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A Functional Developmental Genomics Analysis of RIN4 and Exocyst Genes as They Relate to Glycine Max Defense to the Plant Parasitic Nematode Heterodera Glycines Infection

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A functional developmental genomics analysis of RIN4 and exocyst genes as they relate to *Glycine max* defense to the plant parasitic nematode *Heterodera glycines* infection

By

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A functional developmental genomics analysis of RIN4 and exocyst genes as they relate to *Glycine max* defense to the plant parasitic nematode *Heterodera glycines* infection

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The initial interaction of vesicle and the target membrane prior to their fusion is called vesicle tethering, a process mediated by an octameric protein complex called the exocyst. The exocyst connects vesicles and binds them to phosphatidylinositol 4, 5-biphosphate (PI (4,5) P2), located on the plasma membrane. The exocyst complex is located at the target site, helping to prepare the soluble N-ethylmaleimide-sensitive fusion protein attachment protein (SNAP) REceptor (SNARE) for docking and subsequent release of vesicular contents after fusion. The importance of the exocyst in cellular processes is inevitable since it performs central roles in exocytosis thereby inducing SNARE-mediated membrane fusion. The study presented here is concentrated on the role of exocyst genes during the defense response in *Glycine max* (soybean) against the plant-parasitic nematode *Heterodera glycines* known as the soybean cyst nematode (SCN). Using developmental genomics procedures, *G. max* root cells that have been induced by *H. glycines* through their pathogenic activities to develop into nurse cells known as a syncytium have been isolated by laser capture microdissection (LCM). RNA isolated

from these cells undergoing resistant reactions in two different *G. max* genotypes have been used in gene expression profiling experiments that have led to the identification of the genes employed in this analysis. The results demonstrate the involvement of exocyst components in the defense process that *G. max* has toward *H. glycines*. Related studies also show the involvement of RPM1-INTERACTING PROTEIN 4 (RIN4) functioning in this defense process.

DEDICATION

I would like to dedicate this thesis to my Timilsina Family, My mom Ishwori Timilsina, my wife Tulasa Pandey and my siblings Mina Sharma, Laxman Sharma, Madhu Sudan Sharma and Prashamsha Sharma.

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CHAPTER I

INTRODUCTION

The plant immune system is very complex as it uses its exterior and interior defense mechanisms to defend itself against its pathogens (Chisholm et al. 2006). Plants apply their resistance responses in diverse ways, including systemic acquired resistance (SAR), gene for gene resistance pathways against avirulent pathogens and activation of defense genes against virulent pathogens (Glazebrook et al. 1997; Matsye et al. 2012; Pant et al. 2014). Physical barriers in the plant block entry of pathogens whereas chemical and enzymatic responses limit growth and spread (Glazebrook et al. 1997; Pant et al. 2014). An extracellular attack on the plant cell wall and pathogen entry to the cell membrane boundary can be detected by their extracellular surface receptors that detect pathogen activated molecular patterns (PAMPs), (**Figure1.1)** triggering immunity in plants (Chisholm et al. 2006; Jones and Dangl, 2006). The detection of the pathogen attack induces PAMP triggered immunity (PTI) confining the pathogen to that limited area, thereby blocking their food source, growth and multiplication (Chisholm et al. 2006). However, pathogens have developed various ways to suppress PTI by paralyzing cell receptors and resistance mechanisms by using their effector proteins (**Figure1.1**) (Chisholm et al. 2006). Pathogens inject various enzymes such as cellulase, cutinases,

pectinases, polygalacturonases and xylanases to dissolve cell wall (Hammond-Kosack and Jones, 1996). After the pathogen has been successful in breaking the primary defense

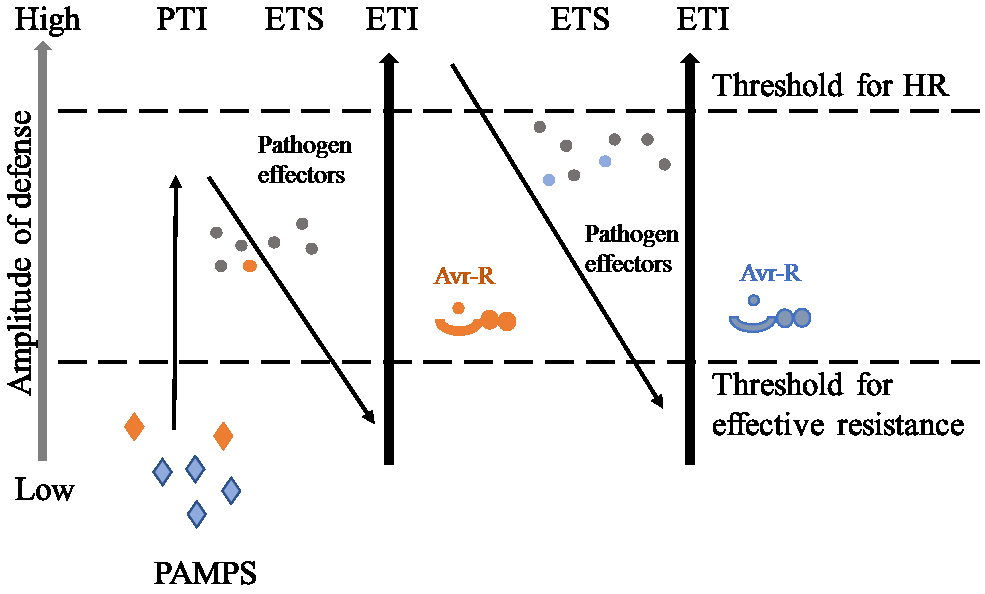

Figure 1.1 A zigzag model showing plant defense system.

Note: The plants become susceptible when plant immune response is parallel to the equation [PTI-ETS+ETI]. The picture depicts the various forms of plant defense strategy in response to the various stages of pathogen attacks. Stage I: Plant detects PAMPS (orange) via RRRs proteins and induces PTI. Stage II: Pathogen deploys effector proteins to suppress PTI to induce effector-triggered susceptibility (ETS). Stage III: The NB-LRR protein detects the pathogen effector (orange) and triggers ETI. Stage IV: Selection of pathogen isolates are made that lost their orange effector and possibly producing new effectors through horizontal gene flow (blue) to suppress ETI. The selection of the isolates activates NB-LRR protein that detects new effectors and induces ETI again to provide defense response (Adapted from Jones and Dangl, 2006).

system, the plant then can deploy effector triggered immunity (ETI) as a more advanced defense response by activating plant resistance (R) proteins (**Figure1.1)** (Chisholm et al. 2006; Jones and Dangl, 2006). The Zigzag model (**Figure 1.1**) shows the series of steps occurring during the pathogen infection and plant defense responses. In stage I, the

pattern recognition receptors (PRRs) at transmembrane sense PAMPS inducing PTI (Zipfel and Felix, 2005; Jones and Dangl, 2006). Plant receptor proteins present at the plasma membrane are the products of resistance (R) genes and are positioned to counter pathogen avirulent gene products (Hammond-Kosack and Jones, 1996; Nimchuk et al. 2003; Jones and Dangl, 2006). In stage II, pathogen induces their virulence by secreting effectors and hijack PTI that leads to effector-triggered susceptibility (ETS) (Chisholm et al. 2006; Jones and Dangl, 2006). During stage III, one of the NBS-LRR proteins recognize a pathogen effector and deploy ETI inducing disease resistance and hypersensitive cell death at the site of pathogen infection (Jones and Dangl, 2006). In stage 4, natural selection facilitates pathogen to manipulates its effector genes or acquire new effector tools to avert ETI whereas the natural selection induces R specific genes that reactivate ETI response (Jones and Dangl, 2006).

The plant defense response includes the production of reactive oxygen species (ROS), R gene transcripts and the biosynthesis of jasmonic acid (JA), salicylic acid (SA), benzoic acid (BA) and ethylene that can induce the transcription of different R genes, various protein coding genes and enzymes (Hammond-Kosack and Jones, 1996). Activation of the hypersensitive response by R proteins mediate cell wall modification thereby limiting pathogen growth and spread (Hammond-Kosack and Jones, 1996). The consequence of these actions is that the plant produces primary and secondary metabolites, activates genes that produce chitinases, thionins, defensins, glucanases, glutathionine-S-transferase (GST), lipoxygenase (LOX), phenylalanine ammonia lyase (PAL) and induce the lignification of plant cell wall (Hammond-Kosack and Jones, 1996; Glazebrook et al. 1997). Through work done primarily in the plant genetic model

Arabidopsis. thaliana, these ETI and PTI levels of pathogen defense have been defined by different receptor systems that exhibit cross-talk (Jones and Dangl, 2006). ETI has been attributed to the coiled coil, nucleotide binding leucine rich repeat resistance protein (CC-NB-LRR R) NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1) (**Figure 1.1**) (Century et al. 1995, 1997; Coppinger et al. 2004). NDR1 activates ETI through its direct interaction with RPM1-INTERACTING PROTEIN 4 (RIN4) (Mackey et al. 2002; Axtell and Staskawicz, 2003; Day et al., 2006). RIN4 also interacts with the CC-NB-LRR protein RESISTANCE TO PSEUDOMONAS SYRINGAE2 (RPS2) and the CC-NB-LRR protein RESISTANCE TO PSEUDOMONAS SYRINGAE PV MACULICOLA1 (RPM1) (Kunkel et al. 1993; Grant et al. 1995). Multiple pathogen effectors impair the function of these proteins, interfering with defense signaling (Mackey et al. 2002; Belkhadir et al. 2004; Kim et al. 2005). Relevant to this dissertation has been the identification of a *Glycine max* (soybean) NDR1 that functions in defense to the plant-parasitic nematode *Heterodera glycines* (soybean cyst nematode [SCN]), showing that it can induce the expression of proven defense genes (McNeece et al. 2017). The ETI membrane receptor toll-interleukin receptor (TIR) NB-LRR R protein RECOGNITION OF PERONOSPORA PARASITICA 4 (RPP4), leads ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1)-driven engagement of defense gene expression involving the production of SA and its employment as a defense signal (Cao et al. 1994; Aarts et al. 1998). Functioning downstream in this SA signaling pathway are NON-EXPRESSOR of PR1 (NPR1) and TGA2 which drive target defense gene expression (Falk et al. 1999; Niggeweg et al. 2000; Kinkema et al. 2000; Fan and Dong, 2002). Relevant to this dissertation has been the demonstration that *G. max* EDS1 and NPR1 functions in defense

to *H. glycines* (Pant et al. 2014). PTI functions through the membrane receptor FLAGELLIN SENSING PROTEIN2 (FLS2) in processes that may lead to mitogen activated protein kinase MAPK signaling (**Figure 1.2**) (Chinchilla et al. 2007; Lin et al. 2014).

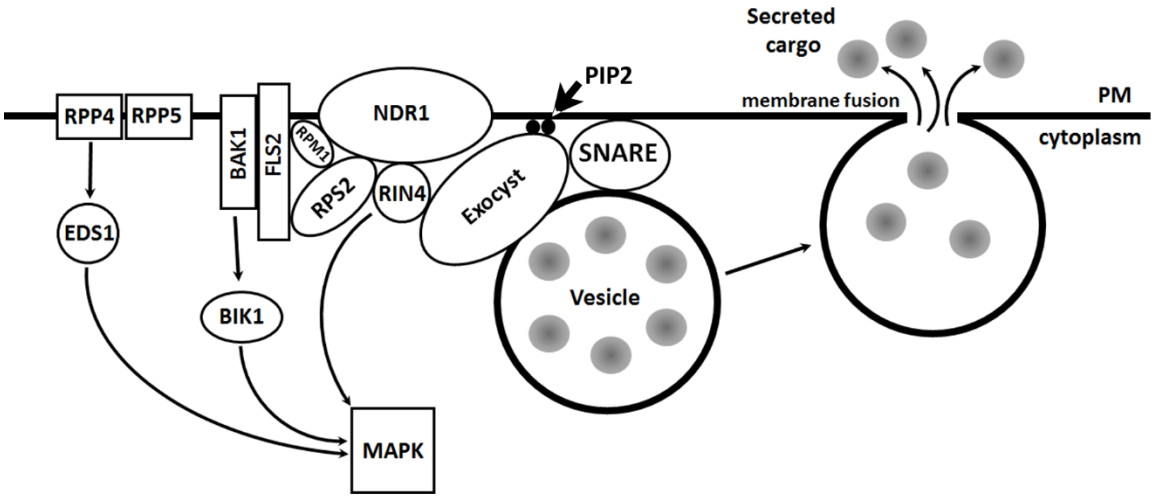

Figure 1.2 The interconnectedness of the NDR1, SNARE and exocyst receptors as it relates to defense.

Note: The exocyst complex, helps prepare SNARE for docking and release of vesicular contents after fusion (TerBush and Novick, 1995). The exocyst complex acts as a signal receiver for various signaling pathways, tethering vesicles at the receptor membrane and mediating fusion by inducing formation of SNARE assembly (He and Guo, 2009; Žárský et al. 2013). Sec3 and Exo70 bind to phosphatidylinositol 4, 5-biphosphate (PI (4,5) P_2) located in the plasma membrane (He et al. 2007; Liu et al. 2007; Zhang et al. 2008). The exocyst has been shown to physically interact with SNARE through and interaction with syntaxin121 and RIN4 and its disruption shown to have a negative impact on its biological function (Sabol et al. 2017). FLS2 binds RPM1 and RPS2, physically linking ETI and PTI with SNARE and the exocyst (Qi et al. 2011). The appropriate references are described in the text (Sollner et al. 1993a, 1993b; Kunkel et al. 1993; Cao et al. 1994; Grant et al. 1995; TerBush et al. 1996; Aarts et al. 1998; Falk et al. 1999; Niggeweg et al. 2000; Kinkema et al. 2000; Fan and Dong, 2002; Mackey et al. 2002; Axtell and Staskawicz, 2003; Day et al., 2006; Veronese et al. 2006; Chinchilla et al. 2007; Qi et al. 2011; Lin et al. 2014; Synek et al. 2017).

FLS2 can activate and physically interact with ETI components, binding RPM1, RPS2 and RPS5, supporting previous observations that has revealed cross-talk occurring between PTI and ETI receptor systems (van der Biezen et al. 2002; Veronese et al. 2006; Zipfel et al. 2006; Thomma et al. 2011; Qi et al. 2011; Liu et al. 2013; Lolle et al. 2017; Jacob et al. 2018). These results are relevant to this dissertation since a *G. max* homolog of the FLS2 activated protein BOTRYTIS INDUCED KINASE1 (BIK1) functions in defense in the *G. max*-*H. glycines* pathosystem (Pant et al. 2014).

The vesicle membrane protein the Rab GTPase (Sec4) connects the exocyst complex and vesicles (Guo et al. 1999; Mizuno-Yamasaki et al. 2012). This defense signaling cascades do not function on their own but instead have been shown to act as part of a larger defense apparatus that is expressed at the genomic level upon pathogen attack (Scheidler et al. 2001). Included in this defense signaling apparatus is the regulon, a unit defined genetically in *A. thaliana* by the penetration mutants (*pen1-pen3*) (Collins et al. 2003). PEN1 is syntaxin 121, a component of the membrane fusion apparatus called soluble N-ethylmaleimide-sensitive fusion protein attachment protein (SNAP) REceptor (SNARE) (Collins et al. 2003). PEN2 is a β-glucosidase (Lipka et al. 2005). PEN3 is an ATP binding cassette (ABC) transporter (Stein et al. 2007). The functions of these regulon proteins converge, resulting in defense (Humphry et al. 2010). Relevant to this dissertation has been the identification of the regulon functioning in *G. max* as it combats *H. glycines* parasitism and that the components are co-regulated in their expression (Sharma et al. 2016; Klink et al. 2017).

The experiments presented by Scheidler et al. (2001) indicates that the defense regulon could be much larger, possibly including other types of receptors. A good

candidate receptor would be the exocyst. The exocyst is composed of 8 proteins and has been shown to function through the NDR1-interacting protein RIN4 and SNARE (TerBush et al. 1996; Mackey et al. 2002; Synek et al. 2017). These observations have indicated that a functional analysis of GmRIN4 and its exocyst complex will provide the basis of an understanding of a very large receptor system including the already studied SNARE and NDR1 that would be complemented by the proposed studies presented here on *G. max* homologs of RIN4 and the exocyst that have previously not been characterized but are hypothesized to function in defense (Sharma et al. 2016; McNeece et al. 2017).

The *G. max-H. glycines* pathosystem

H. glycines Ichinohe is the major pathogen of *G. max*, causing more than a billion dollars in losses in the U.S., annually (Smolik and Draper, 2007; Koenning and Wrather, 2010; Allen, 2017, Wang et al. 2017). *H. glycines* accomplishes infection by puncturing the root with its needle-like mouth apparatus called a stylet (Smolik and Draper, 2007). *H. glycines* infestation of soybean field is a severe agricultural threat with plant infection causing nutrient deficiency syndromes (Wrather et al. 1984; Gao et al 2003). Furthermore, *H. glycines* infected plants are susceptible to secondary diseases such as the fungal pathogen *Macrophomina phaseolina* (charcoal rot) (Todd et al. 1987; Winkler et al. 1994). Some of the consequences of *H. glycines* infection include plants having irregular chlorotic patches, suppression of growth and development, decreased nodule formation and necrosis, however, above ground symptoms may not be visible in all cases (Wrather et al. 1984; Bird, 1990; Niblack, 2005; Chang et al. 2011; Yu, 2011).

The origin of the *H. glycines* is believed to be China while the available sources of resistant cultivars for the pathogen are also from China (Bernard et al. 1988; Liu et al.

1997; Li et al. 2011; Yu, 2011). SCN has been first reported in Japan in 1915 and the pathogen has been first described scientifically in 1952 in Japan as well (Hori, 1915; Ichinohe, 1952; Yu, 2011). The spread of *H. glycines* is believed to have occurred through its hosts (Yu, 2011). *H. glycines* is an invasive pathogen to the U.S. and was first discovered in North Carolina in 1954 (Winstead et al. 1955). Subsequently, it spread to Mississippi by 1957 and 26 other soybean producing states in the U.S., some parts of Canada and countries in South America (Yu, 2011). The spread of this pathogen has been unintentional, due to pathogen overwintering capability and the dormant cyst stage (Riggs, 1977; Wrather et al. 1984). Furthermore, gravid nematodes can produce hundreds of juveniles in a single season, enough to infest large areas under agricultural production (Niblack et al. 2005).

Soybean cyst nematode biology and life cycle

H. glycines is a sedentary endoparasite (Williamson and Hussey, 1996). At the completion of its life cycle, the hardened cyst which is the carcass of the female that encases the eggs has the capability to overwinter if needed. The brown leathery cyst composed of skin made of cuticle protects the viable eggs within (Wrather et al. 2001; Agrios, 2005). The cuticle layer is made up of chitin, a polymer of β-1, 4 linked residues of N-acetyl glucosamine secreted by hypodermis (Spiegel and McClure, 1995; Veronico et al. 2001). *H. glycines* is protected by a surface coat existing outside of their cuticle which is made up of proteins, carbohydrates and lipids that protect against various forms of stress (Brown et al. 1971; Bird and Bird, 1991; Jones et al. 1993; Spiegel and McClure, 1995). Eggs packed inside cyst contain pre-infective juvenile (J2), that hatch when the conditions are favorable (Agrios, 2005). Usually, cysts having viable eggs can

remain in the soil for up to 9 years and J2s can be infective for 7 years whereas it varies with temperature, moisture and other environmental conditions (Inagaki and Tsutsumi, 1971). *H. glycines* has a wide host range that includes at least 100 plant species encompassing legumes, non-legume and weed species (Epps and Chambers 1958; Riggs and Hamblen, 1962, 1966a, 1966b; Baldwin and Mundo-Ocampo, 1991; Yu 2011). The different forms (biotype) of *H. glycines* are termed as races according to their ability to infect and reproduce in different soybean genotypes (Golden, 1970). There have been 16 different *H. glycines* races that have been identified (Golden, 1970; Riggs and Schmitt, 1991; Yu, 2011). From these 16 *H. glycines* races, race 3 is the most prominent in the world (Yu, 2011). *H. glycines* feeds using its stylet to penetrate the root cell and draw nutrients (Davis et al. 2000).

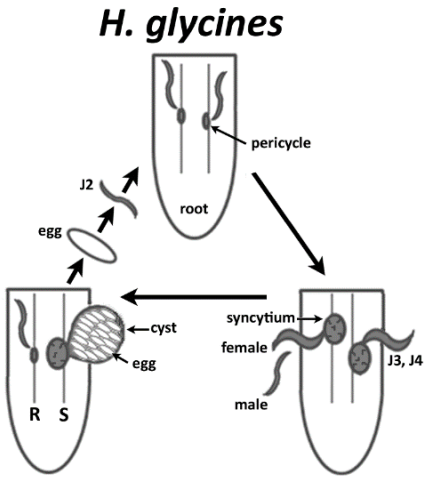

Figure 1.3 The life cycle of *H. glycines.*

Note: Figure showing egg, second juvenile (J2), third juvenile (J3), fourth juvenile (J4) mature female and cyst. Male, female; parasitized pericycle cells develop into a syncytium which during a susceptible (S) reaction is a nursing structure composed from the merged cytoplasm of 200-250 cells. The syncytium also serves as the site of the localized defense response leading to a resistant (R) reaction; cyst, female carcass structure containing the eggs. (Sharma et al. submitted)

They complete their life cycle in three to four weeks during summer days, but the time frame changes according to temperature (Lauritis et al. 1983; Alston and Schmitt, 1988). During its life cycle, *H. glycines* molts through four juvenile stages (J1, J2, J3, J4), followed by an adult stage (Sijmons, 1993). Details of the *H. glycines* life cycle are provided (**Figure 1.3**). *H. glycines* engages its infection processes when the J2 senses root exudates (Tsutsumi and Sakurai, 1966; Tefft and Bone, 1985). This event promotes *H. glycines* migration toward the root by sinusoidal movement. The infective juvenile (i-J2) punctures root epidermal cells with its stylet and bores into the vascular cylinder untill it reaches its preferred cells, the pericycle cells for feeding (Ithal et al. 2006). After 2 days post infection (dpi), the i-J2 reaches the pericycle cells and starts feeding (Endo, 1965, 1991). The plant-parasitic nematodes have developed different processes to parasitize plants (Davis et al. 2000). During infection various parasitic genes are expressed in their esophageal gland producing various cell wall degrading proteins and enzymes (Gao et al. 2003; Lee et al. 2011). The nematode gland secretions change the physiology of the infected cell and dissolve cell walls of the surrounding cell to form a large feeding site described as a syncytium that consists of more than 200 cells sharing a common cytoplasm in which nuclei exist (Davis et al. 2000; Hussey et al. 2002; Niblack et al. 2005; Baum et al. 2007). Consequently, the syncytium is a multinucleate cell having organelles and a dense granular cytoplasm inside a thick wall (Davis et al. 2000; Hussey et al. 2002; Davis et al. 2008). The expression of nematode genes facilitates its ability to overcome host resistance (Hussey et al. 2002). This process changes the structure and physiology of the parasitized cell, allowing *H. glycines* to secure a continuous food supply for their growth and development (Hussey et al. 2002).

The i-J2 becomes sedentary after feeding for certain time and then goes through a series of molts (Williamson and Hussey, 1996). The i-J2 molts to a J3 at 3-5 dpi while their sexual dimorphism is apparent after 5 dpi (Lauritis et al. 1983; Niblack et al. 2005; Ithal et al. 2006). Due to their continuous feeding, their body becomes swollen and projects outside of the root epidermis after 6 dpi (Lauritis et al. 1983). Subsequently, the J3 molts into a J4 after 6-7 dpi (Lauritis et al. 1983; Niblack et al. 2005). The J4 then molts into free adult males at 9-11 dpi while this process occurs in females at 8-10 dpi (Lauritis et al. 1983; Niblack et al. 2005). Ultimately, adult males stop feeding, devoting their time to search for females meanwhile, females continue feeding and their body continues to grow until it protrudes outside of the root boundary to facilitate mating (Lauritis et al. 1983). Males are attracted to females by a pheromone that is released by the female nematode (Chen, 2011). After mating, the female develops 100-300 eggs inside its body (Tefft et al. 1982; Lauritis et al. 1983; Niblack et al. 2005).

Management of SCN

For the effective management of *H. glycines*, a long-term control strategy is necessary to control its outbreak and environmental issues (Trivedi and Barker, 1986; Niblack, 2005). Management practices for *H. glycines* have been performed since the identification of *H. glycines* and has advanced with more research and technologies (Winstead et al. 1955; Spears, 1955; Riggs, 1977; Boerma and Hussey, 1984; Concibido et al. 2004; Smolik and Draper, 2007; Tian et al. 2007; Klink et al. 2007; Matsye et al. 2012; McNeece et al. 2017; Bajwa et al. 2017; Joalland et al. 2017). Research has been concentrated on every aspect of control including plant culture, nematicide production, seed treatments and host resistance (Boerma and Hussey, 1984; Cregan et al. 1999;

Concibido et al. 2004; Klink et al. 2007; Matsye et al. 2011; Matsye et al. 2012). Investigations of *H. glycines* and syncytium has also shown promise in identifying resistance (Matsye et al. 2012).

H. glycines has been shown to use their specific virulence genes to overcome plant resistance (Qui et al. 1997). However, the plant can sense various virulence proteins from numerous pathogens, promoting the expression of their resistance genes to counter their attack (Qui et al. 1997). In *G. max*, several resistant cultivars have been identified that counteract *H. glycines* infection (Bernard et al. 1987). For example, *G. max* [Peking/PI 17852] and *G. max* [PI 88788] have been extensively used in breeding purposes to produce *H. glycines* resistant soybean cultivars (Ross and Brim, 1957; Hartwig, 1985; Rao Arelli, 1994). Furthermore, the study of *G. max* genotypes and mapping have revealed several resistant loci providing resistance to *H. glycines* (Caldwell et al.1960; Matson and Williams, 1965; Rao Arelli, 1994). There are three recessive resistant loci in soybean known as resistance to *Heterodera glycines* (*rhg*) that are *rhg1*, *rhg2* and *rhg3* and two dominant resistant loci *rhg4* and *rhg5* (Caldwell et al.1960; Matson and Williams, 1965; Rao Arelli, 1994). The study of resistance has taken to a new level with sequence analysis and gene expression studies. The *rhg1* and *rhg4* have subsequently been identified (Matsye et al. 2011, 2012; Liu et al. 2012; Sharma et al. 2016).

Plant pathogen interaction and defense

H. glycines parasitism of the plant depends on the physical characteristics of the plant-parasitic nematode, its ability to find roots, infect, parasitize, reproduce and survive (Baum et al. 2007). Microarray analysis and quantitative PCR (qPCR) results have shown various genes that are upregulated in roots after infection when tested at different time

intervals (Ithal et al. 2006; Klink et al 2007, 2007a, 2007b, 2010c; Pant et al. 2014; Sharma et al. 2016). Through these analyses different virulent and avirulent genes expressed by the nematode during parasitism and genes expressed by plants in response to nematode infection have been identified (Ithal et al. 2006; Klink et al. 2010c). Mainly during infection SCN expresses their virulence genes allowing them to attack plant defense mechanisms by altering cell signaling, hormones, metabolism, and cell wall repair processes (Ithal et al. 2006).

The secretions of nematodes are the products of genes that are expressed in their esophageal glands, consisting of one dorsal and two subventral glands (Endo, 1984, Gao et al. 2003; Davis et al. 2000, 2004). *H. glycines* also produce proteins whose activities change the cell wall (Baum et al. 2007). Cell wall modifying proteins have a significant impact in this interaction as nematode secretes them to dissolve the surrounding cell walls so that they can form the syncytium (De Boer et al. 1999; 2002a; 2002b). The expression of parasitism genes produces proteins such as chorismate mutase that functions to deplete the synthesis of plant metabolites such as auxin and SA (Baum et al. 2007). A decrease in SA production alters the defense system in plants, leading to infection (Baum et al. 2007).

The *G. max-H. glycines* pathosystem as a model to study plant defense to root pathogens

In comparison to the plant shoot, very little is understood about plant defense processes in the root. Consequently, the agricultural plant *G. max* has been developed as a model to understand root pathogens with most of those efforts focused on its most significant pathogen, *H. glycines* (**Figure 1.3**) (Wrather et al. 2001; Klink et al. 2005,

2007a, b; 2008; Matsye et al. 2011, 2012). This plant-pathosystem model is an essential tool because it is comparatively easy to perform genetic experiments while at the same time being agriculturally relevant whereby knowledge can be translated directly to improving cultivation. In this manner a root genetic transformation platform has been developed that can allow the experimental induction and suppression of both *G. max* and *H. glycines* genes with many of these already studied and identified through gene expression studies that have used microarray analyses (MA) in various forms (Klink et al. 2007a, b; 2010; Matsye et al. 2011). Related studies have also used RNA sequencing (Matsye et al. 2011). These studies relate to the experiments outlined in this dissertation.

Cytological study of the infected cells

The silencing of plant and nematode genes that relate to a susceptible reaction could have the effect of perturbing parasitism, leading to a successful defense response (Baum et al. 2007). Complimentary DNA (cDNA) libraries and expressed sequence tags (ESTs) have been used to study parasitism in nematodes (Davis et al. 2000). The study of the syncytium cells that are parasitized by *H. glycines* has been used to identify the possible genes induced during infection (Klink et al. 2005, 2007b). Using MAs, unique and differentially expressed genes have been identified from RNAs isolated from syncytium cells that relate to the defense response (**Figure 1.4**) (Klink et al. 2007b). Identifying genes that are uniquely expressed has been accomplished through a developed procedure called detection call methodology (DCM) (Klink et al. 2010c). Identified in those studies have been heat shock proteins (HSP), LOX, superoxidase dismutase (SOD) and genes related to transcription factors and DNA binding proteins, found specifically in syncytia undergoing an incompatible (resistant) reaction (Klink et

al. 2007b). The soybean MA analysis of the infected cells revealed that compatible and incompatible reaction have their own unique genes expressed during pathogen attack, showing few identical genes in both (Klink et al. 2010c).

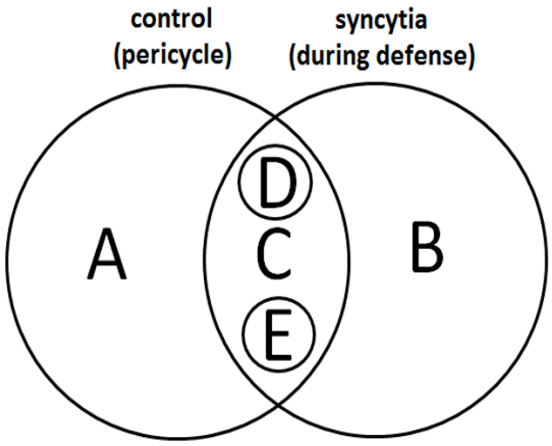

Figure 1.4 DCM. RNA isolated control and syncytia undergoing a resistant reaction have been used in gene expression.

Note: A, represents genes exhibiting measured detection only in the control. B, represents genes exhibiting measured detection only in the syncytia undergoing a resistant reaction. The overlapping region in the middle between A and B represents genes expressed in control and syncytia and is the pool that is used in differential expression studies while A and B pools are discarded because they are uniquely expressed only in one of the two cell types preventing statistical analyses. The B pool has been believed and proven to define the resistant reaction (Matsye et al. 2011, 2012). C, represents the pool of genes that do not exhibit differential expression. D, is a pool of genes that are common to the control and syncytia, representing genes that are increased (induced) in their expression. E, is a subset of expressed genes that are common to the control and syncytia, representing genes that are decreased (suppressed) in their expression studies (Adapted from Klink et al. 2010c; McNeece et al. 2017; Sharma et al. submitted).

The components of the phenylpropanoid pathway were detected with elevated transcripts in both incompatible and the compatible reaction (Klink et al. 2010c). The analyses reveal the expression of various unique genes such as TIR-NBS-LRR protein kinases, WRKY transcription factors, cytochrome P450 protein, kunitz trypsin and

extracellular dermal glycoproteins only in the incompatible reaction (Klink et al. 2010c). Also, in the incompatible reaction after 12 hpi different genes with elevated transcripts have been detected such as transcription factors of no apical meristem (NAM) gene family, WRKY, FYVE, NBS-LRR and LRR gene family, regulators of chromosome condensation (RCC1) and others (Klink et al. 2010c). From the earlier experiments and comparative analysis of WRKY, and R genes show that they are related to defense and provide resistance against nematodes (Hammond-Kosack and Jones 1997; Milligan et al. 1998; Dangl and Jones, 2001; Klink et al. 2010c). The resistance response in G. *max*$_{[Peking/PI548402]}$ induces the formation of cell wall appositions (CWA) that gives structural and chemical defense against nematode penetration forcing nematodes to die in their i-J2 stage (Aist, 1976; Schmelzer 2002; Colgrove and Niblack, 2008; Hardham et al. 2008; Matsye et al. 2011). The defense response is induced simultaneously with the accumulation of the subcellular components, localization of actin at the infected site and the formation of necrotic layer around nematode head thereby separating the syncytium from the surrounding cells (Endo 1964, 1965; Riggs et al. 1973; Kim et al 1987; Kim and Riggs, 1992; Colgrove and Niblack, 2008; Klink et al.2009a; Matsye et al. 20011). This resistance process is induced after 4 dpi in G. *max*$_{[Peking/PI548402]}$ (Matsye et al. 2011). Another potent resistant cultivar G. *max*$_{[PI88788]}$ lacks formation of necrotic layer and cell wall apposition but induces nuclear degeneration and aggregation of rough endoplasmic retculum and cisternae at the infected site and kills nematode during its J3-J4 stages (Acedo et al. 1984; Kim et al. 1987; Colgrove and Niblack, 2008; Matsye et al. 2011).

The comparative analysis of the transcripts identified from the resistant reaction shows that the component of the alpha soluble NSF attachment protein (α-SNAP) in the

rhg1 locus is providing resistance against *H. glycines* (Caldwell et al. 1960; Matsye et al. 2011, 2012; Sharma et al. 2016). The RNA sequencing of the resistance cultivars reveal genes active in SA, shikimate, arachidonic acid, N-glycan biosynthesis, nicotinate and nicotinamide metabolism, glyoxylate and dicarboxylate metabolism and zeatin biosynthesis pathways providing defense (Smigocki et al. 1993; Emmerlich et al. 2003; Galis et al. 2004; Steppuhn et al. 2004; Veronese et al. 2006; Pattison and Amtmann, 2009; Onkokesung et al. 2010; Hanssen et al. 2011; Klink et al. 2010; Matsye et al. 2011). The sequence analysis and Pathway Analysis and Integrated Coloring of Experiments (PAICE) analysis have indicated, JA might induce transcriptional activation of genes functioning as a defense in soybean against parasitic nematodes (Gao et al. 2008; Klink et al. 2007; Klink et al. 2009; Klink et al. 2010; Matsye et al. 2011). Various compounds such as lignin and suberin of phenylpropanoid pathway have been observed in cytological examinations of the root syncytium (Ross, 1958; Klink et al. 2009; Klink et al. 2010b; Matsye et al. 2011). These phenylpropanoid metabolites; chitin, lignin, pectin, and suberin induce the production of a structural barrier as a defense to a root pathogen (Matsye et al. 2011). Other proteins such as S-methionine synthetase, hydroxyproline rich glycoproteins, extensin and peroxidases are also induced in syncytium as a defense response (Klink et al. 2009; Kim et al. 2010; Matsye et al. 2011). The expression of the cell wall associated proteins is concurrent with the production of ROS after the pathogen attack that induce synthesis and cross linking of the cell wall proteins providing resistance against pathogen invasion and growth (Levine et al. 1994; Mellersh et al. 2002; Matsye et al. 2011). Genes related to the production of ROS have been observed to be

induced in syncytium providing defense to a root pathogen (Klink et al. 2007; Matsye et al. 2011).

Vesicular membrane fusion and defense

Structural features that relate to membrane trafficking are involved in *G. max* defense to plant-parasitic nematodes (Matsye et al. 2012; Sharma et al. 2016). These observations related to earlier exeriments that have been done in *A. thaliana* have shown its syntaxin 121 (PEN1) protein functions in defense (Collins et al. 2003). Syntaxin, as a component of the SNARE is involved in the formation of CWA and has been shown to be expressed to higher level after pathogen infection, including defense to plant-parasitic nematodes (Collins et al. 2003; Matsye et al. 2012; Pant et al. 2014). Similarly, other components of the vesicular membrane fusion such as N-ethylmaleimide sensitive factor attachment protein (NSF), α-SNAP, synaptosomal associated protein 25 (SNAP-25), and other the SNARE complex proteins are involved (Novick et al 1980; Clary et al. 1990; Collins et al. 2003; Pajonk et al. 2008; Matsye et al. 2011; 2012; Pant et al. 2014; Sharma et al. 2016). A number of the proteins identified to function during the defense process that *G. max* has toward *H. glycines* are highlighted (**Figure 1.5**). The involvement of the proteins associated with the vesicular membrane fusion machinery in CWA formation during the defense unveils broader concepts about the membrane transport mechanism and their associated proteins (Matsye et al. 2012; Pant et al. 2014; Sharma et al. 2016). These broader concepts include the examination of the exocyst along with RIN4 as presented in this dissertation.

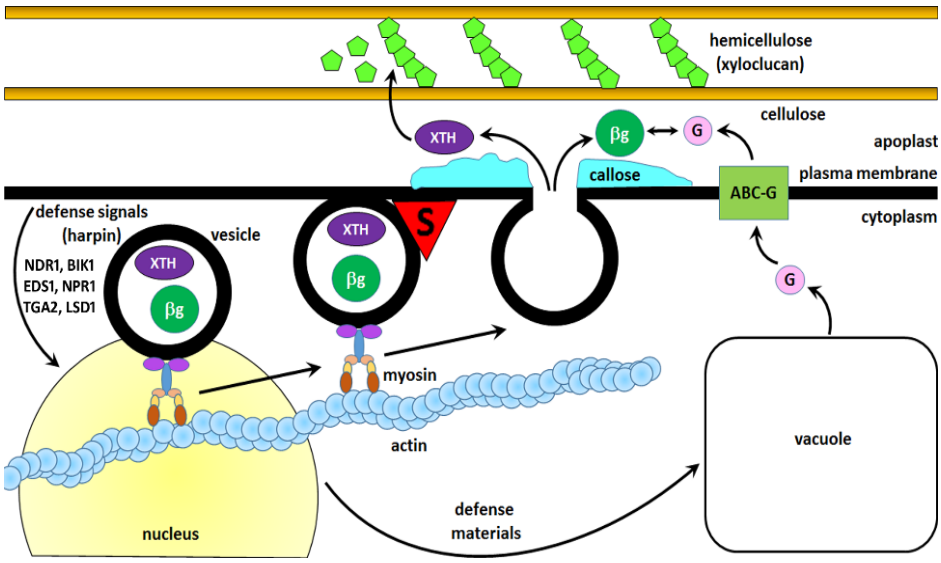

Figure 1.5 Components of the *G. max-H. glycines* defense regulon.

Note: The model presents several tested genes functioning in defense in the *G. max-H. glycines* pathosystem under the described procedures. Defense signals that lead to the propagation of defense include harpin (Aljaafri et al. 2017). Harpin treatment leads to increased transcript levels of a number of genes that have been proven to function in defense. These genes include those signaling both effector triggered immunity (ETI) and pathogen activated molecular pattern (PAMP) triggered immunity (PTI). Harpin increases transcript levels of the coiled-coil nucleotide binding leucine rich repeat (CC-NB-LRR) NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1)/HARPIN INDUCED1 (HIN1) and the cytoplasmic receptor-like kinase BOTRYTIS INDUCED KINASE1 (BIK1). Components of salicylic acid signaling are also increased in their transcript abundance, including the PTI genes ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), NONEXPRESSOR of PR1 (NPR1), TGA2 and LESION SIMULATING DISEASE1 (LSD1). The induced transcription of several secreted proteins that function in defense, including and xyloglucan endotransglycosylase (XTH) and α-hydroxynitrile glycosidase (βg). The secreted proteins would enter the vesicle transport system, experience requisite modifications and become secreted into the apoplast to perform their defense role. S, SNARE-SM, including synaptotagmin; G, conjugated glycoside; ABC-G, ABC-G-type transporter. In this review, data is presented for the involvement of Myosin and CS. Defense proteins not discussed include galactinol synthase, reticuline oxidase and a number of membrane fusion proteins including Sec14, Sec4 and Sec23, an endosomal bromo domain-containing protein1 (Bro1), syntaxin6 (SYP6), SYP131, SYP71, SYP8, Bet1, coatomer epsilon (ε–COP), a coatomer zeta (ζ-COP) an ER to Golgi component (ERGIC) protein (Klink et al. 2017). The image depicts different cargo proteins within the vesicle which may or may not be true only for presentation purposes (Sharma et al. 2016).

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CHAPTER II

THE EXPERIMENTALLY INDUCED EXPRESSION OF *GLYCINE MAX* RPM1-INTERACTING PROTEIN 4 (RIN4) RESULTS IN RESISTANCE IN *GLYCINE MAX* TO *HETERODERA GLYCINES*

Abstract

The plant secretion system is an important regulatory process where many proteins associate together, delivering the cellular cargo to various destinations. RIN4, as an NDR1-interacting protein, has been shown in the plant genetic model *A. thaliana* to tether the exocyst complex at the plasma membrane. This event induces vesicular fusion at the specific targeted site. In the experiments presented here, four GmRIN4 paralogs (GmRIN4-1 through GmRIN4-4) having homology to the *A. thaliana*. RIN4 have been identified in the genome of *G. max*. An analysis of gene expression data has been able to identify the expression of GmRIN4-4 in root cells prior to and during a successful defense response. The identification of this gene expression has been accomplished in two different *G. max* genotypes that are resistant to *H. glycines* parasitism. The results indicate that there is a preformed defense apparatus in place and that experimental conditions that perturb the normal defense response would impair the resistant reaction. In contrast, the expression of GmRIN4-4 would be expected to result in the engineering of a successful defense response in a *G. max* genotype that is normally susceptible to *H. glycines* parasitism. The experimentally induced overexpression of a *G. max* RIN4

GmRIN4-4) in the normally *H. glycines* susceptible genotype *G. max* [Williams 82/PI 518671] has led to a suppression of nematode parasitism. In contrast, RNAi of GmRIN4-4 in the normally *H. glycines*-resistant genotype *G. max*[Peking/PI548402] has led to increased *H. glycines* parasitism. These results have linked the *H. glycines* defense process caused by GmRIN4-4 to results originally presented in *A. thaliana* showing RIN4 functions in resistance by targeting the exocyst to the site of infection. Furthermore, the results are consistent with the demonstration that GmNDR1-1 functions in resistance in the *G. max*-*H. glycines* pathosystem.

Introduction

Experiments in the plant genetic model *A. thaliana* have demonstrated that RIN4 is an important defense protein (Day et al. 2006). RIN4 has relevance to the experiments presented here in Chapter II since in *A. thaliana* it binds to another defense protein, NDR1 to effect defense processes (Mackey et al. 2002, 2003; Day et al. 2006). As shown in earlier experiments, GmNDR1-1 is expressed within root cells undergoing a defense response and functions in defense (McNeece et al. 2017). Consequently, by adapting knowledge from *A. thaliana*, it is possible to identify *G. max* homologs of proteins known to associate with NDR1 and show that they also function in the defense process that *G. max* has toward *H. glycines* parasitism. With this knowledge in place, the experiments provided for GmRIN4-4 can serve as a prerequisite to the understanding of the role that the exocyst has in defense. In *A. thaliana*, NDR1, as a RIN4-binding protein, serves to dock the exocyst through interactions with EXO70 (Sabol et al. 2017). As stated, prior experiments have shown GmNDR1-1 functions in the process of resistance that *G. max* has toward *H. glycines* (McNeece et al. 2017). Consequently, proteins closely

associated with *A. thaliana* NDR1 could be expected to function during the *G. max* defense response to *H. glycines* parasitism as shown in our earlier experiments on membrane fusion and signaling genes in this pathosystem (McNeece et al. 2017).

In *A. thaliana* NDR1 has been shown to be a plasma membrane (PM)-localized, late embryogenesis abundant (LEA) protein, having a topology of a coiled-coil nucleotide binding leucine rich repeat (CC-NB-LRR) resistance (R) protein (Century et al. 1995, 1997; Repetti et al. 2004). NDR1 exhibits structural similarity to animal integrins and has basic functions in plant cell biology in addition to its defense roles (Tamkun et al. 1986; Knepper et al. 2011). Integrins have been most actively studied in animal systems and shown to function as transmembrane adhesion receptors acting in various processes including activating signal transduction processes that mediate aspects of the cell cycle, arrangements of the cytoskeleton and movement of new receptors to the PM (LaFlamme et al. 2018). Consequently, NDR1 and its associated proteins would be expected to perform important roles in plants since these known processes have already been shown to function in defense (Matsye et al. 2012, Pant et al. 2014; Sharma et al. 2016; McNeece et al. 2017; Aljaafri et al. 2017; Klink et al. 2017). Furthermore, the treatment of plant tissues with the pathogen effector protein harpin has been shown to lead to the expression of NDR1, thus its designation as a harpin induced (*HIN*) gene (*NDR1/HIN1*) (Wei and Beer, 1992; Gopalan et al. 1996). Harpins are heat stable, glycine rich proteins found in gram negative plant pathogenic bacteria that are secreted through the bacterial type III secretion system (Wei et al.1993; Bogdanove et al. 1996; Choi et al. 2013). While harpins have been identified to function during the plant HR, leading to plant cell death, they may also function in the absence of an HR reaction by inducing a systemic response that could

function throughout the plant even in tissues that are not treated (Wei and Beer, 1992; Neyt and Cornelis, 1999; Dong et al. 1999, 2004; Lee et al. 2001; Kariola et al. 2003; Fontanilla et al. 2005a, b; Jang et al. 2006; Sohn et al. 2007; Chen et al. 2008a, 2008b; Engelhardt et al. 2009; Chuang et al. 2010; Miao et al. 2010; Pavli et al. 2011). Consequently, these observations indicate foliar application of harpin can lead to systemic defense signals that could function in the root. This effect has been proven to be true (Aljaafri et al. 2017). Furthermore, harpin treatment in *G. max* induces the expression of GmNDR1-1, along with a number of other proven defense signaling genes including those that function in SA signaling (Aljaafri et al. 2017).

Experiments presented in *A. thaliana* have shown NDR1 interacts directly with other proteins. These proteins include RIN4 (**Figure 2.1**) (Mackey et al. 2002, 2003; Day et al. 2006). The transduction of the defense signal happens through the interaction of RIN4 with both RPS2 and RPM1 (Kunkel et al. 1993; Grant et al. 1995) (**Figure 2.1**). Notably, RIN4 has been shown to recruit the exocyst protein EXO70 to the plasma membrane, implicating it also functions to recruit secretory vesicles to the site of infection (Sabol et al. 2017).

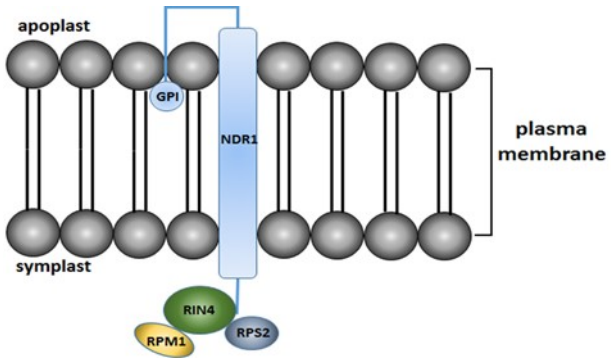

Figure 2.1 The NDR1 plasma membrane receptor and its interacting partners including RIN4, RPM1 and RPS2. The cytoplasmic N-terminus of NDR1 binds RIN4 in the symplast, itself binding to RPM1 and RPS2. The C-terminus, protruding into the apoplast, contains a GPI anchor domain (adapted from McNeece et al. 2017).

The experiments presented here aim to determine if a *G. max* RIN4 gene functions in resistance to *H. glycines* in *G. max*. The experiments presented here identify four *G. max* paralogs that are homologous to the *A. thaliana* RIN4 (Klink et al. 2010). Gene expression experiments have been able to identify that one of the four paralogs are expressed within parasitized cells that are undergoing a resistant reaction in two different *H. glycines* resistant genotypes (Klink et al. 2010). The experimental induction of GmRIN4-4 expression in the *H. glycines*-susceptible genotype *G. max* [Williams 82/PI518671] impairs parasitism, leading to a resistance outcome. In contrast, the experimental suppression of GmRIN4-4 expression in the *H. glycines* resistant genotype *G. max* [Peking/PI 548402] impairs the resistant reaction, leading to a susceptible outcome. The experiments are a prerequisite for experiments aimed at understanding the contribution of the *G. max* exocyst to *H. glycines* that is presented in Chapter III while further reinforcing the importance of the NDR1 receptor to the process of resistance in the *G. max*-*H. glycines* pathosystem.

Materials and methods

Identification of *G. max* RIN4 paralogs for gene expression determination

The identified *A. thaliana* RIN4 protein sequence AT3G25070 has been downloaded from Genebank (Mackey et al. 2002). The *A. thaliana* RIN4 protein sequence has been used in protein blast queries of the *G. max* genome using the default parameters. Gene sequences from the identified *G. max* RIN4 (GmRIN4) genes have been downloaded from the *G. max* genome database (Goodstein et al. 2012). The *G. max* genome accessions have been used in queries of a database that has been compared the *G. max* genome accessions that have corresponding Affymetrix® probe set identifiers for the *G. max* Gene Chip (Klink et al. 2007). From these comparisons, it has been determined that only one of the four GmRIN4 paralogs (GmRIN4-4) had a probe set fabricated on the Affymetrix® soybean Genechip®. Consequently, gene expression data could only be obtained for the GmRIN4-4 paralog.

Identification of GmRIN4-4 expression

The detection call methodology (DCM) has been first published by Klink et al. (2010). In brief, the laser microdissection (LM) procedure has been used to collect control cells (pericycle) at 0 days post infection (dpi) from histological sections (Klink et al. 2010). Furthermore, LM has collected *H. glycines*-induced syncytia undergoing the resistance process at 3 and 6 dpi. For robustness, the experiments were run in triplicate independently in two different *H. glycines*-resistant genotypes. After the production of microarray probes through proprietary procedures, microarray hybridizations were run in triplicate in each genotype. Consequently, 6 different microarrays have been generated independently. The gene has been considered expressed at a given time point (0, 3 or

6dpi), only if the probe signal had been measurable above threshold on all three arrays for both *G. max* [Peking/PI 548402] and *G. max* [PI 88788] (6 total arrays), $p < 0.05$. The analysis procedures have been performed using the Bioconductor implementation of the standard Affymetrix® detection call methodology (DCM) analysis (Klink et al. 2010). The analysis procedure consists of four steps. These steps have included (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. The detection call is (1) present ($p < 0.05$), (2) marginal ($p = 0.05$) or (3) absent ($p > 0.05$) (Klink et al. 2010). From these results, the GmRIN4-4 data has been extracted.

Cloning of GmRIN4-4

The cloning procedures have been adapted from Sharma et al. (2016). The GmRIN4-4 gene primers have been designed from its cDNA in a manner to allow cloning into pENTR/D-TOPO® (Invitrogen®) entry vector and subsequently into the appropriate destination vector (pRAP15-overexpression and pRAP17-RNAi) (**Table 2.1**) (Klink et al. 2009b; Matsye et al. 2012; Sharma et al. 2016). RNA has been isolated from *G. max* [Peking/PI 548402] and converted to cDNA by using Superscript First Strand Synthesis System (Invitrogen®) and used in PCR. The gel purified product is ligated to pENTR/D-TOPO® vector and transformed to chemically competent Top 10® *E. coli* cells (Invitrogen®). The amplified product has been confirmed for correct sequence and ligated to the destination vector using LR Clonase® (Invitrogen®). These destination vectors pRAP15 and pRAP17 are designed for *Agrobacterium rhizogenes* mediated genetic transformations (Tepher, 1984). The enhanced green fluorescent protein (eGFP) gene in the destination vectors is driven by *rol*D promotor (Haseloff et al. 1997; White et

al. 1985; Elmayan and Tepfer, 1995; Collier et al. 2005) that helps in the visual screening of the genetically engineered roots. The pRAP15 vector has a single Gateway (Invitrogen®) compatible *att*R1-*ccd*B-*att*R2 (*att*R) cassette whereas the pRAP17 vector has two (*att*R) subcassettes producing tendem inverted repeats (Klink et al. 2009a; Matsye et al. 2012). Both vectors are driven by firwort mosaic virus sub-genomic transcript (FMV-sgt) promoter and cauliflower mosaic virus 35S terminator (Bhattacharyya et al. 2002; Klink et sl. 2009a; Matsye et al. 2012). In pRAP17 the expression of the first *att*R subcassette is in forward direction producing sense (Watson) strand and the expression of the second subcassette is in reverse direction producing anitisense (Crick) strand (Klink et al. 2009a; Matsye et al. 2012). The pRAP15 and pRAP17 vector has tetracycline resistance genes and the shuttled vector with the desired amplicon has been transformed to chemically competent One Shot TOP10 *E. coli* strain (Invitrogen®) and selected under LB-tetracycline (µg/ml) (Matsye et al. 2012). The purified destination vector has been transformed to chemically competent *Agrobacterium rhizogenes* K599 (K599) strain which has root inducing ability (Tepfer, 1984; Haas et al. 1995; Klink et al. 2009a; Matsye et al. 2012; Sharma et al. 2016). The genetic study has been conducted by gene overexpression in susceptible cultivar and knock out through RNAi in resistant cultivar (Klink et al. 2009a; Matsye et al. 2012).

Table 2.1 Primers used in the analysis

GENE	ACCESSION	Type	Direction	Primer 5'-->3'
GmRIN4-4	Glyma18g36000	OE	**F**	CACCATGGCTCAACGTTCTAATGTTCC
			R	CTCATGACCATTCACAACCCTATT
		RNAi	**F**	CACCATGGCTCAACGTTCTAATGTTCC
			R	GGCCAGCAGAAGAAACTACATCT
		qPCR	**F**	GTTAGGCAAGAGTGAAGAGAATGT
			R	CCAGCAGAAGAAACTACATCTGA
			P	CCAGAAAGGTCAGCCAGGTTCAAAGATGA

Plant genetic transformations

These procedures have been performed according to (Sharma et al. 2016). The K599 culture has been prepared by inoculating LB-tetracycline (5 µg/mL) at 225 rpm over 16 hours at 28° C. Roots of one-week old *G. max* seedlings have been sliced off at the hypocotyl in K599 solution with a clean, sterile razor blade. The base of the cut plants has been placed in Murashige and Skoog (MS) media and vacuum infiltrated for 30 minutes, allowing the K599 to enter the plant tissue through the wound (Murashige and Skoog, 1962). The K599-infected, root-less plants have been replanted in coarse vermiculite at ambient temperature. After 1-week, the plants were transferred to the greenhouse. After 2-3 weeks the plants have been uprooted and screened to determine successful genetic engineering. This determination has been accomplished using the enhanced green fluorescent protein (eGFP) reporter (**Figure 2.2**).

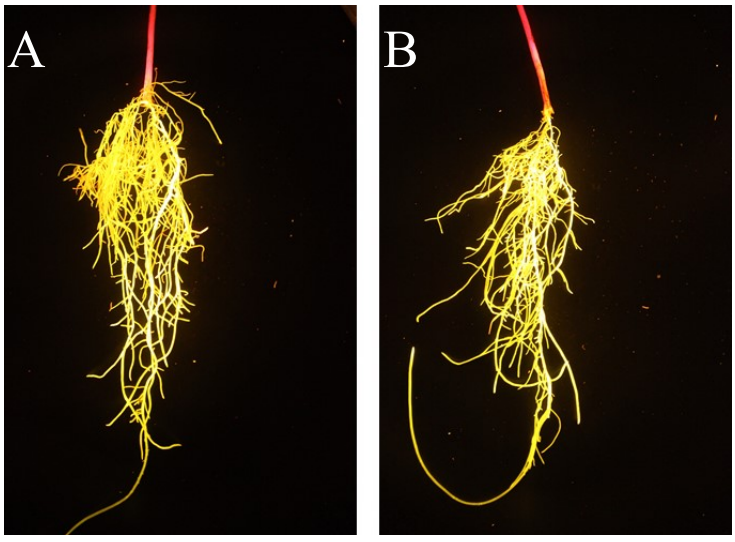

Figure 2.2 The agrobacterium engineered transformed roots with eGFP.

Note: The control overexpression with engineered pRAP15 is conducted in *G. max* [Williams 82/PI518671] (A) and control RNAi with engineered pRAP17 conducted in *G. max* [Peking/PI548402] (B). The percent difference in root mass of the genetically engineered roots

with control and selected genes in both OE and RNAi are not statistically significant ($P<0.05$) by Mann-Whitney-Wilcoxon Rank–Sum Test.

Only green (transformed) roots visible under Dark Reader® spot lamp have been used for further experiment (Klink et al. 2009a; Sharma et al. 2016). The non-transformed roots have been excised leaving transgenic eGFP-expressing roots that are also engineered to have the expression cassette (OE or RNAi). Transgenic plants have then been replanted in autoclaved soil (sand: clay in a 1.6:1 ratio) and infected with *H. glycines* J2s (Klink et al. 2009a; Sharma et al. 2016).

Quality control of engineered roots-quantitative PCR

Genes have been overexpressed in the *H. glycines* susceptible line *G. max* [Williams 82/PI518671] to determine if the candidate gene engages the defense process. In contrast, RNAi has been conducted in the *H. glycines* resistant line, *G. max*[Peking/PI548402] to determine if the construct perturbs the defense process. Target gene expression occurring in the eGFP-expressing roots has been measured by quantitative PCR (qPCR) (Klink et al. 2009; Matsye et al. 2012; Pant et al. 2014; Sharma et al. 2016). RNA was isolated from those roots and reverse transcribed to make cDNA using the Superscript First Strand Synthesis Kit® (Invitrogen®). The cDNA has been reversed transcribed from RNA using oligo dT primer (Invitrogen®). The qPCR reaction has been assembled using 10 µl of a gene expression Master Mix® (Applied Biosystems®), 1 µl of forward (100 µM) and 1µl of reverse (100 µM) and 2 µl of probe (2.5 µM), 3 µl of template cDNA and 3 µl of nuclease free water. Each primer set has TaqMan® 6 carboxyfluorescein (6-FAM) probes with the Black Hole Quencher (BHQ1) (MWG Operon; Birmingham, AL). The total volume of 20 µl is pre-incubated in 50^{o} C for 2 min, followed by 95^{o} C for 10 min

which is preceded by alternating 95° C for 15 sec and 40 cycles of 60° C for 1 min. The control gene that has been used for the qPCR studies is the ribosomal protein gene s21 gene (Klink et al. 2005). The S21 provides the same expression determination as other control genes (Klink et al. 2005). The effect on fold expressions have been calculated statistically by using $2^{-\Delta\Delta C}{}_{t}$ (Livak and Schmittgen 2001). Statistical analysis has been done by calculating the p-value by using t-test (Yuan et al. 2006).

Nematode procurement/infection

The *H. glycines* [NL1-Rhg/HG-type 7/race 3] (*H. glycines*) population has been used in the experiments (Klink et al. 2009a; Matsye et al. 2012; Sharma et al. 2016). The *H. glycines* stock has been presented to the soil at a concentration of 2,000 J2s per pot (plant) (Sharma et al. 2016). After the conclusion of a 30-day infection period, the *H. glycines* cysts have been extracted from pots by dislodging the plants, gently massaging the roots and straining the material through 20 mesh sieves for debris and collected on 100 mesh sieves. These two filtration steps result in the collection of cysts. The number of cysts are counted in controls and experimentally treated plants that have the candidate gene overexpressed or suppressed by RNAi. After extraction, the female index (FI) is calculated (Golden et al. 1970). The FI = (Nx/Ns) X 100, where Nx is number of females in test cultivar and Ns is number of females in control (Golden *et al.* 1970; Klink *et al.* 2009; Sharma et al. 2016). All tests have been done in three replicates for each genetic line and its appropriate control that has incorporated 15 plants per replicate. The FI has been tested statically by using Mann-Whitney-Wilcoxon Rank–Sum Test, $p < 0.05$ (Matsye et al. 2012; Sharma et al. 2016).

Results

Identification of a *G. max* RIN4 homolog

The *G. max* genome has been examined to determine if it has homologs of the *A. thaliana* RIN4. To accomplish this objective, the conceptually translated amino acid sequence of the *A. thaliana* RIN4 (Genbank Accession AT3G25070) has been used in protein database searches of the *G. max* proteome using default parameters. Those searches identified four *G. max* accessions (**Table 2.2**). These accessions then have been used to determine if any of the GmRIN4 genes exhibit expression within *G. max* root cells undergoing the process of resistance to *H. glycines* parasitism. The identification of these accession then has been used to determine from prior gene expression experiments if the genes exhibited expression with in the cells that produce syncytia both prior to and during the resistant reaction.

Table 2.2 *G. max* accessions exhibiting homology to the *A. thaliana* RIN4 protein

Gene	Annotatation
GmRIN4-1	Glyma03g19920
GmRIN4-2	Glyma08g46400
GmRIN4-3	Glyma16g12160
GmRIN4-4	Glyma18g36000

Detection call methodology

Data produced in prior microarray analyses have been used to determine the gene expression that is occurring within syncytia experiencing a resistant reaction as well as pericycle cells from uninfected roots that had served as a control (Klink et al. 2010). The original microarray studies that had been performed used Affymetrix® microarrays did not have complete coverage of the *G. max* genome on their arrays since its genome had

yet to be sequenced (Klink et al. 2007, 2010; Schmutz et al. 2010). During these studies, a database had been generated that identified the soybean genes that had corresponding Affymetrix® probe sets. This database has been used in the analysis presented here to determine any of the GmRIN4 paralogs had corresponding Affymetrix® probe sets and if so, had measurable gene expression.

The genome accessions of the four GmRIN4 paralogs have been queried against the Affymetrix® database as described, resulting the determination that only one of the four accessions had a corresponding Affymetrix® probe set identifier (Gma.5142.1.S1_at) (Klink et al. 2010). From that information, analyses have been performed that extracted the gene expression data for GmRIN4-4. The results have determined that GmRIN4-4 has measurable gene expression in control cells as well as syncytia undergoing the process of defense in two different *H. glycines*-resistant *G. max* genotypes. From these results it was concluded that useful knowledge could be obtained from genetic engineering experiments of GmRIN4-4.

A functional analysis of GmRIN4-4 relating to resistance to *H. glycines* parasitism

Genetic constructs aimed at experimentally inducing the expression (overexpression) of the GmRIN4-4 have been made and genetically engineered into the

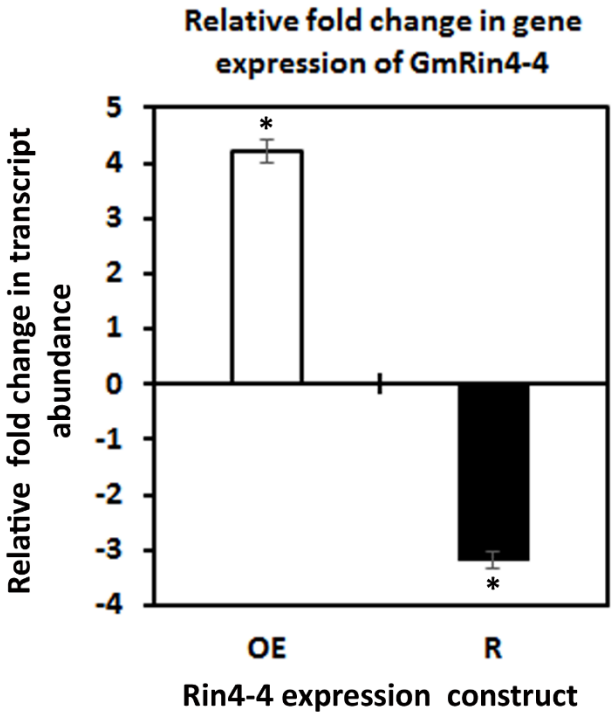

Figure 2.3 The effect that the genetic construction has on the relative transcript abundance of GmRIN4-4. * statistically significant $p < 0.05$.

H. glycines-susceptible genotype *G. max* [Williams 82/PI518671]. In contrast, the experimental suppression of GmRIN4-4 expression in the *H. glycines*-resistant genotype *G. max* [Peking/PI 548402] have been made. The effect that these constructs have on the relative transcript abundance of GmRIN4-4 have been determined, confirming the genetic constructs are functioning as they are supposed to (**Figure 2.3**).

Induced GmRIN4-4 expression decreases *H. glycines* parasitism

Plant resistance refers to the ability of plant to control pathogen infection, spread, growth, spread and their virulence effects by inducing non-host specific and host specific defense response (Uknes et al. 1992; Hammond-Kosack and Jones, 1996; Bent, 1996; Scheel, 1998; Collins et al. 2003; Jones and Dangl, 2006). However, the degree of resistance induced by plant could differ according to species, variety or genotype (Collins

et al. 2003; Jones and Dangl, 2006; Klink et al. 2010, Matsye et al. 2011; Matsye et al. 2012; Pant et al. 2014; Sharma et al. 2016). The plant becomes susceptible if it is unable to detect pathogen effectors or the induced defense response is inadequate (Hammond-Kosack and Jones, 1996). The overexpression study of multiple genes on *G. max* [Williams 82/PI 518671] results varying level of defense responses (Matsye et al. 2012; Pant et al. 2014; Sharma et al. 2016; Klink et al. 2017; Mcneece et al. 2018).

Analyses have been performed aiming at determining from functional experiments whether GmRIN4-4 exhibits characteristics of a resistance gene. To examine such a role, experiments have employed GmRIN4-4 overexpression that have been demonstrated to have the expected altered expression. In three replicate experiments, *G. max* plants engineered to overexpress GmRIN4-4 exhibit a decrease in *H. glycines* parasitism as revealed by the FI (**Figure 2.4**). Earlier studies show that the expression of a gene associated with vesicular membrane fusion induced the expression of the other associated genes as well (Sharm et al. 2016; Klink et al. 2017).

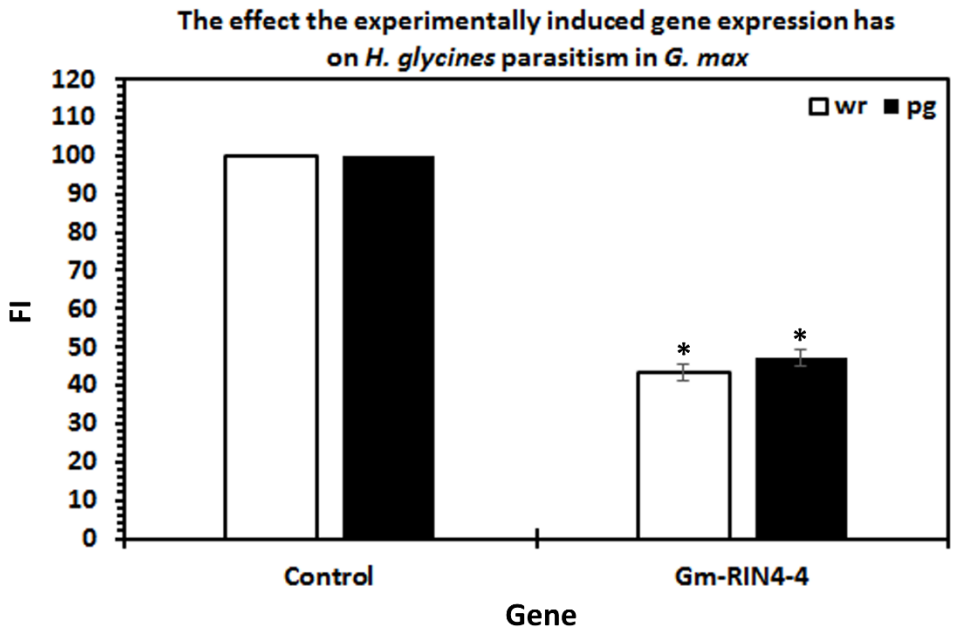

Figure 2.4 *G. max* genetically engineered to experimentally induce GmRIN4-4 expression in the *H. glycines* susceptible genotype *G. max* [Williams 82/PI 518671] impairs the susceptible reaction, leading to an incompatible reaction, $P < 0.05$. Please refer to methods for details

Suppressed GmRIN4-4 expression increases *H. glycines* parasitism

To compliment the overexpression experiments, analyses have been performed to suppress GmRIN4-4 expression in a *G. max* genotype that is normally resistant to *H. glycines* parasitism. In three replicate experiments, *G. max* plants engineered to suppress GmRIN4-4 exhibit an increase in *H. glycines* parasitism as revealed by the FI (**Figure 2.5**). The combination of outcomes presented here showing the experimental induction of GmRIN4-4 expression in the *H. glycines*-susceptible genotype *G. max*[Williams 82/PI518671] impairs parasitism, leading to a resistance outcome and in contrast, the experimental suppression of GmRIN4-4 expression in the *H. glycines*-resistant genotype *G. max*[Peking/PI 548402] impairs the resistant reaction, leading to a susceptible outcome is indicative that the gene functions in the defense process.

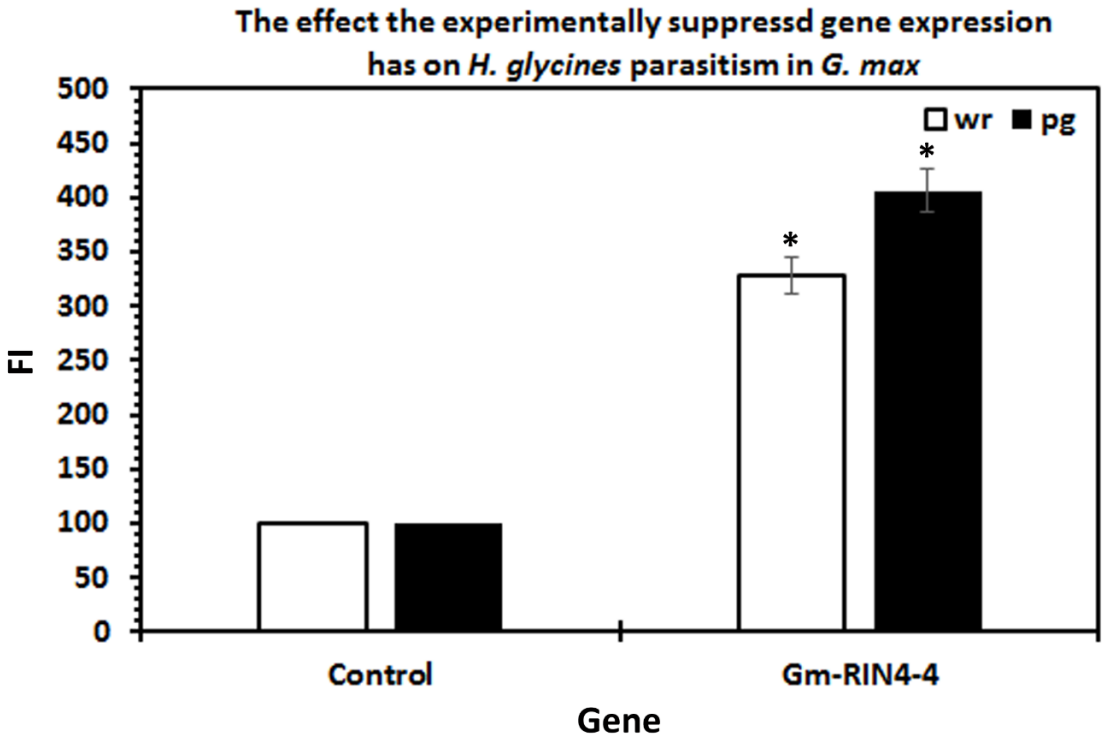

Figure 2.5 *G. max* genetically engineered to experimentally suppress GmRIN4-4 expression in the *H. glycines*-resistant genotype *G. max* [Peking/PI 548402] impairs the resistant reaction, leading to a susceptible outcome, $P < 0.05$. Please refer to methods for details

Discussion

Prior experiments have identified the importance of Gm-NDR1-1 to the defense process that *G. max* has toward *H. glycines* parasitism (McNeece et al. 2017). That work had been reinforced in experiments that revealed the bacterial elicitor harpin, which is known to induce the expression of NDR1, also induced the expression of Gm-NDR1 while functioning in the process of defense in *G. max* to several different genera of plant-parasitic nematodes (Aljaafri et al. 2017). These experiments have revealed the importance of the GmNDR1-1 receptor to parasitic nematode defense. Further experiments have revealed the scope of defense processes that GmNDR1-1 functions in when it had also been revealed to work in defense processes to the charcoal rot pathogen

M. phaseolina in *G. max* (Lawaju et al. 2018). The experiments presented here have aimed to understand the GmNDR1-1 receptor in more detail.

The experiments presented here have used information generated in the plant model genetic system *A. thaliana* to identify orthologs of the NDR1-interacting proteins (Century et al. 1995; Aarts et al. 1998; Day et al. 2006). These experiments began by focusing in on RIN4. Analyses of the *G. max* genome have identified RIN4, but it exists as a gene family having 4 paralogs (Klink et al. 2010). Subsequent analyses of gene expression data generated in *G. max* has first determined that only one of the four paralogs (GmRIN4-4) had corresponding probe sets fabricated onto the Affymetrix® Soybean Gene Chip ® (Klink et al. 2007, 2010). From these results, gene expression data had been extracted and used in the analysis presented here. The study of gene expression in *G. max* root cells by DCM shows that GmRIN4-4 is expressed in the same root cells where GmNDR1-1 is expressed (McNeece et al. 2017). This result is supporting previous studies performed in *A. thaliana* that show RIN4 is expressed within the cells where a defense response occurs (Day et al. 2006). In *A. thaliana* NDR1 induces ETI as a resistance response to its pathogens that occurs through an interaction with RIN4 (Century et al. 1995; Aarts et al. 1998; Coppinger et al. 2004; Day et al. 2006; Knepper et al. 2011; McNeece et al. 2017). Experiments have shown the CC-NB-LRR proteins such as RPM1, RPS2 and RPS5 require NDR1 protein to induce resistance in *A. thaliana* (Day et al. 2006). The membrane bound RIN4 proteins are the target of bacterial type III virulence effector AvRpt2 (Mackey et al. 2003). Consequently, the deactivation of RIN4 by bacterial effectors is an efficient way for the pathogen to disarm plant defense processes (**Figure 2.6)** (Mackey et al. 2002). RIN4 protein is required for the

accumulation and function of the RPM1 proteins but RPM1 is not required for the localization of RIN4 proteins (Mackey et al. 2002). Decrease in RIN4 proteins reduce the level of RPM1 proteins and resistance to pathogens (de Wit, 2002). Phosphorylation of

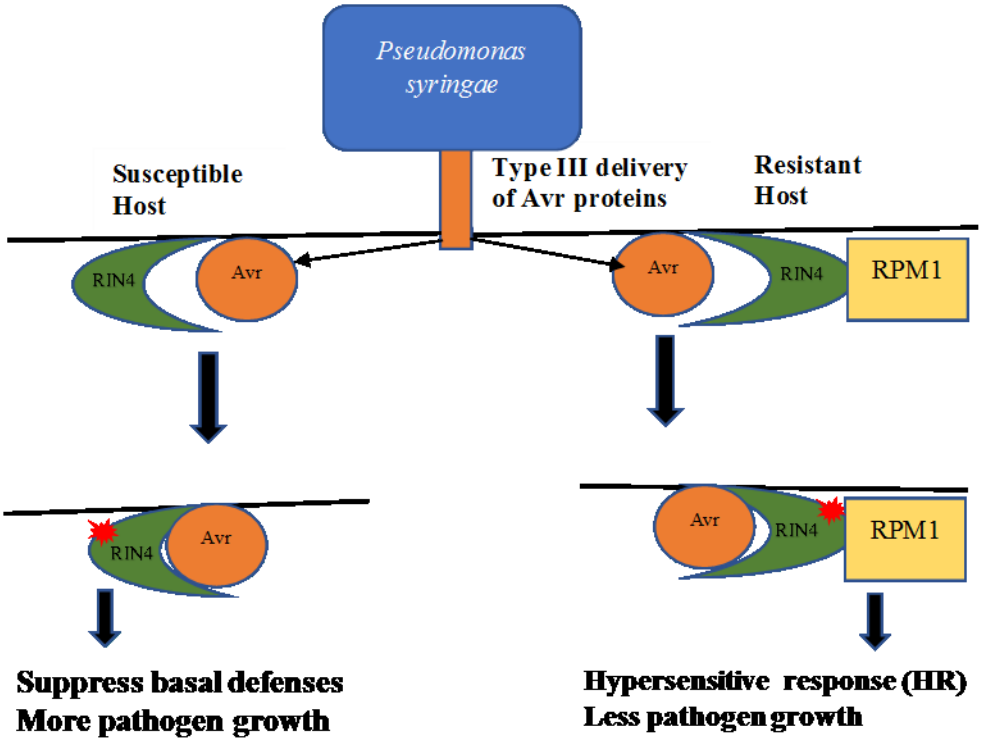

Figure 2.6 RIN4 and RPM1 mediated defense strategy.

Note: RIN4 is targeted by virulence TYPE III effectors (AvRpm1 or AvrB) and is guarded by RPM1 proteins. The effector proteins (orange) are circulated to plant cell through type III secretion syatem. In susceptible host lacking RPM1expression, the effectors bind and phophorelate RIN4 proteins (green) that suppress basal defense and induce more pathogen growth. In resistant host the type III effectors bind and phosphorelate RIN4 proteins that activates its binding with RPM1 proteins (yellow) and induce RPM1 mediated defense response such as HR (Adapted from Mackey et al. 2002).

the RIN4 by bacterial type III effectors induces the RPM1 dependent HR and defense responses (Figure 2.6) (Mackey et al. 2002). In susceptible host when *P. syringae* inject

avirulent proteins (Avr) and phosphorylate RIN4 proteins, it suppresses the basal defense and induce more pathogen growth whereas in resistance host, the effectors events are perceived by RPM1 protein that induces HR proving resistance (Figure:2.6) (Mackey et al. 2002; de Wit, 2002). According to Flor (1971) states that "for each gene that conditions resistance in host there is a corresponding gene that conditions pathogenicity in the pathogen". Previous studies show that RIN4 being the target of bacterial Type III effectors have varying roles switching its gear from pathogenicity to resistance in the presence and absence of RPM1 proteins (Mackey et al. 2002). Our study reveals that their overexpression suppresses SCN population by inducing the incompatible reaction, however, more detail study is needed regarding the interaction of various hosts genotype and pathogens effectors. The resistance response engaged by proteins like RPS2 is negatively regulated by RIN4, suggesting that RPS2 based resistance pathways are induced in the absence of RIN4 proteins (Mackey et al. 2003; Axtell and Staskawicz, 2003). Whereas studies show that the AvrRpt2 does not require RIN4 for its virulence function suggesting that it is not only the target (Belkhadir et al. 2004). However, various defense strategies in *A. thaliana* are induced by the interaction of NDR1 and RIN4 proteins (Day et al. 2006). These effects have been determined in experiments showing the AvrRpt2-induced bacterial growth in *rin4* mutants and, in contrast, *RIN4* overexpression suppressed the bacterial growth, suggesting RIN4 plays important roles in plant defense (Belkhadir et al. 2004). In *A. thaliana*, the RIN4 protein is associated with the exocyst subunit EXO70 (Sabol et al. 2017). This observation indicates that components of the whole exocyst may function in *G. max* defense to *H. glycines*. This hypothesis is the focus of Chapter III. Supporting this hypothesis, co-

immunoprecipitation assays and confocal microscopy experiments performed in *A. thaliana* have shown that RIN4 interacts with the exocyst subunit EXO70B1, recruiting it to the cell membrane (Sabol et al. 2017).

In contrast, in the absence of RIN4, EXO70 is localized in the cytoplasm and nucleus (Sabol et al. 2017). Other experiments have shown RIN4 proteins weakly interact with EXO70B2, while an interaction with other exocysts subunits is not clear (Sabol et al. 2017). Furthermore, EXO70B1 co-localizes with membrane protein syntaxin121 (SYP121) in the plasma membrane (PM) (Sabol et al. 2017). This observation is important because the *A. thaliana* SYP121 is PEN1, a gene identified by mutational studies shown to function in preventing penetration of the fungal pathogens *Blumeria graminis* f. sp. *hordei*, *Erysiphe cichoracearum*, *Golovinomyces orontii* into *A. thaliana* leaves (Collins et al. 2003). These results relate directly to the demonstration of GmSYP121 in defense in *G. max* (Sharma et al. 2016). However, SYP121 does not recruit EXO70B1 to the PM (Sabol et al. 2017). Consequently, the results indicate other proteins and maybe other EXO70-like proteins are involved in this complex process. However, RIN4 appears to play a major role in the recruitment of the exocyst to the cell membrane, a process mediated through EXO70B1(Sabol et al. 2017). To examine this process further, the *G. max* exocyst components have been identified and studied in functional experiments with the aim of determining whether individual exocyst genes exhibit defense functions in *G. max* to combat *H. glycines* parasitism.

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CHAPTER III

THE EXOCYST FUNCTIONS IN *GLYCINE MAX* DEFENSE AGAINST *HETERODERA GLYCINES*

Abstract

The exocyst, an octameric protein complex, plays important roles in exocytosis, thereby directing SNARE-mediated membrane fusion. The exocyst complex acts as a receiver for various signaling pathways, helping to tether vesicles at the receptor membrane and mediating fusion by inducing the formation of the SNARE assembly apparatus. The exocyst complex is located at the target site, helping prepare SNARE for docking and subsequent release of vesicular contents after fusion. The exocyst complex connects with Sec1p/Munc 18 and the t-SNARE Sec9p (SNAP-25) for tethering and fusion of the secretory vesicles. These subunits are coiled-coil proteins, sharing some structural homology with helical bundles that help them interact to promote complex formation. The exocyst complex is a rod-shaped structure with C and N termini occurring at opposite poles, assisting in tethering the vesicles to the plasma membrane and delivering cargos packed in vesicles to the apoplast. The exocyst connects vesicles, with its Sec10 and Sec15 subunits attach to the plasma membrane with Sec3 and Exo70 subunits. Sec3 and Exo70 bind to phosphatidylinositol 4, 5-biphosphate (PI (4, 5) P2) located in the plasma membrane. The movement of vesicles is regulated by Sec4, which encodes a small GTP-binding protein, directs the vesicle to the plasma membrane at the targeted site. Sec4p regulates the assembly of the exocyst through its interaction with

Sec15p. With the demonstration that GmRIN4-4 functions in defense in the *G. max-H. glycines* pathosystem, the functional developmental genomics study presented here is concentrated on identifying the role of exocyst genes has during defense in the *G. max-H. glycines* pathosystem.

Introduction

The secretion system is involved in the *G. max* defense process to *H. glycines* parasitism (Matsye et al. 2012). This conclusion has been determined through transcript mapping of the major resistance locus*, rhg1,* followed by functional studies (Matsye et al. 2011, 2012). The work has led to the demonstration of α-SNAP being present within the locus and functioning in defense (Matsye et al. 2011, 2012). The result has provided important insight into the mechanism of how the defense response functions in this pathosystem. Furthermore, the results have indicated that the defense response that *G. max* has to *H. glycines* parasitism exhibits commonalities to the vesicle transport system identified in *A. thaliana* (Collins et al. 2003). In a broader context, these observations relate to the original experiments that identified genetically in *S. cerevisiae* the components that function in secretion (Novick et al. 1980). In those experiments, the stepwise process of secretion has been shown to be driven by membrane fusion and the secretion related genes (Novick et al.1980, 1981; Esmon et al. 1981; Kaiser and Schekman, 1990). Homologous genes have since been shown to be present in all eukaryotes (Clary et al. 1990; Griff et al. 1992; Gerst, 1997; Payne et al. 2000; Sanderfoot et al. 2000, 2001; Hong et al. 2004; Babcock et al. 2004; Rodríguez et al. 2011). The results have been expanded on in the *G. max-H. glycines* pathosystem,

showing other SNARE components also functioning in the defense process (Matsye et al. 2012; Pant et al. 2014; Sharma et al. 2016).

The identification of secretion functioning in plant defense

The membrane fusion apparatus is composed of two main components. One component is consisting of membrane-bound proteins called the Soluble NSF Attachment Protein Receptor (SNARE) (**Table 3.1**, **Figure 3.1**). SNARE includes syntaxin (SYP)/Suppressor of sec1 (SSO1), a gene homologous to *A. thaliana* PEN1 (Aalto et al. 1993; Collins et al. 2003). Other SNARE components include synaptobrevin (SYB)/YKT6/SEC22 and SNAP-25/SEC9 (Oyler et al. 1989; Baumert et al. 1989; Bennett et al. 1992; Aalto et al. 1993; Sogaard et al. 1994; McNew et al. 1997). Also, additional other SNARE proteins include mammalian uncoordinated-18 (MUNC18/SEC1), (i.e., SM) which may facilitate or inhibit membrane fusion and synaptotagmin (SYT)/Tricalbin-3 (TCB3) which may serve a calcium-sensing role (Burkhardt et al. 2008; Südhof and Rothman, 2009). The aggregate role of the SNARE proteins is to tether the vesicle to the target membrane, SNARE metabolism including its disassembly which is mediated by α-SNAP/Sec17p and the ATPase N-ethylmaleimide-sensitive factor (NSF)/Sec18p (Novick and Schekman, 1980; Jahn and Fasshauer, 2012). The entire SNARE complex, including α-SNAP/Sec17p and NSF/Sec18p, can be isolated biochemically as part a larger 20 S particle that mediates secretion (Söllner et al. 1993a, b). Complimentary studies in animal systems investigating pathogenesis have identified botulinum and tetanus microbial neurotoxin effectors that target SNARE components and thus inhibit secretion and resulting in paralysis (Schiavo et al. 1992a, b, 1994; Pellegrini et al. 1995; Chai et al. 2006; Jin et al. 2006; Strotmeier, 2012; Bennett et al. 2013).

Similar types of effectors have also been identified in plants leading to impaired functionality of 20 S components during defense, confirming the importance of the plant secretion system in the process of defense (Barszczewski et al. 2008; Matsye et al. 2011, 2012; Bekal et al. 2015; Sharma et al. 2016).

Table 3.1 SNARE genes

yeast	Reference	mammal	Reference	*A. thaliana*	Reference	*G. max*
SSO1	1	STX	8	KNOLLE/PEN1*	15, 16, 17	23, 24
SEC1	2	MUNC18	9	KEULE	15, 18	24
SEC9	3	SNAP-25	10	SNAP33	15, 19	24
SEC17	4	α-SNAP	11	α-SNAP	15, 17	24, 25
SEC18	5	NSF	12	NSF	15, 20	24
SEC22	6	VAMP-1	13	VAMP	15, 21	24
TCB3	7	SYT	14	SYT	15, 22	24

Footnote: suppressor of sec1 (SSO1); secretion (SEC); tricalbin-3 (TCB3); mammalian uncoordinated-18 (MUNC18); synaptosomal-associated protein 25 (SNAP-25); alpha-soluble N-ethylmaleimide-sensitive fusion protein associated protein (α-SNAP); N-ethylmaleimide-sensitive fusion protein (NSF); synaptobrevin (SYB); synaptotagmin (SYT); PENETRATION1/syntaxin121 (PEN1/SYP121); synaptosomal-associated protein 33 (SNAP33); vesicle associated membrane protein (VAMP). References: 1, Aalto et al. 1993; 2, Aalto et al. 1991; 3, Brennwald et al. 1994; 4, Griff et al. 1992; 5, Eakle et al. 1988; 6, McNew et al. 1997; 7, Creutz et al. 2004; 8, Bennett et al. 1992; 9, Hata et al. 1993; 10, Oyler et al. 1989; 11, Clary et al. 1990; 12, Wilson et al. 1989; 13, Trimble et al. 1988; 14, Perin et al. 1991; 15, Arabidopsis Genome Initiative, 2000; 16, Lukowitz et al. 1996; 17, Collins et al. 2003; 18, Assaad et al. 2001; 19, Heese et al. 2001; 20, Tanabashi et al. 2018; 21, Kwon et al. 2008; 22, Schapire et al. 2008; 23, Pant et al. 2014; 24, Sharma et al. 2016; 25, Matsye et al. 2012. * Wilson et al. 1992; Sollner et al. 1993a. (adapted from Sharma et al. under review).

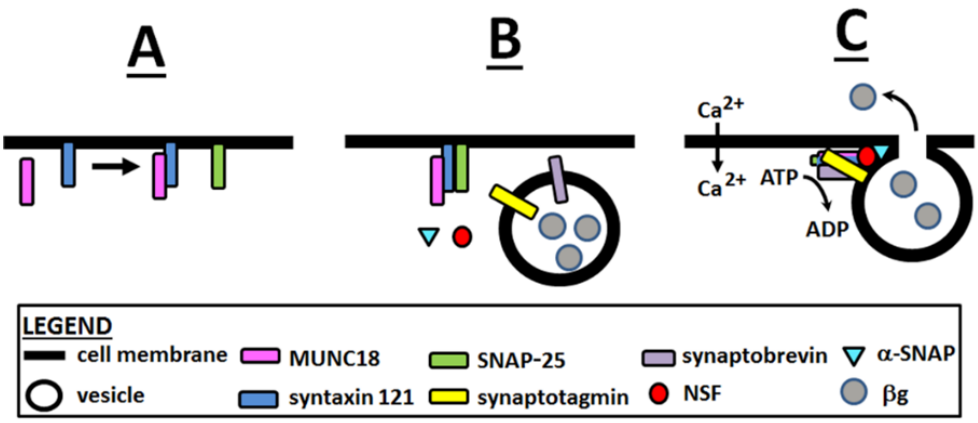

Figure 3.1 The 20 S particle and Beta-glucosidase cargo protein (adapted from Jahn and Fasshauer, 2012; Sharma et al. 2016).

The understanding of *G. max* defense to *H. glycines* is incomplete

Prior experiments performed in the *G. max*-*H. glycines* pathosystem have identified a list of 1,789 genes as being expressed specifically in the root cells undergoing the process of defense (Matsye et al. 2011). Among these genes is the *G. max* homolog of Sec4 (GmSec4). The *S. cerevisae* Sec4p is a Rab GTPase regulates the assembly of the exocyst through its interaction with Sec15p (Guo et al. 1999; Mizuno-Yamasaki et al. 2012). Experiments in *G. max* have shown that overexpressing GmSec4 in the *H. glycines* susceptible genotype *G. max* [Williams 82/PI518671] leads to impaired parasitism (Klink et al. 2017). However, RNAi-driven experiments had not been presented. These observations indicate the exocyst likely also functions in defense, but this aspect of secretion has remained to be examined in detail experimentally.

Several lines of evidence point toward the involvement of the exocyst functioning during the defense response that *G. max* has toward *H. glycines*. These lines include the genetic and transcriptional mapping and functional tests of α-SNAP (Matsye et al. 2011, 2012). Recent experiments performed in *S. cerevisiae* continue to reveal the central role

that Sec17/α-SNAP has in both membrane fusion and recycling of SNARE (Zick et al. 2015, Song et al. 2017; Schwartz et al. 2017; Harner and Wickner, 2018). These observations implicate the involvement of the 20 S particle in defense, furthermore, additional 20 S components of the SNARE complex, including the *G. max* Sec9 homolog SNAP-25, also function in defense **(Figure 3.1**) (Sharma et al. 2016).

As described in Chapter II, more recent experiments have demonstrated a general role that the *G. max* NDR1 has in defense to different plant-parasitic nematode species and that its transcription is induced by harpin (McNeece et al. 2017; Aljaafri et al. 2017). These experiments relate directly to the results presented in Chapter II and the hypothesis presented here of the involvement of the exocyst in the defense response that *G. max* has to *H. glycines* parasitism. Furthermore, as shown in Chapter II, *G. max* homologs of RIN4 (GmRIN4-4) which binds EXO70 in *A. thaliana* are expressed within the cells undergoing the defense process to *H. glycines* (Klink et al. 2010; Matsye et al. 2011; McNeece et al. 2017). As will be shown in Chapter III, components of the exocyst are expressed during the process of defense that *G. max* has toward *H. glycines* parasitism. Related observations have been a good measure of the genes having a role in defense (Matsye et al. 2011, 2012; Sharma et al. 2016; McNeece et al. 2017; Klink et al. 2017). Consequently, this study aims at determining whether the exocyst functions during *G. max* defense to *H. glycines* parasitism using already identified genes and proven methods in the study of the exocyst genes.

The *G. max* exocyst complex and defense response

Exocytosis, an evolutionary conserved biological event is possible due to the fusion of secretory vesicles with the targeted membrane (Novick et al. 1980; He and Guo,

2009; Heider and Munson, 2012). The process also allows the cell to carry out various cellular processes such as driving cell polarity, growth, division, cell migration, ciliogenesis and autophagy (Novick et al. 1980; He and Guo, 2009; Heider and Munson, 2012). The initial interaction of the vesicle and the target membrane occurring before fusion is called vesicle tethering, an event that is mediated by an octameric protein complex called exocyst (TerBush et al. 1996; Guo et al. 1999a; He and Guo, 2009). The exocyst complex consists of eight subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (**Figure 3.2**) (TerBush et al. 1995, 1996; Hsu et al. 1996; Guo et al. 1999a; Lipschutz and Mostov, 2002). These subunits are coiled-coil proteins and share some structural homology with helical bundles that help them interact during complex formation (Haarer et al. 1996; TerBush et al. 1996). The exocyst complex is a rod-shaped structure with C and N termini occurring at opposite poles and help in tethering the vesicles to the plasma membrane and delivering cargos packed in vesicles to the apoplast (TerBush and Novick, 1995; Guo et al. 1999a; Hamburger et al. 2006; He and Guo, 2009; Croteau et al. 2009; Yamashita et al. 2010; Picco et al. 2017).

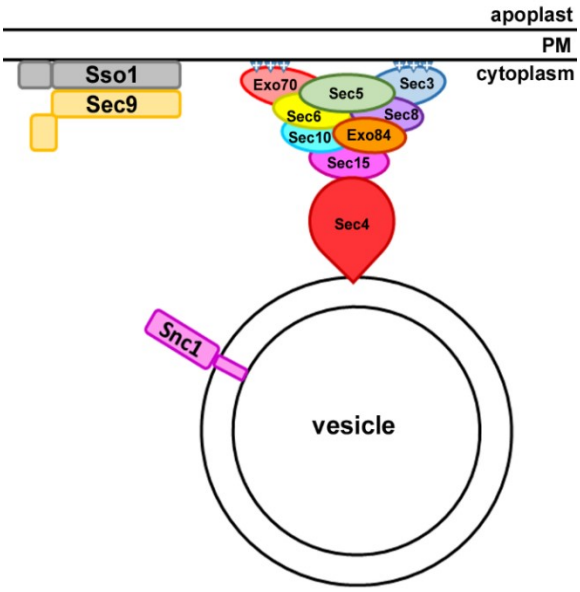

Figure 3.2 The exocyst complex.

Note: The exocyst is composed of 8 subunits, including Exo70, Exo84, Sec3, Sec5, Sec6, Sec8, Sec10 and Sec15. Sso1 and Sec9 (Snap25) are target membrane SNARE proteins that bind Synaptobrevin homolog 1 (Snc1) to bring target and vesicle membranes closer together. Sec4 is a vesicle membrane, Ras-related, GTPase that binds the exocyst. Exo70 and Sec3 bind to the target membrane by the positively charged residues of PI (4, 5) P2 (shown as blue triangles with white +) (Adapted from He and Guo, 2009).

The exocyst connects vesicles with Sec10 and Sec15 and the plasma membrane with Sec3 and Exo70 (Roth et al. 1998; Finger et al. 1998; Guo et al. 1999; Boyd et al. 2004; He and Guo, 2009). Sec3 and Exo70 bind to phosphatidylinositol 4, 5-biphosphate (PI (4, 5) P2) located in the plasma membrane (He et al. 2007; Liu et al. 2007; Zhang et al. 2008). The movement of vesicles is regulated by Sec4 that encodes a small GTP-binding protein and are directed to the plasma membrane at the targeted site (Salminen and Novick, 1987; Bourne, 1988; Goud et al. 1988; Walworth et al. 1989). In *S. cerevisiae*, the Sec4p regulates the assembly of exocyst through its interaction with Sec15p (Guo et al. 1999; Mizuno-Yamasaki et al. 2012). The vesicles that are at the targeted sites fuse to the target membrane with the help of SNARE proteins (Jahn and

Scheller, 2006). The exocyst complex, located at the target site help prepare SNARE for docking and subsequent release of vesicular contents after fusion (TerBush and Novick, 1995). The complex connects with Sec1p/Munc18 and the t-SNAREs Sec9p (SNAP-25) for tethering and fusion of the secretory vesicles (Wiederkehr et al. 2004; Sivaram et al. 2005). During this process the proteins located at vesicles membranes, the SYB also known as VAMP assembles with membrane proteins SYP and SNAP-25, forming a ternary complex (Trimble et al. 1988; Baumert et al. 1989; Oyler et al. 1989; Bennett et al. 1992; Hanson et al. 1997). These proteins are the receptors for NSF and SNAPs and are called SNAREs (Söllner et al. 1993a, 1993b).

These SNARE proteins, located on vesicles (v-SNARE) interact with proteins at the targeted membrane (t-SNARE) and help in fusion (Söllner et al. 1993a; Rothman and Warren, 1994). SNAREs that are aligned parallel to their transmembrane anchor during docking and connect two membranes thereby zippering of v-SNARE and t-SNARE (Otto et al. 1997; Hanson et al. 1997). The ATPase activity of NSF dissociates the ternary SNARE complex leading to the conformational change of associated proteins and induce fusion of secretory vesicle to the target membrane (Söllner et al. 1993a; Hayashi et al. 1995; Hanson et al. 1997). This activity of NSF could be to dock new secretory vesicles thereby recruiting new SNARE complexes (Hanson et al. 1997).

The importance of exocyst in cellular processes is inevitable as it plays a key role in exocytosis, thereby mediating SNARE-mediated membrane fusion (He and Guo, 2009). The exocyst complex acts as a signal receiver for various signaling pathways, helping tether vesicles at the receptor membrane and mediate fusion by inducing the formation of SNARE assembly (He and Guo, 2009; Žárský et al. 2013). Various

experiments have been done to prove its efficacy for growth, migration, repair, and defense by increasing or decreasing proteins, breaking the association among the subunits and its associated proteins that are necessary for this process (Novick et al. 1980; Hala et al. 2002; He et al. 2007; Zhang et al. 2008).

In the experiments presented here, *G. max* homologs of the exocyst complex have been identified, and their expression pattern determined. Candidate genes have been selected based on whether they exhibit gene expression in control cells or during the defense process. Based on those results, candidate genes have been cloned and engineered for overexpression or RNAi analyses.

Materials and methods

Gene selection and cloning

All methods have been performed according Sharma et al. (2016), described in Chapter II. Exocyst genes have been selected for cloning by examining the gene expression data of Klink et al. (2010). Gene sequences from the selected candidate genes have been downloaded from the *G. max* genome database (Goodstein et al. 2012). Candidate defense gene cloning and qPCR primers have been designed in a manner described in Chapter II. PCR primer sequences are presented (**Table 3.2**).

Table 3.2 PCR Primers

GENE	ACCESSION	PRIMER TYPE	FORWARD	REVERSE
Sec3-1	Glyma04g03710	OE	CACCGTTAGATCGATTTGAGATCAGATAGAAG	GGAAAGAAGGCATGTGTATAAACCT
		RNAi	CACCGTTAGATCGATTTGAGATCAGATAGAAG	ACCAAACAGGAGGCGATTT
Sec5-1	Glyma10g36120	OE	CACCCCTCAGATCTAAAATCACACCCA	GCAAAGAATGCAAATTTTCATTAAC
		RNAi	CACCCCTCAGATCTAAAATCACACCCA	TGAAGCCACAAATTTTTGAGG
Sec6-5	Glyma03g03120	OE	CACAATGATGGCTGAGGATCT	GTAGGGTATGAGAATGACGACTTCA
		RNAi	CACAATGATGGCTGAGGATCT	CAATTTCTTTGTCATCGCTCAA
Sec8-1	Glyma10g35190	OE	CACCCGTTTTCGATTCGTTCTTCCC	CAGAAATCTAATTGATGCAAGCACC
		RNAi	CACCCGTTTTCGATTCGTTCTTCCC	AGCGGCAGCATCACGG
Sec10-2	Glyma16g01660	OE	CACCGATTCCTTCCGTGATGAGAGAG	TGGCTGTGGTAGTGGTGATACTAG
		RNAi	CACCGATTCCTTCCGTGATGAGAGAG	CTCAGCAAGTGTCTTCCGATG
Sec15-1	Glyma02g19110	OE	CCCTAAGCTACTTTTCATGCTTC	ATACATCAATTATACCCTACTTCCCA
		RNAi	CCCTAAGCTACTTTTCATGCTTC	AGCTCCAACACCTGTTATACAGTTCT
Sec15-5	Glyma14g00390	OE	CACCATGTGGGAGAAGGGAAGTACT	GGAAGACAGTGATAGCCTGGC
		RNAi	CACCATGTGGGAGAAGGGAAGTACT	AGGTGGTGGTGGAGGGTCT
EXO84-4	Glyma07g34880	OE	CACCGAGGTGAACAGAGTGAGAAAAAGG	GCAAGGGCAAATTTTAAATAATGTAG
		RNAi	CACCGAGGTGAACAGAGTGAGAAAAAGG	GCCAAAAGTACATCAATGGTTTC
EXO70-A1	Glyma20g33590	OE	CACCTGGTTCTCTGAGAAGATTGAGCTTC	TTTCAGCCACCAAATACAACCTC
		RNAi	CACCTGGTTCTCTGAGAAGATTGAGCTTC	CCGCACCTTCGAAGTCCT
EXO70-B1	Glyma02g39790	OE	CACCTATGGTCTCTGCTCTCTGCCTT	AGAACAACTAGTAGCAAGCTTCAACTTC
		RNAi	CACCTATGGTCTCTGCTCTCTGCCTT	GCATCTGCGCCTTTCTCA
EXO70-D2	Glyma07g04600	OE	CACCATGGAGAGCCTCCCGCTT	TCATTCAGCTCTCCTTCTCAAGTG
		RNAi	CACCATGGAGAGCCTCCCGCTT	GAGCGCTGGATTTCGTCG
EXO70-D3	Glyma16g01190	OE	CACCATGTCCCACTCCCACAGGG	TCCCATTGAATCCATCATTCAGA
		RNAi	CACCATGTCCCACTCCCACAGGG	GGACCGTTGGATTTCGTCC
EXO70-E1	Glyma08g26920	OE	CACCGTTGATTATTGTTTGTTGAAGTTTGG	GAGAACAGCATTATTCTTGCCC
		RNAi	CACCGTTGATTATTGTTTGTTGAAGTTTGG	ACCACCGCCATCACACAATTAT
EXO70-F1	Glyma05g03310	OE	CACCTTGCTTTCACACCAATCTCAGAC	CCAAGTAGAAATACACATGACACAGG
		RNAi	CACCTTGCTTTCACACCAATCTCAGAC	AGAGAGGCATCAGCGAGAATC
EXO70-G1	Glyma17g29210	OE	CACCCACCACACCGATTTGGAATC	ACAGTAGCCATCCATCTGATGAG
		RNAi	CACCCACCACACCGATTTGGAATC	ATTGTCTCCCAGGAACCTCAGA
EXO70-H7	Glyma11g15420	OE	TCGGCTTCCCATCGCTCTAATCG	TCACCGTCGATTAGAGCGATGGGAA
		RNAi	TCGGCTTCCCATCGCTCTAATCG	GATTTCGTCGTCGTAATCGGAGACGC

Nematode procurement/infection

The *H. glycines* [NL1-Rhg/HG-type 7/race 3] has been used in the experiments as described in Chapter II (Klink et al. 2009; Matsye et al. 2012; Sharma et al. 2016). The *H. glycines* stock is presented to the soil at a concentration of 2,000 J2s per pot (plant) (Sharma et al. 2016). FI has been calculated as described in Chapter II.

Plant transformations and qPCR

Plant transformation procedures have been described in Chapter II. The expected influence of the expression constructs on gene expression has been confirmed by qPCR. The qPCR primer sequences are provided (**Table 3.3**).

Table 3.3 qPCR primers for exocyst genes

GENE	ACCESSION	FORWARD	REVERSE	PROBE
Sec3-1	Glyma04g03710	CGAAGCACAAGGTCGTTCTC	GAACTTTGGCTTTGGTGCAG	GCACTTGGGGAAAAACTGCTAAGCTTGG
Sec5-1	Glyma10g36120	ATGTCAACTACGGCGGCAA	TTCGGAATCGTCGTCGTCG	GGCAAACTACGTCCAGCCGCTGAAGA
Sec6-5	Glyma03g03120	CCTAAACACAACCTTGAAGGATGTA	AGTTAACCTCTCGTAAGTGTTGACA	CAGCAGAGGCTCGGGATTCTTTGAGC
Sec8-1	Glyma10g35190	ATACCAACCACCACTGCTGT	CACAGATGCTGGCCTATACGAT	CCTCTGTCTCGAAGAACAAGATCACTCAAAGG
Sec10-2	Glyma16g01660	ATAACAAGCCCTCTAAAGCCG	CGTCGGAAGAAGCTCGTTG	TCTCGACGTCGACGATTTCAAGGGAGAC
Sec15-1	Glyma02g19110	GGTGTTATGGAGAACAGTGATGG	CATATATCAGCTGGTGAAGCAGC	GATGTTGGTCCTCTTGTCAGGCTTGCC
Sec15-5	Glyma14g00390	GTGGGAGAAGGGAGTACTGA	GCAAATGGCGGAGGAGAG	ACGACGATGCTCTCCTCCAAACCC
EXO84-4	Glyma07g34880	TGATGTTTCTGAAATTCAGCAAGAAC	TCTAATGCTTCTAATGTTTCTTCAAACTT	CCTTGAGCCCTTACCAAATGAGAGAAATGACA
EXO70-A1	Glyma20g33590	TGGGCAGATTCTTCAGTGC	GAATGCCTTAAACCTGTCTTTCAC	GTGGTGACAGTGGAACTGGAAGCAGTAG
EXO70-B1	Glyma02g39790	CTACTGGAACGGCGAGTCA	ATGACGATATCGAAGCCGGT	AAGAGGAAGCGAGAAACGGAGGAGGAG
EXO70-D2	Glyma07g04600	CCGTCTCCTCCGACAAAGTTA	GGAGGGGTCGAAGGGGTTGGT	GCCCGCTTAGAAGACGAGTTCCGC
EXO70-D3	Glyma16g01190	ATGTCCCACTCCCACAGG	GCGGGAGGCTCTCCATTATT	GCACATACGCAACGCAATTTTACCCAACAAAAC
EXO70-E1	Glyma08g26920	GTAAGGCATATTGTGAAGGCACT	TCATCCTTCCCTTGCCCTT	CTCCATGTCCGTACCTAGTGAGAAGGAGG
EXO70-F1	Glyma05g03310	AGAAGACCTCGACCGCTT	TATGATCTCGTCGACGGCA	TCTCTCCGGCGAGCCTTCGC
EXO70-G1	Glyma17g29210	AGTTGAAGAATCTTCGCGAGTC	GGCACACTATTCTCACTCAACAG	GGAGCTTGATGGAGGGTTGCTGGATG
EXO70-H7	Glyma11g15420	TCTTCACATCAACGCCCAC	GCTGAAAAGTTGCGTTGTTGT	CCGTCAACGCACTTTCTCAGACTCGT

Results

The identification of *G. max* exocyst homologs

The *G. max* genome has been examined for the presence of its exocyst genes using the same procedure used to identify GmRIN4-4. An examination of the *G. max* genome identified 5 Sec3 genes, 2 Sec5 genes, 5 Sec6 genes, 2 Sec8 genes, 2 Sec10 genes, 6 Sec15 genes, 34 Exo70 genes and 8 Exo84 genes (**Table 3.4**) (Klink et al. 2007; 2010). From these data, exocyst genes that exhibit expression within the syncytium have been chosen for further study (**Table 2**).

Table 3.4 The exocyst gene family of *G. max*.

Gene	*G. max* accession	Gene	*G. max* accession	Gene	*G. max* accession
Sec3-1	Glyma04g03710	EXO84-4	Glyma07g34880	EXO70-D1	Glyma11g15420
Sec3-2	Glyma09g18840	EXO84-5	Glyma08g23840	EXO70-D1	Glyma06g22160
Sec3-3	Glyma09g18813	EXO84-6	Glyma10g04516	EXO70-D1	Glyma04g32420
Sec3-4	no accession*	EXO84-7	Glyma13g18766	EXO70-D1	Glyma15g04750
Sec3-5	Glyma17g36540	EXO84-8	Glyma20g02670	EXO70-D1	Glyma13g40680
Sec5-1	Glyma10g36120	EXO70-A1	Glyma20g33590	EXO70-D1	Glyma03g33160
Sec5-2	Glyma20g31490	EXO70-A1	Glyma10g34000	EXO70-D1	Glyma19g35880
Sec6-1	Glyma01g33866	EXO70-A1	Glyma13g05044	EXO70-D1	Glyma10g05280
Sec6-2	Glyma03g03015	EXO70-A1	no accession*	EXO70-D2	Glyma07g04600
Sec6-3	Glyma03g03050	EXO70-B1	Glyma14g37840	EXO70-D3	Glyma16g01190
Sec6-4	Glyma03g03063	EXO70-B1	Glyma02g39790	EXO70-E1	Glyma08g26920
Sec6-5	Glyma03g03120	EXO70-B1	Glyma02g07220	EXO70-F1	Glyma05g03310
Sec8-1	Glyma10g35190	EXO70-B1	Glyma17g13900	EXO70-G1	Glyma17g29210
Sec8-2	Glyma20g32370	EXO70-B1	Glyma07g04600	EXO70-G1	Glyma14g17690
Sec10-1	Glyma07g05160	EXO70-B1	Glyma16g01190	EXO70-G1	Glyma07g00603
Sec10-2	Glyma16g01660	EXO70-B1	Glyma05g03310	EXO70-G1	Glyma08g23790
Sec15-1	Glyma02g19110	EXO70-B1	Glyma19g26830	EXO70-H1	no accession*
Sec15-2	Glyma03g22300	EXO70-B1	Glyma18g50160	EXO70-H1	Glyma19g35880
Sec15-3	Glyma03g37331	EXO70-B1	Glyma16g05710	EXO70-H2	Glyma12g08020
Sec15-4	Glyma10g13870	EXO70-B1	no accession*	EXO70-H3	Glyma10g05280
Sec15-5	Glyma14g00390	EXO70-B1	Glyma08g26920	EXO70-H4	Glyma03g33160
Sec15-6	Glyma16g09730	EXO70-B1	Glyma02g39780	EXO70-H5	Glyma15g04750
EXO84-1	Glyma01g04650	EXO70-B1	Glyma10g44570	EXO70-H6	Glyma13g40680
EXO84-2	Glyma02g02910	EXO70-D1	Glyma10g23810	EXO70-H7	Glyma11g15420
EXO84-3	Glyma07g00570	EXO70-D1	Glyma12g08020	EXO70-H8	Glyma13g40690

Footnote: In yellow are presented the genes that have been used in genetic engineering studies. * no accession means that the gene had not been identified in the original annotation of 2009 while the gene had been identified in a subsequent annotation in 2015. For the purposes of the study, the identification of the gene in a subsequent annotation had no bearing on the analysis presented in Chapter III since genes selected for study were only selected if gene expression data could be obtained which was done by using the original Affymetrix® microarray analyses performed by Klink et al. (2007, 2010).

G. max exocyst genes are expressed within nematode feeding sites undergoing defense

The *G. max* accessions have been queried against the database that identified which *G. max* genome accessions also had Affymetrix®. A summary of the genome accessions having corresponding Affymetrix® probe set identifiers are presented (**Table 3.5**). The gene expression that has been measured is also presented (**Table 3.5**).

Table 3.5 The exocyst genes examined in the proposed study.

Gene	*G. max* accession	Affymetrix	control-0*	3	6
Sec3-1	Glyma04g03710	Gma.6597.1.S1_at	N/M	N/M	N/M::M
Sec3-2	Glyma09g18840	GmaAffx.1023.1.S1_at	N/M	N/M	N/M
Sec5-1	Glyma10g36120	GmaAffx.2818.1.S1_at	M	M	M
Sec6-5	Glyma03g03120	GmaAffx.76206.2.S1_at	N/M	N/M	N/M::M
Sec8-1	Glyma10g35190	GmaAffx.82992.1.S1_at	N/M	N/M	M
Sec10-2	Glyma16g01660	GmaAffx.19843.1.S1_at	N/M	M	M
Sec15-1	Glyma02g19110	GmaAffx.60749.1.A1_at	N/M	N/M	M
Sec15-4	Glyma10g13870	GmaAffx.83570.1.S1_at	N/M	N/M	N/M
Sec15-5	Glyma14g00390	Gma.3621.2.S1_a_at	N/M	N/M	M
EXO84-3	Glyma07g00570	GmaAffx.68128.1.S1_at	N/M	N/M	N/M
EXO84-4	Glyma07g34880	GmaAffx.81372.1.S1_at	N/M	M	N/M::M
EXO84-5	Glyma08g23840	Gma.7614.2.S1_a_at	N/M	N/M	N/M
EXO84-8	Glyma20g02670	GmaAffx.51707.1.S1_at	N/M	N/M	N/M
EXO70-A1	Glyma20g33590	Gma.16874.1.A1_at	M	M	M
EXO70-B1	Glyma14g37840	GmaAffx.62927.1.S1_at	N/M	N/M	N/M
EXO70-B1	Glyma02g39790	Gma.9061.1.S1_at	N/M	M	M
EXO70-B1	Glyma17g13900	GmaAffx.80596.1.S1_at	N/M	N/M	N/M
EXO70-B1	Glyma07g04600	GmaAffx.63420.1.S1_at	M	M	M
EXO70-B1	Glyma16g01190	GmaAffx.8836.1.S1_at	N/M	N/M	N/M
EXO70-B1	Glyma05g03310	Gma.1935.1.S1_at	M	M	M
EXO70-B1	Glyma08g26920	GmaAffx.1096.1.S1_at	N/M	N/M	M
EXO70-B1	Glyma02g39780	GmaAffx.81173.1.S1_at	N/M	N/M	N/M
EXO70-D1	Glyma10g23810	GmaAffx.47243.1.S1_at	N/M	N/M	N/M
EXO70-D1	Glyma11g15420	GmaAffx.48077.1.A1_at	N/M	M	M
EXO70-D1	Glyma04g32420	GmaAffx.85281.1.S1_at	N/M	N/M	N/M
EXO70-D1	Glyma03g33160	GmaAffx.85721.1.S1_at	N/M	N/M	N/M
EXO70-D2	Glyma07g04600	GmaAffx.63420.1.S1_at	M	M	M
EXO70-D3	Glyma16g01190	GmaAffx.8836.1.S1_at	N/M	N/M	N/M::M
EXO70-E1	Glyma08g26920	GmaAffx.1096.1.S1_at	N/M	N/M	M
EXO70-F1	Glyma05g03310	Gma.1935.1.S1_at	M	M	M
EXO70-G1	Glyma17g29210	GmaAffx.81535.1.S1_at	N/M	N/M	M
EXO70-H4	Glyma03g33160	GmaAffx.85721.1.S1_at	N/M	N/M	N/M
EXO70-H7	Glyma11g15420	GmaAffx.48077.1.A1_at	N/M	M	M

Footnotes: Yellow, genes examined in transgenic studies. Blue, genes not measured by DCM (Klink et al. 2010). Red, genes measured by DCM. Orange, genes exhibiting some inconsistent measurement within one of the two resistant genotypes, but are expressed within the parasitized cells of one of the two resistant genotypes.

Exocyst genes are induced in genetically engineered roots

The Affymetrix microarray analysis shows that exocyst genes are expressed within syncytia (Klink et al. 2010; Matsye et al. 2011, 2012). Selected exocyst genes that have been identified from these earlier experiments and presented here have been cloned and tested for their role in genetic resistance using the same procedures as presented in Chapter II. The selected genes have been overexpressed in the susceptible cultivar. In

contrast, the same genes have been silenced in a resistant cultivar through RNAi (Klink et al. 2009a; Sharma et al. 2016). The altered RNA level shows that the exocyst components have been induced in the overexpression lines and decreased in the RNAi lines (**Figure 3.3**).

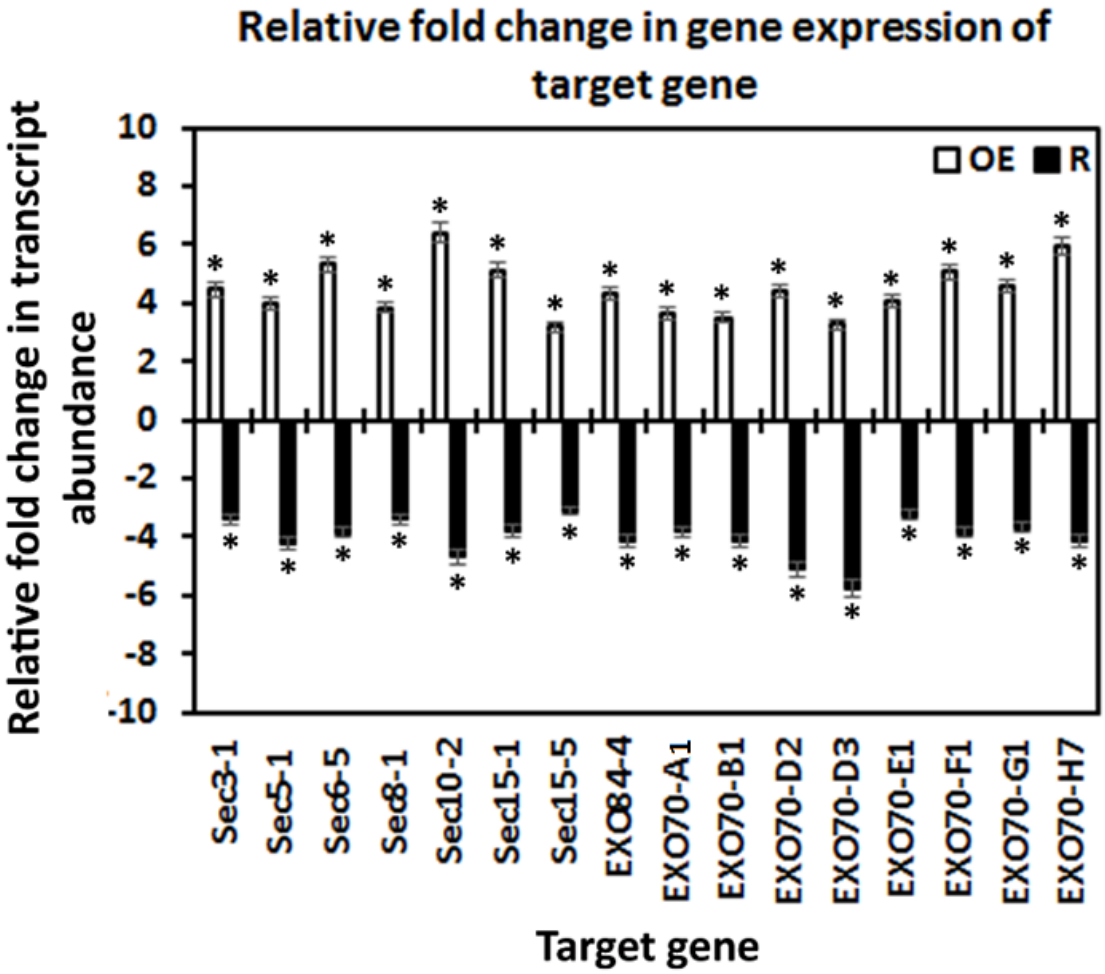

Figure 3.3 qPCR of *G. max* homologs of the exocyst that have been engineered for their overexpression or RNAi. * statistically significant $p < 0.05$.

Genetic analyses of exocyst components-overexpression studies

The genetic expression of the exocyst genes have been tested in comparison to their appropriate controls in overexpression and RNAi studies. The engineered *G. max* roots have been infected with the *H. glycines* and compared with the control (Klink et al. 2009; Matsye et al. 2012; Sharma et al. 2016). The FI shows that the *H. glycines* population has been reduced in analysis of cysts extracted from the whole root (pot) and per gram of tissue as compared to the control roots that have been engineered with the pRAP15 overexpression vector lacking the exocyst gene (**Figure 3.4**).

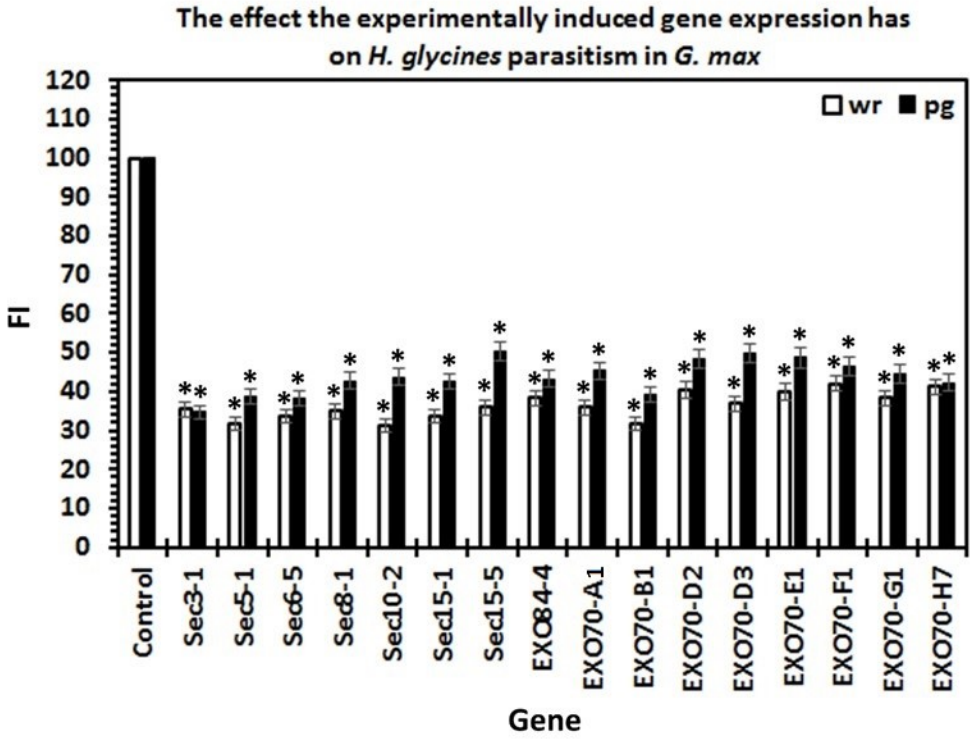

Figure 3.4 Overexpression of the exocyst subunits in *G. max* [Williams 82/PI 518671] induced resistance to *H. glycines*. Where the control population is set to 100 and is used to compare with the tested candidate genes. * statistically significant $p < 0.05$.

Genetic analyses of exocyst components-RNAi studies

In studies complimenting the overexpression analyses, RNAi studies of the candidate exocyst genes have been performed. The results of those analyses are presented (**Figure 3.5**). The results of those experiments show that the elimination of one of the exocyst genes tends to significantly increase the *H. glycines* FI population in analysis of the number of cysts per whole root and the number of cysts per gram of root tissue (**Figure 3.5**).

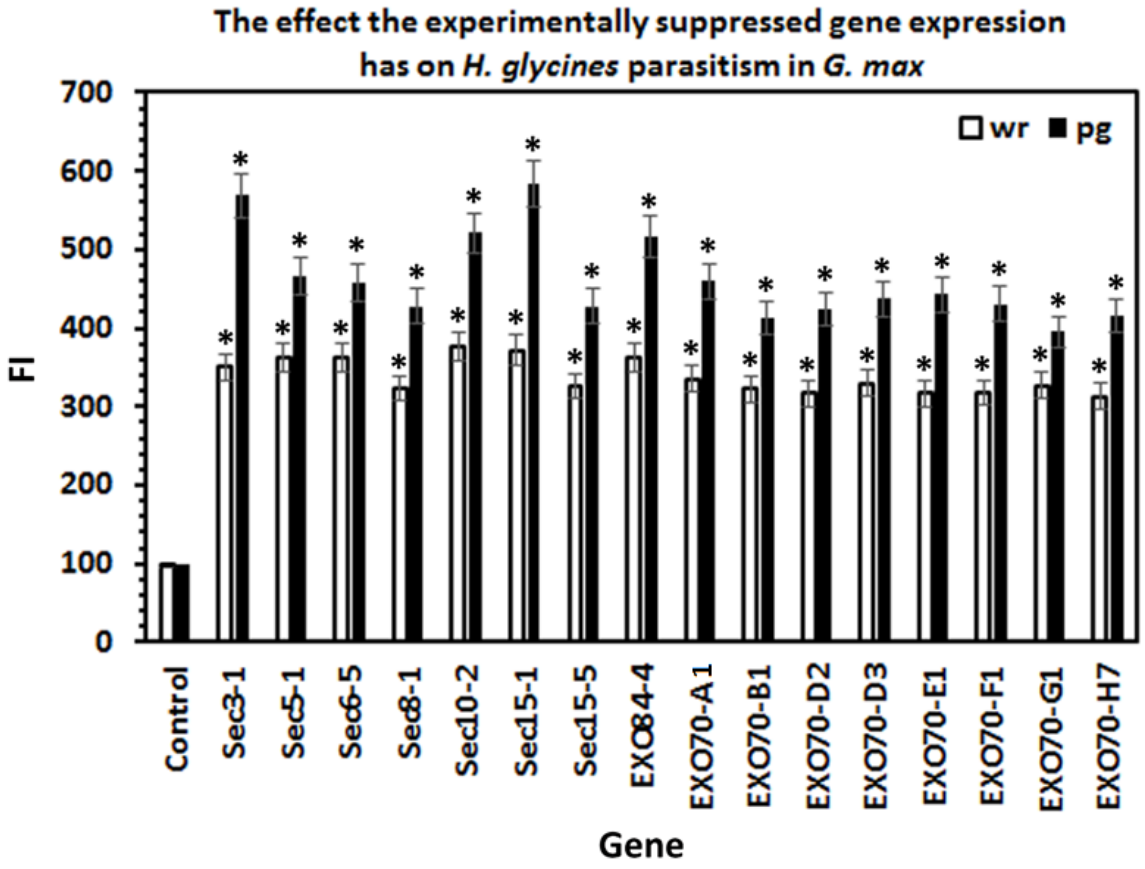

Figure 3.5 RNAi of the exocyst subunits in *G. max* [Peking/PI 548402] induced *H. glycines* infection and multiplication. The control is set to 100 and candidate genes are compared for their significance. * statistically significant $p < 0.05$.

Discussion

In the analysis presented here, the exocyst components present within the genome of *G. max* have been identified. The genome accessions of these genes have been compared against a database that allowed a determination of whether any of the genes are expressed in specific types of *G. max* root cells before and during resistant reactions found in two different genotypes exhibiting resistance to *H. glycines* parasitism (Klink et al 2010). The results have allowed for the identification of *G. max* exocyst genes that are capable of functioning in the process of resistance. At the same time, exocyst genes that do not exhibit measurable expression and those whose expression could not be measured due to the procedures of the analysis have been identified (Klink et al. 2010).

G. max has exocyst genes.

The analysis presented here has aimed at the identification of *G. max* exocyst, a goal that had been expected due to the conserved nature of the complex and its central role in many biological processes (Elias et al. 2003; Synek et al. 2006; Pečenková, et al. 2011, 2017). An examination of the *G. max* genome has resulted in the identification of homologs of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (Klink et al. 2010). These observations indicate an exocyst likely exists in *G. max*. A similar observation has already been made for the SNARE complex in *G. max*, a cytological particle shown through functional studies to have a role in defense and related to earlier work on plant growth and disease resistance that was performed in *A. thaliana* (Lukowitz et al. 1996; Assaad et al. 2001; Collins et al. 2003; Matsye et al. 2012, Pant et al. 2014; Sharma et al. 2016). The SNARE and exocyst complexes are functionally interrelated (He and Guo, 2009; Sabol et al. 2017). For example, experiments performed in *S. cerevisiae* have

shown its Sec1 proteins SSO1 and SSO2 functionally interact with several exocyst components including SEC3, SEC5, and SEC15 (Aalto et al. 1993). These observations are not limited to *S. cerevisiae* since a functional exocyst composing a structural unit of 900 kD has been studied in *A. thaliana* and *Nicotiana benthamiana* (Hala et al. 2008). Furthermore, the exocyst has been shown to physically connect with SNARE through interaction with SYP121 and RIN4 and its disruption shown to have a negative impact on its biological function (Sabol et al. 2017). These processes can involve aspects of plant defense signaling (Ma et al. 2018). The experiments presented here aimed to understand expressed exocyst genes in more detail in the *G. max*-*H. glycines* pathosystem.

The *G. max* exocyst genes are expressed both before and during a defense response

Expression analysis of the exocyst genes has been performed using published data. The results have led to the identification of specific homologs that are sometimes expressed before and during the defense response or specifically during the defense responses found in two different *G. max* genotypes that are capable of a resistant reaction (Klink et al. 2007; Klink et al. 2010). In some cases, no gene expression information could be obtained from the prior transcription studies because of the nature of the gene expression platform used at the time (Klink et al. 2007, 2010). However, from the gene expression studies presented here, comparatively few of the exocyst genes are exhibiting expression before parasitism by *H. glycines*. For example, probe sets for GmSec5-1, GmExo70A1, GmExo70B1, and GmExo70D2 have measured expression in pericycle cells prior to *G. max* infection (**Table3.2**). These results indicate that some aspects of the exocyst that are employed for the defense process that is performed for their expression being present in uninfected tissues and is probably crucial for fundamental aspects of root

biology. In *Zea mays*, the *ROOTHAIRLESS1* gene encodes a Sec3 homolog (Wen et al. 2005). Regarding defense processes, these observations are consistent with those performed in *A. thaliana* showing that its Exo70B1 is important to the defense response (Sabol et al. 2017). Although the gene expression studies before infection does not provide much clue towards when and how genes are expressed during the infection whereas relating the defense response to the zigzag model, the expressed genes (**Table 3.5**) suggest that the exocyst complex is possibly inducing ETI in phase 3 or 4 to control nematode virulence (**Figure1.1**).

Exo70 in plant defense

Regarding plant defense, most of the research has focused in on Exo70, whose protein product mediates the direct interaction with the plasma membrane and subunits Sec5 and Sec6 (He and Guo, 2009; Synek et al. 2006). Exo70 is a large gene family including, having evolved into multiple subfamilies (i.e., A-H), each subdivided further (i.e., A1-A3) which could be employed in a modular manner to conduct various cellular functions while also being recruited to defend plants against various stresses (Synek et al. 2006). Earlier gene expression studies of Exo70A1, E2, and F1 shows that they are expressed in various cells and organs (Synek et al. 2006). In contrast, Exo70B1, B2, D1, D2 D3, E1, G1, and H7 are expressed in sporophyte tissues and organs (Synek et al. 2006). Exo70A1 plays important roles in growth and elongation as their mutants show retarded growth and elongation (Synek et al. 2006). Exo70 interacts with Sec3 to connect the exocyst complex to the plasma membrane for exocytosis (He et al. 2007). The other subunits, Sec5, Sec6, Sec8, Sec10, Sec15, and Exo84 are connected to secretory vesicles during the delivery process (He et al. 2007). Exo70 binds to phosphatidylinositol 4, 5-

biphosphate (PI (4, 5) P2) of the plasma membrane with its positively charged C terminus (He et al. 2007). In these studies, no specific subgroup had been provided. More recent experiments have revealed a number of different family members involved in these processes (Synek et al. 2017; Sekereš et al. 2017).

Functional analysis of the exocyst complex in defense

Plants have a unique defense strategy as it uses various tactics to protect itself from biotic stresses (Withers and Dong, 2017). They use their cellular defense strategies by inducing gene expression and consequent alteration of signaling pathways (McNeece et al. 2017; Withers and Dong, 2017). A process that is central to plant defense is membrane trafficking that plays important roles by limiting cellular growth and related functions (Withers and Dong, 2017; Pecenkova et al. 2017). When facilitated by membrane trafficking, the induction of the resistance process is due to the supportive interaction among the associated proteins for the effective vesicular transport and fusion (Pant et al. 2014; Sharma et al. 2016; Pecenkova et al. 2017; Withers and Dong, 2017). As already noted, interactions between RIN4, Exo70, and PEN1 have been identified in other systems (Synek et al. 2017).

The membrane trafficking apparatus is vast, involving many more proteins than just the SNARE and exocyst components (Novick et al. 1980; Clary et al. 1990; He et al. 2007; Mizuno-Yamasaki, 2012; Jahn and Fasshauer, 2012; Klink et al. 2017). The system also composed of cargo and enzymes could play important roles in the production of secondary metabolites (Kornfeld, 1986; Hammond-Kosack and Jones, 1996; Simons and Ikonen, 1997; Glazebrook et al. 1997). In *A. thaliana*, this vast network has been designated as a regulon that has been genetically delimited by the PEN1, PEN2 and

PEN3 proteins (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006; Humphry et al. 2010). These studies, however, did not examine whether there was any interrelatedness. Subsequent studies have shown in *G. max* that its SNARE-containing regulon components are co-regulated in their expression during defense to *H. glycines* infection (Sharma et al. 2016). The SNARE complex, also known as CATCHER complex, helps fuse vesicles by the zippering action of their helical bundles (Bocket al. 2001; Duman and Forte, 2003; Jahn and Fasshauer, 2012). It is still unclear but the exocyst complex might facilitate the zippering action as it promotes SNARE-mediated membrane fusion (TerBush and Novick, 1995; Collins et al. 2003; Sivaram et al. 2005; He and Guo, 2009; Pecenkova et al. 2011; Jahn and Fasshauer, 2012). Membrane fusion requires many proteins for secretion, transport, and fusion (Novick et al. 1980; Jahn and Fasshauer, 2012; Heider and Munson, 2012). For timely and targeted fusion, expression of these proteins is important in the series of the fusion events (Finger and Novick, 1997; Finger et al. 1998; Jahn and Fasshauer, 2012). From earlier experiments, mutations of Sec3 in yeast, the primary subunit of exocyst complex to connect vesicles with the target membrane, resulting in the accumulation of the secretory vesicle in the cytoplasm as they have been unable to dock with the membrane (Finger and Novick, 1997; Finger et al. 1998). These results are an example showing all the exocyst proteins are important for the fusion process. The results presented here show that a similar condition may exist in the *G. max*-*H. glycines* pathosystem.

During the defense response, transport and fusion are the important processes where exocyst helps in targeting the membrane and preparing SNARE for the fusion (TerBush and Novick, 1995; Pecenkova et al. 2011; Jahn and fasshauer, 2012). Some

exocyst components are associated with the vesicles and others with the target membrane (Novick et al. 1980; He et al. 2007; Zhang et al. 2008; He and Guo, 2009). The exocyst subunits are attached to each other by the helical bundles and form an exocyst complex (Haarer et al. 1996; TerBush et al. 1996; Croteau et al. 2009). The vesicle membrane protein the Rab GTPase (Sec4) connects the exocyst complex and vesicles (Guo et al. 1999; Mizuno-Yamasaki et al. 2012). Meanwhile, the SNARE proteins v-SNARE on vesicle which is Snc1 (VAMP), and t-SNARE on membrane Sso1(syntaxin) and Sec9 (SNAP25) are essential in the process (Söllner et al. 1993a; Wiederkehr et al. 2004; Sivaram et al. 2005; Jahn and Scheller, 2006; Jahn and Fasshauer, 2012). A number of studies have shown that the membrane proteins should be expressed for the successful release, transport, and fusion processes (Novick et al. 1980; Novick et al, 1981; Esmon et al. 1981; Kaiser and Schekman, 1990; Hala et al. 2002; He et al. 2007; Zhang et al. 2008; He and Guo, 2009). The DCM gene expression studies of the *G. max* root cells indicates that condition is likely (klink et al. 2007, 2010). Furthermore, the functional studies suggest that all the subunits tested are important for the defense process.

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CHAPTER IV

CONCLUSION

This dissertation has aimed to build on two different, but related sets of experiments involving the published work on SNARE and the exocyst which has been presented here (Sharma et al. 2016). The experiments have identified a *G. max* homolog of the exocyst receptor (i.e., GmRIN4-4) that is expressed in the pericycle cells of the root (Klink et al. 2010) that will be targeted by *H. glycines* as their site of parasitism. This same site which is known as the syncytium, upon *H. glycines* parasitism, becomes the site of the defense response (Ross et al. 1958). The functional experiments accomplished through a transgenic approach have shown that GmRIN4-4 engages a defense response. With this first aim accomplished, the experiments then moved on to determine if *G. max* had homologs of exocyst genes which, like the observations made for SNARE, has been expected due to the structure's conserved nature (Elias et al. 2003; Synek et al. 2006; Hala et al. 2008; Sharma et al. 2016). The approach presented here used to study the exocyst components has been the same as that presented for GmRIN4-4 in Chapter II. The experiments have identified exocyst subunits and the paralogs having expression within the root pericycle cells before or during the defense process (Klink et al. 2010). The work demonstrates that GmRIN4-4 functions during the defense response to *H. glycines* parasitism. Furthermore, components of the exocyst also function in the defense response.

***G. max* has a RIN4 homolog that functions in the defense process to *H. glycines*.**

Genetic studies performed primarily in *A. thaliana* have revealed ETI and PTI levels of defense (Jones and Dangl, 2006). ETI has been defined by the CC-NB-LRR R protein NDR1 (Century et al. 1995, 1997; Coppinger et al. 2004). NDR1 activates ETI by directly interacting with RIN4 (Mackey et al. 2002; Axtell and Staskawicz, 2003; Day et al., 2006). RIN4 also interacts with the CC-NB-LRR protein RPS2 and the CC-NB-LRR protein RPM1 (Kunkel et al. 1993; Grant et al. 1995). Multiple pathogen effectors impair the function of these proteins and interfere with defense signaling (Mackey et al. 2002; Belkhadir et al. 2004; Kim et al. 2005). A *G. max* homolog of NDR1 has been analyzed (McNeece et al. 2017). Other ETI membrane receptors have also been identified including the toll-interleukin receptor (TIR) nucleotide binding NB-LRR R protein RPP4) (Aarts et al. 1998). RPP4 has been shown to lead EDS1-driven engagement of defense gene expression (Aarts et al. 1998). SA signaling has shown to function through ETI and can be activated by membrane receptors such as RPP4, along with EDS1 (Cao et al. 1994). Proteins functioning in this pathway include NPR1 (Falk et al. 1999). In NPR1-dependent SA signaling, SA binds to NPR1 to stimulate movement into the nucleus where it's copper-dependent binding to the transcription factor TGA2 drives the expression of target genes like those encoding the secreted protein PR-1 (Niggeweg et al. 2000; Kinkema et al. 2000; Fan and Dong, 2002). PTI functions through the FLS2 membrane receptor in processes that may lead to mitogen-activated protein kinase MAPK signaling (Chinchilla et al. 2007; Lin et al. 2014). FLS2 has been shown to activate and also physically interact with components of ETI, binding RPM1, RPS2 and RPS5 (Qi et al. 2011). These experiments support previous observations that have

revealed cross-talk occurring between PTI and ETI receptor systems (van der Biezen et al. 2002; Veronese et al. 2006; Thomma et al. 2011; Zipfel et al. 2006; Liu et al. 2013; Lolle et al. 2017; Jacob et al. 2018).

The GmNDR1-1 was expressed within the cells that undergo a defense response and through transgenic experiments has been shown to function in defense (McNeece et al. 2017). In *A. thaliana*, the expression of NDR1 is induced by the bacterial effector harpin (Wei et al. 1992; Gopalan et al. 1996). Consequently, NDR1 also became known as harpin induced1 (HIN1) (Gopalan et al. 1996). Experiments presented in *G. max* have shown that the topical treatment of harpin led to defense to different plant-parasitic nematode species in *G. max* and *Gossypium hirsutum* (cotton) (Aljaafri et al. 2017). Furthermore, harpin induced the expression of several proven defense genes, including the *rhg1* gene α-SNAP-5 and *rhg4* serine hydroxymethyltransferase-5 (SHMT-5) (Sharma et al. 2016; Aljaafri et al. 2017). Consequently, GmNDR1-1 is placed in an important position regarding *G. max* defense to *H. glycines*. These results further demonstrate the importance of NDR1 to transduce defense signals (McNeece et al. 2017; Aljaafri et al. 2017). As stated, crosstalk and physical interactions between ETI and PTI occurs between these proteins as well (van der Biezen et al. 2002; Veronese et al. 2006; Thomma et al. 2011; Zipfel et al. 2006; Qi et al. 2011; Liu et al. 2013; Lolle et al. 2017; Jacob et al. 2018).

Recent experiments have clarified the role of NDR1 in that as having structural features related to animal integrins that provides structural support to the cell in addition to its signaling functions (Tamkun et al. 1986; Knepper et al. 2011; LaFlamme et al. 2018). Complementary studies have shown that RIN4 serves an anchoring role and that

the anchoring role relates to the exocyst (Synek et al. 2017). Consequently, RIN4 is in a position that bridges the NDR1 membrane receptor and the exocyst (Mackey et al. 2002, 2003; Day et al., 2006; Sabol et al. 2017). Recent experiments performed in *A. thaliana* have demonstrated this function of RIN4 to be true (Synek et al. 2017). These results also confirm experiments performed in studies of the *G. max-H. glycines* pathosystem where the importance of the SNARE protein α-SNAP to defense had been demonstrated as the major *rhg1* related gene α-SNAP mediates fusion of the vesicle and targeted membrane (Matsye et al. 2011, 2012; Pant et al. 2014; Zick et al. 2015; Sharma et al. 2016; Song et al. 2017; Harner and Wickner, 2018).

Experiments presented in *A. thaliana* have shown RIN4 is expressed in the cells where a defense response occurs (Day et al. 2006). NDR1 induces ETI as a resistance response to its pathogens that occurs through an interaction with RIN4 (Century et al. 1995; Aarts et al. 1998; Coppinger et al. 2004; Day et al. 2006; Knepper et al. 2011; McNeece et al. 2017). The CC-NB-LRR proteins such as RPM1, RPS2, and RPS5 require NDR1 protein to induce resistance in *A. thaliana* (Day et al. 2006). The membrane-bound RIN4 proteins are the target of bacterial type III virulence effector AvRpt2 (Mackey et al. 2003). Consequently, the deactivation of RIN4 by bacterial effectors is an efficient way for the pathogen to disarm plant defense processes (Mackey et al. 2002, 2003; Day et al. 2006). The resistance response engaged by proteins like RPS2 is negatively regulated by RIN4, suggesting that RPS2 based resistance pathways are induced in the absence of RIN4 proteins (Mackey et al. 2003; Axtell and Staskawicz, 2003). Whereas studies show that the AvrRpt2 does not require RIN4 for its virulence function suggesting that it is not only the target (Belkhadir et al. 2004). However, various

defense strategies in *A. thaliana* are induced by the interaction of NDR1 and RIN4 proteins (Day et al. 2006). These results have been able to physically link ETI and PTI defense branches, each of which has been shown to function in the *G. max-H. glycines* pathosystem (Pant et al. 2014; Aljaafri et al. 2017; McNeece et al. 2017). The work presented here describes how they function in relation to the exocyst.

The analysis of the exocyst

Experiments performed in *S. cerevisiae* have been able to identify and then functionally link the exocyst and SNARE cytoplasmic structures (Sollner et al. 1993; TerBush and Novick, 1995; Sivaram et al. 2005; Morgera et al. 2012; Shen et al. 2013; Dubuke et al. 2015). The analysis of the exocyst presented here relates to the prior work presented using the *G. max-H. glycines* pathosystem on SNARE genes (Sharma et al. 2016). As shown in *S. cerevisiae* the exocyst mediates the SNARE assembly and their mutants failed to form a SNARE complex (Grote et al. 2000). The mutation of components of the exocyst complex impairs tethering and fusion of the vesicles that results in accumulation of vesicles inside the cell. (Novick et al. 1980; Heider and Munson, 2012).

Consequently, the exocyst mutants show secretion defects and intracellular accumulation of the secretory vesicles (Novick et al. 1980; Guo et al. 1999, 1999a; Zhang et al. 2005, 2008; He et al. 2007; Heider and Munson, 2012). Since SNARE and secretion are important aspects of defense in the *G. max-H. glycines* pathosystem the related processes such as the exocyst should also be relevant (Sharma et al. 2016; Klink et al. 2017). For example, *S. cerevisiae* Sec3 proteins are associated with membrane proteins localized at the site of polarized exocytosis (Finger et al. 1998). Similar observations

have been shown in the root of *Zea mays* (Wen et al. 2005). In the experiments presented here in the *G. max*-*H. glycines* pathosystem, the induced expression of Sec3 has suppressed *H. glycines* parasitism as measured by a decrease in its FI. This is an important observation since genetic and biochemical experiments presented in *S. cerevisiae* have shown Sec3 functions with Exo70 for the targeted fusion (He et al. 2007). Single mutants of Sec3 or Exo70 were less conclusive as compared to their double mutants which impaired membrane anchoring of the vesicles leading to fusion defects (He et al. 2007). These experiments indicate some level of functional redundancy may exist which is not surprising since each subunit is interconnected and involved in binding the plasma membrane and tethering of the secretory vesicle (He et al. 2007). The RNAi results presented here show defects in the resistance response leading to more infection, but levels of susceptibility found in the susceptible *G. max* genotypes have not been obtained. This observation could be explained by the multiple paralogs *G. max* has for each of its exocyst genes, a consequence of its duplicated genome (Schmutz et al. 2010). Sec3 mutants are reported with root hair growth defects that lead to various growth defects in plants (Wen et al. 2005). Earlier experiments show that impairing the binding of Sec3 and Exo70 with PI (4,5) P2 halts the fusion process leading to cell death (He et al. 2007; Zhang et al. 2008). Consequently, it is possible that since *G. max* has multiple paralogs of each exocyst gene, an environment ispresented that allows the experiments to be performed. The observations made with the remaining exocyst genes studied here, all have a similar outcome to those presented for Sec3 and Exo70.

Several biochemical studies have been performed in *S. cerevisiae* aimed at studying the exocyst (TerBush et al. 1995, 1996; Roth et al. 1998; Guo et al. 1999a; Hsu

et al. 2004). The study of the interaction between exocyst subunits through FLAG and haemagglutinin (HA) epitope tagging shows that Sec5 interacts with Sec3, Sec6, Exo70 (TerBush et al. 1995; TerBush et al. 1996; Roth et al. 1998; Guo et al. 1999a; Hsu et al. 2004). In contrast, mutants of Sec3, Sec5, and Sec10 resulted in the disruption of the Sec6/8/15 complex (TerBush et al. 1995; TerBush et al. 1996; Roth et al. 1998; Guo et al. 1999a; Hsu et al. 2004). These observations indicate that Sec5 plays an essential role in the formation of Sec6/8/15 implicating that they might encode other proteins of the exocyst complex and further demonstrate as how Sec5 relates to the formation of the exocyst complex possibly linking with the other cellular functions (TerBush et al. 1995; TerBush et al. 1996; Roth et al. 1998; Guo et al. 1999a; Hsu et al. 2004) such as defense (Du et al. 2015). The results mean that by perturbing one exocyst component that the stability of the whole structure may be compromised (TerBush et al. 1995; TerBush et al. 1996; Roth et al. 1998; Guo et al. 1999a; Hsu et al. 2004; Du et al. 2015). These observations are like the RNAi results obtained here in the *G. max*-*H. glycines* pathosystem. Experiments conducted in *N. benthamiana* relates to these observations, showing Sec5 plays an important role in plant growth and defense (Du et al. 2015). The Sec5 mutants displayed reduced plant growth and expression of pathogen-related (PR) proteins associated with pathogen infection (Du et al. 2015). Experiments have shown the fungal pathogen *Phytophthora infestans* alters the vesicular transport process by impairing exocyst subunits, leading to infection (Du et al. 2015). In these experiments, Sec5 mutants that are susceptible to *P. infestans* appeared to have less reactive oxygen species (ROS) production and callose deposition at the plasma membrane (Du et al. 2015). These experiments relate to a defect in SNARE leading to less callose deposition

at sites of parasitism in the *G. max-H. glycines* pathosystem (Sharma et al. 2016). Plant pathogens as a part of their virulence, attack components of vesicle transport system by impairing secretion of Golgi-derived vesicles, callose deposition and penetration resistance (Driouich et al. 1997; Nielsen et al. 2012; Sharma et al. 2016). The exocyst subunits such as Sec6, Sec8, Sec15b, and Exo70A1 are detected in various stages of cell plate formation (Fendrych et al. 2010). In plants mutation of Sec5, Sec6, Sec8, Sec15a resulted in less pollen germination and reduced growth (Hala et al. 2008). Mutation of Sec8 has shown poor pollen germination and pollen tube growth (Cole et al. 2005). During cytokinesis secretory vesicles are directed to the cell plate formation matrix where the exocyst complex assists in tethering and fusion (Fredrych et al. 2010). After fusion, vesicles are an elongated, projecting like to dumbbell shape (Fendrych et al. 2010). These structures connect and form a perforated layer with a network of tubes and vesicles with callose deposition (Fendrych et al. 2010).

The exocyst subunits Sec6 and Sec8 have been shown to interact with each other and are broadly distributed in tissues, indicating their possible role in membrane trafficking (Ting et al. 1995). These subunits are localized with the transport vesicles assisting in fusion (Hsu et al. 2004). A detailed study on exocyst subunits in plants and their role remains to be done. In the *G. max-H. glycines* pathosystem, the induced exocysts gene expression occurring through overexpression in an *H. glycines*-susceptible cultivar has led to a decrease in the FI while RNAi in a resistant genotype had the opposite effect. The experiment confirms the importance of the exocyst to the process of defense in the *G. max-H. glycines* pathosystem. The results show that the expression of these subunits in the cells that undergo the process of defense presage their function in

defense. Furthermore, the findings relate to earlier work performed on SNARE in the *G. max-H. glycines* pathosystem that revealed its role in defense and led to the identification of its major resistance genes (Matsye et al. 2011, 2012; Pant et al. 2014; Sharma et al. 2016).

Analysis of Exo84

The exocyst subunit Exo84 plays a vital role in the formation of exocyst complex and post Golgi trafficking by targeting the complex at unique sites in plasma membrane for exocytosis (Zhang et al. 2005). Studies of Exo84 mutants in yeast have identified their role in the post-Golgi secretion process (Zhang et al. 2005). Like the work done on the other subunits, Exo84 has a vital role in complex formation which is essential for the docking of the vesicle at the targeted membrane (Zhang et al. 2005; He et al. 2007). The experimentally induced expression of GmExo84-4 induces resistance in *G. max* [Williams 82/PI 518671] to *H. glycines* parasitism.

In contrast, RNAi of this complex increased *H. glycines* parasitism. Previous experiments on Exo84 employing an exo84b mutation has resulted in vesicles accumulating in the cytoplasm, cytokinesis defects and a variable phenotype with retarded growth and sterility (Fendrych et al. 2010). The accrued vesicles in exo84 mutants contain compounds such as pectin and xyloglucan (Fendrych et al. 2010). This is an important observation since in the *G. max-H. glycines* pathosystem, xyloglucan metabolism has been shown to play an important role in the defense process (Pant et al. 2014; Aljaafri et al. 2017; McNeece et al. 2017).

Exo70 is a part of a large gene family

The most duplicated exocyst gene in plants is Exo70 (Žárský et al. 2013), Exo70 has been studied for its role in cell repair and defense response (Žárský et al. 2013). The duplication of this gene into a large gene family might have resulted due to their multiple roles in various cellular functions as well as defense. These multiple functions include exocytosis, cell membrane recycling and autophagy-related transport are carried out by different isoforms of Exo70 (Žárský et al. 2013). The distribution of Exo70 genes in plants is wide, for example *A. thaliana* has 23 paralogs; Sorghum bicolor has 31, *G. max* has 35 while Oryza sativa has 47 (Elias et al. 2003; Synek et al. 2006; Klink et al. 2010; Cvrckova et al. 2012; Žárský et al. 2013). Transcriptional analysis has revealed that the Exo70 paralogs such as Exo70B2 and Exo70H1 are upregulated in *A. thaliana* when infected with the fungal pathogen *B. graminis* f. sp. *hordei* and bacterial pathogen *P. syringae* pv. *maculicola* (Pecenkova et al. 2011). The results of mutant studies have resulted in susceptibility to each pathogen (Pecenkova et al. 2011). Related findings have shown exo70 mutants show defects in secretion of secretory vesicles that transport endoglucanase Bg12 required for cell membrane expansion and cell wall remodeling (He et al. 2007; He and Guo, 2009). During cell division, cell plate formation is carried out by the accumulation of the vesicles at the site of cytokinesis and their fusion by the exocyst complex (Seguí-Simarro et al. 2004; Žárský et al. 2013). Mutation of Exo70A1 shows some defects in cell plate formation, inability in root growth, loss of apical dominance, impaired flower development and smaller organs which proves that they are essential in growth and development process (Fendrych et al. 2010; Synek et al. 2006). Thus, these multiple isoforms of the Exo70 genes regulate exocytosis related to biotic and abiotic

stress and other functions such as membrane recycling, autophagy related vesicular transport (Pečenková et al. 2011; Lin et al. 2013; Žárský et al. 2013). More broadly, mutation of exocyst subunits; Sec5, Sec6, Sec8, Sec15A, Exo70B, and Exo84B in *N. benthamiana* plants have resulted in impaired resistance to *P. infestans* (Du et al. 2017). Also, Sec5, Sec6, and Sec10 mutants in *N. benthamiana* showed more bacterial infection and growth whereas mutants of other subunits did not affect resistance (Du et al. 2017). The results are consistent with the observations presented here of their importance and that to the defense induced by exocyst in the *G. max*-*H. glycines* pathosystem. Presented here, the results have shown that *G. max* homologs of RIN4 and the exocyst play important defense roles related to ailed parasitism attempts by *H. glycines*.

Future directions

The current and previous studies demonstrate that the RIN4 and exocyst being an integral part of the plant secretory system play an important role in plant growth, development and defense responses by assisting transport and fusion of the secretory vesicles (Mackey et al. 2002; Day et al. 2006; Hála et al. 2008; Fendrych et al. 2010; Pečenková et al. 2011; Heider and Munson, 2012; Sabol et al. 2017; Klink et al. 2017; Du et al. 2017). The plant secretory system is vaguely understood and there are many other known and unknown proteins associated with the process. The plant having a unique defense strategy, the study of those known and unknown proteins is necessary as the identification of resistance responses has become broader with the genetic exploitations. As planned, genetic response and functional study of the proposed genes towards *H. glycines* parasitism have been accomplished. Moving forward future research could be directed more towards *cis*, *trans* and intra Golgi network to study more proteins

interaction, vesicular processing, transport, and fusion. More detail and comprehensive study is needed in plant genetic system. This current research could be directed to the study of protein interaction, electron microscopy, expression of two or more proteins and their interaction with other cellular processes such as different cell signaling process to understand better the actual cellular physiology occurring during the defense response. Thus, the study presented here proves the model and provides the fact that selected induced genes from the resistance cultivar could be exploited and expressed in a susceptible variety that could provide more qualitative and quantitative agricultural production in *G. max* and other agricultural commodities. As the farming world is in a challenge to produce higher output in the limited land for the increasing human population, identification of genetic resistance and functional studies could provide an essential clue to scientists, agricultural and biotech companies to further improve the genetic traits of the susceptible cultivars into more potent and high yielding crops.

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