick and Schekman 1979; Novick *et al.* 1980; Lukowitz *et al.* 1996; Sanderfoot *et al.* 2001a, c; Clary *et al.* 1990; Bennett *et al.* 1992; Boyd 1995; Vroemen *et al.* 1996; Lauber *et al.* 1997; Burgess *et al.* 1997; Schulz *et al.* 1997, Neiman 1998; Peter *et al.* 1998; Ramalho-Santos *et al.* 2000, Waizenegger *et al.* 2000; Babcock *et al.* 2004; Hong *et al.* 2004; Perrotta *et al.* 2010; Cotrufo *et al.* 2011; Rodrıguez *et al.* 2011). A variety of studies show membrane fusion to be important to the defense process that plants have toward pathogens as well as different types of defense responses (Collins *et al.* 2003; Assaad *et al.* 2004; An *et al.* 2006a, 2006b; Kalde *et al.* 2007; Kwon *et al.* 2008; Patel and Dinesh-Kumar 2008, Hofius *et al.* 2009; Lenz *et al.* 2011; Lai *et al.* 2011; Pant *et al.* 2014). While the list of functions that the membrane fusion and vesicle transport proteins have is large, it is less clear whether the proteins also are engaged in other, but related functions.

Recent experiments in *G. max* have demonstrated that α-SNAP contributes to the resistance of *G. max* to the plant parasitic nematode, *Heterodera glycines* (Cook *et al.* 2011; Matsye *et al.* 2012). The α-SNAP gene was first identified in *S. cerevisiae* as Sec17p in a genetic screen for temperature sensitive secretion (*sec*) mutants (Novick *et al.* 1980). Subsequent research has demonstrated Sec17p is required for vesicle transport from the endoplasmic reticulum (ER) to the Golgi apparatus as mutants accumulated 50

nm vesicles (Novick *et al.* 1981; Esmon *et al.* 1981). The results presented by Matsye *et al.* (2012) identified the existence of a role for α-SNAP that went beyond membrane fusion. Matsye *et al.* (2012) examined the effect that the overexpression of an α-SNAP gene had on genes associated with different types of hormonal signaling that have known defense functions. While not comprehensive, these genes included an analysis of the SA-regulated cysteine rich secretory protein gene, pathogenesis-related 1 (PR1) (Antoniw and Pierpoint 1978). Furthermore, the study examined the transcriptional activity of other genes whose protein products are secreted. These genes included the ethylene responsive β-1,3-glucanase, PR2 (Kauffmann *et al.* 1987), the ethylene and jasmonic acid (JA) responsive chitinase gene, PR3 (Legrand *et al.* 1987) and the SA-responsive thaumatin, PR5 (Kauffmann *et al.* 1990). In those experiments, Matsye *et al.* (2012) demonstrated α-SNAP overexpression causes induced expression of PR1, PR2 and PR5. Thus, the induced expression of components of the membrane fusion and vesicular transport machinery (α-SNAP) appears to influence the expression of genes that are vesicle cargo. To expand on this concept further, related experiments have been performed analyzing the effect that the overexpression of the α-SNAP binding partner, syntaxin 31 has on transcription (Pant *et al.* 2014). In these experiments, the overexpression of α-SNAP or SYP38 also results in the transcriptional induction of the SA signaling genes *EDS1* and *NPR1* (Pant *et al.* 2014). In *A. thaliana*, SA biosynthesis and signaling occurs through a well-understood pathway including the *EDS1* protein binding to the lipase *PHYTOALEXIN DEFICIENT 4* (*PAD4*) (Zhou *et al.* 1998; Falk *et al.* 1999; Feys *et al.* 2001). This heterodimer functions upstream of SALICYLIC-ACID-INDUCTION DEFICIENT2 (SID2), a putative chloroplast-localized isochorismate synthase, its allelic

EDS16, along with the multidrug and toxin extrusion (MATE) efflux transporter EDS5 to activate SA biosynthesis (Nawrath and Me´traux 1999; Wildermuth *et al.* 2001; Nawrath *et al.* 2002). Downstream, a complex composed of SA, the SA hormone receptor protein *NPR1*, copper ions and the transcription factor TGA2 forms (Niggeweg *et al.* 2000; Wu *et al.* 2012). The complex binds to a DNA promoter sequence composed of TGACG which results in the induction of PR1 transcription (Cao *et al.* 1994; Delaney *et al.* 1995; Glazebrook *et al.* 1996; Shah *et al.* 1997; Pieterse and Van 2004; Wu *et al.* 2012). Another gene that relates to SA signaling in *A. thaliana* is *LESION SIMULATING DISEASE1* (*LSD1*) (Dietrich *et al.* 1994). In *A. thaliana*, the *LSD1* gene is a negative regulator of programmed cell death (PCD) and its activity is antagonized by a related positive regulator of cell death gene called *LSD1-like* (*LOL1*) (Jabs *et al.* 1996; Dietrich *et al.* 1997; Kliebenstein *et al.* 1999; Epple *et al.* 2001; Wituszynska *et al.* 2013). Currently, it is unknown whether the *G. max LSD1* functions in defense. However, its involvement in establishing a tight boundary between cells targeted and not targeted for apoptosis makes it an intriguing candidate.

In the analysis presented here, the relationship between the *G. max* α-SNAP, Gm-SYP38 and SA signaling is examined further, adding to information generated in prior experiments (Pant *et al.* 2014). Gene expression experiments have identified induced levels of Gm-LSD1 (Gm-LSD1-2) in roots engineered to overexpress α-SNAP or SYP38. These results further strengthen a link between vesicle transport and SA signaling. Genetic engineering experiments reveal that the overexpression of Gm-LSD1-2 results in engineered resistance. In contrast, RNAi of Gm-LSD1-2 in a *G. max* genotype that is normally resistant to *H. glycines* infection results in roots that permit parasitism at

a higher frequency. It is shown the Gm-LSD1-2 overexpression positively influences the transcriptional activity of *G. max* SYP38, *EDS1*, *NPR1* and *BIK1*. Furthermore, the overexpression of Gm-LSD1-2 also results in the induction of the expression of the hemicellulose-modifying, vesicle-cargo gene XTH43. In contrast, their expression is suppressed in roots expressing an LSD1-2 RNAi construct. The experiments presented here identify an antiapoptotic aspect of defense in the *G. max -H. glycines* pathosystem.

Methods

Gene cloning

The candidate gene overexpression study presented here was done according to our published procedures using the pRAP15 and pRAP17 vectors (Matsye *et al.* 2012; Pant *et al.* 2014). The primers used to clone Gm-LSD1-2 (Glyma08g13630) are provided (Appendix Table B.1). The nature of the hairy root system is that each transgenic root system functions as an independent transformant line (Tepfer 1984; Pant *et al.* 2014). Amplicons, representing the gene of interest (GOI) generated by PCR were gel purified in 1.0% agarose using the Qiagen® gel purification kit, ligated into the directional pENTR/D-TOPO® vector and transformed into chemically competent *E. coli* strain One Shot TOP10. Chemical selection was done on LB-kanamycin (50 μg/ml) according to protocol (Invitrogen®). Amplicons were confirmed by sequencing and comparing the sequence to its original Genbank accession. The *G. max* amplicon was shuttled into the pRAP15 or pRAP17 destination vector using LR clonase (Invitrogen®). The engineered pRAP15 or pRAP17 vector was transformed into chemically competent *A. rhizogenes* strain K599 (K599) (Haas *et al.* 1995) using the freeze-thaw method (Hofgen and Willmitzer 1988) on LB-tetracycline (5 μg/ml).

The infection of *G. max* by *H. glycines*

Genetic transformation overexpression experiments were performed according to Pant *et al.* (2014) in the functionally hypomorphic *rhg1*$^{-/-}$ genetic background of *G. max*[Williams 82/PI 518671], lacking a defense response to *H. glycines* parasitism. In contrast, RNAi studies were performed in the *rhg1*$^{+/+}$ genetic background of *G. max* [Peking/PI 548402] according to Pant *et al.* (2014). Female *H. glycines*[NL1-Rhg/HG-type 7/race 3] were purified by sucrose flotation (Jenkins 1964; Matthews *et al.* 2003). Each root was inoculated with one ml of nematodes at a concentration of 2,000 second stage juveniles (J2s)/ml per root system (per plant), infected for 30 days and confirmed by acid fuchsin staining (Byrd *et al.* 1983). At the end of the experiment, the cysts (fully matured females) were collected over nested 20 and 100-mesh sieves (Pant *et al.* 2014). Furthermore, the soil was washed several times and the rinse water sieved to assure collection of all cysts (Pant *et al.* 2014). The accepted assay to accurately reflect if a condition exerts an influence on *H. glycines* development is the female index (FI) (Golden *et al.* 1970). The FI were calculated in a double blind analysis as FI = (Nx/Ns) x 100, where Nx is the average number of females on the test cultivar and Ns is the average number of females on the standard susceptible cultivar (Golden *et al.* 1970). Nx is the pRAP15-transformed line that had the engineered GOI. Ns is the pRAP15 control in their *G. max* [Williams 82/PI 518671]. The effect of the overexpressed gene on parasitism was tested statistically using the Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, $p < 0.05$ (Pant *et al.* 2014).

Histology

Histological observation was according to Klink et al. (2005), presented in Chapter 2. Briefly, tissue was fixed in Farmer's solution (FS) composed of 75% ethanol,

25% acetic acid (Sass 1958; Klink et al. 2005). Serial sections of roots were made on an American Optical 820® microtome (American Optical Co®.; Buffalo, NY, U.S.A.) at a section thickness of 10 μm. Sections were stained in Safranin O (Fisher Scientific Co.; Fair Lawn, NJ, U.S.A.) in 50% ETOH and counter-stained in Fast Green FCF (Fisher Scientific Co.) (Klink *et al*., 2005). For histological analyses, the tissue was permanently mounted in Permount® (Fisher Scientific Co.).

RNA-seq

Exon sequencing (RNA seq) was performed according to our original published work with modifications (Matsye *et al.* 2011). RNA was extracted from *G. max* roots using the UltraClean® Plant RNA Isolation Kit (Mo Bio Laboratories®, Inc.; Carlsbad, CA) and treated with DNase I to remove genomic DNA (Matsye *et al.* 2012, Pant *et al.* 2014). RNA-seq analyses were performed using the Illumina® HighSeq 2500® platform (Eurofins MWG Operon; Huntsville, Alabama). The RNAseq procedures that identified transcript (tag) counts and chromosomal coordinates of the *G. max* genome (Schmutz *et al.* 2010) along with the associated gene ontology (GO) annotations (Harris *et al.* 2004) were outlined here, subsequently. The qualities of raw reads were checked using program FASTQC. The updated genome sequence and annotation of *G. max (*Schmutz *et al.* 2010) were obtained from Phytozome v9.0 (dated: Nov 27, 2011). The abundance of transcripts across all samples was measured and compared (Trapnell *et al.* 2012) and default setting of the programs used unless specified. Briefly, the raw reads for each sample were mapped on *G. max* genome using TopHat v2.0.6 (Trapnell *et al.* 2009). Then, Cufflinks v2.0.2 (Trapnell *et al.* 2010) program was used to assemble the mapped reads into transcripts. The FPKM values were calculated for all genes in all samples and their

differential transcript expression (log base 2) computed using program Cuffdiff (Trapnell *et al.* 2010).

Quantitative real-time PCR (qPCR)

The qPCR experiments examining LSD-1-2 overexpression were performed according to Pant *et al.* (2014). The same root mRNA used in Pant *et al.* (2014) was used here for the qPCR analyses of roots overexpressing *G. max* SYP38, α-SNAP, EDS1-2, NPR1-2, XTH43, BIK1-6. The RNA was treated with DNase I to remove genomic DNA. The cDNA was reversed transcribed from RNA. This procedure was done using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen®) with oligo d(T) as the primer according to protocol (Invitrogen®). Genomic DNA contamination was assessed by PCR by using β-conglycinin primer pair (Appendix Table A.1) that amplify across an intron, thus yielding different sized DNA fragments based on the presence/absence of that intron (contaminating DNA). No contaminating genomic DNA existed in the cDNA as demonstrated in PCR reactions containing no template and reactions using RNA processed in parallel but with no Superscript® reverse transcriptase that also served as controls, producing no amplicon.

Primers used in qPCR gene expression experiments were provided in Appendix Table B.1. The experiments used the ribosomal protein gene S21 as a control (Klink *et al.* 2005; Matsye *et al.* 2012). Gene expression were tested in relation to several different classes of pathogenesis related (PR) genes, and defense genes (Table 3.2). The qPCR experiments used Taqman® 6-carboxyfluorescein (6-FAM) probes and Black Hole Quencher (BHQ1) (MWG Operon; Birmingham, AL). The qPCR differential expression

tests were performed according to Livak and Schmittgen (Livak and Schmittgen 2001). The qPCR reaction conditions were prepared according to Pant *et al.* (2014) and included a 20 µl Taqman Gene Expression Master Mix (Applied Biosystems; Foster City, CA), 0.9 µl of µM forward primer, 0.9 µl of 100 µM reverse primer, 2 µl of 2.5 µM 6-FAM (MWG Operon®) probe and 9.0 µl (900 ng) of template DNA. The qPCR reactions were executed on an ABI 7300 (Applied Biosystems®). The qPCR conditions included a preincubation of 50° C for 2 min, followed by 95° C for 10 min. This step was followed by alternating 95° C for 15 sec followed by 60° C for 1 min for 40 cycles.

Results

Gm-LSD1 is expressed in roots overexpressing α-SNAP, SYP38 and genes relating to SA signaling

Deep sequencing experiments show that the overexpression of the *G. max* Gm-SYP38 results in the induction of five α-SNAP paralogs, including the *rhg1* component Glyma18g02590 and Glyma11g35820 (Table 3.1). This result strengthened prior observations of the importance of α-SNAP to the process of defense (Matsye *et al.* 2012). Furthermore, Pant *et al.* (2014) has demonstrated that along with the involvement of Gm-SYP38 during the defense of *G. max* to *H. glycines*, its overexpression also results in induced levels of the SA signaling gene *EDS1*. The demonstration that SA signaling genes function in the defense of *G. max* to *H. glycines* has led to an analysis showing that Gm-LSD1 (Gm-LSD1-2) is induced in roots overexpressing Gm-SYP38 (Table 3.2). During parasitism, a well demarcated boundary is established between parasitized and non-parasitized cells in the *G. max* -*H. glycines* pathosystem (Figure 3.1).

Table 3.1 Deep sequencing of mRNA isolated from uninfected Gm-SYP38 overexpressing roots reveals altered transcriptional activity of the *rhg1* resistance gene, α-SNAP (Glyma18g02590) and paralogs of α-SNAP

α-SNAP	log2(fold change)	p-value	q-value	Significant
Glyma18g02590	0.396298	0.0204	0.03961	yes
Glyma11g35820	0.39959	0.0192	0.0375849	yes
Glyma14g05920	0.936435	5.00E-05	0.0001755	yes
Glyma02g42820	2.64661	0.00365	0.0086688	yes
Glyma09g41590	1.31903	5.00E-05	0.0001755	yes

The expression was statistically significant, $p < 0.05$. The expression was further tested using a false discovery rate (FDR) adjusted p-value (q-value) of 0.05, meaning that the correct call is made 95% of cases.

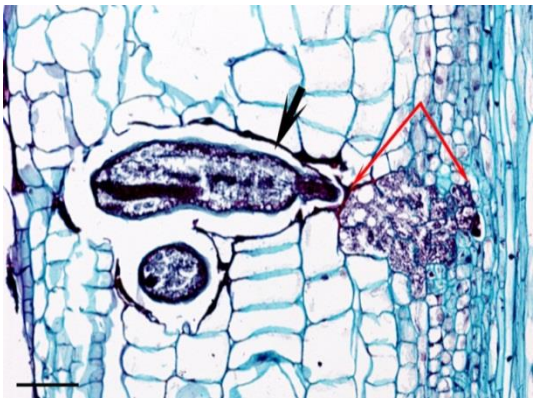


Figure 3.1 A 3 dpi image of *H. glycines* successfully parasitizing a root of *G. max*[Williams 82/PI 518671].

A *G. max*[Williams 82/PI 518671] root stained with Safranin O and counter-stained in Fast Green FCF. Please refer to the Materials section in Chapter II for details regarding the processing of the root specimen. Black arrow, nematode; red arrows, boundary of the nurse cell (syncytium). Bar = 100 μm

To understand the nature of Gm-LSD1-2 in relation to resistance (Figure 3.2), qPCR experiments have been performed using cDNA template from genetically engineered *G. max* roots that acquired the ability to defend itself from *H. glycines* parasitism. Roots genetically engineered to overexpress *G. max* α-SNAP, SYP38, *NPR1*, *EDS1*, *BIK1* or XTH43 exhibit induced levels of LSD1-2 (Table 3.2). The association of Gm-LSD1-2 expression in roots undergoing defense indicates that it may be performing

an important role in the process. To test this hypothesis, the susceptible *G. max*[Williams 82/PI 518671] has been engineered to overexpress Gm-LSD1-2 (Figure 3.3). No statistically significant effect is observed in root growth (Appendix Figure B.1). In experiments presented here, the overexpression of the Gm-LSD1-2 results in a significant reduction in parasitism (Figure 3.4).

Table 3.2 Gene expression analysis of *G. max* roots either overexpressing LSD1-2 or genetically engineered with a RNAi construct targeting LSD1-2

	Transgenic lines	
	LSD1-2 OE	**LSD1-2 RNAi**
Gene tested	**0 dpi**	**0 dpi**
LSD1	293.784	-1.851
EDS1	40.129	-2.094
NPR1	145.11	-2.346
SYP38	581.545	-1.889
α-SNAP	-3.104	N/A
BIK1	161.048	-1.359
XTH43	37.536	-1.223
PR1	4.276	3.192
PR2	159.282	-3.222
PR3	3.206	1.388
PR5	-2.005	1.2

The experiments used the ribosomal S21 gene as a control to standardize the qPCR experiments. The gene expression presented as fold change. N/A: expression not detected. An arbitrary cutoff of +/- 1.5 fold, $p < 0.05$ was used for differential expression.

To examine the specificity of the overexpression experiments, the expression of an RNAi cassette for Gm-LSD1-2 in the normally resistant genotype *G. max*[Peking/PI 548402] was done (Figure 3.3). No statistically significant effect is observed in root growth (Appendix Figure B.1). The expression of an RNAi cassette for Gm-LSD1-2 in the normally resistant genotype *G. max*[Peking/PI 548402] results in an increased capability of *H. glycines* to parasitize the resistant *G. max*[Peking/PI 548402] (Figure 3.5).

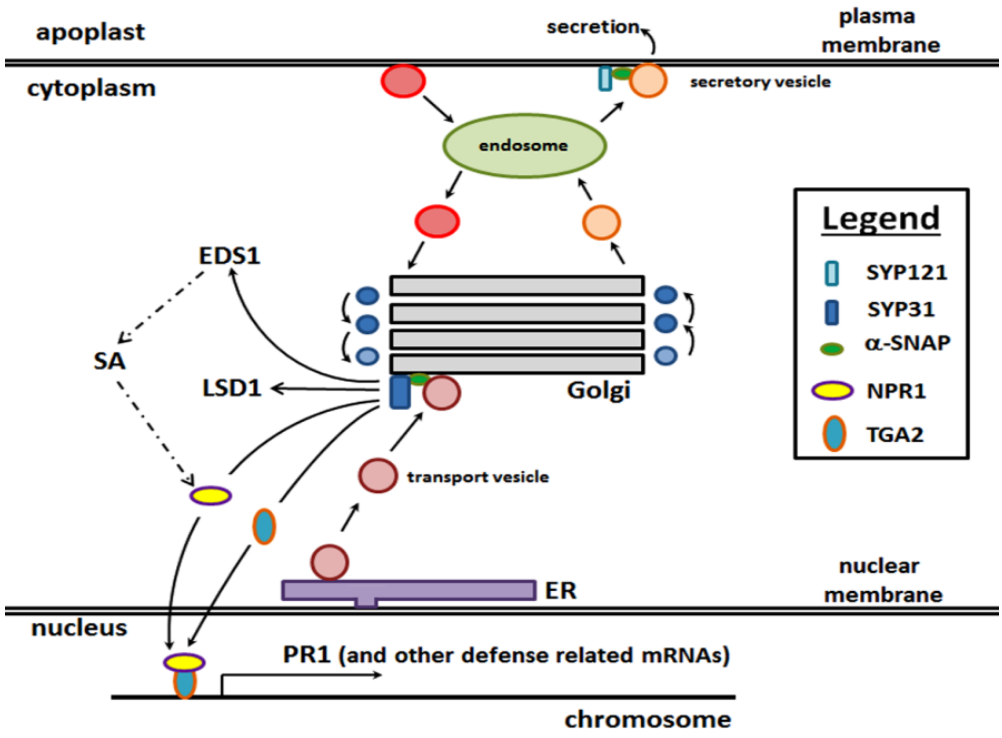


Figure 3.2 Framework showing position of *LSD1* and other tested genes

The Golgi apparatus serves a central role in resistance as a defense engine, processing proteins for their eventual transport. The overexpression of α-SNAP resulted in engineered resistance (Matsye *et al.* 2012). Furthermore, α-SNAP overexpression results in the induction of Gm-SYP38 transcription (Pant *et al.* 2014). In reciprocal experiments, Gm-SYP38 overexpression results in the transcriptional activation of α-SNAP and its paralogs (Table 3.3). The overexpression of Gm-SYP38 results in the transcriptional activation of *EDS1* which functions upstream of SA biosynthesis (dashed lines). The overexpression of Gm-SYP38 also results in the transcriptional activation of the SA receptor, *NPR1*, the DNA binding α-ZIP transcription factor TGA2 and the GATA-like transcription factor *LSD1*. The binding of SA to *NPR1* results in its translocation to the nucleus. *NPR1* and TGA2 are directly involved in the transcriptional activation of PR1 and PR5. For presentation purposes, on the right side of the Golgi apparatus are shown vesicles undergoing anterograde transport while those on the left are undergoing retrograde transport. Vesicles are shown released from the *trans*-Golgi network, moving toward the endosome. Ultimately, secretory vesicles fuse with the plasma membrane to deliver receptor components and secrete contents into the apoplast. Some of these secreted contents, like Gm-XTH43, play important roles in defense (Pant *et al.* 2014). In contrast, vesicles emerge from the plasma membrane and fuse with the endosome, recycling contents. Not shown, Gm-SYP38 and α-SNAP overexpression results in induced expression of the cytoplasmic receptor-like kinase *BIK1* that is important for defense (Adapted from Pant *et al.* 2014).

Gm-LSD1-2 overexpression induces the expression of genes relating to membrane fusion and SA signaling

To understand the relationship between Gm-LSD1-2 and resistance, a series of qPCR analyses have been performed using cDNA synthesized from RNA isolated from

roots overexpressing Gm-LSD1-2 (Table 3.3). qPCR was performed using primers designed specifically against LSD1-2. The experiments used the ribosomal S21 gene (Matsye *et al.* 2012) as a control to standardize the experiments. The gene expression analysis demonstrates that Gm-LSD1-2 overexpression results in induced mRNA levels of LSD1-2 as well as EDS1-2, NPR1-2, BIK1-6, XTH43 and SYP38.

Table 3.3 Gene expression analysis of *G. max* roots overexpressing defense-related genes at 0 dpi

Transgenic lines	Gene expression (fold change)
EDS1-OE	47.679
NPR1-OE	82.061
SYP38-OE	335.571
α-SNAP-OE	228.011
BIK1-OE	89.195
XTH43-OE	190.915

The experiments used the ribosomal S21 gene as a control to standardize the qPCR experiments. An arbitrary cutoff of +/- 1.5 fold, $p < 0.05$ was used for differential expression.

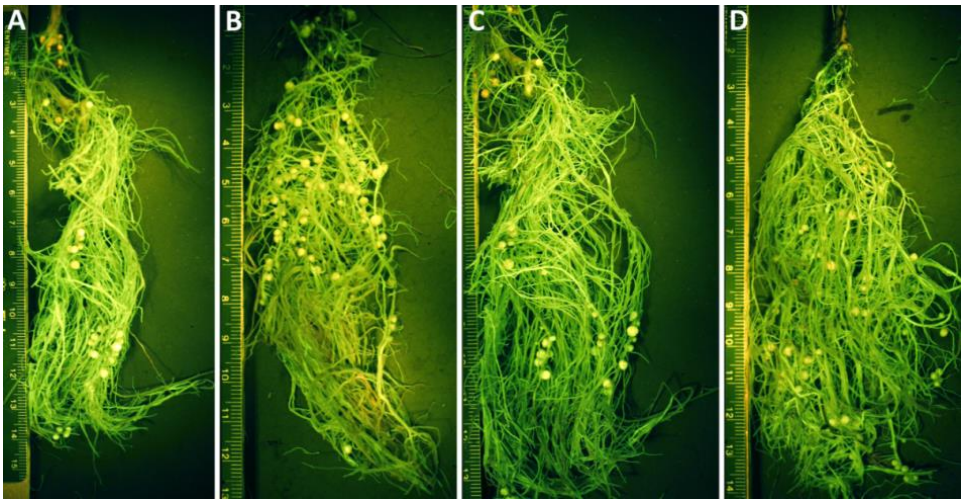


Figure 3.3 Representative control and transgenic LSD1-2 overexpressing and LSD1-2 RNAi *G. max* plants

A. Control susceptible *G. max*[Williams 82/PI 518671] plant. B. Genetically engineered *G. max*[Williams 82/PI 518671] overexpressing Gm-LSD1-2. C. Control resistant *G. max*[Peking/PI 548402] plant. D. A resistant *G. max*[Peking/PI 548402] plant genetically engineered to express an LSD1-2 RNAi construct. Scale provided on left of each image.

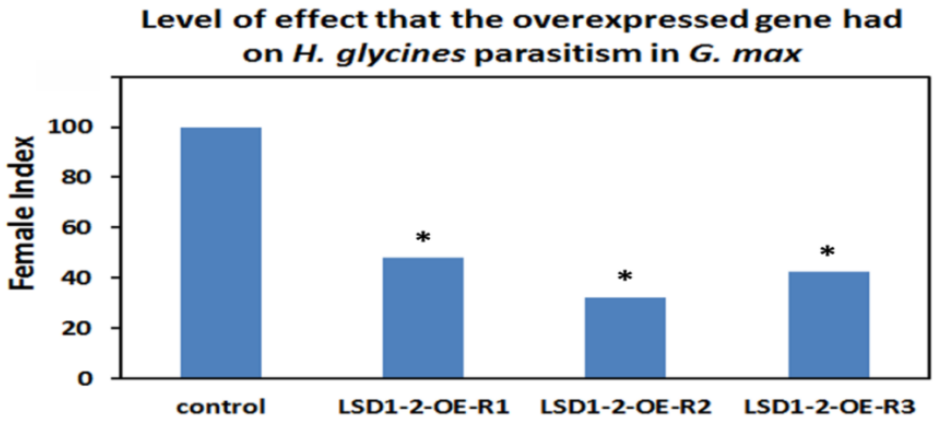


Figure 3.4 The female index for transgenic *G. max* plants genetically engineered to overexpress Gm-LSD1-2 and infected with *H. glycines*.

Replicate 1 (R1) control plants had 28.39 cysts per gram (12 plants); LSD1-2-R1-overexpressing plants (LSD1-2-R1: oe) had 13.66 cysts per gram (12 plants). The FI = 47.92; p-value = 0.0216541 which is statistically significant ($p < 0.05$). R2 control plants (replicate 2) had 30.40 cysts per gram (16 plants); LSD1-2-R2-overexpressing plants (LSD1-2-R2: oe) had 9.85 cysts per gram (12 plants). The FI = 32.4; p-value = 0.000059234 which is statistically significant ($p < 0.05$). R3 control plants had 32.98 cysts per gram (20 plants); LSD1-2-R3 overexpressing plants (LSD1-2-R3: oe) had 14.07 cysts per gram (18 plants). The FI = 42.662; p-value = 3.36219e-06 which is statistically significant ($p < 0.05$). * = statistically significant $p < 0.05$.

In contrast, Gm-LSD1-2 overexpression results in suppressed levels of α-SNAP prior to infection. This result is not surprising since recent experiments have shown that α-SNAP becomes highly induced later during the resistant reaction (Pant *et al.* 2014). In reciprocal experiments, the expression of an RNAi cassette for Gm-LSD1-2 in the normally resistant genotype *G. max*[Peking/PI 548402] results in suppressed transcriptional activity for LSD1-2 as well as EDS1-2, NPR1-2, BIK1-6, XTH43 and SYP38 (Table 3.3). Expression of α-SNAP was not detected under the experimental conditions. The results confirm and provide further context for the existence of a link between the membrane fusion gene SYP38 and SA signaling.

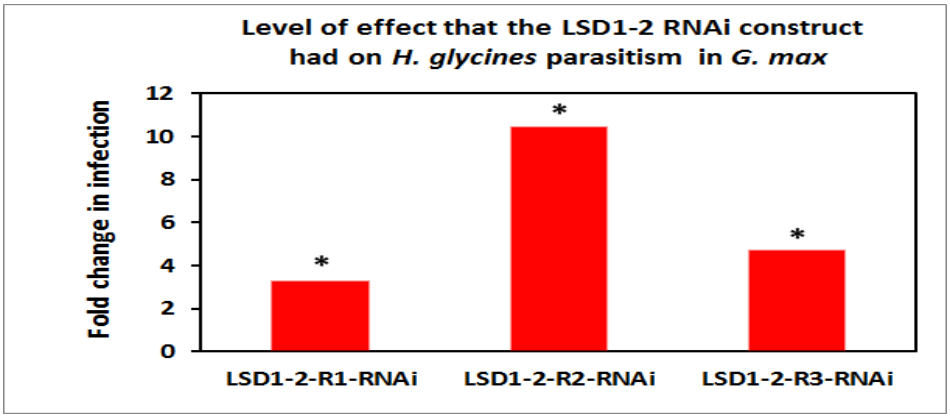


Figure 3.5 *G. max* plants genetically engineered for RNAi of Gm-LSD1-2 and infected with *H. glycines* have an increased capability, shown as fold change, for parasitism

Replicate 1 (R1) control plants (resistant *G. max*$_{[Peking/PI\ 548402]}$) had average 1.98 cysts per gram (10 plants). LSD1-2-RNAi-R1 (LSD1-2-R1: RNAi) in resistant *G. max*$_{[Peking/PI\ 548402]}$) had average 6.41 cysts per gram (11 plants). The results were statistically significant (p = 0.00255251). Replicate 2 (R2) control plants (resistant *G. max*$_{[Peking/PI\ 548402]}$) had average 0.79 cysts per gram (12 plants). LSD1-2-RNAi-R2 (LSD1-2-R2: RNAi) in resistant *G. max*$_{[Peking/PI\ 548402]}$) had average 8.63 cysts per gram (5 plants). The results were statistically significant (p = 0.0117053). Replicate 3 (R3) control plants (resistant *G. max*$_{[Peking/PI\ 548402]}$) had average 2.51 cysts per gram (10 plants). LSD1-2-RNAi-R3 (LSD1-2-R3: RNAi) in resistant *G. max*$_{[Peking/PI\ 548402]}$) had average 11.7 cysts per gram (7 plants). The results were statistically significant (p = 0.0120138). * = statistically significant $p < 0.05$.

Discussion

LSD1 was first discovered in *A. thaliana* in a forward genetic screen designed to identify spontaneous lesion simulating mutants (Dietrich *et al.* 1997). The five identified *lsd* mutants have been divided into two classes. One class forms spontaneous necrotic lesions that are determinate in nature (Dietrich *et al.* 1997). In this class, the expansion of necrosis into adjacent tissue is limited (Dietrich *et al.* 1997).

Furthermore, lesion formation is not influenced by pathogens or chemicals such as SA and the non SA-inducing 2,6-dichloroisonicotinic acid (INA) that induce the onset of systemic acquired resistance (SAR) (Vernooij *et al.* 1995; Dietrich *et al.* 1997). The second class of *lsd* mutants, defined by *LSD1*, is described as a feedback or propagation mutant (Dietrich *et al.* 1997). The *lsd1* mutant forms spontaneous lesions under long day

growth conditions (Dietrich *et al.* 1997). In contrast, lesion formation is suppressed under short days (Dietrich *et al.* 1997). These characteristics indicate that light influences the process at some level. The *lsd1* mutant is characterized by indeterminate lesions that eventually consume the whole leaf or plant (Dietrich *et al.* 1997). Another characteristic of *lsd1* mutants is that plants grown under permissive short day conditions develop lesions that eventually consume the whole plant when switched to long day (Dietrich *et al.* 1997). Furthermore, the *lsd1* mutant initiates lesion formation by fungal or bacterial pathogens and inducers of SAR, including SA and INA (Dietrich *et al.* 1997). Related experiments using *lsd1* mutants demonstrate that superoxide (O_2^-) accumulates in the cells adjacent to the cells undergoing cell death (Jabs *et al.* 1996). This result demonstrates that O_2^- is both necessary and sufficient to initiate lesion formation and promote its spreading into adjacent cells (Jabs *et al.* 1996). This result also identifies a link between photorespiration and lesion development.

It is clear from these studies that *lsd1* mutants are impaired in their ability to establish a boundary beyond which the neighboring cells are not consumed in the wave of cell death. Sequence analysis of *LSD1* demonstrates it to be a novel zinc finger, GATA-type transcription factor (Dietrich *et al.* 1997). In this regard, the data presented here provides an example of a GATA-type transcription factor involved in *G. max* defense against *H. glycines*. From observations made in *A. thaliana* it has been hypothesized that *LSD1* is responsible either to negatively regulate a pro-death pathway or activate a repressor of cell death (Dietrich *et al.* 1997). As a regulator, *LSD1* would function very early in the process. In *A. thaliana*, *LSD1* has since been shown to function in relation to genes composing the SA signaling pathway, including *EDS1*, *PAD4* and *NPR1* as well as

the signaling molecule SA (Kliebenstein *et al.* 1999; Rusterucci *et al.* 1999, Aviv *et al.* 2002). Notably, *LSD1* as an antiapoptotic gene, functions in the cells adjacent to the infected cell that is undergoing cell death (Kliebenstein *et al.* 1999; Rusterucci *et al.* 1999, Aviv *et al.* 2002). Experiments have shown that runaway cell death was dependent on SA and *NPR1* in *lsd1* mutants (Aviv *et al.* 2002). In contrast, *LSD1* has been shown to negatively regulate SA and *NPR1*-independent basal disease resistance (Aviv *et al.* 2002). From these studies, it has been proposed that SA and *NPR1* function in runaway cell death in the *lsd1* mutant through their participation in a signal amplification loop that promotes apoptosis (Rusterucci *et al.* 1999, Aviv *et al.* 2002). It has been shown that an important component of runaway cell death is the generation of reactive oxygen intermediates (ROI) such as O_2^- (Jabs *et al.* 1996; Kliebenstein *et al.* 1999; Rusterucci *et al.* 1999, Aviv *et al.* 2002). Additional studies further link the *lsd1* mutant to impaired photorespiration, leading to the accumulation of excess excitation energy and subsequent cell death (Mateo *et al.* 2004). In contrast, cell death is prevented in the *lsd1* mutants by impeding conditions that lead to photorespiration (Mateo *et al.* 2004). These results explain the link between the *lsd1* and photo-oxidative damage. Thus, it has been proposed that the *LSD1* protein functions like a rheostat whereby above a ROI threshold, the cell would undergo cell death (Jabs *et al.* 1996; Dietrich *et al.* 1997; Kliebenstein *et al.* 1999; Rusterucci *et al.* 1999, Aviv *et al.* 2002; Mateo *et al.* 2004). In contrast, below a certain threshold, the cell would survive. From this work, a signal potentiation loop has been coined to describe how in the absence of *LSD1* protein, the accumulation of signaling components leads to runaway apoptosis (Aviv *et al.* 2002).

These experiments focused in on the above portions of *A. thaliana*. Subsequently, a number of experiments examining *LSD1* have examined specific aspects of root biology. Under certain adaptive environmental circumstances (i.e. water saturated conditions and low oxygen [hypoxia]), root cells become targeted for apoptosis through a process called lysigeny. As a consequence of this process, the roots develop aerenchyma which increases the ability of roots to maintain higher O_2 levels. Experiments in *A. thaliana* have shown that lysigeny is under the control of *LSD1* (Muhlenbock *et al.* 2007). Under conditions of hypoxia, *LSD1, EDS1* and *PAD4* function upstream of H_2O_2 production and ethylene signaling events that lead to lysigeny (Muhlenbock *et al.* 2007). Under normal conditions in *A. thaliana*, *LSD1* functions as a negative regulator of the apoptosis-promoting *EDS1* and *PAD4*. In contrast, under hypoxia, *LSD1* is negatively regulated, permitting *EDS1* and *PAD4* to promote cell death in *A. thaliana* (Muhlenbock *et al.* 2007). To understand how H_2O_2 production could be regulated in the roots, earlier experiments performed on aerial portions of *A. thaliana* demonstrated that *LSD1* controls H_2O_2 production through SA-regulated transcription of CuSOD (Kliebenstein *et al.* 1999). This is an important finding since plants can produce the highly toxic O_2^- during plant defense by the activities of NADPH oxidase (Desikan *et al.* 1996).

Recent findings performed in *A. thaliana* have shown a direct link between NADPH oxidase and *BIK1* (Kadota *et al.* 2014). In those experiments, *BIK1* directly phosphorylates NADPH oxidase to produce O_2^- and activate defense pathways. Plants then detoxify O_2^- to H_2O_2 through major antioxidant enzymes like CuSOD. Thus, certain aspects of *LSD1* function in *A. thaliana* are similar between the shoot and root. Furthermore, recent findings in *A. thaliana* have also revealed *LSD1* has many functions

with regard to basic aspects of plant growth, development and its ability to function under different environmental conditions and stresses (Wituszynska *et al.* 2013). These observations place some context into the observation that Gm-BIK1 functions in defense in the *G. max* -*H. glycines* pathosystem (Pant *et al.* 2014).

LSD1 transcription is induced in *G. max* roots overexpressing the membrane fusion gene α-SNAP

Two major *H. glycines* resistance loci have been identified from screening ecological collections of *G. max* (Caldwell *et al.* 1960; Matson and Williams 1965). These loci, the recessive *rhg1* and the dominant *Rhg4*, have been mapped and cloned through traditional means and aided further by transcriptomics and candidate gene approaches (Caldwell *et al.* 1960; Matson and Williams 1965; Esmon *et al.* 1981; Kim *et al.* 2010; Matsye *et al.* 2011; Cook *et al.* 2012, 2014; Liu *et al.* 2012). Genetic crosses of *rhg1* and *Rhg4*-containing genotypes leads to progeny with further-enhanced, nearly full resistance. The additive effect that these loci have, regarding *H. glycines* resistance, indicate that the genes function in different genetic pathways that converge on the same outcome (resistance). The *rhg1* locus, depending on the resistant genotype examined, is composed of multiple tandem repeated copies of 3 or 4 genes. These genes include an amino acid transporter, α-SNAP, a wound inducible protein and in some genotypes, a gene known as placenta-specific gene 8 protein (PLAC8) (Schmutz *et al. 2010*; Matsye *et al.* 2011; Cook *et al.* 2012, 2014). Among these genes, the overexpression of α-SNAP has been shown to yield a resistant reaction when overexpressed on its own. As part of the secretory pathway, α-SNAP would function in many essential cellular processes (Novick *et al.* 1980). The other resistance gene, *Rhg4*, gene is a SHMT which plays a role

in photorespiration. In overexpression studies, SHMT suppresses the ability of *H. glycines* to parasitize *G. max* (Liu *et al.* 2012; Matthews *et al.* 2013).

The overexpression of α-SNAP leads to an increase in expression of its binding partner, syntaxin 31 (Gm-SYP38). Syntaxin 31 functions at the *cis* face of the Golgi apparatus to facilitate the fusion of transport vesicles transported from the endoplasmic reticulum (Novick *et al. 1980;* Novick et al. 1981; Esmon et al. 1981; Banfield et al. 1995, Bubeck et al. 2008; Melser *et al.* 2009*;* Chatre *et al.* 2009). In *G. max*, the overexpression of α-SNAP and Gm-SYP38 results in induced levels of the SA signaling genes *EDS1*, *NPR1* and PR1 (Pant *et al.* 2014). While the observation of an influence of vesicle transport on SA signaling is not a new concept (Zhang *et al.* 2007), the results of Pant *et al.* (2014) indicates that SA signaling may be important to the process of defense in the *G. max -H. glycines* pathosystem. To test this hypothesis, the overexpression of Gm-EDS1 and *NPR1* has been shown to lead to resistance (Pant *et al.* 2014). In related experiments, the overexpression of *EDS1* and *NPR1* in *G. max* leads to induced levels of SHMT prior to infection (Pant *et al.* 2014). Furthermore, the overexpression of *G. max* syntaxin 31 leads to slightly induced levels of *EDS1* and SHMT during infection (Pant *et al.* 2014). While these experiments were not comprehensive, they indicate that genes composing the *rhg1* locus can influence the expression of *Rhg4*.

The observation in *G. max* that *EDS1* and *NPR1* function in resistance to *H. glycines* indicated other genes relating to them may also function in the process. An obvious candidate is Gm-LSD1. In qPCR experiments examining *G. max* roots overexpressing α-SNAP, it is shown that Gm-LSD1-2 transcription is induced. Complimentary experiments presented here show that Gm-LSD1-2 is also induced in

roots engineered to overexpress Gm-SYP38. Furthermore, Gm-LSD1-2 transcription is also induced in roots overexpressing *BIK1*, *EDS1*, *NPR1* or XTH. The strong association of Gm-LSD1-2 with engineered forms of resistance led to the idea that it may perform a direct role in the process. Since *A. thaliana LSD1* is known to play roles in establishing and maintaining a tight boundary around the cells and tissues involved in pathogen infection, it is possible that the expression of Gm-LSD1-2 could be performing an important role in regulating the expansion and/or initial survival of parasitized cells. The *H. glycines*-parasitized root cells undergo a slow process taking days to conclude that ultimately leads to resistance (Endo 1965). During this time, the parasitized root cell would have time to synthesize and secrete molecules in the vicinity of the nematode to neutralize its activities while fortifying the parasitized area. One such enzyme is Gm-XTH43. Notably, XTH contains a signal peptide and is transported through the vesicle transport machinery to the apoplast where it modifies hemicellulose (Yokoyama and Nishitani 2001; Pant *et al.* 2014). Furthermore, the parasitized cell may produce O_2^- whose subsequent metabolism to H_2O_2 has been shown in *A. thaliana* to be under regulation by *LSD1* (Jabs *et al.* 1996; Kliebenstein *et al.* 1999; Vernooij *et al.* 1995; Rusterucci *et al.* 2001; Aviv *et al.* 2002; Mateo *et al.* 2004; Muhlenbock *et al.* 2007). In the analysis presented here, the overexpression of Gm-LSD1-2 in *G. max*[Williams 82/PI 518671] roots that are otherwise susceptible to *H. glycines* parasitism, resulted in ~52 to 68% reduction in nematode parasitism. Roots overexpressing Gm-LSD1-2, when tested for the expression of markers of resistance (i.e. XTH43, SYP38, *NPR1*, *EDS1* and *BIK1*) show that each is induced in its expression prior to *H. glycines* infection. In examining molecular markers of different signaling processes, highly induced levels of PR2 were

observed in Gm-LSD1-2 overexpressing roots prior to their infection by *H. glycines*. The induction of PR2 transcription indicates ethylene may also be a component of in Gm-LSD1-2-mediated resistance. The contribution of PR2 to resistance has been demonstrated, linking ethylene to the process (Matthews *et al.* 2013). In contrast, RNAi of Gm-LSD1-2 in the resistant genotype *G. max*$_{[Peking/PI\ 548402]}$ demonstrates specificity. In these experiments, the normally resistant *G. max*$_{[Peking/PI\ 548402]}$ roots engineered with the Gm-LSD1-2 RNAi cassette lacked the induction of LSD1-2 expression and exhibited an increase in parasitism capability. These results provide direct evidence that Gm-LSD1-2 plays an important role in the ability of *G. max* to prevent parasitism by *H. glycines*, contrasting with recent heterologous expression studies (Matthews *et al.* 2014). In examining this discrepancy between the heterologous expression of *A. thaliana LSD1* and Gm-LSD1-2 further, the conceptually translated At-*LSD1* gene studied in Matthews *et al.* (Matthews *et al.* 2014) is 66.5% identical to the tested *G. max* LSD1-2 protein (Glyma08g13630) presented here. Thus, part of the difference observed between the capability of At-LSD1 and Gm-LSD1-2 proteins to function in *G. max* may arise from gene sequence variation. To reinforce our observation that Gm-LSD1-2 functioned in resistance, we present through a double-blind analysis experimental and biological replicates in both the Gm-LSD1-2 overexpression and RNAi experiments.

Spatial and temporal aspects regarding *LSD1*

The demonstration that Gm-LSD1-2 is important to the defense process clarifies the paradox that parasitized *G. max* root cells tolerate the establishment and maintenance of the attacked cell early during *H. glycines* parasitism prior to the commitment of the parasitized cell for demise. The association of *LSD1* with the antiapoptotic activities of

photorespiration in *A. thaliana* links its function to *G. max Rhg4*-mediated defense (Jabs *et al.* 1996; Kliebenstein *et al.* 1999; Vernooij *et al.* 1995; Rusterucci *et al.* 2001; Aviv *et al.* 2002; Mateo *et al.* 2004). The demonstration that induced levels of Gm-LSD1-2 transcription in roots overexpressing the *rhg1* gene α-SNAP and SYP38 links *LSD1* to the process of vesicle transport at some level. At this point, many details remain concerning the genetic program responsible for the establishment and maintenance parasitized cell and surrounding root cells. From these observations, it is plausible that Gm-LSD1-2 functions initially in both the parasitized cell and surrounding cells to prevent cell death and establish a boundary. The demonstration that Gm-BIK1 is important to resistance implicates NADPH oxidase performing a role in the process (Kadota *et al.* 2014; Pant *et al.* 2014). NADPH oxidase would provide the O_2^- that could antagonize *H. glycines*. During this time, as the cell is protected from apoptosis, the vesicle transport machinery including the *rhg1* gene α-SNAP would function to deliver antimicrobials, cell wall modifying enzymes and other substances to the site of parasitism. However, the process of resistance is not limited to this framework.

References

An, Q., Ehlers, K., Kogel, K.H., van Bel, A.J., Hückelhoven, R. (2006a) Multivesicular compartments proliferate in susceptible and resistant MLA12-barley leaves in response to infection by the biotrophic powdery mildew fungus. New Phytologist 172: 563-57.

An, Q., Hückelhoven, R., Kogel, K.H., van Bel, A.J. (2006b) Multivesicular bodies participate in a cell wall-associated defence response in barley leaves attacked by the pathogenic powdery mildew fungus. Cell Microbiology 8:1009-1019.

Antoniw, J.F., Pierpoint, W.S. (1978) The purification and properties of one of the 'b" proteins from virus-infected tobacco plants. Journal of General Virology 39: 343-350.

Assaad, F.F., Qiu, J.L., Youngs, H., Ehrhardt, D., Zimmerli, L., Kalde, M., Wanner, G., Peck, S.C., Edwards, H., Ramonell, K., Somerville, C.R., Thordal-Christensen, H. (2004) The *PEN1* syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Molecular Biology of the Cell 15: 5118-5129.

Aviv, D.H., Rusterucci, C., Holt III, B.F., Dietrich, R.A., Parker, J.E., Jeffery, L. Dangl, J.L. (2002) Runaway cell death, but not basal disease resistance, in lsd1 is SA- and NIM1/*NPR1*-dependent. Plant Journal 29: 381-391.

Babcock, M., Macleod, G.T., Leither, J., Pallanck, L. (2004) Genetic analysis of soluble N ethylmaleimide-sensitive factor attachment protein function in Drosophila reveals positive and negative secretory roles. Journal of Neuroscience 24: 3964-3973.

Banfield, D.K., Lewis, M.J., Pelham, H.R. (1995) A SNARE-like protein required for traffic through the Golgi complex. Nature 375: 806-809.

Bennett, M.K., Calakos, N., Scheller, R.H. (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257: 255-259.

Boyd, R.S., Duggan, M.J., Shone, C.C., Foster, K, A. (1995) The effect of botulinum neurotoxins on the release of insulin from the insulinoma cell lines HIT-15 and RINm5F. Journal of Biological Chemistry 270: 18216-18218.

Bubeck, J., Scheuring, D., Hummel, E., Langhans, M., Viotti, C., Foresti, O., Denecke, J., Banfield, D.K., Robinson, D.G. (2008) The syntaxins SYP31 and SYP81 control ER-Golgi trafficking in the plant secretory pathway. Traffic 9: 1629-1652.

Burgess, R.W., Deitcherm D.L., Schwarz, T.L. (1997) The synaptic protein syntaxin1 is required for cellularization of Drosophila embryos. Journal of Cell Biology 138: 861-875.

Byrd, D.W. Jr, Kirkpatrick, T., Barker, K.R. (1983) An improved technique for clearing and staining plant tissue for detection of nematodes. Journal of Nematology 15: 142-143.

Caldwell, B.E., Brim, C.A., Ross, J.P. (1960) Inheritance of resistance of soybeans to the soybean cyst nematode, Heterodera glycines. Agronomy Journal 52: 635-636.

Cao, H., Bowling, S.A., Gordon, A.S., Dong, X. (1994) Characterization of an *Arabidopsis* mutant that is non-responsive to inducers of systemic acquired resistance. Plant Cell 6: 1583-1592.

Chatre, L., Wattelet-Boyer, V., Melser, S., Maneta-Peyret, L., Brandizzi, F., Moreau, P. (2009) A novel di-acidic motif facilitates ER export of the syntaxin SYP31. Journal of Experimental Botany 60: 3157-3165.

Clary, D.O., Griff, I.C., Rothman, J.E. (1990) SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. Cell 61: 709-721.

Collins, N.C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J.L., Hückelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S.C., Schulze-Lefert, P. (2003) SNARE-protein mediated disease resistance at the plant cell wall. Nature 425**:** 973-977.

Cook, D.E., Lee, T.G., Guo, X., Melito, S., Wang, K., Bayless, A., Wang, J., Hughes, T.J., Willis, D,K., Clemente, T., Diers, B.W., Jiang, J., Hudson, M.E., Bent, A.F. (2012) Copy Number Variation of Multiple Genes at *Rhg1* Mediates Nematode Resistance in Soybean. Science 338: 1206-1209.

Cook, D.E., Bayless, A.M., Wang, K., Guo, X., Song, Q., Jiang, J., Bent, A.F. (2014) Distinct copy number, coding sequence and locus methylation patterns underlie *Rhg1*-mediated soybean resistance to soybean cyst nematode. Plant Physiology 165: 630-647.

Cotrufo, T., Pérez-Brangulí, F., Muhaisen, A., Ros, O., Andrés, R., Baeriswyl, T., Fuschini, G., Tarrago, T., Pascual, M., Ureña, J., Blasi, J., Giralt, E., Stoeckli, E.T., Soriano, E. (2011) A signaling mechanism coupling netrin-1/deleted in colorectal cancer chemoattraction to SNARE-mediated exocytosis in axonal growth cones. Journal of Neuroscience 31: 14463-14480.

Delaney, T.P., Friedrich, L., Ryals, J.A. (1995) *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. Proceedings of the Natioonal Academy of Sciences USA 92: 6602-6606.

Desikan, R., Hancock, J.T., Coffey, M.J., Neill, S.J. (1996) Generation of active oxygen in elicited cells of Arabidopsis thaliana is mediated by a NADPH oxidase-like enzyme. FEBS Letters 11: 213-217.

Dietrich, R.A., Delaney, T.P., Uknes, S.J., Ward, E.R., Ryals, J.A., Dangl, J, L. (1994) Arabidopsis mutants simulating disease resistance response. Cell 77: 565-577.

Dietrich, R.A., Richberg, M.H., Schmidt, R., Dean, C., Dangl, J.L. (1997) A novel zinc finger protein is encoded by the Arabidopsis *LSD1* gene and functions as a negative regulator of plant cell death. Cell 88: 685-694.

Endo, B.Y. (1965) Histological responses of resistant and susceptible soybean varieties, and backcross progeny to entry development of *Heterodera glycines*. Phytopathology 55: 375-381.

Epple, P., Mack, A.A., Morris, V.R., Dangl, J.L. (2001) Antagonistic control of oxidative stress-induced cell death in Arabidopsis by two related, plant-specific zinc finger proteins. Proceedings of the National Academy of Sciences USA 100: 6831-6836.

Esmon, B., Novick, P., Schekman, R. (1981) Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. Cell 25: 451-460.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J., Parker, J.E. (1999) *EDS1*, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proceedings of the National Academy of Sciences USA 96: 3292-3297.

Feys, B.J., Moisan, L.J., Newman, M.A., Parker, J.E. (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, *EDS1* and PAD4. EMBO Journal 20: 5400-5411.

Glazebrook, J., Rogers, E.E., Ausubel, F.M. (1996) Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics 143: 973-998.

Golden, A.M., Epps, J.M., Riggs, R.D., Duclos, L.A., Fox, J.A., Bernard, R.L. (1970) Terminology and identity of infraspecific forms of the soybean cyst nematode *(Heterodera glycines).* Plant Disease Reports 54: 544-546.

Haas, J.H., Moore, L.W., Ream, W., Manulis, S. (1995) Universal PCR primers for detection of phytopathogenic *Agrobacterium* strains. Applied Environmental Microbiology 61: 2879-2884.

Hardwick, K.G., Pelham, H.R. (1992) SED5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. Journal of Cell Biology 119: 513-521.

Harris, M.A., Clark, J., Ireland, A., Lomax, J., Ashburner, M., Foulger, R., Eilbeck, K., Lewis, S., Marshall, B., Mungall, C., Richter, J., Rubin, G.M., Blake, J.A., Bult, C., Dolan, M., Drabkin, H., Eppig, J.T., Hill, D.P., Ni, L., Ringwald, M., Balakrishnan, R., Cherry, J.M., Christie, K.R., Costanzo, M.C., Dwight, S.S., Engel, S., Fisk, D.G., Hirschman, J.E., Hong, E.L., Nash, R.S., Sethuraman, A., Theesfeld, C.L., Botstein, D., Dolinski, K., Feierbach, B., Berardini, T., Mundodi, S., Rhee, S.Y., Apweiler, R., Barrell, D., Camon, E., Dimmer, E., Lee, V., Chisholm, R., Gaudet, P., Kibbe, W., Kishore, R., Schwarz, E.M., Sternberg, P., Gwinn, M., Hannick, L., Wortman, J., Berriman, M., Wood, V., de la, Cruz, N., Tonellato, P., Jaiswal, P., Seigfried, T., White, R. (2004) Gene Ontology Consortium.. The Gene Ontology (GO) database and informatics resource. Nucleic Acids Research 32: D 258-261.

Hofgen, R., Willmitzer, L. (1988) Storage of competent cells for Agrobacterium transformation. Nucleic Acids Research 16: 9877.

Hofius, D., Schultz-Larsen, T., Joensen, J., Tsitsigiannis, D.I., Petersen, N.H., Mattsson, O., Jørgensen, L.B., Jones, J.D., Mundy, J., Petersen, M. (2009) Autophagic components contribute to hypersensitive cell death in Arabidopsis. Cell 137: 773-783.

Hong, K–K., Chakravarti, A., Takahashi, J.S. (2004) The gene for soluble N-ethylmaleimide sensitive factor attachment protein a is mutated in hydrocephaly with hop gait (hyh) mice. Proceedings of the National Academy of Sciences USA 101: 1748-1753.

Jabs, T., Dietrich, R.A., Dangl, J.L. (1996) Initiation of runaway cell death in an Arabidopsis mutant by extracellular superoxide. Science 273: 1853-1856.

Jahn, R., Fasshauer, D. (2012) Molecular machines governing exocytosis of synaptic vesicles. Nature 490: 201-207.

Jenkins, W.R. (1964) A rapid centrifugal flotation technique for separating nematodes from soil. Plant Disease Reports 48: 692.

Kadota, Y., Sklenal, J., Derbyshire, P., Stransfeld, L., Asai, S., Ntoukakis, V., Jones, J.D.G. Shirasu K, Menke F, Jones A Zipfel C. 2014. Direct Regulation of the NADPH Oxidase RBOHD by the PRR-Associated Kinase *BIK1* during Plant Immunity. Molecular Cell 54: 43-55.

Kalde, M., Nühse, T.S., Findlay, K., Peck, S.C. (2007) The syntaxin SYP132 contributes to plant resistance against bacteria and secretion of pathogenesis-related protein 1. Proceedings of the National Academy of Sciences USA 104: 11850-11855.

Kauffmann, S., Legrand, M., Geoffroy, P., Fritig, B. (1987) Biological function of 'pathogenesis-related" proteins: four PR proteins of tobacco have 1, 3-β-glucanase activity. EMBO Journal 6: 3209-3212.

Kauffmann, S., Legrand, M., Fritig, B. (1990) Isolation and characterization of six pathogenesis-related (PR) proteins of Samsun NN tobacco. Plant Molecular Biology 14: 381-390.

Kim, M., Hyten, D.L., Bent, A.F., Diers, B.W. (2010) Fine mapping of the SCN resistance locus *rhg1-b* from PI 88788. The Plant Genome 3: 81-89.

Kliebenstein, D.J., Dietrich, R.A., Martin, A.C., Last, R.L., Dangl, J.L. (1999) *LSD1* regulates salicylic acid induction of copper zinc superoxide dismutase in Arabidopsis thaliana. Molecular Plant Microbe Interactions 12: 1022-1026.

Kwon, C., Neu, C., Pajonk, S., Yun, H.S., Lipka, U., Humphry, M., Bau, S., Straus, M., Kwaaitaal, M., Rampelt, H., El Kasmi, F., Jürgens, G., Parker, J., Panstruga, R., Lipka, V., Schulze-Lefert, P. (2008) Co-option of a default secretory pathway for plant immune responses. Nature 451: 835-840.

Lai, Z., Wang, F., Zheng, Z., Fan. B., Chen, Z. (2011) A critical role of autophagy in plant resistance to necrotrophic fungal pathogens. Plant Journal 66: 953-968.

Lauber, M.H., Waizenegger, I., Steinmann, T., Schwarz, H., Mayer, U., Hwang, I., Lukowitz, W., Jürgens, G. (1997) The Arabidopsis KNOLLE protein is a cytokinesis-specific syntaxin. Journal of Cell Biology 139: 1485-1493.

Legrand, M., Kauffman, S., Geoffroy. P., Fritig, B. (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis related proteins are chitinases. Proceedings of the National Academy of Sciences USA 84: 6750-6754.

Lenz, H.D., Haller, E., Melzer, E., Kober, K., Wurster, K., Stahl, M., Bassham, D.C., Vierstra, R.D., Parker, J.E., Bautor, J., Molina, A., Escudero, V., Shindo, T., van der Hoorn, R.A., Gust, A.A., Nürnberger, T. (2011) Autophagy differentially controls plant basal immunity to biotrophic and necrotrophic pathogens. Plant Journal 66: 818-830.

Liu. S., Kandoth, P.K., Warren, S.D., Yeckel, G., Heinz, R., Alden, J., Yang, C., Jamai, A., El-Mellouki, T., Juvale, P.S., Hill, J., Baum, T.J., Cianzio, S., Whitham, S.A., Korkin, D., Mitchum, M.G., Meksem, K. (2012) A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens. Nature 492: 256-260.

Livak, K.J., Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408.

Lobingier, B.T., Nickerson, D.P., Lo, S-Y, Merz, A.J. (2014) SM proteins Sly1 and Vps33 co-assemble with Sec17 and SNARE complexes to oppose SNARE disassembly by Sec18. eLife 10:02272.

Lukowitz, W., Mayer, U., Jürgens, G. (1996) Cytokinesis in the Arabidopsis embryo involves the syntaxin-related KNOLLE gene product. Cell 84: 61-71.

Lupashin, V.V., Pokrovskaya, I.D., McNew, J.A., Waters, M.G. (1997) Characterization of a novel yeast SNARE protein implicated in Golgi retrograde traffic. Molecular Biology of the Cell 8: 2659-2676.

Mateo, A., Muhlenbock. P., Rusterucci, C., Chang, CC-C., Miszalski, Z., Karpinska, B., Parker, J.E., Mullineaux, P.M., Karpinski, S. (2004) LESION SIMULATING DISEASE 1 is required for acclimation to conditions that promote excess excitation energy. Plant Physiology 136: 2818-2830.

Matson, A.L., Williams, L.F. (1965) Evidence of a fourth gene for resistance to the soybean cyst nematode. Crop Science 5: 477.

Matsye, P.D., Kumar, R., Hosseini, P., Jones, C.M., Tremblay, A., Alkharouf, N.W., Matthews, B.F., Klink, V.P. (2011) Mapping cell fate decisions that occur during soybean defense responses. Plant Molecular Biology 77: 513-528.

Matsye, P.D., Lawrence, G.W., Youssef, R.M., Kim, K-H., Matthews, B.F., Lawrence, K.S., Klink, V.P. (2012) The expression of a naturally occurring, truncated allele of an α-SNAP gene suppresses plant parasitic nematode infection. Plant Molecular Biology 80: 131-155.

Matthews, B., MacDonald, M.H., Thai, V.K., Tucker, M.L. (2003) Molecular characterization of argenine kinase in the soybean cyst nematode (*Heterodera glycines*). Journal of Nematology 35: 252-258.

Matthews, B.F., Beard, H., Brewer. E., Kabir, S., MacDonald, M.H., Youssef, R.M. (2014) Arabidopsis genes, At*NPR1*, AtTGA2 and AtPR-5, confer partial resistance to soybean cyst nematode (*Heterodera glycines*) when overexpressed in transgenic soybean roots. BMC Plant Biology 14: 96.

Matthews, B.F., Beard, H., MacDonald, M.H., Kabir, S., Youssef, R.M., Hosseini, P., Brewer, E. (2013) Engineered resistance and hypersusceptibility through functional metabolic studies of 100 genes in soybean to its major pathogen, the soybean cyst nematode. Planta 237: 1337-1357.

Mayer, U., Torres, Ruiz R.A., Berleth, T., Misera , S., Jurgens, G. (1991) Mutations affecting body organization in the *Arabidopsis* embryo. Nature 353: 402-407.

Melser, S., Wattelet-Boyer, V., Brandizzi, F., Moreau, P. (2009) Blocking ER export of the Golgi SNARE SYP31 affects plant growth. Plant Signaling and Behaviour 4: 962-964.

Muhlenbock, P., Plaszczyca,, Mal., Plaszczyca, Mar., Mellerowicz, E. Karpinski, S. (2007) Lysigenous aerenchyma formation in arabidopsis is controlled by *LESION SIMULATING DISEASE1*. Plant Cell 19: 3819-3830.

Nawrath, C., Heck, S., Parinthawong, N., Me´traux, J-P. (2002) EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. Plant Cell 14: 275-286.

Nawrath, C., Me´traux, J.P. (1999) Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell 11: 1393-1404.

Neiman, A.M. (1998) Prospore membrane formation defines a developmentally regulated branch of the secretory pathway in yeast. Journal of Cell Biology 140: 29-37.

Niggeweg, R., Thurow, C., Kegler, C., Gatz, C. (2000) Tobacco transcription factor TGA2.2 is the main component of as-1-binding factor ASF1 and is involved in salicylic acid- and auxin-inducible expression of as-1-containing target promoters. Journal of Biological Chemistry 275: 19897-19905.

Novick, P., Schekman, R. (1979) Secretion and cell surface growth are blocked in a temperature sensitive mutant of Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences USA 76: 1858-1862.

Novick, P., Field, C., Schekman, R. (1980) The identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 21: 205-215.

Novick, P., Ferro, S., Schekman, R. (1981) Order of events in the yeast secretory pathway. Cell 25: 461-469.

Palade, G.E . (1975) Intracellular aspects of protein secretion. Science 189: 347-358.

Pant, S.R., Matsye, P.D., McNeece, B.T., Sharma, K., Krishnavajhala, A., Lawrence, G.W., Klink, V.P. (2014) Syntaxin 31 functions in *Glycine max* resistance to the plant parasitic nematode *Heterodera glycines* Plant Molecular Biology 85: 107-121.

Patel, S., Dinesh-Kumar, S.P. (2008) *Arabidopsis* ATG6 is required to limit the pathogen-associated cell death response. Autophagy 4: 20-27.

Perrotta, C., Bizzozero, L., Cazzato, D., Morlacchi, S., Assi E., Simbari, F., Zhang, Y., Gulbins, E., Bassi, M.T., Rosa, P., Clementi, E. (2010) Syntaxin 4 is required for acid sphingomyelinase activity and apoptotic function. Journal of Biological Chemistry 285: 40240-40251.

Peter, F., Wong, S.H., Subramaniam, V.N., Tang, B.L., Hong, W. (1998) A-SNAP but not gamma-SNAP is required for ER-Golgi transport after vesicle budding and the Rab1-requiring step but before the EGTA-sensitive step. Journal of Cell Science 111: 2625-2633.

Pieterse, C.M.J., Van Loon, L.C. (2004) *NPR1*: the spider in the web of induced resistance signaling pathways. Current Opinion in Plant Biology 7: 456-464.

Ramalho-Santos, J., Moreno, R.D., Sutovsky, P., Chan, A.W., Hewitson, L., Wessel, G.M., Simerly, C.R., Schatten, G. (2000) SNAREs in mammalian sperm: possible implications for fertilization. Developmental Biology 223: 54-69.

Rodrıguez, F., Bustos, M.A., Zanetti, M.N., Ruete, M.C., Mayorga, L.S., Tomes, C.N. (2011) α-SNAP prevents docking of the acrosome during sperm exocytosis because it sequesters monomeric syntaxin. PLoS ONE 6: e21925.

Rusterucci, C., Aviv, D.H., Holt, B.F. 3rd, Dangl, J.L., Parker, J.E. (2001) The disease resistance signaling components *EDS1* and PAD4 are essential regulators of the cell death pathway controlled by *LSD1* in Arabidopsis. Plant Cell 13: 2211-2224.

Sanderfoot, A.A., Farhah, F., Assaad, F.F., Natasha, V., Raikhel. N.V. (2001a) The Arabidopsis genome. An abundance of soluble N-ethylmaleimide-sensitive factor adaptor protein receptors. Plant Physiology 124: 1558-1565.

Sanderfoot, A.A., Pilgrim, M., Adam, L., Raikhel, N.V. (2001b) Disruption of individual members of Arabidopsis syntaxin gene families indicates each has essential functions. Plant Cell 13: 659-666.

Sanderfoot, A.A., Kovaleva, V., Bassham, D.C., Raikhel, N.V. (2001c) Interactions between Syntaxins Identify at Least Five SNARE Complexes within the Golgi/Prevacuolar System of the Arabidopsis Cell. Molecular Biology of the Cell 12: 3733-3743.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D.L., Song, Q., Thelen, J.J., Cheng, J., Xu, D., Hellsten, U., May, G.D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M.K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, D., Shu, S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X.C., Shinozaki, K., Nguyen, H.T., Wing, R.A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R.C., Jackson, S.A. (2010) Genome sequence of the palaeopolyploid soybean. Nature 463: 178-183.

Schulz, J.R., Wessel, G.M., Vacquier, V.D. (1997) The exocytosis regulatory proteins syntaxin and VAMP are shed from sea urchin sperm during the acrosome reaction. Developmental Biology 191: 80-87.

Shah, J., Tsui, F., Klessig, D.F. (1997) Characterization of a salicylic acid insensitive mutant (sai1) of Arabidopsis thaliana, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. Molecular Plant Microbe Interactions 10: 69-78.

Tepfer, D. (1984) Transformation of several species of higher plants by Agrobacterium rhizogenes: sexual transmission of the transformed genotype and phenotype. Cell 37: 959-967.

Trapnell, C., Pachter, L., Salzberg, S.L. (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25: 1105-1111.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., Pachter, L, (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature Biotechnology 28: 511-515.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., Pachter, L. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols 7: 562-578.

Vernooij, B., Friedrich, L., Ahl Goy, P., Staub, T., Kessmann, H. and Ryals, J. (1995) 2,6-Dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. Molecular Plant Microbe Interactions 8: 228-234.

Vroemen, C.W., Langeveld. S., Mayer, U., Ripper, G., Jurgens, G., Van Kammen, A., De Vries, S.C. (1996) Pattern Formation in the Arabidopsis Embryo Revealed by Position-Specific Lipid Transfer Protein Gene Expression. Plant Cell 8: 783-791.

Waizenegger, I., Lukowitz, W., Assaad, F., Schwarz, H., Jürgens, G., Mayer, U. (2000) The Arabidopsis KNOLLE and KEULE genes interact to promote vesicle fusion during cytokinesis. Current Biology 2: 1371-1374.

Wildermuth, M.C., Dewdney, J., Wu, G., Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defense. Nature 414: 562-565.

Wituszynska, W., Slesak, I., Vanderauwera, S., Szechynska-Hebda, M., Kornas, A., Van Der Kelen, K., Mühlenbock, P., Karpinska, B., Mackowski, S., Van Breusegem, F., Karpinski, S. (2013) *LESION SIMULATING DISEASE1, ENHANCED DISEASE SUSCEPTIBILITY1* and *PHYTOALEXIN DEFICIENT4* conditionally regulate cellular signaling homeostasis, photosynthesis, water use efficiency, and seed yield in Arabidopsis. Plant Physiology 161: 1795-1805.

Wu, Y., Zhang, D., Chu, J.Y., Boyle, P., Wang, Y., Brindle, I.D., De Luca, V., Despre, C. (2012) The Arabidopsis *NPR1* protein is a receptor for the plant defense Hormone salicylic acid. Cell Reports 1: 639-647.

Yokoyama, R., Nishitani, K. (2001) Endoxyloglucan transferase is localized both in the cell plate and in the secretory pathway destined for the apoplast in tobacco cells. Plant Cell Physiology 42: 292-300.

Zhang, Z., Feechan, A., Pedersen, C., Newman, M.A., Qiu, J.L., Olesen, K.L., Thordal-Christensen, H. (2007) A SNARE-protein has opposing functions in penetration resistance and defence signalling pathways. Plant Journal 49:302-312Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F., Glazebrook, J. (1998) *PAD4* functions upstream from salicylic acid to control defense responses in Arabidopsis. Plant Cell 10: 1021-1030.

CHAPTER IV

THE INVOLVEMENT OF ALPHA-HYDROXYNITRILE LYASE (AHL) AND AN ATP BINDING CASSETTE (ABC) FUNCTIONING DURING GLYCINE MAX DEFENSE TO THE ROOT PARASITE HETERODERA GLYCINES

Abstract

Genes functioning in membrane fusion were originally identified genetically in *Saccharomyces cerevisiae* and are found in all eukaryotes. Components of the unit, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE), function in the plant genetic model *Arabidopsis thaliana* during its defense to shoot pathogens. Regarding defense, little is understood about SNARE in roots or its regulation. Experiments in *Glycine max* (soybean) have provided an opportunity to perform such studies, revealing that SNARE genes are expressed under natural conditions in root cells undergoing defense to parasitism by the nematode *Heterodera glycines*. Presented here, the *G. max* homolog of *S. cerevisiae* suppressor synaptobrevin/vesicle associated membrane protein/*YKT6*/*SEC22* (*SYB*/*VAMP*/*YKT6/SEC22*) functions in resistance. In contrast, a coatomer zeta/retrieval3 (Cζ/*RET3*) homolog known to function in retrograde transport within and between the Golgi and endoplasmic reticulum (ER) does not appear to function in resistance. Experiments show that a β-glucosidase related to alpha-hydroxynitrile lyase (AHL) and an ATP binding cassette (ABC) transporter also function in defense.

Introduction

Secretion is a central component of natural physiological processes of all eukaryotic cells (Zhou *et al.* 2015). The process of secretion examined genetically, beginning with studies in the model organism *Saccharomyces cerevisiae* (yeast), have resulted in the identification of the *Secretion* phenotype from which the *sec* mutant alleles have been determined (Novick *et al.* 1980, 1981). The protein products of the *SEC* genes function in an orderly stepwise manner, mediating membrane fusion (Novick *et al.* 1980, 1981). The functional unit responsible for membrane fusion is the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) (reviewed in Jahn and Fasshauer 2012). SNARE homologs have been identified in all eukaryotes, functioning in cellular stasis (Clary *et al.* 1990; Lukowitz *et al.* 1996; Geelen *et al.* 2002; Zhou *et al.* 2015).

Genetic studies in the plant genetic model *Arabidopsis thaliana* have revealed SNARE components also function in defense to a shoot fungal pathogen (Collins *et al.* 2003; Inada and Ueda 2014). The *PENETRATION1* (*PEN1*) gene originally identified in the *A. thaliana* genome as syntaxin 121 (SYP121) functions in defense to *Blumeria graminis* f. sp. *hordei* (Sanderfoot *et al.* 2000; Collins *et al.* 2003). The SYP121 protein, homologous to the *S. cerevisiae* suppressor of Sec1 protein (Sso1p), is responsible for fusion of trans-Golgi network (TGN) derived vesicles with the plasma membrane (PM) (Bennett *et al.* 1992; Aalto *et al.* 1993; Geelen *et al.* 2002). SYP121 forms a complex on the PM in association with two vesicle-associated membrane proteins (VAMPs), VAMP721 and VAMP722 (Collins *et al.* 2003; Kwon *et al.* 2008). VAMP721 and VAMP722 exhibit homology to the rat (*Rattus norvegicus*) synaptobrevin (SYB) and *S.*

cerevisiae Ykt6p and Sec22p (Baumert *et al.* 1989; Dujon *et al.* 1994; Søgaard *et al.* 1994; McNew *et al.* 1997; Sanderfoot *et al.* 2000; Collins *et al.* 2003; Lipka *et al.* 2007; Kwon *et al.* 2008; Kim *et al.* 2014). Therefore, 4 VAMP protein classes exist (Lipka *et al.* 2007). SYP121 also functions with the 33 kilodalton (kD) soluble N-ethylmaleimide-sensitive factor (NSF) adaptor protein (SNAP33), related to the mouse (*Mus musculus*) SNAP-25 and *S. cerevisiae* Sec9p (Oyler *et al.* 1989; Collins *et al.* 2003; Kwon *et al.* 2008; Kim *et al.* 2014). *PEN1* functions in the formation of a membranous defense apparatus called a cell wall apposition (CWA) (Aist 1976; Collins *et al.* 2003).

Subsequent genetic analyses in *A. thaliana* have demonstrated the involvement of additional components functioning in defense, including the secreted signal peptide-containing β-thioglucoside glucohydrolase gene *PENETRATION2* (*PEN2*) which is part of a large family of β-glycosidases (Lipka *et al.* 2005; Stein *et al.* 2006). Plants produce a vast number of secondary compounds known as β-glycosides that are conjugated to various sugar moieties to increase solubility and inactivate the molecule for storage. The conjugated β-glycoside is part of a binary system that requires its cognate β-glycosidase to activate the compound. The presence of a signal peptide is consistent with *PEN2* entering the secretion system (Lipka *et al.* 2005; Stein *et al.* 2006).

The transport of glycosides to the apoplast is mediated by the eukaryotic ATP-binding cassette (ABC) superfamily of proteins. The roles of ABC transporters in plants are diverse, including pathogen resistance, lead tolerance, resistance to antimicrobials, resistance to auxin-perturbing herbicides, volatile compound production and rhizosphere signaling. The vast majority of ABC transporters are membrane bound and have been divided into 8 subfamilies (ABC A-H) (Verrier *et al.* 2008). In particular, the ABC-G

subfamily has undergone extensive diversification in plants. Early work in *A. thaliana* on the ABC-G subgroup revealed a function in the secretion of cuticular wax (Pighin *et al.* 2004; Bird *et al.* 2007). Genetic and molecular analyses have shown that the plasma membrane localized ABC-G type transporter PENETRATION3 (*PEN3*) resistance protein functions in the export of a toxic glucoside known as a glucosinolate to the fungal penetration site, neutralizing the barley powdery mildew *Blumeria graminis* f. sp *hordei* pathogen (Lipka *et al.* 2005; Stein *et al.* 2006). Furthermore, the *PEN3* protein functions with *PEN1* and *PEN2* during a race specific defense reaction (Johansson *et al.* 2014). These studies explain the long-known involvement of a two component system functioning in legume shoots against various herbivores, identified from natural genetic variants (Armstrong *et al.* 1913; Ware 1925; reviewed in Hughes, 1991). From these studies and the genetic analyses involving *A. thaliana PEN1*, *PEN2* and *PEN3*, a cell biological framework called a regulon has been coined to describe the defense system (Humphry *et al.* 2010). However, the intricacies and extent of how these genes interact genetically are not well understood. Furthermore, experiments in *Oryza sativa* (rice) have demonstrated a role for an ABC half transporter playing essential roles in mycorrhizal arbuscule formation in *Oryza sativa* (rice) (Gutjahr *et al.* 2012). This observation indicates that ABC-G type transporters function in both symbiotic relationships in the root as well as events that aid in antagonizing plant-pathogen interactions in the shoot. Little information exists for an involvement of these genes in plant resistance to root pathogens except for the identification of a natural variant of α-SNAP functioning in some capacity in the defense of *Glycine max* (soybean) to its root pathogen the parasitic nematode *Heterodera glycines* (Matsye *et al.* 2012). In this pathosystem, the

overexpression of the α-SNAP variant is accompanied by elevated transcript levels of syntaxin 31 which resides on the *cis* face of the Golgi apparatus (Hardwick and Pelham 1992; Lupashin *et al.* 1997; Bubeck *et al.* 2008; Matsye *et al.* 2012; Pant *et al.* 2014). Therefore, SNARE components function in the defense of *G. max* to *H. glycines* parasitism and are co-regulated. However, the extent of this co-regulation has yet to be demonstrated and the functionality of the other SNARE components tested.

In the analysis presented here, an examination of data from published gene expression experiments that have detected the presence of *G. max* transcripts in *H. glycines*-parasitized feeding sites known as syncytia undergoing the natural process of resistance in roots have aided in candidate gene selection (Klink *et al.* 2005, 2007, 2009a, 2010a, b, 2011; Matsye *et al.* 2011). The experiments presented here have examined a gene that is related to SYB, known in *A. thaliana* as *VAMP721* which functions in defense (Gm-VAMP721-2) (Collins *et al.* 2003; Wang *et al.* 2007; Klink *et al.* 2010b, 2011; Matsye *et al.* 2011; Kim *et al.* 2014). Experiments show a resistance outcome occurs when the relative transcript levels are increased for Gm-VAMP721-2. In contrast, by decreasing the relative transcript abundance in RNAi lines for Gm-VAMP721-2, the defense reaction in the normally *H. glycines*-resistant *G. max*$_{[\text{Peking/PI 548402}]}$ is impaired. The extent of the importance of the secretion system during defense to *H. glycines* parasitism has been examined by identifying the contribution of *G. max* homologs of the *A. thaliana PEN2* and *PEN3* genes.

Materials and Methods

Selection of candidate genes

The selection of candidate genes has been aided by mining data from published gene expression experiments (Klink *et al.* 2007, 2009a, 2010a, b, 2011; Matsye *et al.* 2011). This procedure is an effective means to identify genes that function in *G. max* defense to *H. glycines* parasitism, proven further in independently-performed genetic mutational analyses (Matsye *et al.* 2012; Liu *et al.* 2012; Matthews *et al.* 2013, 2014; Pant *et al.* 2014, 2015a). To summarize those published experimental procedures, *G. max*[Peking/PI 548402] and *G. max*[PI 88788] were infected with *H. glycines*[NL1-Rhg/HG-type 7/race 3], resulting in a resistant reaction as proven histologically in unengineered roots which is the natural resistance response found in these *G. max* genotypes (Ross 1958; Endo 1965, 1991; Klink *et al.* 2007, 2009a, 2010a, b, 2011). Roots were then processed for histology and laser microdissection (LM), a procedure that was used to collect syncytia undergoing the defense response (Klink *et al.* 2005, 2007, 2009a, 2010a, b, 2011). The mRNA was isolated from the syncytia and converted to probe according to the manufacturer's procedures (Affymetrix). These methods were performed by the National Cancer Institute, Frederick, MD resulting in the generation of labeled probe used for hybridization onto the Affymetrix® Soybean GeneChip® (Klink *et al.* 2007, 2009a, 2010a, b, 2011; Matsye *et al.* 2011). The hybridizations were run in triplicate (arrays 1-3) using probe derived from RNA isolated from LM-collected syncytia obtained from 3 independent replicate experiments each run independently in the two different *H. glycines*-resistant genotypes (Klink *et al.* 2007, 2009a, 2010a, b, 2011). For the gene to be considered expressed at a given time point (3 or 6 days post infection [dpi]), probe

signal had to be measurable above threshold on all three arrays for both *G. max*[Peking/PI 548402] and *G. max*[PI 88788] (6 total arrays), $p < 0.05$ (Klink *et al.* 2007, 2009a, 2010a, b, 2011). The original analysis procedure was performed as follows; the measurement for a particular probe set (gene) transcript on a single array was determined using the Bioconductor implementation of the standard Affymetrix® detection call methodology (DCM) (Klink *et al.* 2007, 2009a, 2010a, b, 2011). DCM consists of four steps, including (1) removal of saturated probes, (2) calculation of discrimination scores, (3) p-value calculation using the Wilcoxon's rank test, and (4) making the detection call (present [P]/marginal [M]/absent [A]). Ultimately, the algorithm determined if the presence of a gene transcript is provably different from zero (P), uncertain or marginal (M), or not provably different from zero or absent (A) (Klink *et al.* 2007, 2009a, 2010a, b, 2011; Matsye *et al.* 2011). The mined data used in the analysis is presented (Supplemental Table 3.1). From these data, genes used in the analysis were selected for functional experiments and/or qPCR.

Gene cloning

G. max root mRNA was isolated according to Matsye *et al.* (2012) using the UltraClean® Plant RNA Isolation Kit according to the manufacturer's instructions (Mo Bio Laboratories®, Inc.; Carlsbad, CA). Genomic DNA was removed from the mRNA with DNase I according to the manufacturer's instructions (Invitrogen®, Carlsbad, California.). The cDNA was synthesized from mRNA using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen®) with oligo d(T) as the primer (Invitrogen®) according to the manufacturer's instructions. The accession numbers and DNA primer sequences for the genes examined in the study are provided in Table 4.1. Genomic DNA

contamination was assessed by PCR by using β-conglycinin primer pair that amplifies DNA across an intron, thus yielding different sized products based on the presence or absence of that intron (Klink *et al.* 2009b).

Table 4.1 PCR and qPCR primer information

Gene name	*G. max* Gene	Accession	Primer type	Primer 5'-->3'
Cζ-2		Glyma08g22580	PCR-F-OE	CACCATGATCCTTGCGGTGCTGT
			PCR-R-OE	TCAAAACTCTGTTGGAGGCTTTAAC
			PCR-F-RNAi	CACCATGATCCTTGCGGTGCTGT
			RNAi	TCAAAACTCTGTTGGAGGCTTTAAC
			qPCR-F	GGCAATATCCTCATCGAACGT
			qPCR-R	TCTTCGTTTTTGACACCCTTAAGAT
			qPCR probe	AGCGTCTGCACTGGCGTTCATTC
VAMP721	Gm-VAMP721-2	Glyma08g47040	PCR-F-OE	CACCATGGGACAGCAATCGTTGATC
			PCR-R-OE	TCATTTACCACAGTTGAAGCCAC
			PCR-F-RNAi	CACCCTTCGCTCTCAGGCTCAAGA
			PCR-R-RNAi	ACCACAGTTGAAGCCACCAC
			qPCR-F	TTATCCTCGCGGAGTACACC
			qPCR-R	ATCGACGAGGTAGTTGAAGGTG
			qPCR probe	CCCTTCCTCCAACAACAAGTTCACCT
α-hydroxynitrile glucosidase	Gm-βg-4	Glyma11g13810	PCR-F-OE	CACCATGGCATTCAAAGGTTATTTCCT
			PCR-R-OE	CTATTTATTGGAGCCATAAAGTTTGG
			PCR-F-RNAi	CACCATGGCATTCAAAGGTTATTTCCT
			PCR-R-RNAi	CCAAATTCCCTGAAGCAAAG
			qPCR-F	AAGGTTATTTCCTTCTCGGCC
			qPCR-R	TCTGGGAAGCTCTTCCGACT
			qPCR probe	GGTCTTCCAAAGTTATATGCGAAGAAGCAG
Cytochrome P450 79 D 4 (CYP79D4)	Gm-CYP79D4-3	Glyma13g06880	PCR-F-OE	CACCATGGCTCACTCCCCTTTTCT
			PCR-R-OE	GAGCATATGTGGCTTCATGTTC
			PCR-F-RNAi	CACCATGGCTCACTCCCCTTTTCT
			PCR-R-RNAi	CTTCATTTTCTTCCATTGGGCT

Table 4.1 (Continued)

			qPCR-F	CACCATTGCGAGGGAGTTCTT
			qPCR-R	TGCGGGGAAAGCAAATCAT
			qPCR probe	ATTTTTGGGCCCTTTGGAGCCCAAT
ABC-G	Gm-ABC-G-26	Glyma17g04360	PCR-F-OE	ATGGCACAGCTGGCAGGTG
			PCR-R-OE	TTACCTCTTCTGGAAATTGAGGTTTCC
			PCR-F-RNAi	CACCGAGCAGCCTTCAGACCGACTAT
			RNAi	CCCACTGTCCTCAAAGAACTCA
			qPCR-F	GGCAGGTGCGGATGAGATA
			qPCR-R	GTTATCAACTTCTTGTTGCACAGGA
			qPCR probe	GAAGTCATGCCTCTAGTTTCCAGAGCG
Ribosomal S21		Expressed sequence tag	qPCR-F	ATGCAGAACGAGGAAGGACAG
			qPCR-R	GAAGCATGGTCCTTAGCG
			qPCR probe	CCTAGGAAGTGCTCTGCCACAAAC
α-tubulin folding cofactor B		Glyma05g38210	qPCR-F	CTTCGAGCATCCAACAAGTGG
			qPCR-R	TCCAGAGCTTGTCTTTGACGG
			qPCR probe	AACCTTCGCCTCCGACATCCG
eGFP			PCR	GAATTTGTTTCGTGAACTATTAGTTGCGG
			PCR	GCATGCCTGCAGGTCACTGGATTTTG
Ar-VirG			PCR	ATGCGCCATCTTATTACCGAGTATTTAAC
			PCR	TCAGGCCGCCATCAGACC
β-conglycinin			PCR	5'-CCATGCTGACGCTGATTACCTC
			PCR	5'-CTACCAGGCTTGTTAACGGGTATGG

F: Forward primer, R: Reverse primer

G. max genetic transformation

The pRAP plant transformation system used here has been designed and tested specifically for studying the interaction between *G. max* and *H. glycines* (Klink *et al.* 2008, 2009b; Matsye *et al.* 2012; Matthews *et al.* 2013, 2014). The pRAP plant transformation system has been proven independently in other labs to obtain the same outcomes (resistance to *H. glycines* parasitism) as genetic mutational analyses and virus

induced gene silencing (VIGS) (Liu *et al.* 2012; Matthews *et al.* 2013). The pRAP vector system that has been proven to function in *G. max* is based off of the published Gateway® cloning vector platform that has been developed and proven to work in other plant systems (Curtis and Grossniklaus 2003; Matsye *et al.* 2012; Matthews *et al.* 2013). The published pRAP vector platform uses an enhanced green fluorescent protein (eGFP) transgenic reporter system (Haseloff *et al.* 1997). The pRAP vector platform, depending on the integrated cassette, is used to activate or suppress the transcription of a targeted gene (Jefferson *et al.* 1987; Fire *et al.* 1998; Collier *et al.* 2005; Klink *et al.* 2009b; Matsye *et al.* 2012; Matthews *et al.* 2013, 2014; Pant *et al.* 2014, 2015a). The expression of the gene cassettes is driven by the figwort mosaic virus subgenomic transcript promoter (FMV-sgt) promoter (Bhattacharyya *et al.* 2002). The FMV-sgt promoter has been proven to drive gene expression in transgenic *G. max* roots throughout the life cycle of *H. glycines* (Klink *et al.* 2008). The activation of transcription of a targeted gene is accomplished using the pRAP15 vector which has been designed for and has been proven to result in an increase in the relative transcript levels of the gene of interest (GOI) (Matsye *et al.* 2012; Youssef *et al.* 2013; Matthews *et al.* 2013, 2014; Pant *et al.* 2014, 2015a, b). The pRAP17 vector has been designed for and proven to result in a decrease in the relative transcript levels of the GOI (Klink *et al.* 2009b; Pant *et al.* 2014, 2015a). Between the left and right border of the pRAP15 and pRAP17 vectors exists the attR homologous recombination sites of the Gateway® system (Invitrogen®) where the GOI integrates (Klink *et al.* 2009b; Matsye *et al.* 2012; Pant *et al.* 2015b). Thus, roots exhibiting the expression of the eGFP visual reporter will also possess the GOI, each with

their own promoter and terminator sequences (Collier *et al.* 2005; Klink *et al.* 2009b; Matsye *et al.* 2012; Matthews *et al.* 2013; Pant *et al.* 2015b).

The amplicons representing the GOI were cloned from *G. max*[Williams 82/PI 518671] and ligated into the directional pENTR/D-TOPO® Gateway®-compatible vector (Invitrogen®) according to the manufacturer's instructions. The reaction contents then were transformed into chemically competent *E. coli* strain One Shot TOP10® and selected on kanamycin (50 μg/ml) according to the manufacturer's instructions (Invitrogen®). Gene sequences were confirmed by matching them to the *G. max*[Williams 82/PI 518671] genome accession (Schmutz *et al.* 2010). Amplicons representing full length genes were cloned into the pRAP15 overexpression vector (Matsye *et al.* 2012; Pant *et al.* 2015b). Alternatively, full length genes or subcloned portions of genes were engineered into the pRAP17 RNAi vector (Klink *et al.* 2009b). This approach was proven effective for RNAi studies in plants (Klink and Wolniak 2001). In the overexpression studies, the amplicons were ligated into the pRAP15 destination vector using LR Clonase® (Invitrogen®) according to the manufacturer's instructions (Matsye *et al.* 2012). The pRAP15-*ccd*B control and engineered pRAP15 vector containing the GOI were used to transform chemically competent *Agrobacterium rhizogenes* K599 (K599) (Hofgen and Willmitzer 1988; Haas *et al.* 1995; Collier *et al.* 2005). The transformation mix then was plated on LB-agar, selecting with tetracycline (5μg/ml) according to Matsye *et al.* (2012). A PCR reaction using pRAP15 primers that amplify the 717 bp eGFP gene and the 690 bp *A. rhizogenes* root inducing (Ri) plasmid (EU186381) *Vir*G gene (VirG) were used to confirmed that the K599 contained both plasmids prior to transformation. The pRAP15 vector containing the GOI was confirmed by PCR using primers for the respective genes

and DNA sequencing. Genetic transformation experiments resulting in gene overexpression in *G. max* roots were performed according to Matsye *et al.* (2012) in *H. glycines*-susceptible genetic background of *G. max*[Williams 82/PI 518671] (Concibido *et al.* 2004; Schmutz *et al.* 2010). Genetic transformation experiments designed to decrease the level of target gene mRNA was performed according to Klink *et al.* (2009b). This procedure used the pRAP17 RNAi vector in the functionally *H. glycines*-resistant genetic background of *G. max*[Peking/PI 548402] (Concibido *et al.* 2004). The procedure for making genetically engineered plants that were used in overexpression or RNAi experiments involved the co-cultivation of 7-9 day old *G. max*[Williams 82/PI 518671] (overexpression experiments) or *G. max*[Peking/PI 548402] (RNAi experiments) with the K599 engineered to harbor the appropriate genetic construct. The roots of these plants were excised while the cut plants were immersed in Murashige and Skoog (MS) media containing the K599 harboring the engineered pRAP15-*ccd*B or pRAP17-*ccd*B controls while at the same time different plants were cut and transformed with K599 harboring the engineered pRAP15-GOI or pRAP17-GOI experimental constructs (Murashige and Skoog 1962; Klink *et al.* 2009b; Matsye *et al.* 2012; Pant *et al.* 2014). Due to the way K599 transfers the DNA cassettes situated between the left and right borders of the plasmid into the root cell chromosomal DNA, the subsequent growth and development of the stably transformed genetically engineered cell into a transgenic root results in the production of a plant that is a genetic mosaic called a composite plant (Collier *et al.* 2005). These composite, genetically mosaic plants have the entire shoot being non-transgenic and the entire root being transgenic (Haas *et al.* 1995; Collier *et al.* 2005; Klink *et al.* 2008, 2009b; Matsye *et al.* 2012; Matthews *et al.* 2013; Pant *et al.* 2014). In these studies, therefore, each

individual transgenic root system functions as an independent transformant line (Tepfer, 1984; Matsye *et al.* 2012; Matthews *et al.* 2013; Pant *et al.* 2014, 2015a). Quantitative PCR (qPCR) were used to confirm the relative levels of transcript abundance in the pRAP15-GOI engineered overexpressing lines or the pRAP17-GOI-engineered RNAi lines.

Quantitative PCR

The DNA sequences for the qPCR primers used in quantitative gene expression experiments are provided (Table 4.1). The experiments involving *G. max* used three different control genes for monitoring the relative levels of transcript abundance, (1) ribosomal protein gene S21 (S21), (2) α-tubulin folding cofactor B and (3) coatomer zeta (Cζ). The Gm-S21 gene was tested and used as a control in prior studies (Klink *et al.* 2005; Matsye *et al.* 2012; Pant *et al.* 2014, 2015). S21 is a highly conserved gene proven to be transcribed into mRNA and translated into protein (Morita-Yamamuro *et al.* 2004). With regard to assessing the relative abundance in transcript levels in qPCR experiments, prior qPCR analyses had shown that the Gm-S21 control performs in the same manner as elongation initiation factor protein 3 (Matsye *et al.* 2012). Therefore, Gm-S21 was selected to serve as the control for the qPCR experiments presented here. Added gene expression controls were performed using the *G. max* α-tubulin folding cofactor B, selected because in other biological systems it has been determined in genomics analyses to be an effective control gene (Caracausi *et al.* 2015). The α-tubulin folding cofactor B gene is transcribed and translated, but functions in the cytosol by direct protein-protein interaction during α-tubulin stasis (Radcliffe and Toda 2000; Dhonukshe *et al.* 2006). A

third control gene proven to be transcribed and translated into protein that has also been used in functional transgenic experiments presented here, is Cζ of which there are three in the genome of *G. max* (Kuge *et al.* 1993). Cζ acts in retrograde transport, functioning in retrieval between the Golgi and endoplasmic reticulum (ER) (Kuge *et al.* 1993; Yamazaki *et al.* 1996; Cosson *et al.* 1996).

The qPCR experiments used Taqman® 6-carboxyfluorescein (6-FAM) probes and Black Hole Quencher (BHQ1) (MWG Operon; Birmingham, AL). The qPCR differential expression tests were performed using mRNA samples isolated from three independent replicates. The qPCR reaction conditions included a 20 µl Taqman Gene Expression Master Mix (Applied Biosystems; Foster City, CA), 0.9 µl of 100 µM forward primer, 0.9 µl of 100 µM reverse primer, 2 µl of 2.5 µM 6-FAM (MWG Operon®) probe and 9.0 µl (100 ng/µl) template DNA. The qPCR reactions were performed on an ABI 7300 (Applied Biosystems®). The qPCR conditions included a preincubation of 50° C for 2 min, followed by 95° C for 10 min. This step was followed by alternating 95° C for 15 sec followed by 60° C for 1 min for 40 cycles. The accepted universal standard for qPCR statistical analysis, using $2^{-\Delta\Delta CT}$ to calculate fold change, was followed according to the derived formula presented in Livak and Schmittgen (2001) (Klink *et al.* 2005; Matsye *et al.* 2012; Pant *et al.* 2014, 2015a).

The infection of *G. max* by *H. glycines*

H. glycines[NL1-Rhg/HG-type 7/race 3] have been proven to generate a susceptible reaction in unengineered and pRAP15-*ccd*B control-engineered *G. max*[Williams 82/PI 518671] (Klink *et al.* 2007, 2009a, 2010a, b; 2011; Matsye *et al.* 2011, 2012; Youssef *et al.* 2013;

Matthews *et al.* 2013, 2014). In contrast, *H. glycines*[NL1-Rhg/HG-type 7/race 3] have been proven to generate a resistant reaction in unengineered and pRAP17-*ccd*B control-engineered *G. max*[Peking/PI 548402] (Klink *et al.* 2007, 2009a, 2010b, 2011; Matsye *et al.* 2011; Pant *et al.* 2014, 2015a). Female *H. glycines*[NL1-Rhg/HG-type 7/race 3] used in the analysis presented here were purified by sucrose flotation (Jenkins, 1964; Matthews *et al.* 2003; Klink *et al.* 2007, 2009b, 2011; Matsye *et al.* 2012; Pant *et al.* 2014, 2015a). Each root was inoculated with one ml of *H. glycines* at a concentration of 2,000 second stage juveniles (J2s)/ml per root system (per plant) and infected for 30 days according to Matsye *et al.* (2012). Infection was confirmed by acid fuchsin staining and histology (Byrd *et al.* 1983; Klink *et al.* 2005). At the end of the experiment, the cysts (female carcass containing the eggs) were collected over nested 20 and 100-mesh sieves (Matsye *et al.* 2012; Pant *et al.* 2014, 2015a). Furthermore, the soil was washed several times and the rinse water sieved to assure collection of all cysts (Matsye *et al.* 2012; Matthews *et al.* 2013; Pant *et al.* 2014, 2015a).

The accepted assay to accurately reflect if a condition exerts an influence on *H. glycines* development is the female index (FI) (Golden *et al.* 1970). The FI were calculated in a double blind analysis as FI = (Nx/Ns) x 100, where Nx is the average number of females on the test cultivar and Ns is the average number of females on the standard susceptible cultivar (Golden *et al.* 1970). Nx is the pRAP15-transformed line that had the engineered GOI. Ns is the pRAP15 control in their *G. max* [Williams 82/PI 518671]. The effect of the overexpressed gene on parasitism was tested statistically using the Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, $p < 0.05$ (Pant *et al.* 2014).

Results

Selection of candidate genes for genetic analyses

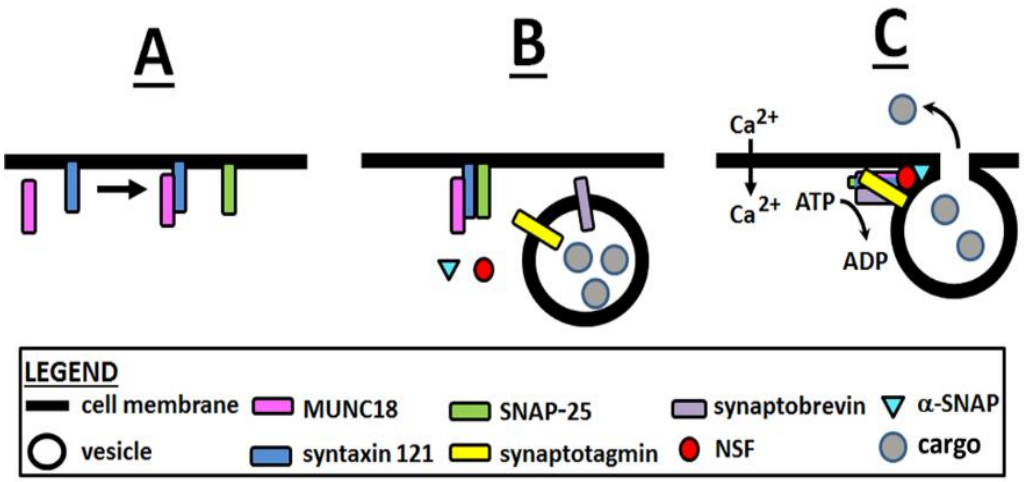


Figure 4.1 The process of membrane fusion and genes involve in the process

The 5 main processes of vesicle fusion have been combined into three steps (**A-C**). **A**, recruitment of MUNC18; **B**, priming; **C**, triggering, activation of the SNARE acceptor complex and fusion. Fusion results in the delivery and release of cargo contents. Footnote: all proteins involved in membrane fusion have not been presented. (Adapted from Jahn and Fasshauer 2012).

In *A. thaliana*, the *PEN1* SNARE protein functions in defense (Figure 4.1), functioning in concert with *PEN2* and *PEN3*. Prior work has demonstrated the involvement of the SNARE homologs SYP121 (*PEN1*/Sso1p), MUNC18 (Sec1p), SNAP-25 (Sec9p), SYB (VAMP/Ykt6p/Sec22p), SYT (Tcb3p), NSF (Sec18p) and α-SNAP (Sec17p) function in the defense to *G. max* to *H. glycines* parasitism (Sharma *et al.* under review). Presented here, the identified Gm-VAMP721-2 gene is being studied to determine if it performs a role in defense analogous to that observed in *A. thaliana***.** *G. max* candidate genes examined here have been selected from published gene expression experiments analyzing the natural defense responses of *G. max*$_{[\text{Peking/PI 548402}]}$ and *G. max*$_{[\text{PI 88788}]}$ (Klink *et al.* 2007, 2009a, 2010a, b, 2011; Matsye *et al.* 2011). In the analysis

presented here, the gene is considered expressed in syncytia undergoing defense if the probe set representing the gene measures probe in all 6 examined arrays (3 arrays for *G. max*$_{[Peking/PI\ 548402]}$ and *G. max*$_{[PI\ 88788]}$) at a statistically significant level above background ($p < 0.05$) for a given time point (3 or 6 dpi) (Table 4.2; Appendix Table C.1) (Klink *et al.* 2010a, b, 2011; Matsye *et al.* 2011).

Table 4.2 The genes originally identified by detection call methodology (DCM) and studied here in the functional analyses

Gene name	**Time point 0 dpi**	***G. max* : Genotype 1 p-value: Peking/PI 548402**			***G. max* : Genotype 2 p-value: PI 88788**		
		Array 1	**Array 2**	**Array 3**	**Array 1**	**Array 2**	**Array 3**
Cζ-2	N/M	0.0204298	0.186972	0.06533	0.00382	0.00818	0.00382
VAMP721-2	M	0.0016728	0.010397	0.03768	0.00382	0.00292	0.00292
CYP79D4-3	n/a	n/a	n/a	n/a	n/a	n/a	n/a
βg-4	N/M	0.2968558	0.211798	0.16403	0.21179	0.04558	0.04558
ABC-G-26	N/M	0.0022196	0.211798	0.00167	0.00167	0.00167	0.00167
Gene name	**Time point 3 dpi**	***G. max* : Genotype 1 p-value: Peking/PI 548402**			***G. max* : Genotype 2 p-value: PI 88788**		
		Array 1	**Array 2**	**Array 3**	**Array 1**	**Array 2**	**Array 3**
Cζ-2	M	0.0016728	0.001672	0.02043	0.002219	0.002219	0.002219
VAMP721-2	M	0.0029235	0.002219	0.00167	0.001672	0.002219	0.002923
CYP79D4-3	n/a	n/a	n/a	n/a	n/a	n/a	n/a
βg-4	M	0.0016728	0.001672	0.01642	0.001672	0.010397	0.008184
ABC-G-26	N/M	0.0029235	0.003822	0.09115	0.004962	0.53542	0.008184
Gene name	**Time point 6 dpi**	***G. max* : Genotype 1 p-value: Peking/PI 548402**			***G. max* : Genotype 2 p-value: PI 88788**		
		Array 1	**Array 2**	**Array 3**	**Array 1**	**Array 2**	**Array 3**
Cζ-2	M	0.00222	0.00221	0.00222	0.00221	0.00221	0.00221
VAMP721-2	M	0.00496	0.00221	0.00167	0.00222	0.00221	0.00221
CYP79D4-3	n/a	n/a	n/a	n/a	n/a	n/a	n/a
βg-4	M	0.00496	0.00382	0.00639	0.00167	0.00167	0.00222
ABC-G-26	M	0.00639	0.00382	0.00382	0.00292	0.00818	0.002221

For the gene to be considered expressed, the probe set for the accompanying gene had to detect probe above threshold in all three arrays in each *G. max* genotype (*G. max*$_{[Peking/PI\ 548402]}$ and *G. max*$_{[PI\ 88788]}$); $p < 0.05$, Wilcoxon's rank test. M, measurable expression (red); N/M no measurable expression (blue); n/a, not applicable (gray).

Expression in control cells did not preclude the genes from consideration since SNARE genes have important functions in normal root cells (Table 4.2) (Arpat *et al.* 2012). In most cases, the gene transcript is detected in the samples collected from cells undergoing the process of defense at both time points in each genotype. In some cases the transcript is detected in the control cells. The results show the candidate genes exhibit expression under natural, unengineered conditions in syncytia that have been induced to form by *H. glycines*[NL1-Rhg/HG-type 7/race 3] during defense.

G. max SNARE Gm-VAMP721-2 functions in defense in the root

The full length Gm-VAMP721-2 has been cloned and engineered into the pRAP15 vector to drive its overexpression in the *H. glycines*-susceptible *G. max*[Williams 82/PI 518671]. In complementary studies, Gm-VAMP721-2 has been engineered into the pRAP17 RNAi vector to suppress its relative transcript level in the *H. glycines*-resistant *G. max*[Peking/PI 548402]. Gm-VAMP721-2-OE and RNAi roots, respectively, have then been infected with *H. glycines*. The FI of Gm-VAMP721-2-OE overexpressing roots in *G. max*[Williams 82/PI 518671] reveals suppressed parasitism (Table 4.3).

In complementary studies, Gm-VAMP721-2-RNAi lines exhibit an impairment of resistance in *G. max*[Peking/PI 548402] (Table 4.4). The results presented here demonstrate that the overexpression of the candidate membrane fusion gene results in a suppressed capability for *H. glycines* to parasitize *G. max*[Williams 82/PI 518671]. In contrast, the results presented here demonstrate that the RNAi of the candidate membrane fusion gene results in an impaired capability of *G. max*[Peking/PI 548402] to suppress *H. glycines* parasitism.

Table 4.3 Suppressed parasitism is observed when overexpressing the candidate resistance gene in the susceptible *G. max*[Williams 82/PI 518671].

Gene	Accession	# of independent transformant control plants	# of independent transformant OE plants	FI (wr)	P-value (wr)	FI (pg)	P-value (pg)
Cζ-2	**Glyma08g22580**	Rep 1: 12 Rep 2: 15 Rep 3: 12	Rep 1: 10 Rep 2: 13 Rep 3: 10	91 99.2 99.3	0.287578 0.371333 0.482511	85.3 118.4 106.4	0.28237 0.567662 0.448382
VAMP721-2	**Glyma08g47040**	Rep 1: 15 Rep 2: 15 Rep 3: 11	Rep 1: 11 Rep 2: 11 Rep 3: 12	28.8 9.5 19.1	0.000019846 0.000039692 2.77E-05	11.5 9.7 23.1	0.00068 0.00042 4.09E-05
βg-4	**Glyma11g13810**	Rep 1: 8 Rep 2: 12 Rep 3: 16	Rep 1: 17 Rep 2: 12 Rep 3: 20	46.2 48.4 14.8	0.000238541 5.14024E-05 1.36839E-10	53 58.1 11.4	0.00241315 0.0715839 1.36839E-10
CYP79D4-3	**Glyma13g06880**	Rep 1: 20 Rep 2: 12 Rep 3: 10	Rep 1: 15 Rep 2: 8 Rep 3: 7	18.9 21.1 18.1	3.07887E-10 0.00013 0.00038	54.4 46.4 33.1	0.003059 0.00494 0.00038
ABC-G-26	**Glyma17g04360**	Rep 1: 11 Rep 2: 12 Rep 3: 10	Rep 1: 6 Rep 2: 16 Rep 3: 12	21.8 49.2 28.6	8.08016E-05 1.92693E-08 4.63932E-05	26.5 33.3 25.9	0.000323206 4.5691E-06 1.85573E-05

The calculated female index (FI) for the cysts per whole root (wr) and cyst per gram (pg) analyses is presented for the overexpressed targeted candidate genes. The accession represents the gene name provided in the *G. max* genomeIn the columns entitled "# of independent transformant control plants" and "# of independent transformant OE plants" "Rep" represents replicate. Three independent replicates are shown for each experiment. . Statistically significant $p < 0.05$ by Mann-Whitney-Wilcoxon Rank Sum Test.

A *G. max* homolog of *PEN2* function in defense in the root

A. thaliana PEN1 delivers the β-glycosidase *PEN2* to the infection site of *B. graminis* f. sp *hordei* to activate resistance, demonstrating the importance of delivered cargo to resistance and that SNARE mediates the process (Stein *et al.* 2006). In the legume *Lotus japonicus*, a β-glycosidase (LjBGD7) that exhibits homology to the *PEN2* gene is expressed in root. Two *L. japonicus* LjBGD7 paralogs that have been shown to be expressed in the shoot, LjBGD2 and LjBGD4, exhibit homology to α-hydroxynitrile glucosidase. Experiments have shown α-hydroxynitrile glucosidase functions effectively in defense through their role as part of a biochemical pathway resulting in the biogenesis of hydrogen cyanide (HCN) (Figure 4.2).

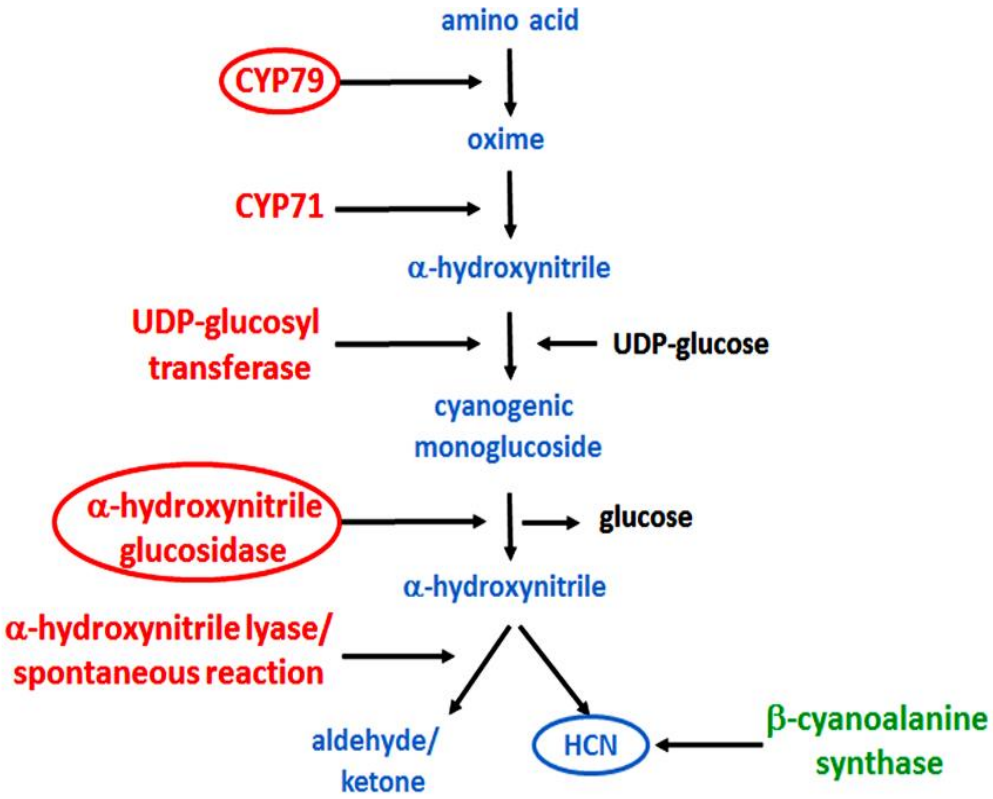


Figure 4.2 The α-hydroxynitrile glucoside metabolic pathway

Active α–hydroxynitrile glucosides are produced through a pathway involving CYP79D4, CYP71, UDP-glucosyltransferase. Subsequent activity by α–hydroxynitrile lyase or a spontaneous event results in the production of toxic HCN (encircled in blue) that is later detoxified by β-cyanoalanine synthase. Functional studies for Gm-CYP79D4-3 and α-hydroxynitrile glucosidase, encircled in red, are presented here. (Adapted from Gleadow and Møller, 2014).

A *G. max* homolog related to the root-expressed LjBGD7 is Gm-βg-4 (Glyma11g13810), sharing 68.3% amino acid (aa) identity with Gm-βg-4 (Table 4.5). Gm-βg-4 transcript has been detected in syncytia undergoing the resistant reaction (Table 4.2). The homology that Gm-βg-4 has to the secreted *L. japonicus* LjBGD7 indicates it may function in the defense process.

Table 4.4 Increased parasitism is observed when suppressing the expression of the candidate resistance gene in the resistant *G. max*[Peking/PI 548402].

Gene	# of independent transformant control plants	# of independent transformant RNAi plants	FI (wr)	FD (wr)	P-value (wr)	FI (pg)	FD (pg)	P-value (pg)
Cζ-2	Rep 1: 12	Rep 1:10	120	1.2	0.214397	105.5	1.1	0.402558
	Rep 2: 13	Rep 2: 10	85.1	0.85	0.12525	95.8	0.96	0.120332
	Rep 3: 12	Rep 3: 11	120	1.2	0.395985	100	1	0.518207
VAMP721-2	Rep 1: 11	Rep 1: 14	157.1	1.6	0.0330708	188.3	1.9	0.0311111
	Rep 2: 12	Rep 2: 8	525	5.3	0.00677733	403.1	4	0.0139453
	Rep 3: 12	Rep 3: 10	150	1.5	0.034619	197.8	2	0.0276443
βg-4	Rep 1: 11	Rep 1: 5	513	5.1	0.01732	1261.7	12.6	0.00222
	Rep 2: 12	Rep 2: 6	300	3	0.04444	214.5	2.1	0.00758
	Rep 3: 12	Rep 3: 6	480	4.8	0.01314	799	8	0.00988
CYP79D4-3	Rep 1: 11	Rep 1: 15	146.7	1.5	0.037983	154.7	1.5	0.036055
	Rep 2: 12	Rep 2: 10	625	6.3	0.033719	180	1.8	0.031386
	Rep 3: 12	Rep 3: 14	150	1.5	0.0231194	305.1	3.1	0.0231194
ABC-G-26	Rep 1: 12	Rep 1: 12	474.8	4.7	0.00071631	507.4	5.1	0.0027798
	Rep 2: 11	Rep 2: 18	445.3	4.5	0.00181831	839.5	8.4	0.0017075
	Rep 3: 12	Rep 3: 12	360.1	3.6	0.00181831	554.6	5.5	0.00225653

The calculated female index (FI) for the cysts per whole root (wr) and cyst per gram (pg) analyses is presented as a fold difference (FD) with the control being 1 fold. The accession represents the gene name provided in the *G. max* genome. Statistically significant $p < 0.05$ by Mann-Whitney-Wilcoxon Rank Sum Test. In the columns entitled "# of independent transformant control plants" and "# of independent transformant OE plants" "Rep" represents replicate. Three independent replicates are shown for each experiment. Statistically significant $p < 0.05$ by Mann-Whitney-Wilcoxon Rank Sum Test.

In *L. japonicus*, the biochemical pathway leading to the production of HCN begins upstream of α-hydroxynitrile glucosidase (Figure 4.2). An analysis of the *G. max* genome resulted in the identification of 5 genes whose conceptually translated protein products share 53.7-66.9% amino acid identity to LjCYP79D4 (Table 4.5). Of the 5 *G. max* protein homologs of LjCYP79D4, Gm-CYP79D4-3 (Glyma13g06880) is most closely related sharing 66.9% amino acid identity (Table 4.5). An analysis of genes proven to have detectable levels of transcript within syncytia undergoing the defense response has been performed. Except for Gm-CYP79D4-1 that has a corresponding probe set fabricated on the Affymetrix® GeneChip, but did not measure detectable levels of transcript, the other *G. max* CYP79D4 paralogs lack corresponding probe sets (Supplemental Table 3.1).

Table 4.5 *G. max* homologs of *Lotus japonicus* α-hydroxynitrile glucosidase and CYP79D4 with amino acid identity and similarity

		LjBGL7	
***G. max* homolog**	**Accession**	**Identity**	**Similarity**
Gm-βg-1	Glyma09g00550	62.6	73.1
Gm-βg-2	Glyma11g13780	68.3	82.1
Gm-βg-3	Glyma11g13800	68.9	82.5
Gm-βg-4	Glyma11g13810	68.3	82.2
Gm-βg-5	Glyma11g13820	69.5	82.6
Gm-βg-6	Glyma11g13850	67.2	81.5
Gm-βg-7	Glyma11g13863	66.8	80.3
Gm-βg-8	Glyma12g05770	66.1	78.3
Gm-βg-9	Glyma12g05780	69.5	82.3
Gm-βg-10	Glyma12g05790	69.3	81.5
Gm-βg-11	Glyma12g05800	68.1	81.7
Gm-βg-12	Glyma12g05811	N/A*	N/A*
Gm-βg-13	Glyma12g05821	70	84.5
Gm-βg-14	Glyma12g05830	68.2	82.8
Gm-βg-15	Glyma12g15620	67	80.4
Gm-βg-16	Glyma12g36870	62	73.3
Gm-βg-17	Glyma13g41800	N/A	N/A
Gm-βg-18	Glyma15g03610	N/A	N/A
Gm-βg-19	Glyma15g03620	64.7	72.3
Gm–βg-20	Glyma15g42570	N/A	N/A
Gm–βg-21	Glyma15g42590	55.9	71.8
Gm-βg-22	Glyma20g03210	51.1	69.3
		LjCYP79D4	
***G. max* homolog**	**Accession**	**Identity**	**Similarity**
GmCYP79-1	Glyma11g31120	66.5	79.2
GmCYP79-2	Glyma11g31151	64.1	77.1
GmCYP79-3	Glyma13g06880	66.9	79.9
GmCYP79-4	Glyma18g05860	53.7	64.4
GmCYP79-5	Glyma20g15960	59.4	74.1

*not applicable

Therefore, transcript measurements could not be made for the remaining Gm-CYP79D4 paralogs (Klink *et al.* 2010a, b, 2011). The Gm-βg-4 and Gm-CYP79D4-3 genes closely related to LjBGD7 and LjCYP79D4, respectively, have been cloned and genetically engineered for overexpression in *G. max*[Williams 82/PI 518671] or RNAi in *G.*

max[Peking PI 548402]. An examination of Gm-βg-4 and CYP79D4-3 overexpressing roots in *G. max*[Williams 82/PI 518671] identified suppressed *H. glycines* parasitism (Table 4.3).

In contrast, Gm-βg-4 and Gm-CYP79D4-3 RNAi lines in *G. max*[Peking/PI 548402] exhibit an increase in *H. glycines* parasitism (Table 4.4). The results show that homologs of components representing enzymatic steps in the α-hydroxynitrile glucosidase metabolic pathway function effectively in resistance when engineered into the *H. glycines* susceptible genotype *G. max*[Williams82/PI 518671]. In contrast, their RNAi results in an impaired capability of *G. max*[Peking/PI 548402] to suppress *H. glycines* parasitism.

A *G. max ABC*-type transporter related to *PEN3* functions in defense in the root

In *A. thaliana*, the *PEN1* and *PEN2* genes function in concert genetically with *PEN3* to mediate defense against *B. graminis* f. sp *hordei* (Figure 4.3) (Stein *et al.* 2006; Johansson *et al.* 2014). Examination of the *G. max* genome shows it contains 35 ABC-G-type transporters. Among them, Gm-ABC-G-26 (Glyma17g04360) exhibits detectable levels of transcript in syncytia undergoing the natural process of resistance to *H. glycines* parasitism in unengineered *G. max*[Peking/PI 548402] and *G. max* [PI 88788] (Klink *et al.* 2010b, 2011; Matsye *et al.* 2011) (Table 4.2, Supplemental Table 3.1). The Gm-ABC-G-26 cDNA has been cloned and overexpressed in *G. max*[Williams 82/PI 518671] or engineered as RNAi lines in *G. max*[Peking/PI 548402]. Gm-ABC-G-26 overexpression in *G. max*[Williams 82/PI 518671] roots suppresses *H. glycines* parasitism (Figure 4.2). In contrast, Gm-ABC-G-26 RNAi lines suppress resistance in *G. max*[Peking/PI 548402], resulting in increased parasitism by *H. glycines* (Figure 4.3).

The results presented here show that there are *G. max* ABC-G type transporters that exhibit detectable levels of transcript abundance within syncytia undergoing the process of resistance. When overexpressed in *G. max*[Williams 82/PI 518671], Gm-ABC-G-26 functions effectively in suppressing *H. glycines* parasitism. In contrast, when Gm-ABC-G-26 is genetically engineered to decrease its relative transcript abundance by RNAi, *G. max*[Peking/PI 548402] exhibits an impaired capability to suppress *H. glycines* parasitism.

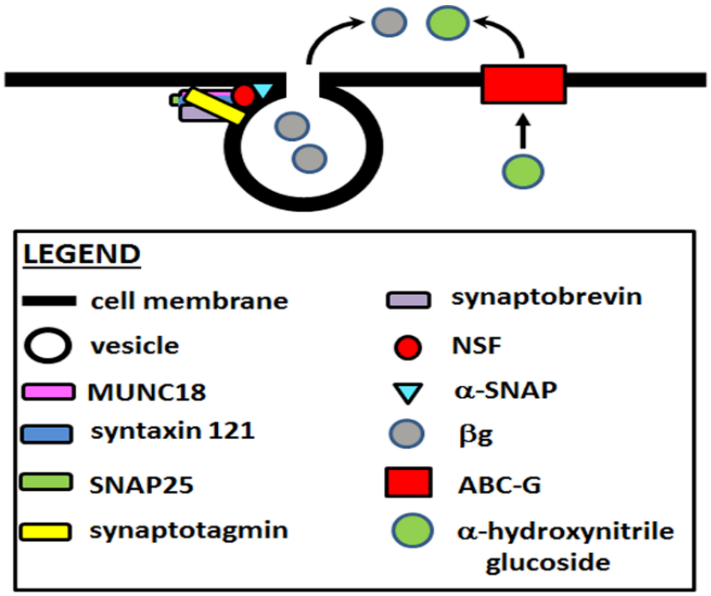


Figure 4.3 Illustration of binary system that relates to the regulon and the protein components.

The regulon is composed of membrane fusion components, cargo, metabolites and an ABC-G transporter. The analysis presented here investigates SYP121, MUNC18, SNAP-25, SYB, SYT, NSF and α-SNAP. Also included are β glucosidase (βγ), ABC-G and α-hydroxynitrile glucoside (Adapted in part from Jahn and Fasshauer 2012).

The results presented here show that there are *G. max* ABC-G type transporters that exhibit detectable levels of transcript abundance within syncytia undergoing the process of resistance. When overexpressed in *G. max*[Williams 82/PI 518671], Gm-ABC-G-26 functions effectively in suppressing *H. glycines* parasitism. In contrast, when Gm-ABC-

G-26 is genetically engineered to decrease its relative transcript abundance by RNAi, *G. max*[Peking/PI 548402] exhibits an impaired capability to suppress *H. glycines* parasitism.

Co-regulation of *G. max* homologs of SNARE, *PEN2* and *PEN3* occurs during the defense reaction

Humphry *et al.* (2010), further supported by Johansson *et al.* (2014), presented analyses whereby the *A. thaliana PEN1*, *PEN2* and *PEN3* genes function during defense as a regulon. These observations led to the hypothesis presented here that the *G. max* SNARE components, including its *PEN1* homolog Gm-SYP121-1, glycoside metabolizing genes, including the *PEN2* homolog βg-4 and the *PEN3* homolog ABC-G-26 may be co-regulated during its process of defense.

In the analysis presented here, qPCR was used to examine cDNA synthesized from RNA isolated from the overexpressing lines and the RNAi lines at 0 dpi. At 0 dpi, the overexpressing lines are accompanied by an increase in relative transcript levels of the remaining genes examined in this study (Figure 4.4). The effect is specific since the relative transcript abundances of α-tubulin folding cofactor B and Cζ-2 control genes are not affected (Figure 4.4). As expected, RNAi of the target gene is accompanied by a decrease in relative transcript abundance of the remaining genes examined in this study while the relative transcript abundances of the control genes are not affected (Figure 4.4).

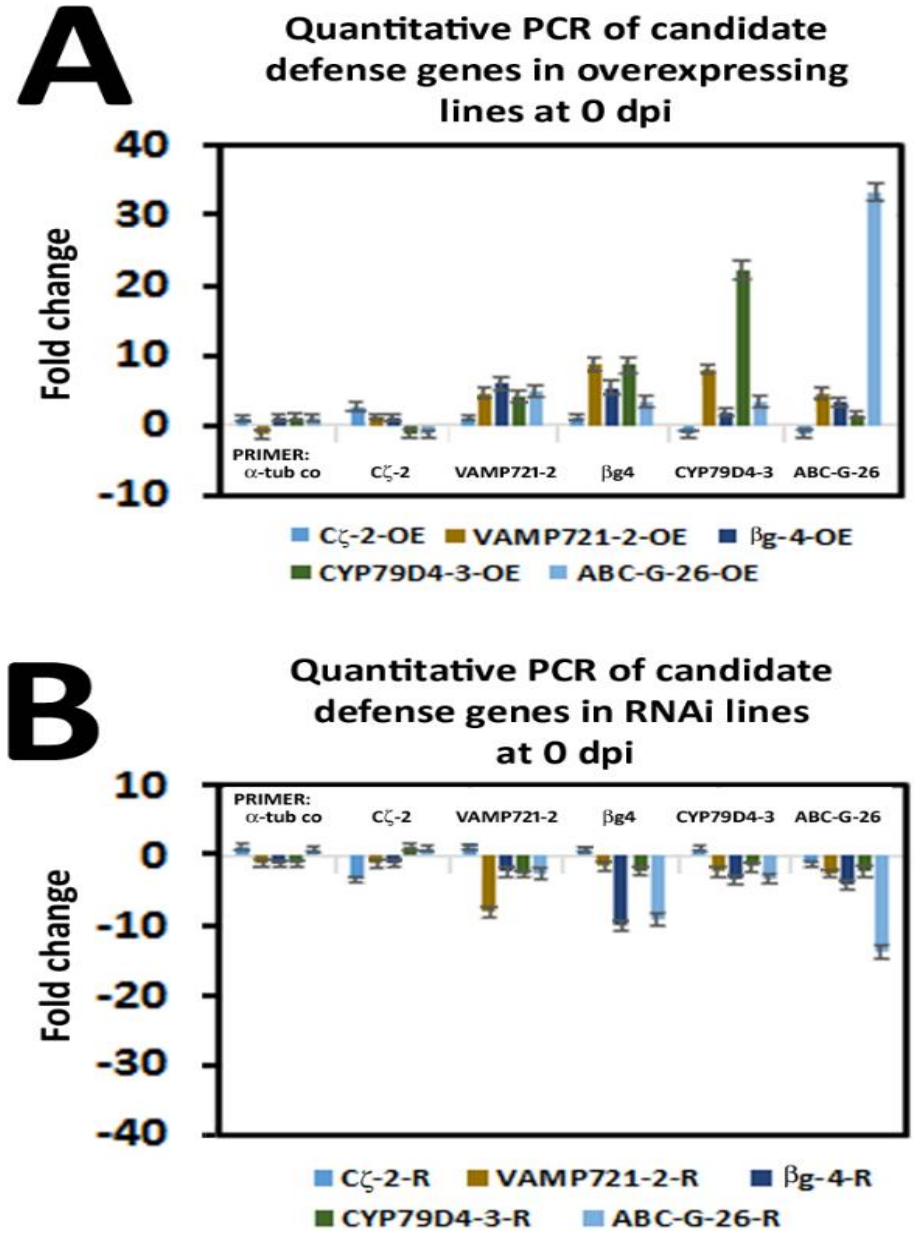


Figure 4.4 Relative transcript abundance of genes under study in overexpression and RNAi lines

Cζ (functional and qPCR control), VAMP721-2, βg-4, CYP79D4-3, Gm-ABC-G-26 in relation to the roots engineered for overexpression (OE) or RNAi (R) at 0 dpi. An additional qPCR control gene, α-tubulin cofactor has also been employed. A, overexpression experiments at 0 dpi; B, RNAi experiments at 0 dpi; An increase or decrease of relative transcript abundance is considered as a fold change ±1.25, respectively. Standard deviation values for the overexpressing and RNAi lines are indicated.

Discussion

Prior experiments have demonstrated the functioning of the *G. max* syntaxin 31 (Gm-SYP38), which is homologous to the *S. cerevisiae* suppressors of the erd2-deletion 5 protein (Sed5p), in the root during its resistance to *H. glycines* parasitism (Hardwick and Pelham 1992; Sanderfoot *et al.* 2000; Pant *et al.* 2014, 2015a). The results of those experiments have led to the development of a model predicting the involvement of other SNARE genes including the *G. max* homolog of *PEN1* (Pant *et al.* 2014). However, a functional test of Gm-SYP121-1 had not been presented. The experiments presented here have expanded that model of defense, reinforced in functional analyses of *G. max* homologs of VAMP721 as well as homologs of *PEN2* and *PEN3*. These functional analyses have been followed by the demonstration of co-regulation of the *G. max* SNARE gene VAMP721 and homologs of *PEN2* and *PEN3* during the defense process.

SNARE functions in defense in the *G. max* root

The experiments presented here have focused on analyzing SNARE, employing gene overexpression and RNAi to examine its relationship to the *G. max* -*H. glycines* root pathosystem. The specificity of the plant transformation platform used here has been reported elsewhere, used in large scale genetic screens to study plant-pathogen interactions (Matthews *et al.* 2013, 2014). We have demonstrated further the specificity of the experimental procedure by examining Gm-Cζ-2. Cζ has been first isolated from bovine (*Bos taurus*) and is related to the *S. cerevisiae* YCZ1 and Ret3p (Kuge *et al.* 1993; Yamazaki *et al.* 1996; Cosson *et al.* 1996). Cζ is part of a 600 kD heptameric coat protein complex I (COPI) that is involved in many cellular processes, functioning during retrograde trafficking between the Golgi and ER, the maturation of endosomes and

autophagy (Kuge *et al.* 1993; Cosson *et al.* 1996; Razi *et al.* 1999; Beck *et al.* 2009). The Gm-Cζ gene family is composed of three members (Gm-Cζ-1-3) with Cζ-2 and Cζ-3 having measurable transcript levels in syncytia undergoing defense (Klink *et al.* 2010b, 2011; Matsye *et al.* 2011). In a control experiment examining Gm-Cζ-2, overexpression and RNAi experiments, supported by prior gene expression studies, histological observations and a FI analysis demonstrate no obvious role for Gm-Cζ-2 in relation to *H. glycines* parasitism (Klink *et al.* 2010b; Matsye *et al.* 2011). The experiments show at the molecular level that the plant transformation system used in the overexpression and RNAi experiments presented here functions in a specific manner on the targeted gene while also lacking an observable effect on *H. glycines* parasitism. The lack of an observable effect on *H. glycines* parasitism found here in experiments targeting Gm-Cζ-2 may be due to the remaining gene family members functioning redundantly. Redundancy for Cζ occurs in other biological systems (Wegmann *et al.* 2004; Moelleken *et al.* 2007; Shtutman *et al.* 2011). These results indicate the effect observed in the experiments presented here reflect the actual role that the tested genes perform in defense.

The results presented here showing the involvement of Gm-VAMP721-2 in *G. max* defense to *H. glycines* parasitism corroborates earlier experiments that demonstrated Gm-SYP121-1 exhibits detectable levels of transcript in syncytia in unengineered roots undergoing their natural process of resistance (Klink *et al.* 2007, 2010b, 2011; Matsye *et al.* 2011). The functional experiments presented here demonstrate that Gm-VAMP721-2 acts in resistance, indicating that part of the defense process in the *G. max* -*H. glycines* pathosystem employs some of the same components that function in *A. thaliana* shoots (Collins *et al.* 2003). This observation is consistent with the identification of *G. max*

homologs of *A. thaliana* defense genes functioning in its resistance to *H. glycines* (Matthews *et al.* 2013; Pant *et al.* 2014, 2015a). The results presented here also show the involvement of *G. max* homologs of *PEN2* and *PEN3* functioning in the process.

References

Aalto, M.K., Ronne, H. and Keränen, S. (1993) Yeast syntaxins Sso1p and Sso2p belong to a family of related membrane proteins that function in vesicular transport. EMBO Journal 12: 4095-4104.

Aist, J.R. (1976) Papillae and related wound plugs of plant cells. Annual Review Phytopathology 14: 145-163.

Armstrong, H.E., Armstrong, E.F. and Horton E. (1913) Herbage studies. II. Variation in Lotus corniculatus and Trifolium repens: (cyanophoric plants). Proceedings of the Royal Socociety of London Serial B 86: 262-269.

Arpat, A.B., Magliano, P., Wege, S., Rouached, H., Stefanovic, A. and Poirier, Y. (2012) Functional expression of PHO1 to the Golgi and *trans*-Golgi network and its role in export of inorganic phosphate. Plant Journal 71: 479-491.

Assaad, F.F., Qiu, J.L., Youngs, H., Ehrhardt, D., Zimmerli, L., Kalde, M., Wanner, G., Peck, S.C., Edwards, H., Ramonell, K. and Somerville, C.R. (2004). The *PEN1* syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Molecular Biology of the Cell 15: 5118-5129.

Baena-González, E., Rolland, F., Thevelein, J.M. and Sheen, J. (2007) A central integrator of transcription networks in plant stress and energy signalling. Nature 448: 938-942.

Baumert, M., Maycox, P.R., Navone, F., De Camilli, P. and Jahn, R. (1989) Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. EMBO Journal 8: 379-384.

Beck, R., Rawet, M., Wieland, F.T. and Cassel, D. (2009) The COPI system: Molecular mechanisms and function. FEBS. Letters 583: 2701-2709.

Beek, J.T., Guskov, A. and Slotboom, D.J. (2014) Structural diversity of ABC transporters. Journal of General Physiology 143: 419-435.

Bednarek, P., Piślewska-Bednarek, M., Svatoš, A., Schneider, B., Doubský, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A. and Molina, A. (2009). A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 323: 101-106.

Bennett, M.K., Calakos, N. and Scheller, R.H. (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science 257: 255-259.

Bhattacharyya, S., Dey, N. and Maiti, I.B. (2002) Analysis of cis-sequence of from the Figwort mosaic virus and comparison of promoter activity with the cauliflower mosaic virus promoters in monocot and dicot cells. Virus Research 90: 47-62.

Bird, D., Beisson, F., Brigham, A., Shin, J., Greer, S., Jetter, R., Kunst, L., Wu, X., Yephremov, A. and Samuels, L. (2007). Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. Plant Journal 52: 485-498.

Bjarnholt, N., Rook, F., Motawia, M.S., Cornett, C., Jørgensen, C., Olsen, C.E., Jaroszewski, J.W., Bak, S. and Møller, B.L. (2008). Diversification of an ancient theme: hydroxynitrile glucosides. Phytochemistry 69: 1507-1516.

Boller, T. and Felix, G. (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annual Review of Plant Biology 60: 379-406.

Bubeck, J., Scheuring, D., Hummel, E., Langhans, M., Viotti, C., Foresti, O., Denecke, J., Banfield, D.K. and Robinson, D.G. (2008) The syntaxins SYP31 and SYP81 control ER–Golgi trafficking in the plant secretory pathway. Traffic 9: 1629-1652.

Caillaud, M.C., Wirthmueller, L., Sklenar, J., Findlay, K., Piquerez, S.J., Jones, A.M., Robatzek, S., Jones, J.D. and Faulkner, C. (2014) The plasmodesmal protein PDLP1 localises to haustoria-associated membranes during downy mildew infection and regulates callose deposition. PLoS Pathogogy 10:e1004496.

Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X. (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6: 1583-1592.

Caracausi, M., Rigon, V., Piovesan, A., Strippoli, P., Vitale, L. and Pelleri, M.C. (2016) A quantitative transcriptome reference map of the normal human hippocampus. Hippocampus 26: 13-26.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D., Felix, G. and Boller, T. (2007). A flagellin-induced complex of the receptor *FLS2* and *BAK1* initiates plant defence. Nature 448: 497-500.

Clary, D.O., Griff, I.C. and Rothman, J.E. (1990) SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. Cell 61: 709-721.

Clay, N.K., Adio, A.M., Denoux, C., Jander, G. and Ausubel, F.M. (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response. Science 323: 95-101.

Collier, R., Fuchs, B., Walter, N., Lutke, K.W. and Taylor, C.G. (2005) Ex vitro composite plants: an inexpensive, rapid method for root biology. Plant Journal 43: 449–457.

Collins, N.C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J.L., Hückelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S.C. and Schulze-Lefert, P. (2003). SNARE-protein-mediated disease resistance at the plant cell wall. Nature 425: 973-977.

Concibido, V.C., Diers, B.W. and Arelli, P.R. (2004) A decade of QTL mapping for cyst nematode resistance in soybean. Crop Science 44: 1121-1131.

Cosson, P., Demolliere, C., Hennecke, S., Duden, R. and Letourneur, F. (1996) γ- and ζ-COP, two coatomer subunits homologous to clathrin-associated proteins, are involved in ER retrieval. EMBO Journal 15: 1792-1798.

Curtis, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiology 133: 462-469.

Dacks, J.B., Poon, P.P. and Field, M.C. (2008) Phylogeny of endocytic components yields insight into the process of nonendosymbiotic organelle evolution. Proceedings of the National Academy of Sciences USA 105: 588-593.

Dhonukshe, P., Bargmann, B.O. and Gadella, T.W. Jr. (2006) Arabidopsis tubulin folding cofactor B interacts with α-tubulin in vivo. Plant Cell Physiology 47: 1406-1411.

Dóczi, R., Brader, G., Pettkó-Szandtner, A., Rajh, I., Djamei, A., Pitzschke, A., Teige, M. and Hirt, H. (2015) The Arabidopsis mitogen-activated protein kinase kinase MKK3 is upstream of Group C mitogen-activated protein kinases and participates in pathogen signaling. Plant Cell 19: 3266-3279.

Dujon, B., Alexandraki, D., André, B., Ansorge, W., Baladron, V., Ballesta, J.P., Banrevi, A., Bolle, P.A., Bolotin-Fukuhara, M. and Bossier, P. (1994). Complete DNA sequence of yeast chromosome XI. Nature 369: 371-378.

Ellinger, D., Naumann, M., Falter, C., Zwikowics, C., Jamrow, T., Manisseri, C., Somerville, S.C. and Voigt, C.A. (2013). Elevated early callose deposition results in complete penetration resistance to powdery mildew in Arabidopsis. Plant Physiology 161: 1433-1444.

Endo, B.Y. (1965) Histological responses of resistant and susceptible soybean varieties, and backcross progeny to entry development of *Heterodera glycines*. Phytopathology 55: 375-381.

Endo, B.Y. (1991) Ultrastructure of initial responses of susceptible and resistant soybean roots to infection by *Heterodera glycines*. Revue of Nématology. 14: 73-84.

Enns, L.C., Kanaoka, M.M., Torii, K.U., Comai, L., Okada, K. and Cleland, R.E. (2005) Two callose synthases, GSL1 and GSL5, play an essential and redundant role in plant and pollen development and in fertility. Plant Molecular Biology 58: 333-349.

Ernst, R., Kueppers, P., Klein, C.M., Schwarzmueller, T., Kuchler, K. and Schmitt, L. (2008) A mutation of the H-loop selectively affects rhodamine transport by the yeast multidrug ABC transporter Pdr5. Proceedings of the National Academy of Sciences USA. 105: 5069-5074.

Eschrich, W. and Currier, H. (1964) Identification of callose by its diachrome and fluochrome reactions. Stain Technogy 39: 303-307.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J. and Parker, J.E. (1999) *EDS1*, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proceedings of the National Academy of Sciences USA 96: 3292-3297.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Potent and specific genetic interference by double stranded RNA in Caenohrabditis elegans. Nature 391: 806-811.

Forslund, K., Morant, M., Jørgensen, B., Olsen, C.E., Asamizu, E., Sato, S., Tabata, S. and Bak, S., (2004). Biosynthesis of the nitrile glucosides rhodiocyanoside A and D and the cyanogenic glucosides lotaustralin and linamarin in Lotus japonicus. Plant Physiology 135: 71-84.

Geelen, D., Leyman, B., Batoko, H., Gian-Pietro, D.S., Moore, I. and Blatt, M.R. (2002) The abscisic acid-related SNARE homolog NtSyr1 contributes to secretion and growth: evidence from competition with its cytosolic domain. Plant Cell 14: 387-406.

Gleadow, R.M. and Møller, B.L. (2014) Cyanogenic glycosides: synthesis, physiology, and phenotypic plasticity. Annual Revue Plant Biology 65: 155-85.

Golden, A.M., Epps, J.M., Riggs, R.D., Duclos, L.A., Fox, J.A. and Bernard, R.L. (1970) Terminology and identity of infraspecific forms of the soybean cyst nematode *(Heterodera glycines).* Plant Disease Reports 54: 544-546.

Gutjahr, C., Radovanovic, D., Geoffroy, J., Zhang, Q., Siegler, H., Chiapello, M., Casieri, L., An, K., An, G., Guiderdoni, E. and Kumar, C.S. (2012). The half-size ABC transporters STR1 and STR2 are indispensable for mycorrhizal arbuscule formation in rice. Plant Journal 69: 906-920.

Haas, J.H., Moore, L.W., Ream, W. and Manulis, S. (1995) Universal PCR primers for detection of phytopathogenic Agrobacterium strains. Applied Environment Microbiology 61: 2879-2884.

Hardwick, K.G. and Pelham, H.R. (1992) SED5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. Journal of Cell Biology 119: 513-521.

Hofgen, R. and Willmitzer, L. (1988) Storage of competent cells for Agrobacterium transformation. Nucleic Acids Research 16: 9877.

Hughes, M.A. (1991) The cyanogenic polymorphism in Trifolium repens L. (white clover). Heredity 66: 105-115.

Humphry, M., Bednarek, P., Kemmerling, B., Koh, S., Stein, M., Göbel, U., Stüber, K., Piślewska-Bednarek, M., Loraine, A., Schulze-Lefert, P. and Somerville, S. (2010). A regulon conserved in monocot and dicot plants defines a functional module in antifungal plant immunity. Proceedings of the National Academy of Sciences USA 107: 21896-21901.

Inada, N. and Ueda, T. (2014) Membrane Trafficking Pathways and their Roles in Plant–Microbe Interactions Plant Cell Physiology 55: 672–686.

Jahn, R. and Fasshauer, D. (2012) Molecular machines governing exocytosis of synaptic vesicles. Nature 490: 201-207.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO Journal 6: 3901-3907.

Jenkins, W.R. (1964) A rapid centrifugal flotation technique for separating nematodes from soil. Plant Disease Reports 48: 692.

Johansson, O.N., Fantozzi, E., Fahlberg, P., Nilsson, A.K., Buhot, N., Tör, M. and Andersson, M.X. (2014). Role of the penetration-resistance genes *PEN1*, *PEN2* and *PEN3* in the hypersensitive response and race-specific resistance in *Arabidopsis thaliana*. Plant Journal 79: 466-476.

Kalde, M., Nühse, T.S., Findlay, K. and Peck, S.C. (2007) The syntaxin SYP132 contributes to plant resistance against bacteria and secretion of pathogenesis-related protein 1. Proceedings of the National Academy of Sciences USA 104: 11850-11855.

Kim, D.G., Riggs, R.D. and Mauromoustakos, A. (1998) Variation in Resistance of Soybean Lines to Races of *Heterodera glycines*. Journal of Nematology 30: 184-191.

Kim, H., O'Connell, R., Maekawa-Yoshikawa, M., Uemura, T., Neumann, U. and Schulze-Lefert, P. (2014) The powdery mildew resistance protein RPW8.2 is carried on VAMP721/722 vesicles to the extrahaustorial membrane of haustorial complexes. Plant Journal 79: 835-847.

Klink, V.P. and Wolniak, S.M. (2001) Centrin is necessary for the formation of the motile apparatus in spermatids of Marsilea. Molecular Biology of the Cell 12: 761-776.

Klink, V.P., Alkharouf, N., MacDonald, M. and Matthews, B.F. (2005) Laser capture microdissection (LCM) and analysis of Glycine max (soybean) syncytial cells formed by the soybean cyst nematode Heterodera glycines. Plant Molecular Biology 59: 969-983.

Klink, V.P., Overall, C.C., Alkharouf, N., MacDonald, M.H. and Matthews, B.F. (2007) Laser capture microdissection (LCM) and comparative microarray expression analysis of syncytial cells isolated from incompatible and compatible soybean roots infected by soybean cyst nematode (Heterodera glycines). Planta 226: 1389-1409.

Klink,V.P., MacDonald, M.H., Martins, V.E., Park, S.C., Kim, K.H., Baek, S.H. and Matthews, B.F. (2008) MiniMax, a new diminutive Glycine max variety, with a rapid life cycle, embryogenic potential and transformation capabilities. Plant Cell Tissue and Organ Culture 92: 183-195.

Klink, V.P., Hosseini, P., Matsye, P.D., Alkharouf. N.W. and Matthews, B.F. (2009a) A gene expression analysis of syncytia laser microdissected from the roots of the *Glycine max* (soybean) genotype PI 548402 (Peking) undergoing a resistant reaction after infection by *Heterodera glycines* (soybean cyst nematode). Plant Molecular Biology 71: 525-567.

Klink, V.P., Kim. K.H., Martins, V.E., MacDonald, M.H., Beard, H.S., Alkharouf, N.W., Lee, S.K., Park, S.C. and Matthews, B.F. (2009b) A correlation between host-mediated expression of parasite genes as tandem inverted repeats and abrogation of the formation of female Heterodera glycines cysts during infection of Glycine max. Planta 230: 53-71.

Klink, V.P., Hosseini, P., Matsye, P.D., Alkharouf, N.W. and Matthews, B.F. (2010a) Syncytium gene expression in *Glycine max*[PI 88788] roots undergoing a resistant reaction to the parasitic nematode *Heterodera glycines*. Plant Physiology and Biochemistry 48: 176-193.

Klink, V.P., Overall, C.C., Alkharouf, N.W., MacDonald, M.H. and Matthews, B.F. (2010b) Microarray detection calls as a means to compare transcripts expressed within syncytial cells isolated from incompatible and compatible soybean (*Glycine max*) roots infected by the soybean cyst nematode (*Heterodera glycines*). Journal of Biomedicine and Biotechnology 1-30.

Klink, V.P., Hosseini, P., Matsye, P.D., Alkharouf, N.W. and Matthews, B.F. (2011) Differences in gene expression amplitude overlie a conserved transcriptomic program occurring between the rapid and potent localized resistant reaction at the syncytium of the *Glycine max* genotype Peking (PI 548402) as compared to the prolonged and potent resistant reaction of PI 88788. Plant Molecular Biology 75: 141-165.

Kuge, O., Hara-Kuge, S., Orci, L., Ravazzola, M., Amherdt, M., Tanigawa, G., Wieland, F.T. and Rothman, J.E. (1993). zeta-COP, a subunit of coatomer, is required for COP-coated vesicle assembly. Journal of Cell Biology 123: 1727-1734.

Kwon, C., Neu, C., Pajonk, S., Yun, H.S., Lipka, U., Humphry, M., Bau, S., Straus, M., Kwaaitaal, M., Rampelt, H. and El Kasmi, F. (2008). Co-option of a default secretory pathway for plant immune responses. Nature 451: 835-840.

Levy, A., Erlanger, M., Rosenthal, M. and Epel, B.L. (2007) A plasmodesmata-associated β-1,3-glucanase in Arabidopsis. Plant Journal 49: 669-682.

Li, J., Todd, T.C., Oakley, T.R., Lee, J. and Trick, H.N. (2010) Host derived suppression of nematode reproductive and fitness genes decreases fecundity of *Heterodera glycines*. Planta 232: 775-785.

Liberali, P., Snijder, B., Pelkmans, L. (2014) A hierarchical map of regulatory genetic interactions in membrane trafficking. Cell 157: 1473-1487.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D. and Llorente, F. (2005). Pre-and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science 310: 1180-1183.

Lipka, V., Kwon, C. Panstruga, R. (2007) SNARE-ware: the role of SNARE-domain proteins in plant biology. Annual Revue of Cell and Developmental Biology 23: 147-174.

Liu, X., Liu, S., Jamai, A., Bendahmane, A., Lightfoot, D.A., Mitchum, M.G. and Meksem, K. (2011). Soybean cyst nematode resistance in soybean is independent of the *Rhg4* locus LRR-RLK gene. Functional and Integrative Genomics 11: 539-549.

Liu, S., Kandoth, P.K., Warren, S.D., Yeckel, G., Heinz, R., Alden, J., Yang, C., Jamai, A., El-Mellouki, T., Juvale, P.S. and Hill, J. (2012). A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens. Nature 492: 256-260.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408.

Lukowitz, W., Mayer, U. and Jürgens, G. (1996) Cytokinesis in the Arabidopsis embryo involves the syntaxin-related KNOLLE gene product. Cell 84: 61-71.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L. and He, P. (2010) A receptor-like cytoplasmic kinase, *BIK1*, associates with a flagellin receptor complex to initiate plant innate immunity. Proceedings of the National Academy of Sciences USA 107: 496-501.

Lupashin, V.V., Pokrovskaya, I.D., McNew, J.A. and Waters, M.G. (1997) Characterization of a novel yeast SNARE protein implicated in Golgi retrograde traffic. Molecular Biology of the Cell 8: 2659-2676.

Maicas, S. and Mateo, J.J. (2005) Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. Applied Microbiology and Biotechnology 67: 322-335.

Mangin, L. (1895) Recherches sur les Péronosporées. Bulletin de la Société d'Histoire Naturelle d'Autun 8: 55-108.

Matsye, P.D., Kumar, R., Hosseini, P., Jones, C.M., Tremblay, A., Alkharouf, N.W., Matthews, B.F. and Klink, V.P. (2011). Mapping cell fate decisions that occur during soybean defense responses. Plant Molecular Biology 77: 513-528.

Matsye, P.D., Lawrence, G.W., Youssef, R.M., Kim, K.H., Lawrence, K.S., Matthews, B.F. and Klink, V.P. (2012). The expression of a naturally occurring, truncated allele of an α-SNAP gene suppresses plant parasitic nematode infection. Plant Molecular Biology 80: 131-155.

Matthews, B., MacDonald, M.H., Thai, V.K. and Tucker, M.L. (2003) Molecular characterization of argenine kinase in the soybean cyst nematode (*Heterodera glycines*). Journal of Nematology 35: 252-258.

Matthews, B.F., Beard, H., MacDonald, M.H., Kabir, S., Youssef, R.M., Hosseini, P. and Brewer, E. (2013). Engineered resistance and hypersusceptibility through functional metabolic studies of 100 genes in soybean to its major pathogen, the soybean cyst nematode. Planta 237: 1337-1357.

Matthews, B.F., Beard, H., Brewer, E., Kabir, S., MacDonald, M.H. and Youssef, R.M. (2014) Arabidopsis genes, At*NPR1*, AtTGA2 and AtPR-5, confer partial resistance to soybean cyst nematode (Heterodera glycines) when overexpressed in transgenic soybean roots BMC Plant Biology 14: 96.

Mazarei, M., Elling, A.A., Maier, T.R., Puthoff, D.P. and Baum, T.J. (2007) GmEREBP1 is a transcription factor activating defense genes in soybean and Arabidopsis Molecular Plant Microbe Interactions 20: 107-119.

McLean, M.D., Hoover, G.J., Bancroft, B., Makhmoudova, A., Clark, S.M., Welacky, T., Simmonds, D.H. and Shelp, B.J. (2007) Identification of the full-length *Hs1*$^{pro-1}$ coding sequence and preliminary evaluation of soybean cyst nematode resistance in soybean transformed with *Hs1*$^{pro-1}$ cDNA.Canadian Journal of Botany 85: 437-441.

McNew, J.A., Søgaard, M., Lampen, N.M., Machida, S., Ye, R.R., Lacomis, L., Tempst, P., Rothman, J.E. and Söllner, T.H. (1997) Ykt6p, a prenylated SNARE essential for endoplasmic reticulum-Golgi transport. Journal of Biological Chemistry 272: 17776-17783.

Melito, S., Heuberger, A., Cook, D., Diers, B., MacGuidwin, A. and Bent A. (2010) A nematode demographics assay in transgenic roots reveals no significant impacts of the *Rhg1* locus LRR-Kinase on soybean cyst nematode resistance. BMC Plant Biology 10: 104.

Meyer, D., Pajonk, S., Micali, C., O'Connell, R. and Schulze-Lefert P. (2009) Extracellular transport and integration of plant secretory proteins into pathogen-induced cell wall compartments. Plant Journal 57: 986-999.

Morant, A.V., Bjarnholt, N., Kragh, M.E., Kjaergaard, C.H., Jorgensen, K., Paquette, S.M., Piotrowski, M., Imberty, A., Olsen, C.E., Møller, B.L. and Bak, S. (2010) The β-glucosidases responsible for bioactivation of hydroxynitrile glucosides in *Lotus japonicus*. Plant Physiology Plant Physiol 147: 1072-1091.

Morita-Yamamuro, C., Tsutsui, T., Tanaka, A. and Yamaguchi, J. (2004) Knock-out of the plastid ribosomal protein S21 causes impaired photosynthesis and sugar-response during germination and seedling development in *Arabidopsis thaliana*. Plant and Cell Physiology 45: 781-788.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Niblack, T.L., Arelli, P.R., Noel, G.R., Opperman, C.H., Orf, J.H., Schmitt, D.P., Shannon, J.G. and Tylka, G.L. (2002) A revised classification scheme for genetically diverse populations of Heterodera glycines. Journal of Nematology 34: 279.

Nielsen, M.E., Feechan, A., Böhlenius, H., Ueda, T. and Thordal-Christensen H. (2012) Arabidopsis ARF-GTP exchange factor, GNOM, mediates transport required for innate immunity and focal accumulation of syntaxin *PEN1*. Proceedings of the National Academy of Sciences USA 109: 11443-11448.

Nishimura, M.T., Stein, M, Hou, B.H., Vogel, J.P., Edwards, H. and Somerville, S.C. (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science 301: 969-972.

Novick, P., Field, C. and Schekman, R. (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell 21: 205-215.

Novick, P., Ferro, S. and Schekman, R. (1981) Order of events in the yeast secretory pathway. Cell 25: 461-469.

Ostergaard, L., Petersen, M., Mattsson, O. and Mundy, J. (2002) An Arabidopsis callose synthase. Plant Molecular Biology 49: 559-566.

Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E. and Wilson, M.C. (1989) The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. The Journal of Cell Biology 109: 3039-3052.

Pajonk, S., Kwon, C., Clemens, N., Panstruga, R. and Schulze-Lefert, P. (2008) Activity determinants and functional specialization of Arabidopsis *PEN1* syntaxin in innate immunity. Journal of Biological Chemistry 283: 26974-26984.

Pant, S.R., Matsye, P.D., McNeece, B.T., Sharma, K., Krishnavajhala, A., Lawrence, G.W. and Klink, V.P. (2014) Syntaxin 31 functions in Glycine max resistance to the plant parasitic nematode Heterodera glycines. Plant Molecular Biology 85: 107-121.

Pant, S.R., Krishnavajhala, A., McNeece, B.T., Lawrence, G.W. and Klink, V.P. (2015) The syntaxin 31-induced gene, LESION SIMULATING DISEASE1 (*LSD1*), functions in *Glycine max* defense to the root parasite *Heterodera glycines*. Plant Signaling and Behaviour 10: 1, e977737.

Pighin, J.A., Zheng, H., Balakshin, L.J., Goodman, I.P., Western, T.L., Jetter, R., Kunst, L. and Samuels, A.L. (2004) Plant cuticular lipid export requires an ABC transporter. Science 306: 702–704.

Radcliffe, P.A. and Toda, T. (2000) Characterisation of fission yeast alp11 mutants defines three functional domains within tubulin-folding cofactor B. Molecular and General Genetics 263: 752-760.

Razi, M., Chan, E.Y. and Tooze, S.A. (2009) Early endosomes and endosomal coatomer are required for autophagy. Journal of Cell Biology 185: 305-321.

Riggs, R.D. and Schmitt, D.P. (1988) Complete characterization of the race scheme for Heterodera glycines. Journal of Nematology 20: 392-395.

Riggs, R.D. and Schmitt, D.P. (1991) Optimization of the *Heterodera glycines* race test procedure. Journal of Nematology 23: 149-154.

Ross, J.P. (1958) Host-Parasite relationship of the soybean cyst nematode in resistant soybean roots. Phytopathology 48: 578-579.

Sanderfoot, A.A., Farhah, F., Assaad, F.F. and Raikhel, N.V. (2000) The Arabidopsis genome. An abundance of soluble N-ethylmaleimide-sensitive factor adaptor protein receptors. Plant Physiology 124: 1558-1569.

Sanderfoot, A.A., Kovaleva, V., Bassham, D.C. and Raikhel, N.V. (2001) Interactions between Syntaxins identify at least five SNARE complexes within the Golgi/prevacuolar system of the Arabidopsis cell. Molecular Biology of the Cell 12: 3733-3743.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D.L., Song, Q., Thelen, J.J., Cheng, J. and Xu, D. (2010) Genome sequence of the palaeopolyploid soybean. Nature 463: 178-183.

Schuette, S., Wood, A.J., Geisler, M., Geisler-Lee, J., Ligrone, R. and Renzaglia, K.S. (2009) Novel localization of callose in the spores of Physcomitrella patens and phylogenomics of the callose synthase gene family. Annals of Botany 103: 749-756.

Shanks, S.G., Carpp, LN., Struthers, M.S., McCann, R.K. and Bryant, N.J. (2012) The Sec1/Munc18 Protein Vps45 Regulates Cellular Levels of Its SNARE Binding Partners Tlg2 and Snc2 in Saccharomyces cerevisiae. PLoS ONE 7: e49628. doi:10.1371/journal.pone.0049628.

Shirakawa, M., Ueda, H., Shimada, T., Koumoto, Y., Shimada, T.L., Kondo, M., Takahashi, T., Okuyama, Y., Nishimura, M. and Hara-Nishimura, I. (2010) Arabidopsis Qa-SNARE SYP2 proteins localized to different subcellular regions function redundantly in vacuolar protein sorting and plant development. Plant Journal 64: 924-635.

Søgaard, M., Tani, K., Ye, R.R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J.E. and Söllner, T. (1994) A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. Cell 78: 937-948.

Steeves, R.M., Todd, T.C., Essig, J.S. and Trick, H.N. (2006) Transgenic soybeans expressing siRNAs specific to a major sperm protein gene suppress Heterodera glycines reproduction. Functional Plant Biology 33: 991-999.

Stein, M., Dittgen, J., Sánchez-Rodríguez, C., Hou, B.H., Molina, A., Schulze-Lefert, P., Lipka, V. and Somerville, S. (2006) Arabidopsis *PEN3*/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18: 731-746.

Stutman, M., Baig, M., Levina, E., Hurteau, G., Lim, C.U., Broude, E., Nikiforov, M., Harkins, T.T., Carmack, C.S., Ding, Y. and Wieland, F. (2011) Tumor-specific silencing of COPZ2 gene encoding coatomer protein complex subunit ζ2 renders tumor cells dependent on its paralogous gene COPZ1. Proceedings of the National Academy of Sciences USA 108: 12449-12454.

Sun, L., Zhu, L., Xu, L., Yuan, D., Min, L. and Zhang, X. (2014) Cotton cytochrome P450 CYP82D regulates systemic cell death by modulating the octadecanoid pathway. Nature Communications 5: 5372.

Takos, A.M., Knudsen, C., Lai, D., Kannangara, R., Mikkelsen, L., Motawia, M.S., Olsen, C.E., Sato, S., Tabata, S., Jørgensen, K. and Møller, B.L. (2011) Genomic clustering of cyanogenic glucoside biosynthetic genes aids their identification in Lotus japonicus and suggests the repeated evolution of this chemical defence pathway. Plant Journal 68: 273-286.

Tepfer, D. (1984) Transformation of several species of higher plants by Agrobacterium rhizogenes: sexual transmission of the transformed genotype and phenotype. Cell 37: 959-967.

Veronese, P., Nakagami, H., Bluhm, B., AbuQamar, S., Chen, X., Salmeron, J., Dietrich, R.A., Hirt, H. and Mengiste, T. (2006). The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in Arabidopsis resistance to necrotrophic and biotrophic pathogens. Plant Cell 18: 257-273.

Verrier, P.J., Bird, D., Burla, B., Dassa, E., Forestier, C., Geisler, M., Klein, M., Kolukisaoglu, Ü., Lee, Y., Martinoia, E. and Murphy, A. (2008) Plant ABC proteins–a unified nomenclature and updated inventory. Trends in Plant Science 13: 151-159.

Wang, W., Devoto, A., Turner, J.G. and Xiao S. (2007) Expression of the membrane-associated resistance protein RPW8 enhances basal defense against biotrophic pathogens. Molecular Plant Microbe Interactions 20: 966-976.

Ware, W.M. (1925) Experiments and observations on forms and strains of Trifolium repens. Journal of Agricultural Science 15: 47-67.

Wegmann, D., Hess, P., Baier, C., Wieland, F.T. and Reinhard, C. (2004) Novel isotypic gamma/zeta subunits reveal three coatomer complexes in mammals. Molecular and Cellular Biology 24: 1070-1080.

Yamazaki, S., Harashima, S., Sakaguchi, M. and Mihara, K. (1997) Identification and functional characterization of yeast zeta-COP. Journal of Biochemistry 121: 8-14.

Yang, R., Hou, Y-X., Campbell, C.A., Palaniyandi, K., Zhao, Q., Bordner, A.J. and Chang, X-B. (2011) Glutamine residues in Q-loops of multidrug resistance protein MRP1 contribute to ATP binding via interaction with metal cofactor. Biochimica and Biophysica Acta 1808: 1790-1796.

Yim, K.O. and Bradford, K.J. (1998) Callose deposition is responsible for apoplastic semipermeability of the endosperm envelope of muskmelon seeds. Plant Physiology 118: 83-90.

Youssef, R.M., MacDonald, M.H., Brewer, E.P., Bauchan, G.R., Kim K-H. and Matthews B.F. (2013) Ectopic expression of AtPAD4 broadens resistance of soybean to soybean cyst and root-knot nematodes. BMC Plant Biology 13: 67.

Xiao, S., Ellwood, S., Calis, O., Patrick, E., Li, T., Coleman, M. and Turner, J.G. (2001) Broad-spectrum mildew resistance in Arabidopsis thaliana mediated by RPW8. Science 291: 118-120.

Xiao, S., Brown, S., Patrick, E., Brearley, C. and Turner, J.G. (2003) Enhanced transcription of the Arabidopsis disease resistance genes RPW8.1 and RPW8.2 via a salicylic acid-dependent amplification circuit is required for hypersensitive cell death. Plant Cell 15: 33-45.

Zicka, M., Orra, A., Schwartz, M.L., Merz, A.J. and Wickner, W.T. (2015) Sec17 can trigger fusion of trans-SNARE paired membranes without Sec18. Proceedings of the National Academy of Sciences USA E2290-E2297.

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S. and Mengiste, T. (2010) Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a Pseudomonas syringae effector. Cell Host and Microbe 7: 290-301.

Zhou, C., Zhang, L., Duan, J., Miki, B. and Wu, K. (2005) HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in Arabidopsis. Plant Cell 17: 1196-1204.

Zhou, Q., Lai, Y., Bacaj, T., Zhao, M., Lyubimov, A.Y., Uervirojnangkoorn, M., Zeldin, O.B., Brewster, A.S., Sauter, N.K., Cohen, A.E. and Soltis, S.M. (2015) Architecture of the synaptotagmin-SNARE machinery for neuronal exocytosis. Nature 525: 62-67.

CHAPTER V

CONCLUSION

The work presented here, along with other recent results in the *G. max -H. glycines* pathosystem demonstrate the involvement of the *PEN1*-containing SNARE and homologs of *PEN2* and *PEN3* in defense (Matsye *et al.* 2012, Pant *et al.* 2014). The results presented here show the involvement of the *G. max* homolog of SYP31 (Gm-SYP38). In *A. thaliana*, SYP31 functions at the *cis* face of the Golgi apparatus. This observation indicates that multiple syntaxins likely function in defense in the *G. max -H. glycines* pathosystem because in contrast to SYP31, the *PEN1 SYP121* gene functions at the plasma membrane.

Framework of defense

In *Nicotiana benthamiana*, its SYP132 homolog functions in the secretion of the defense protein PATHOGENESIS RELATED 1 (PR-1) and other apoplastic proteins (Kalde *et al.* 2007). Furthermore, NbSYP132 has been shown to be involved in basal and salicylic acid (SA)-associated defense (Kalde *et al.* 2007). This observation is in agreement with our results showing the involvement of the SA signaling proteins ENHANCED DISEASE SUSCEPTIBILITY1 (*EDS1*) and NONEXPRESSOR OF PR1 (*NPR1*) and the expression of *PR1* gene during defense in the *G. max -H. glycines* pathosystem (Cao *et al.* 1994; Falk *et al.* 1999; Matsye *et al.* 2012; Pant *et al.* 2014). These observations support diverse roles for plant syntaxins (Sanderfoot *et al.* 2001;

Shirakawa *et al.* 2010). Furthermore, it is likely that other regulatory components of this secretion apparatus function in defense as has been shown for the ADP ribosylation factor (ARF)-GTP exchange factor, GNOM (Nielsen *et al.* 2012). GNOM delivers SYP121 and callose to the plasma membrane during resistance to *B. graminis* f.sp. *hordei* (Nielsen *et al.* 2012). These results are consistent with observations of callose synthase being expressed within syncytia undergoing the process of defense. These experiments have been followed by the examination of other SNARE components including VAMP721-2 showing they function in resistance. Presented here, specificity of the genetically engineered cassettes is demonstrated in the control experiments whereby *G. max*[Williams 82/PI 518671] engineered with the pRAP15-*ccd*B overexpression cassette and *G. max*[Peking/PI 548402] engineered with the pRAP17-*ccd*B RNAi cassette exhibit levels of infection that are comparable to unengineered control plants (Klink *et al.* 2009; Matsye *et al.* 2012; Matthews *et al.* 2013, 2014; Pant *et al.* 2014, 2015a).

In *A. thaliana*, *PEN1* protein functions in the shoot in one pathway leading to resistance by forming a complex on the plasma membrane with VAMP721/VAMP722 and SNAP33 and mediating the secretion of PR1 to the apoplast (Collins *et al.* 2003; Assaad *et al.* 2004; Kalde *et al.* 2007; Kwon *et al.* 2008; Pajonk *et al.* 2008; Kim *et al.* 2014). Those results clearly show the *A. thaliana* SNARE components function in secretion in the shoot during resistance. The *A. thaliana* SNAP33 protein is homologous to the Gm-SNAP-25-3. For comparative purposes, we have included in the analysis presented here an examination of a *G. max* SYB homolog of the *A. thaliana* *VAMP721*/*VAMP722* gene (Gm-VAMP721-2). The results show Gm-VAMP721-2 plays a role in resistance of *G. max* to *H. glycines* parasitism. In *A. thaliana*, VAMP721 co-

immunoprecipitates with PLASMODESMATA-LOCATED PROTEIN 1 (PDLP1) and regulates callose deposition at developing encasements at *Hyaloperonospora arabidopsidis* infection sites during defense (Caillaud *et al.* 2014). In *A. thaliana* VAMP721 protein also plays an important role in the delivery of the resistance (R) protein RESISTANCE TO POWDERY MILDEW8 (RPW8) paralog, RPW8.2, to the extrahaustorial membrane of *Golovinomyces orontii* (Kim *et al.* 2014). The RPW8.2 and VAMP721 proteins function along with *PEN1* and SNAP33 during infection by *G. orontii* to accomplish defense (Kim *et al.* 2014). Therefore, as presented by Kim *et al.* (2014), vesicles deliver R proteins to the site of defense and this fusion of vesicle and plasma membranes is mediated by SNARE. In this regard, the experiments presented here help in explaining our prior observations of the involvement of the membrane-bound *G. max* homolog of the *A. thaliana BOTRYTIS INDUCED KINASE1* (*BIK1*) functioning in resistance (Veronese *et al.* 2006; Pant *et al.* 2014). In *A. thaliana*, *BIK1* is a PM-tethered receptor-like cytoplasmic kinase that becomes activated by phosphorylation stimulated by bacterial flagellin (flg22) peptide (Veronese *et al.* 2006; Lu *et al.* 2010; Zhang *et al.* 2010). Flg22 activates FLAGELLIN SENSING PROTEIN2 (*FLS2*) protein and transphosphorylation of BRASSINOSTEROID ACTIVATED KINASE1 (*BAK1*) which then phosphorylates *BIK1* to induce downstream signaling events (Chinchilla *et al.* 2007). In *A. thaliana*, the *FLS2* pathway activates defense processes including, but not limited to, SA signaling and callose deposition (Boller and Felix 2009). In *A. thaliana*, the *RPW8.2* gene has been identified along with *RPW8.1* functioning to confer broad-spectrum resistance to diverse species of powdery mildew fungi (Xiao *et al.* 2001; Wang *et al.* 2007). The protein products of the *RPW8.1* and *RPW8.2* R genes transduce their

signal through the SA signaling pathway by activating *EDS1* (Falk *et al.* 1999; Xiao *et al.* 2001, 2003). As stated, the *G. max* homologs of *EDS1* and *NPR1* genes have already been shown to function effectively during resistance to *H. glycines* parasitism (Cao *et al.* 1994; Matsye *et al.* 2012; Pant *et al.* 2014, 2015a). The activation of these signaling pathways is consistent with the observation of transcripts for hundreds of genes becoming increased in their relative abundance in syncytia undergoing the process of defense (Klink *et al.* 2007, 2009a, 2010a, b, 2011; Matsye *et al.* 2011). It is also consistent with the involvement of the GATA-type transcription factor *LESION SIMULATING DISEASE1* (*LSD1*), which is associated with SA signaling during defense. Furthermore, in *A. thaliana* the transcription of the callose synthase AtGsl5 is induced by SA in wild type plants (Ostergaard *et al.* 2002). This observation is important from the standpoint that in *A. thaliana*, complete resistance to *G. cichoracearum* and *B. graminis* f. sp. *hordei* is mediated by the callose synthase gene *PMR4* (*GSL5*) although this response was not SA-dependent (Ellinger *et al.* 2013). Therefore, the cellular machinery that facilitates the defense of *A. thaliana* against multiple shoot pathogens also appears to function at least in part in the defense of the *G. max* root under parasitism by *H. glycines*. The experiments presented here have also examined the relative changes in transcript abundance of SNARE, demonstrating that the genes appear to be co-regulated. The co-regulation of different vesicle components observed here in this system has been seen in other organisms, some of them non-plant systems, and functional genomics screens have revealed this co-regulation can be quite extensive (Shanks *et al.* 2012; Liberali *et al.* 2014; Pant *et al.* 2014, 2015a; Zicka *et al.* 2015). However, very little published data is available in plants.

A homolog of *PEN2* functions in defense in the *G. max* root

The involvement of SNARE in the root during the resistance of *G. max* to *H. glycines* parasitism has led to the hypothesis that homologs of *A. thaliana PEN2* gene are involved in the process since it has been demonstrated in *A. thaliana* that the *PEN2* protein functions during an inducible pre-invasion resistance process (Lipka *et al.* 2005; Stein *et al.* 2006; Clay *et al.* 2009). In *A. thaliana*, the *PEN2* genetic pathway functions in the extracellular deposition of callose, working in concert with *PEN3* gene (Collins *et al.* 2003; Lipka *et al.* 2005; Kwon *et al.* 2008; Bednarek *et al.* 2009; Clay *et al.* 2009; Johansson *et al.* 2014). In contrast, a protein functioning very effectively in defense in the legume *L. japonicus* is a *PEN2* homolog belonging to a family of glucosidases known as α-hydroxynitrile glucosidase (Morant *et al.* 2008; Takos *et al.* 2010). Bioinformatics analyses presented here show that the conceptually translated Gm-βg-4 is most closely related to the root-specific *L. japonicus* α-hydroxynitrile glucosidase LjBGD7, belonging to a small family of enzymes involved in the production of cyanogenic α-hydroxynitrile glucosides (Morant *et al.* 2008; Takos *et al.* 2010). While Gm-βg-4 likely functions differently than the *PEN2* gene in *A. thaliana*, overexpression and RNAi experiments show Gm-βg-4 functions in the *G. max* root during defense. In *L. japonicus*, the production of cyanogenic α-hydroxynitrile glucosides involves CYP79, CYP71, UDP-glucosyl transferase, α-hydroxynitrile glucosidase and α-hydroxynitrile lyase with cyanide detoxification occurring through the activity of β-cyanoalanine synthase (Gleadow and Moller 2014). Except for Gm-CYP79D4 where 4 of its 5 paralogs lack the fabrication of corresponding probe sets on the Affymetrix® soybean GeneChip®, each of

these genes in this pathway exhibit measurable levels of transcript syncytia undergoing the process of resistance (Klink *et al.* 2010b, 2011; Matsye *et al.* 2011). The experiments are further supported by overexpression and RNAi of CYP79D4-3, an enzyme which has been shown in other systems to function at the initial conversion of amino acids to oximes (Gleadow and Moller 2014). The production of the α-hydroxynitrile glucosides is accomplished by specific cytochrome P450 enzymes including CYP79D3 and CYP79D4, respectively (Forslund *et al.* 2004; Bjarnholt *et al.* 2008). Morant *et al.* (2008) has demonstrated increased relative levels of expression of LjCYP79D3 in aerial parts of *L. japonicus* plants which is also where LjBGD2 and LjBGD4 are expressed. In contrast, LjCYP79D4 has been shown to have increased relative levels of expression exclusively in the roots where LjBGD7 occurs (Forslund *et al.* 2004). The results presented by Morant *et al.* (2008) have demonstrated the co-expression of α-hydroxynitrile glucoside and their cognate hydrolyzing α-hydroxynitrile glucosidase. We have presented a similar observation here for Gm-βg-4 and CYP79D4-3. Furthermore, in *L. japonicus*, the heterologous expression of a *Manihot esculenta* (cassava) CYP79D2 driven by the cauliflower mosaic virus 35S promoter resulted in the accumulation of cyanogenic α-hydroxynitrile glucosides (Forslund *et al.* 2004). From the presented gene expression experiments of the syncytium, it is likely that other β-glucosidases and biochemical pathways requiring their activity are involved in defense and function in parallel (Klink *et al.* 2007, 2009a, 2010a, b, 2011; Matsye *et al.* 2011).

A *PEN3* homolog functions in defense in the *G. max* root

The involvement of *G. max* homologs of *PEN1* and *PEN2* genes implicate the involvement of a *G. max* homolog of the *A. thaliana PEN3* functioning in resistance to *H. glycines*. Genetic experiments in *A. thaliana* have shown this to be true for race-specific defense processes occurring in the shoot (Johansson *et al.* 2014). One of the functions of *PEN3* in defense is to export toxins to the penetration site to neutralize *B. graminis* f. sp *hordei* (Stein *et al.* 2006; Clay *et al.* 2009 Meyer *et al.* 2009). Therefore, the hypothesis that a *G. max* homolog of the *PEN3* gene functions in defense to *H. glycines* parasitism as presented here has merit. The *G. max* genome has 35 ABC-G transporters and some exhibit detectable levels of transcript abundance in syncytia undergoing the process of resistance (Klink *et al.* 2010b, 2011; Matsye *et al.* 2011). Through overexpression and RNAi experiments, the *G. max PEN3* homolog Gm-ABC-G-26 is shown to function in its root during resistance to *H. glycines* parasitism. The results presented here establish the involvement of full ABC-G type transporters functioning in defense in the root.

The regulation of the regulon

Based on ecological genetic variants and how *PEN1*, *PEN2* and *PEN3* genes function in *A. thaliana*, the cellular apparatus acting in resistance is described as a binary system composed of two parallel pathways called a regulon that converge on defense (Humphry *et al.* 2010; Johansson *et al.* 2014). In this manner, the defense apparatus identified here that acts during *G. max* resistance to *H. glycines* functions like the regulon described for *A. thaliana* and the ecological variants identified in other plant systems over a century ago (Armstrong *et al.* 1913; Ware 1925; reviewed in Hughes, 1991; Humphry *et al.* 2010; Johansson *et al.* 2014). The experiments presented here

provide context to the observation of the functionality of a number of membrane bound and secreted proteins, SA signaling and transcription factors in defense in the *G. max* -*H. glycines* pathosystem. Through these experiments it is shown that it is possible to recapitulate at least part of the defense response found naturally in *G. max* that is utilized as it defends itself from *H. glycines* parasitism.

References

Armstrong, H.E., Armstrong, E.F. and Horton E. (1913) Herbage studies. II. Variation in Lotus corniculatus and Trifolium repens: (cyanophoric plants). Proceedings of the Royal Society of London. Series B 86: 262-269.

Assaad, F.F., Qiu, J.L., Youngs, H., Ehrhardt, D., Zimmerli, L., Kalde, M., Wanner, G., Peck, S.C., Edwards, H., Ramonell, K. and Somerville, C.R. (2004) The *PEN1* syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. Molecular Biology of the Cell 15: 5118-5129.

Bednarek, P., Piślewska-Bednarek, M., Svatoš, A., Schneider, B., Doubský, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A. and Molina, A. (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 323: 101-106.

Bjarnholt, N., Rook, F., Motawia, M.S., Cornett, C., Jørgensen, C., Olsen, C.E., Jaroszewski, J.W., Bak, S. and Møller, B.L. (2008) Diversification of an ancient theme: hydroxynitrile glucosides. Phytochemistry 69: 1507-1516.

Boller, T. and Felix, G. (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annual Review in Plant Biology 60: 379-406.

Caillaud, M.C., Wirthmueller, L., Sklenar, J., Findlay, K., Piquerez, S.J., Jones, A.M., Robatzek, S., Jones, J.D. and Faulkner, C. (2014) The plasmodesmal protein PDLP1 localises to haustoria-associated membranes during downy mildew infection and regulates callose deposition. PLoS Pathogogy 10:e1004496.

Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X. (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6: 1583-1592.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D., Felix, G. and Boller, T. (2007) A flagellin-induced complex of the receptor *FLS2* and *BAK1* initiates plant defence. Nature 448: 497-500.

Clay, N.K., Adio, A.M., Denoux, C., Jander, G. and Ausubel, F.M. (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response Science 323: 95-101.

Collins, N.C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J.L., Hückelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S.C. and Schulze-Lefert, P. (2003) SNARE-protein-mediated disease resistance at the plant cell wall. Nature 425: 973-977.

Ellinger, D., Naumann, M., Falter, C., Zwikowics, C., Jamrow, T., Manisseri, M., *et al.* (2013) Elevated early callose deposition results in complete penetration resistance to powdery mildew in Arabidopsis. Plant Physiology 161: 1433-1444.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J. and Parker, J.E. (1999) *EDS1*, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proceedings of the National Academy of Sciences USA 96: 3292-3297.

Forslund, K., Morant, M., Jørgensen, B., Olsen, C.E., Asamizu, E., Sato, S., Tabata, S. and Bak, S., (2004) Biosynthesis of the nitrile glucosides rhodiocyanoside A and D and the cyanogenic glucosides lotaustralin and linamarin in Lotus japonicus. Plant Physiology 135: 71-84.

Gleadow, R.M. and Møller, B.L. (2014) Cyanogenic glycosides: synthesis, physiology, and phenotypic plasticity. Annual Review in Plant Biology 65: 155-85.

Hughes, M.A. (1991) The cyanogenic polymorphism in Trifolium repens L. (white clover). Heredity 66: 105-115.

Humphry, M., Bednarek, P., Kemmerling, B., Koh, S., Stein, M., Göbel, U., Stüber, K., Piślewska-Bednarek, M., Loraine, A., Schulze-Lefert, P. and Somerville, S. (2010) A regulon conserved in monocot and dicot plants defines a functional module in antifungal plant immunity. Proceedings of the National Academy of Sciences 107: 21896-21901.

Johansson, O.N., Fantozzi, E., Fahlberg, P., Nilsson, A.K., Buhot, N., Tör, M. and Andersson, M.X. (2014) Role of the penetration-resistance genes *PEN1*, *PEN2* and *PEN3* in the hypersensitive response and race-specific resistance in *Arabidopsis thaliana*. Plant Journal 79: 466-476.

Kalde, M., Nühse, T.S., Findlay, K. and Peck, S.C. (2007) The syntaxin SYP132 contributes to plant resistance against bacteria and secretion of pathogenesis-related protein 1. Proceedings of the National Academy of Sciences USA 104: 11850-11855.

Kim, H., O'Connell, R., Maekawa-Yoshikawa, M., Uemura, T., Neumann, U. and Schulze-Lefert, P. (2014) The powdery mildew resistance protein RPW8.2 is carried on VAMP721/722 vesicles to the extrahaustorial membrane of haustorial complexes. Plant Journal 79: 835-847.

Klink, V.P., Overall, C.C., Alkharouf, N., MacDonald, M.H. and Matthews, B.F. (2007) Laser capture microdissection (LCM) and comparative microarray expression analysis of syncytial cells isolated from incompatible and compatible soybean roots infected by soybean cyst nematode (Heterodera glycines). Planta 226: 1389-1409.

Klink, V.P., Hosseini, P., Matsye, P.D., Alkharouf, N.W. and Matthews, B.F. (2009a) A gene expression analysis of syncytia laser microdissected from the roots of the *Glycine max* (soybean) genotype PI 548402 (Peking) undergoing a resistant reaction after infection by *Heterodera glycines* (soybean cyst nematode). Plant Molecular Biology 71: 525-567.

Klink, V.P., Overall, C.C., Alkharouf, N.W., MacDonald, M.H. and Matthews, B.F. (2010b) Microarray detection calls as a means to compare transcripts expressed within syncytial cells isolated from incompatible and compatible soybean (Glycine max) roots infected by the soybean cyst nematode (Heterodera glycines). Journal of Biomedicine and Biotechnology 1-30.

Klink, V.P., Hosseini, P., Matsye, P.D., Alkharouf, N.W. and Matthews, B.F. (2011) Differences in gene expression amplitude overlie a conserved transcriptomic program occurring between the rapid and potent localized resistant reaction at the syncytium of the *Glycine max* genotype Peking (PI 548402) as compared to the prolonged and potent resistant reaction of PI 88788. Plant Molecular Biology 75: 141-165.

Kwon, C., Neu, C., Pajonk, S., Yun, H.S., Lipka, U., Humphry, M., Bau, S., Straus, M., Kwaaitaal, M., Rampelt, H. and El Kasmi, F. (2008) Co-option of a default secretory pathway for plant immune responses. Nature 451: 835-840.

Liberali, P., Snijder, B., Pelkmans, L. (2014) A hierarchical map of regulatory genetic interactions in membrane trafficking. Cell 157: 1473-1487.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D. and Llorente, F. (2005) Pre-and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science 310: 1180-1183.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L. and He, P. (2010) A receptor-like cytoplasmic kinase, *BIK1*, associates with a flagellin receptor complex to initiate plant innate immunity. Proceedings of the National Academy of Sciences USA 107: 496-501.

Matsye, P.D., Kumar, R., Hosseini, P., Jones, C.M., Tremblay, A., Alkharouf, N.W., Matthews, B.F. and Klink, V.P. (2011) Mapping cell fate decisions that occur during soybean defense responses. Plant Molecular Biology 77: 513-528.

Matsye, P.D., Lawrence, G.W., Youssef, R.M., Kim, K.H., Lawrence, K.S., Matthews, B.F. and Klink, V.P. (2012) The expression of a naturally occurring, truncated allele of an α-SNAP gene suppresses plant parasitic nematode infection. Plant Molecular Biology 80: 131-155.

Matthews, B.F., Beard, H., MacDonald, M.H., Kabir, S., Youssef, R.M., Hosseini, P. and Brewer, E. (2013) Engineered resistance and hypersusceptibility through functional metabolic studies of 100 genes in soybean to its major pathogen, the soybean cyst nematode. Planta 237: 1337-1357.

Matthews, B.F., Beard, H., Brewer, E., Kabir, S., MacDonald, M.H. and Youssef, R.M. (2014) Arabidopsis genes, At*NPR1*, AtTGA2 and AtPR-5, confer partial resistance to soybean cyst nematode (*Heterodera glycines*) when overexpressed in transgenic soybean roots. BMC Plant Biology 14: 96.

Meyer, D., Pajonk, S., Micali, C., O'Connell, R. and Schulze-Lefert P. (2009) Extracellular transport and integration of plant secretory proteins into pathogen-induced cell wall compartments. Plant Journal 57: 986-999.

Morant, M., Bak, S., Møller, B.L. and Werck-Reichhart, D. (2003) Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation. Current Opinion in Biotechnology 14:151-162.

Nielsen, M.E., Feechan, A., Böhlenius, H., Ueda, T. and Thordal-Christensen H. (2012) Arabidopsis ARF-GTP exchange factor, GNOM, mediates transport required for innate immunity and focal accumulation of syntaxin *PEN1*. Proceedings of the National Academy of Sciences USA 109: 11443-11448.

Ostergaard, L., Petersen, M., Mattsson, O. and Mundy, J. (2002) An Arabidopsis callose synthase. Plant Molecular Biology 49: 559-566.

Pant, S.R., Matsye, P.D., McNeece, B.T., Sharma, K., Krishnavajhala, A., Lawrence, G.W. and Klink, V.P. (2014) Syntaxin 31 functions in Glycine max resistance to the plant parasitic nematode Heterodera glycines. Plant Molecular Biology 85: 107-121.

Pant, S.R., Krishnavajhala, A., McNeece, B.T., Lawrence, G.W. and Klink, V.P. (2015) The syntaxin 31-induced gene, LESION SIMULATING DISEASE1 (*LSD1*), functions in *Glycine max* defense to the root parasite *Heterodera glycines*. Plant Signaling & Behaviour 10: 1 e977737.

Sanderfoot, A.A., Kovaleva, V., Bassham, D.C. and Raikhel, N.V. (2001) Interactions between Syntaxins identify at least five SNARE complexes within the Golgi/prevacuolar system of the Arabidopsis cell. Molecular Biology of the Cell 12: 3733-3743.

Shanks, S.G., Carpp, LN., Struthers, M.S., McCann, R.K. and Bryant, N.J. (2012) The Sec1/Munc18 Protein Vps45 Regulates Cellular Levels of Its SNARE Binding Partners Tlg2 and Snc2 in Saccharomyces cerevisiae. PLoS ONE 7: e49628. doi:10.1371/journal.pone.0049628.

Shirakawa, M., Ueda, H., Shimada, T., Koumoto, Y., Shimada, T.L., Kondo, M., Takahashi, T., Okuyama, Y., Nishimura, M. and Hara-Nishimura, I. (2010) Arabidopsis Qa-SNARE SYP2 proteins localized to different subcellular regions function redundantly in vacuolar protein sorting and plant development. Plant Journal 64: 924-635.

Stein, M., Dittgen, J., Sánchez-Rodríguez, C., Hou, B.H., Molina, A., Schulze-Lefert, P., Lipka, V. and Somerville, S. (2006) Arabidopsis *PEN3*/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18: 731-746.

Takos, A.M., Knudsen, C., Lai, D., Kannangara, R., Mikkelsen, L., Motawia, M.S., Olsen, C.E., Sato, S., Tabata, S., Jørgensen, K. and Møller, B.L. (2011) Genomic clustering of cyanogenic glucoside biosynthetic genes aids their identification in Lotus japonicus and suggests the repeated evolution of this chemical defence pathway. Plant Journal 68: 273-286.

Veronese, P., Nakagami, H., Bluhm, B., AbuQamar, S., Chen, X., Salmeron, J., Dietrich, R.A., Hirt, H. and Mengiste, T. (2006) The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in Arabidopsis resistance to necrotrophic and biotrophic pathogens. Plant Cell 18: 257-273.

Wang, W., Devoto, A., Turner, J.G. and Xiao S. (2007) Expression of the membrane-associated resistance protein RPW8 enhances basal defense against biotrophic pathogens. Molecular Plant Microbe Interactions 20: 966-976.

Ware, W.M. (1925) Experiments and observations on forms and strains of Trifolium repens. Journal of Agricural Science 15: 47-67.

Xiao, S., Ellwood, S., Calis, O., Patrick, E., Li, T., Coleman, M. and Turner, J.G. (2001) Broad-spectrum mildew resistance in Arabidopsis thaliana mediated by RPW8. Science 291: 118-120.

Xiao, S., Brown, S., Patrick, E., Brearley, C. and Turner, J.G. (2003) Enhanced transcription of the Arabidopsis disease resistance genes RPW8.1 and RPW8.2 via a salicylic acid-dependent amplification circuit is required for hypersensitive cell death. Plant Cell 15: 33-45.

Zicka, M., Orra, A., Schwartz, M.L., Merz, A.J. and Wickner, W.T. (2015) Sec17 can trigger fusion of trans-SNARE paired membranes without Sec18. Proceedings of the National Academy of Sciences USA E2290-E2297.

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S. and Mengiste, T. (2010) Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a Pseudomonas syringae effector. Cell Host and Microbe 7: 290-301

APPENDIX A.

SUPPLEMENTARY TABLES AND FIGURES OF CHAPTER II

Table A.1 PCR Primer information

Gene name	Type	Primer (5'-3')
Xyloglucan endotransglycosylase	overexpression	F-CACCATGGCTTCTACCTTCTCTCGAAG
	overexpression	R-CTAGGAGTGTTTGCATTCGAGTG
	qPCR	F-GGGAGATGGTCGTGCTAAAATA
	qPCR	R-TATTCGTTTTTGGATTGGAAGC
	qPCR	P-CGAAAATCTTCTCACTCTCTCCCTTGACA
Syntaxin	overexpression	F-CACCATGGCTTCCTCATACCGTGAC
	overexpression	R-TTAGGCGACAAAGAATATGAAG
	RNAi	F-CACCATGGCTTCCTCATACCGTG
	RNAi	R-GGTGGAATGCACAATCGTATC
	qPCR	F-ATGGCTTCCTCATACCGTGAC
	qPCR	R-CTCGAATACGACTCGCCATG
	qPCR	P-CGGTTATTATTGGAGACTCTGAAGAAGATCG
NONEXPRESSOR OF PR1	overexpression	F-CACCATGGCTTATTCAGCCGAACCC
	overexpression	R-TTACACTTTCCTAGCCTTGTAATGTACA
	qPCR	F-TGATGCTGACCTTGTTGTCG
	qPCR	R-ATGACCCCTTCTCCCTCTTG
	qPCR	P-CATCGATGTATTCTGGCCTCTAGGAGTAAG
ENHANCED DISEASE SUSCEPTIBILITY 1	overexpression	F-CACCATGACTCAAGTGATGAGAGGAG
	overexpression	R-TCACTCTCTAATAAGAGTTTTAATGC
	qPCR	F-TGATGAGAGGAGAGGTGATTGAG
	qPCR	R-TCTTGAGGGTCGTTTCTGTTGA
	qPCR	P-CACAAGTCCCCAGACAAGCCTTACC
BOTRYTIS INDUCED KINASE 1	overexpression	F-CACCATGGGGTGCTGCTTAAGTGC
	overexpression	R-TCACTTCCTTGTTGTTTCATGTTGTC
	RNAi	F-CACCGCCAGGATCAAAGCTGAGAG
	RNAi	R-TTCACTGTGTCCCTGAAGAC
	qPCR	F-ACTCTTGCCATTCAATGCCTATC
	qPCR	R-ATGTTGTCTAGGGCCACTCCTTC
	qPCR	P-GATGGATGAAGTGGTGAGAGCATTGG
Basic pathogenesis-related protein 1	qPCR	F-CTCACCAACAGACTATGTTAATGC
	qPCR	R-CGAGTTTGCAGTCACCTTTG
	qPCR	P-CCAAATATAGTTTGGGATAACGCAGTCG
β-1,3-glucanase 1	qPCR	F-ATGGCTAAGTATCATTCAAGTGG
	qPCR	R-GTGCCTGTATAAGTGATTAGAAGG
	qPCR	P-CTTCCATGACTGCTATAGCCTTCCTG
Basic chitinase	qPCR	F-ATGAAAAACATGAAATTGTGTTCG
	qPCR	R-CTGCAACATAATCTATTTGGGC

Table A.1 (Continued)

	qPCR	P-GCAGAACAATGTGGCACACAAGC
Pathogenesis-related thaumatin superfamily protein	qPCR	F-ACTTCTACGACGTGAGCCTG
	qPCR	R-GTAGCTGCATTTTCCGGAT
	qPCR	P-CAACCTACCCATCTCCATCACCC
Amino acid transporter	qPCR	F-ATGAAGGTTCTCGGCGTAGTTC
	qPCR	R-AACCGCATCAGGAAGTCCAC
	qPCR	P-ATGATTCTCGTGGTGGCCGTG
α-SNAP	qPCR	F-GCTGTAACCAATGCATTAGAAC
	qPCR	R-CAATGTCCAAAACTAGTGACCTAACG
	qPCR	P-GATCCAACATTTTCAGGAACACGTG
Wound inducible protein	qPCR	F-GATTCGTTCCGCAGTCCATC
	qPCR	R-GTGAGGGCGGTGTTGAAGTA
	qPCR	P-CAACATTGCCTGGGTCCACGC
Serine hydroxynethyltransferase 4	qPCR	F-ATCTCCGCCACCTCCATTTACT
	qPCR	R-GGCCTGAAGTCTAGGGCTTTTT
	qPCR	P-GTAAACTCCACCACCGGCTACATCG
	qPCR	F-ATGCAGAACGAGGAAGGACAG
	qPCR	R-GAAGCATGGTCCTTAGCG
	qPCR	P-CCTAGGAAGTGCTCTGCCACAAAC
Root inducing plasmid gene sequence	PCR	F-TCAGCCTCCCCGCCGGATG
	PCR	R-ATGCAAAAGACAGGATTGATCGCA
Enhanced green fluorescent protein	PCR	F-GAATTTGTTTCGTGAACTATTAGTTGCGG
	PCR	R-GCATGCCTGCAGGTCACTGGATTTTG
	PCR	F-CCATGCTGACGCTGATTACCTC
	PCR	R-CTACCAGGCTTGTTAACGGGTATGG

F: Forward primer, R: Reverse primer, P: Probe

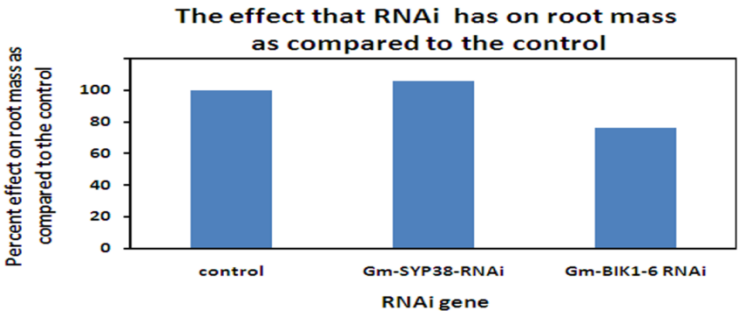


Figure A.1 Effect of G. max SYP38 and BIK1-6 RNAi on root growth

For all experiments, * = statistically significant $p < 0.05$. Control, roots transformed with the pRAP17 RNAi vector. SYP38-RNAi (n = 19); SYP38-RNAi roots, p = 0.499081; BIK1-6-RNAi (n = 19); BIK1-RNAi roots, p = 0.354595. Note: RNAi had no statistically significant effect.

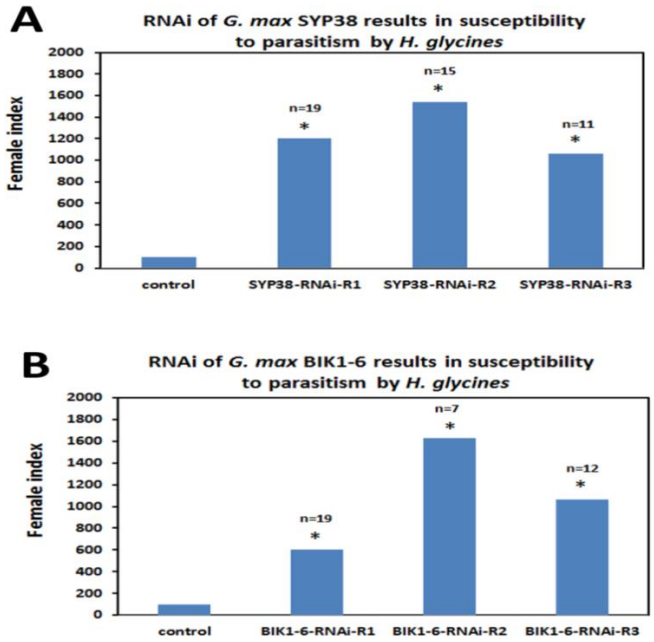


Figure A.2 RNAi of G. max SYP38 and BIK1-6 results in susceptibility to parasitism by H. glycines

G. max plants genetically engineered for RNAi of Gm-BIK1 and SYP38, and infected with H. glycines, have an increased capability, shown as fold change, for parasitism.
For all experiments, * = statistically significant $p < 0.05$. SYP38-RNAi-R1 (n = 19); SYP38-RNAi-R1, FI = 1,200.00; p-value = 0.009937*. SYP38-RNAi-R2 (n = 15); SYP38-RNAi-R2, FI = 1,538.00; p-value = 0.00197416*. SYP38-RNAi-R3 (n = 11); SYP38-RNAi-R3, FI = 1063.64; p-value = 0.0298544*. BIK1-6-RNAi-R1 (n = 19); BIK1-RNAi-R1, FI = 600.00; p-value = 0.0174306*. BIK1-6-RNAi-R2 (n = 7); BIK1-RNAi-R2, FI = 1628.58; p-value = 0.0175829*. BIK1-6-RNAi-R3 (n = 12); BIK1-RNAi-R3, FI = 1063.64; p-value = 0.0348612*, R1: Replicate1, R2: Replicate 2, R3: Replicate 3

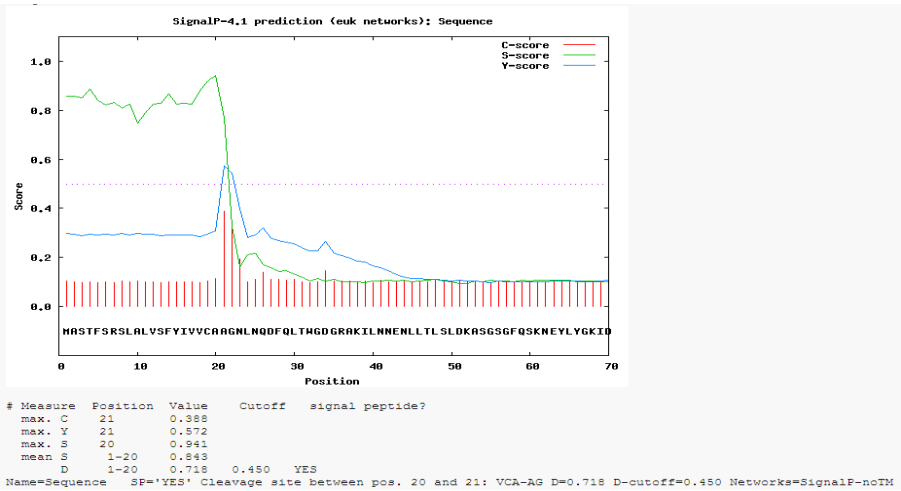
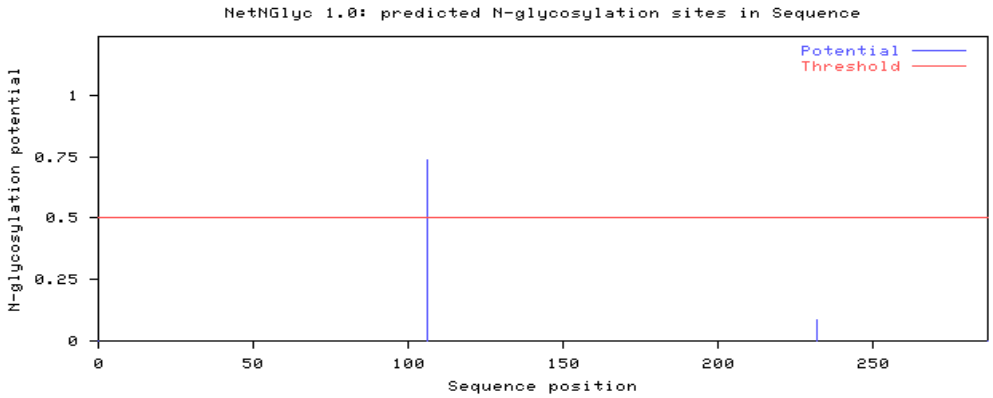


Figure A.3 Signal peptide prediction for GmXTH43

Signal peptide was predicted using SignalP-4.1 (http://www.cbs.dtu.dk/services/SignalP/) on default (Petersen et al. 2011).



(Threshold=0.5)

SeqName	Position	Potential	Jury agreement	N-Glyc result
-----Sequence	106	NLSG 0.7378	(9/9)	++

Figure A.4 N-glycosylation prediction for Gm-XTH43

N-glycosylation prediction using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) on default. N-glycosylation was predicted for Gm-XTH43.

Gm-BIK1-6: Glyma14g07460.1
MGCCLSARIKAESPPRNGLSSKDGNKEEDGLSSKVSTPSDPPTPRTEGEILKSSNMKSFNFSE
LKTATRNFRPDSVVGEGGFGCVFKGWIDEQTLAPVRPGTGMVIAVKRLNQEGLQGHSEWL
TEINYLGQLRHPNLVKLIGYCLEDDQRLLVYEFLTKGSLDNHLFRRASYFQPLSWNFRMKV
ALDAAKGLAYLHSDEAKVIYRDFKASNILLDSNYNAKLSDFGLAKDGPAGDKSHVSTRVM
GTYGYAAPEYMATGHLTKKSDVYSFGVVLLEIMSGKRALDSNRPSGEHNLIEWAKPYLSN
KRRIFQVMDARIEGQYTLRESMKVANLAIQCLSVEPRFRPKMDEVVRALEELQDSEDRAGG
VGSSRDQTARRSGHSSSSSGPRQHRGRQHETTRK

Gm-BIK1-1: Glyma01g24150.1
MGACWSSRIKAVSPSNTGFTSRSVSRDGHDIQSSSRNSSASIPMTPRSEGEILQFSNLKSYSYN
ELKMATKNFCPDSVLGEGGFGSVFKGWIDEHSLAVTRPGTGMVIAVKKLNQDSFQGHKEW
LAEINYLGQLQNPNLVKLIGYCLEDQHRLLVYEYMPKGSVENHLFRRGSHFQQLSWTLRLK
ISLGAARGLAFLHSTETKVIYRDFKTSNILLDTNYNAKLSDFGLARDGPTGDKSHVSTRVMG
THGYAAPEYLATGHLTAKSDVYSFGVVLLEMLSGRRAIDKNRPSGEQCLVEWAKPYLSNK
RRVFRVMDSRLEGQYSLTQAQRAATLAFQCLSVEPKYRPNMDEVVKALEQLRESNDKVKN
GDHKKCRVSGSGLGHPNGLPASTSKGSIDAAKKFNYPRPSASLLYS

Gm-BIK1-2: Glyma02g41490.1
MGCCLSARIKAESPPRNGLSSKDGNKEEDGLSSKASTPSVPPTPRTEGEILKSSNMKSFNFSE
LKTATRNFRPDSVVGEGGFGCVFKGWIDEQTLAPVRPGTGMVIAVKRLNQEGLQGHSEWL
TEINYLGQLRHPNLVKLIGYCLEDDHRLLVYEFLTKGSLDNHLFRRASYFQPLSWNIRMKV
ALDAAKGLAYLHSDEAKVIYRDFKASNILLDSNYNAKLSDFGLAKDGPAGDKSHVSTRVM
GTYGYAAPEYMATGHLTKKSDVYSFGVVLLEIMSGKRALDSNRPSGEHNLIEWAKPYLSSK
RRIFQVMDARIEGQYMLREAMKVATLAIQCLSVEPRFRPKMDEVVRALEELQDSDDRVGG
VGSSRDQTTRRSGPRQHRGRQHETTRK

Gm-BIK1-3: Glyma03g09870.1
MGACWSSRIKSVSPSNTGFTSRSVSRDGYDIHSNSRNSSASIPMTPRSEGEILQSSNLKSYSYN
ELKMATKNFCPDSVLGEGGFGSVFKGWIDEHSLAVTRAGTGMVVAVKKLNQESFQGHKE
WLAEINYLGQLQHPNLVKLIGYCLEDQHRLLVYEYMPKGSVENHLFRRGSHFQQLSWTLR
LKISLGAARGLAFLHSTETKVIYRDFKTSNILLDTNYNAKLSDFGLARDGPTGDKSHVSTRV
MGTHGYAAPEYLATGHLTAKSDVYSFGVVLLEMLSGRRAIDKNRPSGEQCLVEWAKPYLS
NKRRVFRVMDSRLEGQYSLTQAQRAATLAFQCLAVEPKYRPNMDEVVRALEQLRESNND
QVKNGDHKKRSRVSGSGLGHHNGLPASTSKGSIDAAKKFNYPRPSASLLY

Gm-BIK1-4: Glyma07g15890.1
MGACWSNRIKSVSPSNTGITSRSVSRSGHDVSSNSRSSSASISVASRSEGEILQSSNLKSFSYN
ELRAATRNFRPDSVLGEGGFGSVFKGWIDEHSLAATKPGIGMIVAVKRLNQDGFQGHREW
LAEINYLGKLQHPNLVRLIGYCFEDEHRLLVYEFMPKGSMENHLFRRGSYFQPFSWSLRMKI
ALGAAKGLAFLHSTEPKVIYRDFKTSNILLDTNYSAKLSDFGLARDGPTGDKSHVSTRVMG
THGYAAPEYLATGHLTTKSDVYSFGVVLLEMISGRRAIDKNQPTGEHNLVDWAKPYLSNK
RRVFRVIDPRLEGQYLQSRAQAAAALAIQCLSIEARCRPNMDEVVKALEQLQESKNMQRKG
ADHKQHHVRNSGPGRSNGGNGGSDVPRKASAYPRPSASLLRG

Figure A.5 Gm-BIK1 paralogs having the MGXXXS/T N-myristoylation consensus sequence (highlighted in cyan).

Accessions identified from http://phytozome.net/

Gm-BIK1-5: Glyma13g41130.1
MGVCLSAQIKAESPFNTVFNSKYVSTDGNDLGSTNDKVSANSVPQTPRSEGEILQSSNLKSF
TLSELKTATRNFRPDSVLGEGGFGSVFKGWIDENSLTATKPGTGIVIAVKRLNQDGIQGHRE
WLAEVNYLGQLSHPHLVRLIGFCLEDEHRLLVYEFMPRGSLENHLFRRGSYFQPLSWSLRL
KVALDAAKGLAFLHSAEAKVIYRDFKTSNVLLDSKYNAKLSDFGLAKDGPTGDKSHVSTR
VMGTYGYAAPEYLATGHLTAKSDVYSFGVVLLEMLSGKRAVDKNRPSGQHNLVEWAKPF
MANKRKIFRVLDTRLQGQYSTDDAYKLATLALRCLSIESKFRPNMDQVVTTLEQLQLSNVN
GGPRVRRRSADVNRGHQNPSSVNGSRVRRRSADDISRLETPNAYPRPSASPLYT

Gm-BIK1-7: Glyma15g04280.1
MGVCLSAQIKAESPYNTGFNSKYVSTDGNDFGSTNDKVSANSIPQTPRSEGEILRSSNLKSFP
LSELKTATRNFRPDSVLGEGWIDENSLTATKPGTGIVIAVKRLNQDGIQGHREWLAEVNYL
GQLSHPHLVRLIGFCLEDEHRLLVYEFMPRGSLENHLFRILTWEVCITLAICIVVTGGSYFQPL
SWSLRLKVALDAAKGLAFLHSAEAKVIYRDFKTSNILLDSKYNAKLSDFGLAKDGPTGDKS
HVSTRVMGTYGYAAPEYLATGHLTAKSDVYSFGVVLLEMLSGKRAVDKNRPSGQHNLVE
WAKPYLANKRKIFRVLDTRLEGQYSTDDACKLATLALRCLSIESKFRPNMDEVVTTLEQLQ
VPNVNGGHQNGSRVRRRSADVNRGYQNPSVNGSRVRRRSADDISPMETPTAYPRPSASPLY
T

Gm-BIK1-8: Glyma18g04340.1
MGCFFSVPSKIKAESPPRNGLNSKDGSKEENDLSCLSSKVSSSAMLLTPQSEDEILQASNLKN
FTFNELRTATRNFRPDSMVGEGGFGCVFKGWIDEHTLAPTKPGTGMVIAVKRLNQESNQGH
IEWLAEINYLGQLSHPNLVKLIGYSLEDDHRILVYEFVAKGSLDNHLFRRGSYFQPLSWNIR
MKVALDAAKGLAFLHSDEVDVIYRDFKTSNILLDSDYNAKLSDFGLAKNGPEGDKSHVSTR
VMGTYGYAAPEYIATGHLTKKSDIYSFGVVLLELMSGKRALDDNRPSGEHSLVEWAKPLLT
NKHKISQVMDARIEGQYSKREAKRIAHLAIQCLSTEQKLRPNINEVVRLLEHLHDSKDTSSSS
NATPNPSLSPSPLRS

Gm-BIK1-9: Glyma18g39820.1
MGACWSNRIKAVSPSNTGITSRSVSRSGHDISSNSRSSSASIPVTSRSEGEILQSSNLKSFSYHE
LRAATRNFRPDSVLGEGGFGSVFKGWIDEHSLAATKPGIGKIVAVKKLNQDGLQGHREWL
AEINYLGQLQHPNLVKLIGYCFEDEHRLLVYEFMPKGSMENHLFRGGSYFQPFSWSLRMKI
ALGAAKGLAFLHSTEHKVIYRDFKTSNILLDTNYNAKLSDFGLARDGPTGDKSHVSTRVMG
TRGYAAPEYLATGHLTTKSDVYSFGVVLLEMISGRRAIDKNQPTGEHNLVEWAKPYLSNKR
RVFRVMDPRLEGQYSQNRAQAAAALAMQCFSVEPKCRPNMDEVVKALEELQESKNMQRK
GADHKQHHVRNSGPGRTNGGDGGSDAPRKASAYPRPSASLLRG

Figure A.5 (Continued)

APPENDIX B

SUPPLEMENTARY TABLES AND FIGURES OF CHAPTER III

Table B.1 PCR and qPCR Primer information

Gene name	Type	Primer (5'-3')
LESION SIMULATING DISEASE1 (Gm-LSD1-2)	overexpression	F-CACCATGCAGAGCCAAGTTGTGTGC
	overexpression	R-TTATTTCTTATCTGTTGTAACCCCAAC
	qPCR	F-ATGCAGAGCCAAGTTGTGTG
	qPCR	R-TACAACCTCCACAATAAAGTTGAGAC
	qPCR	P-AATGTCTGTTGTGCATTGTGCAACAC
ENHANCED DISEASE SUSCEPTIBILITY 1 (Gm-EDS1-2)	qPCR	F-TGATGAGAGGAGAGGTGATTGAG
	qPCR	R-TCTTGAGGGTCGTTTCTGTTGA
	qPCR	P-CACAAGTCCCCAGACAAGCCTTACC
NONEXPRESSOR OF PR1 (Gm-NPR1-2)	qPCR	F-TGATGCTGACCTTGTTGTCG
	qPCR	R-ATGACCCCTTCTCCCTCTTG
	qPCR	P-CATCGATGTATTCTGGCCTCTAGGAGTAAG
Syntaxin 31 (Gm-SYP38)	qPCR	F-ATGGCTTCCTCATACCGTGAC
	qPCR	R-CTCGAATACGACTCGCCATG
	qPCR	P-CGGTTATTATTGGAGACTCTGAAGAAGATCG
Gm-α-SNAP	qPCR	F-GCTGTAACCAATGCATTAGAAC
	qPCR	R-CAATGTCCAAAACTAGTGACCTAACG
	qPCR	P-GATCCAACATTTTCAGGAACACGTG
BOTRYTIS INDUCED KINASE 1 (Gm-BIK1-6)	qPCR	F-ACTCTTGCCATTCAATGCCTATC
	qPCR	R-ATGTTGTCTAGGGCCACTCCTTC
	qPCR	P-GATGGATGAAGTGGTGAGAGCATTGG
Xyloglucan endotransglycosylase (Gm-XTH43)	qPCR	F-GGGAGATGGTCGTGCTAAAATA
	qPCR	R-TATTCGTTTTTGGATTGGAAGC
	qPCR	P-CGAAAATCTTCTCACTCTCTCCCTTGACA
Basic pathogenesis-related protein 1 (PR1)	qPCR	F-CTCACCAACAGACTATGTTAATGC
	qPCR	R-CGAGTTTGCAGTCACCTTTG
	qPCR	P-CCAAATATAGTTTGGGATAACGCAGTCG
β-1,3-glucanase 1 (PR2)	qPCR	F-ATGGCTAAGTATCATTCAAGTGG
	qPCR	R-GTGCCTGTATAAGTGATTAGAAGG
	qPCR	P-CTTCCATGACTGCTATAGCCTTCCTG
Basic chitinase (PR3)	qPCR	F-ATGAAAAACATGAAATTGTGTTCG
	qPCR	R-CTGCAACATAATCTATTTGGGC
	qPCR	P-GCAGAACAATGTGGCACACAAGC
Pathogenesis-related thaumatin superfamily protein (PR5)	qPCR	F-ACTTCTACGACGTGAGCCTG
	qPCR	R-GTAGCTGCATTTTCCGGAT
	qPCR	P-CAACCTACCCATCTCCATCACCC

F: Forward primer, R: Reverse primer, P: Probe

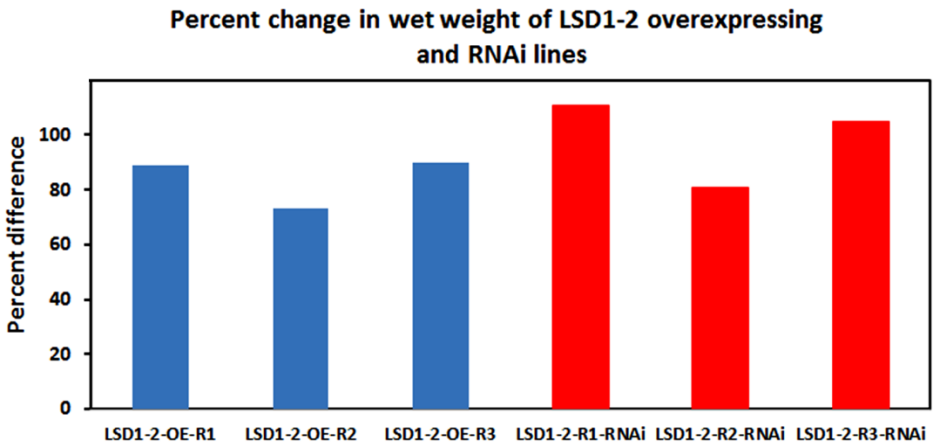


Figure B.1 Percent change in wet weight of LSD1-2 overexpressing and RNAi lines

Blue histograms are the comparisons of the LSD1-2 overexpressing lines. Red histograms are the comparisons of the LSD1-2 RNAi lines. A total of 12 transgenic roots were included in each of the replicate. There are no statistically significant changes between the LSD1-2 overexpressing or RNAi lines as compared to the controls ($p > 0.05$).