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A Functional Genomics Analysis of Glycine Max Vesicle Membrane Fusion Genes in Relation to Infection by Heterodera Glycine

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A functional genomics analysis of *Glycine max* vesicle membrane fusion genes in

relation to infection by *Heterodera glycines*

By

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Soybean cyst nematode (SCN), a major pathogen of soybean worldwide, causes huge losses in soybean production. Various approaches including cloning of genes to combat this devastating disease help to better understand the cellular function and immune responses of plants. Membrane fusion genes are the important regulatory parts of vesicular transport system, which works through packaging of intracellular compounds and delivering them to apoplast or nematode feeding sites to induce an incompatible reaction. The incompatible nature of membrane fusion proteins such as SNAP25, Munc18, Syntaxin, Synaptobrevin, NSF, Synaptotagmin and alpha-SNAP are conserved in eukaryotes and regulate the intracellular function to combat abiotic and biotic stress in plants. Overexpression of these genes in *G. max* [Williams 82(PI518671)] which is a susceptible cultivar of soybean to nematodes resulted in a reduction of the SCN population providing further insights of molecular and genetic approaches to solve the SCN problems in agriculture.

DEDICATION

I would like to dedicate this thesis to my Timilsina Family, My parents Lalchan Sharma, Ishwori Sharma and my siblings Mina Sharma, Laxman Sharma, Madhu Sudan Sharma and Prashamsha Sharma.

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CHAPTER I

INTRODUCTION

The defense of plants to pathogens

Plants are under constant attack by a variety of different pathogens and herbivores. To combat these insults, plants have developed various strategies. In contrast to animal cells, plant cells have rigid cell wall that adds extra security towards pathogen infection and also provides chemical cues during pathogen attack (Chisholm et al. 2006). Plants have no circulatory system and defensive mobile cells therefore, they have to work through their physiological approaches to combat the pathogen attacks (Kwon and Yun, 2014). Plants respond by activating their defense in three ways; (1) functional pathways that limit spread of the pathogens, (2) systemic acquired resistance (SAR) pathway that gives potential resistance against different pathogens and (3) gene for gene resistance pathway (Glazebrook et al. 1997). In gene for gene resistance interaction the avirulence gene of pathogen induces ligand binding to receptors of plants resistance gene (R gene) that regulates resistance responses confining spread and multiplication of pathogens (Glazebrook et al. 1997). As part of these processes, plants have an innate immunity system that identifies and responds to pathogen attacks (Dodds and Rathjen, 2010). This process uses pathogen activated molecular pattern (PAMP)-triggered immunity (PTI) and effector triggered immunity (ETI) as their defense strategies (Chisholm et al. 2006; Dodds and Rathjen, 2010). Plants detect PAMPs through their extracellular surface

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receptors which induce PTI to suppress and arrest the pathogen attacks (Chisholm et al. 2006). Pathogens attempt to alter PTI by modifying surface receptors by injecting their virulence factors to change signaling in plants (Chisholm et al. 2006). After the pathogen controls the initial defense, the plants develop more advanced ETIs which detect their invasion (Chisholm et al. 2006). ETI uses plant resistance (R) proteins to identify pathogen proteins and induce R protein related resistance response to confine pathogen spread (Chisholm et al. 2006). The defense response includes chemical and enzymatic defense mechanisms that limit pathogen metabolism by activating numerous genes related to chitinases, phytoalexins, defensins, glucanases, glutathione-S-transferases, thionins, lipoxygenase, phenylalanine, ammonia lyase, and various other enzymes for secondary metabolism and by synthesizing reactive species, antimicrobial secondary metabolites and inducing thickening of cell wall (Hammond-Kosack and Jones, 1996; Glazebrook et al. 1997). For example, salicylic acid (SA) induces signaling in plants and promotes SAR (Durrant and Dong, 2004). The transcription level of plant secretory components is induced for SAR (Wang et al. 2005, 2006). SAR is a signal transduction pathway that acts as a defense response to pathogen attack (Ryals et al. 1996). The acquired resistance that occurs after pathogen attack forms necrosis in infected areas (Uknes et al. 1992). Plants, as their defense response, try to defend against external stress (abiotic and biotic) through their different signaling pathways that regulate expression of genes within the roots and shoots (Glazebrook et al. 1997; Knight and Knight, 2001). The expression of stress related genes increased in roots compared to other parts (Chen et al. 2002). In some cases, accompanying the process of resistance is a dramatic increase in the deposition of cell wall material at infection sites undergoing the process of resistance.

This observation indicated that the plant was secreting materials at the site of infection and this secretion would be mediated by the process of vesicle transport, involving the process of membrane fusion.

Membrane fusion

The vesicular transport system is an essential characteristic of eukaryotic organisms that consists of conserved proteins found in all eukaryotes (Novick et al. 1980; Clary et al. 1990; Sanderfoot et al. 2001; Jahn and Fasshauer, 2012). The proteins function in membrane fusion events at the endoplasmic reticulum (ER) and at various locations within the cell, including the plasma membrane with their action contributing to a functional secretion system (Novick et al. 1980, 1981; Hay and Scheller, 1997; Hodel, 1998; Bock et al. 2001; Collins et al. 2003; Jahn and Fasshauer, 2012; Hu et. al 2003). Vesicles function by containing biochemical products within membrane bound organelles (ER, Golgi body, endosomes and lysosomes) and transferring them to other organelles and the cell membrane for different purposes (Bock et al. 2001). These vesicles are formed by budding of the donor organelle which is then released and subsequently transferred to the acceptor organelle or plasma membrane that has Soluble N-ethylmaleimide-sensitive fusion (NSF) Attachment Protein (SNAP) REceptor (SNARE) complex known as catcher complex present to dock the vesicle to the membrane (Bock et al. 2001; Jahn and Fasshauer, 2012). The docking and subsequent membrane fusion event between the donor and receptor membrane allows for the release of vesicular contents. While many proteins and their paralogs function in the process, certain proteins (Jahn and Fasshauer, 2012) are central to this process (Table 1.1).The process of membrane fusion occurs through five steps including (1) recruitment of Munc18, syntaxin and SNAP-25,

(2) activation of the SNARE acceptor complex, (3) priming, (4) triggering and (5) fusion. Each step is described below.

Table 1.1   The core proteins involved in membrane fusion

Human Gene	Yeast Homologue	Soybean Homologs
Syntaxin (i.e. syntaxin 121 (SYP121)	Suppressors of Sec One [Sso1p]	Glyma02g35210, Glyma03g36120, Glyma02g35230, Glyma10g10210, Glyma10g10200, Glyma19g38770
Synaptosomal-associated protein 25 (SNAP25)	Secretion 9 [Sec9p]	Glyma04g32710, Glyma06g21560, Glyma05g00640, Glyma17g08450, Glyma02g12821, Glyma01g06860
Mammalian uncoordinated-18 (Munc18)	Secretion 1 [Sec1]	Glyma17g14450, Glyma05g03970, Glyma11g03230, Glyma01g42140, Glyma03g02740, Glyma01g34340
Synaptotagmin (SYT)	Tricalbin-3 [Tcb3p]	Glyma11g11470, Glyma12g03620, Glyma06g00610, Glyma14g40290, Glyma17g37850, Glyma10g35410, Glyma20g32110
Synaptobrevin (SYB)/ Vesicle associated membrane protein (VAMP)	YKT6	Glyma07g04740, Glyma16g01330
N-ethylmaleimide-sensitive fusion protein (NSF)	Secretion 18 [Sec18]	Glyma13g24850, Glyma07g31570

Note: an extensive literature search did not produce what the YKT6 acronym represents.

Step 1: Recruitment of Munc18, Syntaxin 121 and SNAP-25

The syntaxin protein (i.e. syntaxin 121) consists of an amino terminal domain that has three antiparallel helices with conserved hydrophobic grooves forming a bundle (the Ha, Hb and Hc domains). The Hb and Hc domains are connected with another membrane fusion gene, synaptotagmin (Lerman et al. 2000). This hydrophobic groove interacts with the C terminus of an intact protein to form a closed conformation that might regulate interactions with other proteins (Lerman et al. 2000). One protein that syntaxin interacts with is Munc18. Furthermore, the carboxy-terminal end of syntaxin binds with both SNAP-25 and synaptobrevin (Hanson et al. 19997a). Thus, an early step in membrane fusion is the recruitment of Munc18 to syntaxin.

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Step 2: Activation of the SNARE acceptor complex

The SNAP-25 protein has Qb and Qc domains that are connected by palmitoylated linker without carboxy-terminal transmembrane region (TMR) (Jahn and Fasshauer, 2012). It is an evolutionary conserved hydrophilic protein essential for exocytosis and is found on the cytoplasmic face of plasma membrane and secretory vesicles (Hodel, 1998). SNAP-25 forms a ternary complex with syntaxin and synaptobrevin and helps in exocytosis (Hanson et al. 19997a). Also early in the process of membrane fusion, an area of undefined active zone proteins accumulates around Munc18, syntaxin and SNAP-25. In several organisms excluding plants, an additional protein, Munc13, is present among these proteins.

Syntaxin mediates exocytosis through its association with Munc18 (Bock et al. 2001). The vesicle transport system consists of different SNARE complexes linking synaptotagmin, SNAP-25, synaptobrevin, syntaxin, Munc18 and Munc13 (Jahn and Fasshauer, 2012). Munc18 binds the N-terminal of syntaxin, keeping it closed and inactive. This binding prevents premature SNARE formation and helps the SNARE complex to form (Jahn and Fasshauer, 2012). The exocytosis membrane fusion process of synaptic vesicle with presynaptic plasma membrane is favored by SNARE proteins including synaptobrevin, syntaxin and SNAP-25 (Sutton et al. 1998; Stein et al. 2007; Puchkov and Haucke, 2013; Jahn and Fasshauer, 2012). SNAREs are an extended coiled–coil structure having 60-70 amino acid residues known as SNARE motifs (Stein et al. 2007; Jahn and Fasshauer, 2012). There are four SNARE motifs known as Qa, Qb, Qc and R. These motifs in syntaxin, synaptobrevin and in some SNAREs are connected to a carboxy-terminal transmembrane region by a short linker (Jahn and Fasshauer, 2012).

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Synaptobrevin is located in the membrane of vesicle (v-SNARE), and SNAP-25 and syntaxin are located on targeted plasma membrane (t-SNARE) (Huang et al. 2008). There are four parallel α-helices in the SNARE complex: two from SNAP-25, one from syntaxin and one from synaptobrevin (Sutton et al. 1998; Ernst and Brunger, 2003). The associations of α-helices form four shallow grooves on surface of synaptic fusion complex that can be used by alpha soluble N-ethylmaleimide-sensitive factor attachment protein (α-SNAP) or complexin as specific binding sites to form a helical bundle (Sutton et al. 1998). These four stable helical bundles are stabilized by leucine-zipper interactions which help to transport the vesicle to the plasma membrane inducing membrane fusion and neurotransmitter release (Sutton et al. 1998; Ernst and Brunger, 2003; Huang et al. 2008).

The process of membrane fusion is an important process in the vesicle transport system where two separate lipid bilayers merge to form a single continuous bilayer depending on time and site (Jahn et al. 2003). Synaptotagmin is important for vesicle fusion and has a single transmembrane domain functioning as a type I signal–anchor sequence in its N terminus and two calcium binding (C2A and C2B) domains in its C terminus (Jahn and Fasshauer, 2012). In the plant genetic model *Arabidopsis thaliana*, the synaptotagmin homolog SYT1 functions where the C2 domain plays an important role in SYT1 localization to plasma membrane (Yamazaki et al. 2010). The C2 domain of SYT1 causes calcium (Ca^{2+}) dependent membrane curvature induction and subsequent membrane fusion (Martens et al. 2007).

There are two phases in the formation of the SNARE-Sec1/Munc18-like (SM) complex. In Phase 1, during fusion, the N peptide of syntaxin binds to Munc18 and

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guides itself to the SNARE complex. This process results in the formation of the SNARE-SM fusion complex. Syntaxin is locked in by Munc18 in a closed complex that is incompatible to SNARE zippering. The role of Munc18 in SNARE nucleation is currently unknown (Jahn and Fasshauer, 2012). In Phase 2, the merging of intracellular membrane bilayers is mediated by fusion complex of SNARE and SM protein without further involvement of N-peptide. The carboxy terminal portion of syntaxin binds with SNAP-25 and synaptobrevin (Hanson et al. 1997a). Synaptotagmin is connected to the vesicle by a single transmembrane region, while synaptobrevin and synaptotagmin proteins are connected to membranes by flexible linkers. The C2 domain of synaptotagmin is linked to syntaxin alone or to a syntaxin-containing SNARE complex. Membranes go through the fusion process due to zippering of v-SNARE and t-SNARE (Duman and Forte, 2003). The fusion of vesicles with membrane occurs in the active zone site having active zone proteins whose function is unknown.

Step 3: Priming

Priming is the process by which α-SNAP-bound cis-SNARE complexes are disassembled by the ATPase activity of NSF. This action releases α-SNAP and the soluble SNARE (Mayer et al. 1996; Boeddinghaus et al. 2002). Currently, there are two hypothesis of how priming occurs, referred to as Priming I and Priming II (Reviewed in Jahn and Fasshauer, 2012). In Priming I there is an arrest of a partially zippered SNARE complex with bound Munc18, Munc13, and synaptotagmin. Further zippering of the SNARE complex leads to the fusion of vesicle and membranes (Jahn and Fasshauer, 2012). Like Munc13, a plant complexin homolog has not been identified (Klink, unpublished). In Priming II, the active zone components help in arresting and positioning

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of the SNARE with possible contact of synaptotagmin with phosphatidylinositol 4,5-bisphosphate [PI (4,5)P2] in the plasma membrane. There is no contact between the SNAREs in Priming II (reviewed in Jahn and Fasshauer, 2012).

Step 4: Triggering

Triggering is the process by which calcium mediates the fusion of two apposed membranes, enabling the release of vesicular contents. In Triggering I, Binding of synaptotagmin to the SNARE complex I and to the plasma membrane is triggered by calcium influx with displacement of complexin and (possibly) Munc18 and/or Munc13. In Triggering II, the calcium dependent Ca^{2+} triggering helps in pulling the vesicle closer through synaptotagmin-mediated cross-linking. This action results in the formation of a SNARE complex with a fully open syntaxin and bound complexin which displaces Munc18 (Jahn and Fasshauer, 2012).

Step 5: Fusion

The assembly of the SNAP-25-syntaxin-synaptobrevin complex is then dissociated by the catalytic activity of the cytosolic ATPase NSF and α-SNAP (Hodel, 1998). The outcome is the completion of fusion of the vesicle membrane with its target membrane and release of its contents in targeted destinations. Fusion of these vesicles is mediated by ATP-dependent cycle that regulates formation and dissociation of SNAREs (Sudhof and Rothman, 2009). The process of membrane fusion that folds SNARE proteins is exergonic and the ATPase (NSF) dependent dissociation is endergonic (Sudhof and Rothman, 2009). Once unfolded the vesicles are reused for the same process (Sudhof and Rothman, 2009). As a whole, SNAREs, SNAPs and ATPase (NSF) are

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essential for the intracellular membrane fusion (Sudhof and Rothman, 2009). However, since SNAPs and NSF dissociate SNARE complexes (v-SNARE and t-SNARE) after fusion, they are not required after fusion in cell free systems.

Vesicle transport and defense

Plant homologs of the membrane fusion proteins have been identified (Sanderfoot et al. 2001). Furthermore, mutational studies have revealed the involvement of some of these proteins in plant defense to pathogen attack (Collins et al. 2003). A mutant screen in *A. thaliana* identified the penetration 1 mutant (PEN1) (Collins et al. 2003). The analysis identified that the plant was mutant for the plasma membrane protein syntaxin 121 (Collins et al. 2003). Thus, Penetration 1 (PEN1) is syntaxin 121 (SYP121). The role of SYP121 was demonstrated in resistance to the leaf fungal pathogen *Blumeria graminis* f. sp. *hordei* (Collins et al. 2003). SYP121 forms a complex on the plasma membrane with the vesicle-associated membrane protein (VAMP)721/VAMP722 (synaptobrevin) and the soluble N-ethylmaleimide-sensitive factor (NSF) adaptor protein SNAP-25 known in *A. thaliana* as SNAP33 (Collins et al. 2003; Assaad et al. 2004; Kalde et al. 2007; Kwon et al. 2008a; Pajonk et al. 2008). The SNARE complex of PEN1/SYP122/SYP132-SNAP33-VAMP721/722 functions as a basic secretory pathway in plants and plays a supportive role by helping them in their growth and defense (Kwon and Yun, 2014). The defense function of SYP132 against bacterial pathogens and SYP122 against fungal pathogens suggests their role as a fundamental aspect of the plant resistance system (Assaad et al. 2004; Kalde et al. 2007; Yun et al. 2013). The double mutants of SYP121 and SYP122 also results in reduced growth indicating they have an added basic function in plant development (Assaad et al. 2004; Zhang et al. 2007). The

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SYP132 mutants are defective in localizing pathogenesis related (PR) proteins in the cell wall that results in reduced growth and lethality in plants (Kalde et al. 2007). The wheat SNARE homologs function in resistance against stripe rust by inducing membrane fusion indicating a conserved function between monocots and dicots (Wang et al. 2014). The pathogens described here attack the aerial portions of the plant. No function has been determined for root pathogens.

Plant Parasitic Nematodes

One of the most destructive of plant pathogen is the plant parasitic nematode (PPN). PPN are successful pathogens, found in many ecological niches. They are very important with regard to agriculture, responsible for approximately $100-125 billion annual agricultural loss worldwide (Sasser and Freckman, 1987; Chitwood, 2003) and have different modes of feeding on plant tissue. However, many destructive PPN cause problems by feeding on living plant cellular contents, altering root cellular structure and function (Jung and Wyss, 1999). These biotrophic parasitic nematodes greatly affect plant growth. Some PPN feed by inserting their mouthpart called stylet into the plant cell, delivering virulence factors called effectors that they produce in their secretory glands (Jones and Dangl, 2006). Nematode parasitism sometimes results in the production of enlarged cells called giant cells that affect growth and development of surrounding root tissue. This process results in the development of galls or root knots (Dangl and Jones, 2001). In contrast, parasitized cells called syncytia are made by the localized breakdown of cells walls caused by the nematode effectors, followed by the merging of their cytoplasm. Therefore, these effectors that are produced in the esophageal glands of nematode, delivered to plant cell through stylet, dissolve cell walls forming a syncytium.

The dissolution of these cell walls happens because of the cutinases and cell wall hydrolyzing enzymes such as cellulase, pectinase, polygalacturonases and xylanases (Hammond-Kosack and Jones, 1996). In these cases where the nematode successfully parasitizes the plant cell, the interaction is called a compatible reaction and the plant is susceptible to the pathogen. In contrast, when the plant responds to the pathogen attack by a successful defense response that suppresses their infection, the interaction is incompatible and the host is resistant (Glazebrook et al. 1997).

Soybean and Soybean Cyst Nematode

Glycine max (soybean) is an important world-wide crop, ranking as the second largest crop grown in United States and is the most important export crop (USDA, 2013). Soybean has tremendous use, including its great value in nutrition. Soybean extracts are also used in different products, including biofuel (Pimentel and Patzek, 2005; Hartman et al. 2011). However, soybean production is hampered by abiotic and biotic factors. Abiotic factors include extremities in water, temperature and nutrients while biotic factors include diseases that reduce its production and yield (Hartman et al. 2011). The most significant pathogen of soybean is the parasitic nematode, *Heterodera glycines* (Wrather et al. 2001; Wrather and Koenning, 2006). *Heterodera glycines*, known as soybean cyst nematode (SCN), was first described in Japan even though the center of SCN distribution is believed to be China (Ichinohe, 1952). SCN causes more economic loss in soybean than rest of its pathogens combined, resulting 7-10% yield loss world-wide and ~1 billion dollars in the U.S. each year (Wrather and Koenning, 2006; Smolik and Draper, 2007; Koenning and Wrather, 2010). SCN was first reported in the U.S. in 1954 in North Carolina (Winstead et al. 1955) and in 1957 in Mississippi (Spears, 1957).

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SCN is primarily associated with *G. max*. However, SCN has been reported to reproduce in 97 different legume and 63 non-legume species (Epps and Chambers, 1958; Riggs and Hamblen, 1962, 1966a, b). Subsequent studies have shown that SCN infects nearly 400 plant species (Niblack et al. 2002). External symptoms of SCN infection may not be visible under low nematode population (Smolik and Draper, 2007). However, higher populations of SCN infection results in chlorotic patches in leaves, root necrosis and suppression of growth and development, indicating highly altered plant cell physiological processes (Gao et al. 2003; Chang et al. 2011). The problem caused by SCN is further complicated because the species is comprised of a complex of at least 16 different races that can even infect soybean cultivars that are considered resistant. Thus, identifying sources of plant resistance from which the mechanism of defense can be understood are urgently sought.

Life cycle of *Heterodera glycines*

SCN is the major pest of soybean which is one of the major causes for yield reduction (Inagaki and Tsutsumi, 1971; Wrather et al. 2001, Wrather and Koenning, 2006). SCN can survive in soil for up to 9 years depending on moisture conditions and can maintain their infective property up to 7 years (Inagaki and Tsutsumi, 1971). Eggs survive in structures called cysts which is the carcass of the senesced female. Eggs lose their hatching capability after 11 years (Inagaki and Tsutsumi, 1971). The number of eggs per cyst is around 200 on average (Lauritis et al. 1983). The hatching property of eggs are regulated by environmental factors. In field conditions they delay hatching and infection, whereas the constant temperature and moisture increase rate of hatching and infection in greenhouse or culture condition (Masler et al. 2008). Hatching is low in

encysted eggs compared to free eggs and low temperature exposure reduces hatching of eggs (Masler and Roger, 2011; Masler et al. 2013). Plant roots exude primary and secondary metabolites that consist of nicotinic acid, oxalic acid, salicylic acid, vanillic acid and other compounds that might be the indicator for juveniles to sense root for infection (Badri et al. 2012). Hatching is influenced by the chemicals present in exudates of host roots and the watery extract of cysts and broken eggs of the same species (high effect) and different species (low effect) of nematodes (Tsutsumi and Sakurai, 1966; Okada, 1973). After hatching, juveniles move randomly in the soil until it finds roots for feeding.
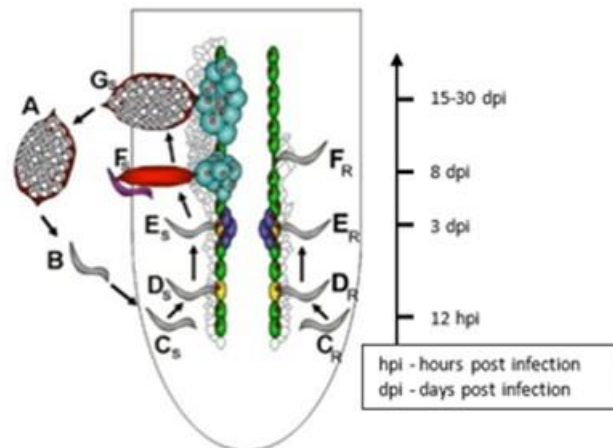
Figure 1.1   Life cycle of Heterodera glycines.

Note: A, Cysts. B, pi-J2s (gray) hatch and migrate toward the root of soybean. C_S and C_R, i-J2 nematodes burrow into the root and migrate toward the pericycle (green). D_S and D_R, i-J2s select a cell (yellow) for feeding site establishment. E_S, i-J2 nematodes have molted into J3. E_R, i-J2 nematodes do not increase in size. F_S, the J3s undergo a subsequent molt into J4 nematodes. Meanwhile, the female continues to grow circumferentially as it feeds. The male discontinues feeding at the end of its J3 stage. Male and female J4 nematodes become adults. The vermiform male (magenta) burrows outside the root and copulates with the female. F_R, the syncytium collapses and the nematodes do not grow. G_S, after approximately 30 days, the female with eggs is clearly visible and emerging from the root. (Adapted from Klink et. al. 2009a).

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Identification of resistance in Soybean

Resistance of *G. max* to SCN has been identified (Ross and Brim, 1957). This identification was accomplished by examining the collection of *G. max* accessions made from wild populations (accessions) of soybean made by the USDA (Bernard and Cremeens, 1988). Resistant accessions were identified from which many commercial resistance varieties have been made subsequently (Ross and Brim, 1957; Endo, 1965; Riggs et al. 1973; Acedo et al. 1984). From these accessions three major recessive resistance loci known as resistance to *Heterodera glycines* (rhg) are: rhg1, rhg2 and rhg3 (Caldwell et al. 1960) and two dominant resistant loci rhg4 (Matson and Williams, 1965) and rhg5 (Rao-Arelli, 1994) were identified through genetic mapping efforts. Subsequent studies that examined gene expression that occurs during the resistant and susceptible reactions led to the identification of the resistance gene alpha soluble N-ethylmaleimide-sensitive factor attachment protein (α-SNAP) (Matsye et al. 2011, 2012). The identification of α-SNAP functioning in soybean resistance to SCN implicated the process of vesicle transport and membrane fusion being important to the defense process. Other studies have shown that the overexpression (OE) of other candidate resistance genes including the vesicle transport gene syntaxin 31 (SYP38) also results in resistance (Pant et al. 2014, 2015). In contrast, RNA interference (RNAi) (Fire et al. 1998) of SYP38 resulted in engineered susceptibility in soybean genotype that is normally resistance to SCN (Pant et al. 2014). In other biological systems, α-SNAP and syntaxin bind directly (Clary et al. 1990; Lupashin et al. 1997). These results indicated that components of the vesicle transport system, in particular, are central to membrane fusion

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would be important in the resistance of soybean to SCN. However, the extent of their role was not examined in detail. This is the central premise of this thesis.

Membrane Fusion and vesicular Transport

The central premise of this thesis is that the core components of the membrane fusion apparatus function in the resistance of soybean to the SCN. The core components of the vesicle transport machinery would be involved in the delivery of secreted materials to the infection site to prevent infection and/or parasitism. Furthermore, it is believed that the components studied here may also influence the transcriptional activity of the other membrane fusion components and is known as coexpression. Coexpression has been observed for α-SNAP and SYP38 in the soybean-SCN pathosystem, but has not been determined for other core members of the membrane fusion machinery (Pant et al. 2014).

Vesicle transport as it relates to plant defense

Membrane fusion genes work in concert as the Soluble NSF Attachment Protein (SNAP) REceptor (SNARE) complex (Bock et al. 2001). Since many proteins and paralogs are involved in vesicle transport, membrane fusion is carried out by SNAREs that consist of small and membrane anchored proteins and their conformation changes due to an assembly and disassembly process (Jahn and Scheller, 2006; Barszewski et al. 2008; Jahn and Fasshauer, 2012). The intracellular fusion events are important for cellular mechanisms, transport of hormones and response (Rathore et al. 2010). The membrane fusion process is mediated by the zippering of v-SNAREs and t-SNAREs together (Jahn and Scheller, 2006). Binding of t-SNARE and v-SNARE occurs before vesicular fusion (Weber et al. 1998).

In *A. thaliana*, SYP122 expression is induced after pathogen attack by the powdery mildew fungus *Erysiphe cichoracearum*, bacterium *Xanthomonas compestris* and tobacco mosaic virus [TMV] (Assaad et al. 2004). PEN1 (SYP121) induces polarized secretion that forms papillae against fungal infections (Assaad et al. 2004). Double mutants of PEN1 and SYP122 results dwarf and necrotic plants (Assaad et al. 2004). The study of cytokinesis in *A. thaliana* root cells revealed involvement of VAMP721/722 in cell plate formation (Zhang et al. 2011) and their double mutants seedling found problematic in cytokinesis and yielded stunted growth and lethal seedlings (Zhang et al. 2011). The *A. thaliana* (At) AtSNAP33 interacts with the syntaxin Knolle (KN) and the secretion 1 (sec1) homologue KEULE (KEU) for cell plate formation (Heese et al. 2001). The mutational analysis of AtSNAP33, a SNAP-25 homologue, yielded dwarf and necrotic plants that ultimately died (Heese et al. 2001). The study of vesicle genes and the above results indicated the involvement of VAMP genes, (VAMP721 and VAMP722) in vesicular transport through the trans Golgi network (TGN)/ early endosomal compartment to cell membrane for membrane fusion contributing to cytokinesis to form cell plate (Zhang et al. 2011).

The involvement of the core machinery for vesicle fusion in *G. max* resistance to *H. glycines*

Recent studies, in addition to those of Matsye et al. (2012) on α-SNAP, have shed light on the involvement of these membrane fusion genes in soybean resistance to SCN. For example, OE of a soybean homolog of the plant syntaxin 31 (Gm-SYP38) which localizes to the cis face of the Golgi apparatus in both yeast and plants results in engineered resistance (Pant et al. 2014). The plant syntaxin 31 was originally identified

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as suppressors of the erd2-deletion 5 (Sed5p) in yeast (Hardwick and Pelham, 1992; Banfield et al. 1995; Lupashin et al. 1997; Leyman et al. 1999; Sanderfoot et al. 2001; Peng and Gallwitz, 2004; Bubeck et al. 2008). While Pant et al. (2014) demonstrated that OE of the Gm-SYP38 in the SCN-susceptible *G. max* [Williams 82(PI518671)] led to engineered resistance, suppression of the expression of Gm-SYP38 in the resistant *G. max* [Peking/PI548402] by RNAi, resulted in engineered susceptibility in a soybean genotype that is normally resistant. The experiments of Matsye et al. (2012) and Pant et al. (2014) demonstrated the importance of membrane fusion genes in resistance of soybean to SCN. The experiments proposed here build on those observations by characterizing additional genes that are part of the core membrane fusion complex (Jahn and Fasshauer, 2012). However, there exist other core components of the membrane fusion machinery whose involvement has not yet been studied. Those proteins are Munc18, syntxin121 synaptobrevin, synaptotagmin, SNAP-25 and NSF (Table 1.1) and are central to this thesis. α-SNAP, while studied earlier (Matsye et al. 2012) is examined here in a series of gene expression experiments. In this thesis, core soybean homologs of components of the membrane fusion apparatus are identified. The genes are genetically engineered to be overexpressed in a soybean genotype that is normally susceptible to SCN infection. It is hypothesized that the susceptible genotype will become resistant to SCN infection. In contrast, these same genes are engineered in a manner that will suppress their expression in a genotype that is normally resistant to SCN infection. It is hypothesized that the resistant genotype will become susceptible because of the inactivation of that gene through RNAi.

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CHAPTER II

MATERIALS AND METHODS

Gene Isolation

α-SNAP was identified as a resistance gene (Matsye et al. 2011, 2012). This observation led to the examination of the role of other core membrane fusion genes in resistance. Candidate gene nucleotide sequences were exported from the soybean genome housed at (http://www.phytozome.net/) (Schmutz et al. 2010) and used to design PCR primers for cloning experiments (Table 2.1). Genes were amplified through PCR using cDNA from *G. max* root RNA. Amplicons were excised from 1% agarose gel and purified using Qiagen gel purification kit and then ligated into pENTR vector (Invitrogen®), followed by transformation to chemically competent Top 10 *Escherichia coli* (*E. coli*) and selected in Lysogeny Broth with Kanamycin 5 µg/ml (Invitrogen protocol). Colony PCR was done to check amplicons and plasmid extracted from *E. coli* was sequenced to confirm correct sequence by matching with its original gene accession. After conformation the gene of interest (GOI) was further ligated to destination vector pRAP15 for OE or pRAP17 for RNAi with LR clonase reaction (Invitrogen®). The LR reaction was followed by bacterial transformation using chemically competent Top 10 *E. coli* strain (Invitrogen®). Selection was done by using LB tetracycline (5µg/ml) plates and further confirmation through colony PCR.

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Table 2.1   PCR primers used for cloning of Genes (OE)

GENE TYPE	ACCESSION	PRIMER DIRECTION	PRIMER SEQUENCES	GC%	LENGTH	Tm
SYT3	Glyma10g35410	Forward	5'CACCATGGGGGTTCTTGAGCAGTTTCTT 3'	48.1	27	66.1
		Reverse	5' TTAAACCACCTTCCATCTTATCTCAAC 3'	37	27	61.6
Munc18-1	Glyma11g03230	Forward	5' CACCATGTCTATGTCCGATTCCGATTC 3'	48.1	27	66.1
		Reverse	5' TCATATCTGGATATCATCTAATGAAAGC 3'	32.1	28	60.2
SYNTAXIN 121	Glyma02g35210	Forward	5' CACCATGAACGACTTGTTCTCCGG 3'	54.2	24	66.3
		Reverse	5' TCAATTTCTTAGAACTATAGGGAGGACT 3'	35.7	28	61.7
SNAP25-4	Glyma17g08450	Forward	5' CACCATGTTTGGTTCAAAGAAATCTCCTTT 3'	36.7	30	63.3
		Reverse	5' TTATTTTCCGAGCAAACGACG 3'	42.9	21	58.7
NSF-1	Glyma13g24850	Forward	5' CACCATGTTCGGCTTATCGTCTTCG 3'	52	25	66.2
		Reverse	5' TTATCTAACAACATCCTGGAGGC 3'	43.5	23	61
VAMP-2	Glyma07g04740	Forward	5' CACCATGGTGAAGTTGACTATGATTGCC '	46.4	28	66.1
		Reverse	5' TCACCATAGTTTATTTTTGATCCAGAA 3 '	29.6	27	58.5

Table 2.2   PCR primers used in cloning of genes (RNAi)

GENE TYPE	ACCESSION	PRIMER DIRECTION	PRIMER SEQUENCES	GC%	LENGTH	Tm
SYT3	Glyma10g35410	Forward	5' CACCTGGGCTGGTAATCCAAACATAG 3'	50	26	66.2
		Reverse	5' TTGCTGGAAGTTTGTCTCCTGT 3'	45.5	22	60.8
Munc18-1	Glyma11g03230	Forward	5' CACCTTCCGATTCCTCTTCTTACGCC 3'	53.8	26	67.7
		Reverse	5' TTGTGATGAAACCCTGGCTGTC 3'	50	22	62.7
SYNTAXIN 121	Glyma02g35210	Forward	5' CACCATGAACGACTTGTTCTCCGG 3'	54.2	24	66.3
		Reverse	5' TCAATTTCTTAGAACTATAGGGAGGACT 3'	35.7	28	61.7
SNAP25-4	Glyma17g08450	Forward	5' CACCAACCCCTTTGATGATGGCACTG 3'	53.8	26	67.7
		Reverse	5' AGTCAGCCCCAACTTCTCTCTT 3'	50	22	62.7
NSF-1	Glyma13g24850	Forward	5' CACCGCTTATCGTCTTCGTCTTCCTCTG 3'	53.6	28	69
		Reverse	5' TCTGTTCACTCTTACTCCCCTTTTT 3'	40	25	61.3
VAMP-2	Glyma07g04740	Forward	5' CACCATGGTGAAGTTGACTATGATTGCC '	46.4	28	66.1
		Reverse	5' TCACCATAGTTTATTTTTGATCCAGAA 3 '	29.6	27	58.5

Vector Pipeline

The pRAP15 vector is designed to express full length genes (Matsye et al. 2012).

The pRAP17 vector is designed to suppress transcription through RNA interference

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(RNAi) (Klink et al. 2009b). Both vectors are especially designed for *Agrobacterium rhizogenes* mediated root transformation (Tepfer, 1984). The expression of GOI in pRAP15 and pRAP17 is driven by the figwort mosaic virus sub-genomic transcript (FMV-sgt) (Fig 2.1). The enhanced green fluorescent protein (eGFP) is used as a visual reporter for transgenic roots.
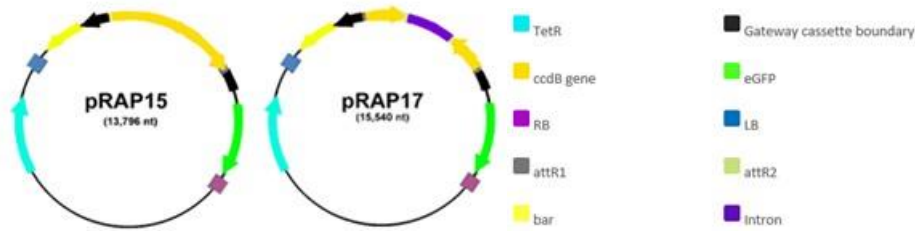
Figure 2.1   pRAP15, overexpression; pRAP17, RNAi Vectors.

Note: Legend of vector components; functional cassette is between left and right border (LB, RB). Cyan, tetracycline resistance; blue, LB; black, Gateway cassette border; gray, attR1; orange, ccdB gene; olive, attR2; purple, intron; green, eGFP; yellow, bar gene; mauve, RB.

Plant growth and greenhouse management

All plant transformation procedures were similar for *G. max* [Williams 82/PI 518671] and *G. max* [Peking/PI 548402]. Seedlings were grown for 7-9 days in greenhouse at ambient temperatures. Plant transformation was done by cutting plants near junction of root and stem (at soil surface) with a clean, sharp razor blade in Murashige and Skoog (MS) media. The plant and recombinant *Agrobacterium rhizogenes* were then cocultivated overnight and then replanted in fresh coarse vermiculite 3-4cm deep in 50 holes flats. Replanted plants were kept in culture room under ambient temperatures for 1 week with light watering and supplement of light. The eGFP expressing root primordia were evident after 5 days. At this point, the plants were moved to the greenhouse and grown under

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ambient temperatures and natural sunlight. Plants were fertilized with commercially available Miracle Grow® twice a week. Plants were uprooted and non-transformed roots were excised after putting in greenhouse for 20-25 days. The transformed roots expressing the eGFP reporter were seen under Dark Reader® Spot Lamp (Clare Chemical Research, Dolores, CO, USA). Plants having genetically engineered roots were planted in a pot with autoclaved soil with 1: 1 mixture of sand and clay.

Infection

Unengineered *G. max* [Williams 82/PI 518671] are susceptible to the SCN race *H. glycines* [NL1-Rhg/HG-type 7/race 3] (Klink et al. 2005; Pant et al. 2014). SCN females are separated from soil and plant debris through sucrose flotation (Jenkins, 1964; Matthews et al. 2003; Matsye et al. 2012) and were crushed to release eggs. Eggs separated from debris by passing through 200 mesh sieve nested on 500 mesh sieves. Contents of 500 mesh sieve consisting of eggs were collected in beakers. Second stage juveniles J2s were obtained from eggs and its concentration of 2000 J2/ml were used to infect each plant (Matsye et al. 2012). A 1 cm wider hole, 4-5 cm deep was made near plant stem directed toward the root. The inoculum was administered into the hole. The holes were then covered with soil right after inoculation. Acid-fuschin staining of roots was done after inoculation to confirm nematode infection (Byrd et al. 1983).

Cyst Extraction

Cyst extraction was done after 30 days of infection by massaging individual roots in water to separate them from roots in individual buckets (Klink et al. 2009b). The soil of individual root system was washed three times by stirring to further induce cyst

flotation in water. Nematodes were harvested by using a 20-mesh sieve to separate debris followed by 100-mesh sieve.

Female Index calculation

The female index (FI) is calculated according to the root mass and cyst count obtained from each plant (Golden et al. 1970). Three biological replicates were performed for each treatment. The average number of females in test cultivar is denoted by Nx and the average number of females in control with empty vector is denoted by Ns. FI is calculated as FI= (Nx/Ns) x 100 (Golden et al. 1970; Riggs and Schmitt, 1988, 1991; Niblack et al. 2002; Klink et al. 2009b; Matsye et al. 2012; Matthews et al. 2013; Pant et al. 2014). The FI analysis was done statistically using the Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test, $p < 0.05$ (Matsye et al. 2012).

Quantitative real-time PCR (qPCR)

The effect of the OE or RNAi cassette was confirmed by qPCR. RNA was isolated from the root samples collected at 0, 3 and 6 dpi. Gene expression was confirmed using primers designed toward the target gene using ribosomal S21 gene as a control (Klink et al. 2005; Alkarhouf et al. 2006) (Table 2.3). The experiment was conducted using Taqman 6 carboxyfluorescein (6-FAM). The qPCR reaction conditions include a 20 µl Taqman Gene Expression Master Mix (Applied Biosystems; Foster City, CA), 0.9 µl of µM forward primer, 0.9 µl of 100 µM reverse primer, 2 µl of 2.5 µM 6-FAM (MWG Operon®) probe and 9.0 µl of template cDNA in (6-FAM) probes and Black Hole Quencher (BHQ1) (MWG Operon; Birmingham, AL). The qPCR reactions were performed on an ABI 7300 (Applied Biosystems®). The statistical analysis was done

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using $2^{-\Delta\Delta CT}$ to determine fold change using derived formula as described in Livak and

Schmittgen (2001).

Table 2.3   Primers used in qPCR expression studies

GENE TYPE		PRIMERS SEQUENCES	PROBE SEQUENCES
SNAP25-4	Forward	5' AACTACAAATTCAGTCACAAATTGTTTG 3'	5' AGGCTACCCAGACACTAGTCACCCTTCATC 3'
	Reverse:	5' CCCCGAGTTAGATCATGATCG 3'	
Munc18-1	Forward:	5' GTGAAGATAATGTCTCACTCATGCA 3'	5' GGTTGAAGACATATACAAGAGAAGGCAGCC 3'
	Reverse:	5' GAAATATATAGCATCCATGGTGGG 3'	
NSF-1	Forward:	5' CCCGGTCACAACAACCTCTAC 3'	5' TGACAACATAGGCAGCGGCCAG 3'
	Reverse:	5' TCGCCGGAAGAAACTTTGAC 3'	
SYT-3	Forward:	5' GGTTTTTGGTTTTGCCGTTG 3'	5' CTCTTGGCCTCTTGGTTGGGTTCTTTC 3'
	Reverse	5' GGGCCTAATTCACTAATTGGCC 3'	
VAMP-2	Forward:	5' GAAGAGCTGAGGAATGAGTTTGAG 3'	5' GGTCTCAAATTGAAACTGCTGCTAGACCT 3'
	Reverse	5' CGCTGAGTATGTGTATCCTGGTAA 3'	
SYNTAXIN 121	Forward:	5' AGGGAAGCGTGAATCTCGAA 3'	5' CTAGAGCGTCTCCATGAAAATCTGCGT 3'
	Reverse:	5' GAAGTTCCTTCACAGCTTTGGC 3'	
Ribosomal protein gene (S21 gene)	Forward:	5' ATGCAGAACGAGGAAGGACAG 3'	5' CCTAGGAAGTGCTCTGCCACAAC 3'
	Reverse:	5' GAAGCATGGTCCTTAGCG 3'	

CHAPTER III

RESULTS

Gene selection and validation

Prior studies, in soybean have shown α-SNAP and syntaxin 31 function in the

defense process of soybean toward SCN (Matsye et al. 2012, Pant et al. 2014). However,

all of the core components have not yet been examined. To obtain a clearer picture of the

involvement of the core membrane fusion apparatus and defense, homologs of the

remaining gene family members were identified. During the course of the analysis, it was

observed that there were a number of related genes for SYP121, SNAP-25, Munc18,

VAMP, SYT and NSF.

RNA sequencing was done in *G. max* [Peking/PI 548402] from desired cell type which

shows pool of genes that are active transcriptionally during defense response (Matsye et

al. 2011). The induced level of transcription is shown as tag count as they are detected by

the specific probe sets designed for the gene (Table 3.1). The tag count provides basis for

cloning membrane fusion genes which is supposed to be induced during resistance

reaction. To narrow down the number of genes, RNA sequencing data was analyzed from

soybean roots overexpressing the syntaxin 31 gene which had resulted in engineered

resistance to SCN (Pant et al. 2014, 2015) (Table 3.1). The experiment demonstrated the

presence of the candidate gene expressed in the roots undergoing the resistant reaction.

PCR primers were designed for cloning selected candidate genes. After the candidate

genes were cloned into the pRAP15 or pRAP17 vectors, the genes were genetically

engineered into soybean roots (Figure 3.1). qPCR was used to confirm that the genes

were overexpressed in the SCN susceptible cultivar *G. max* [Williams 82/PI 518671] (Table 3.2).

In contrast, roots engineered for undergoing RNA interference for the candidate genes

were performed in the SCN resistant cultivar *G. max* [Peking/PI 548402] (Table 3.3).

Table 3.1   RNA sequencing data used in candidate gene selection

Gene	Locus	Tag Count
Syntaxin 121	Glyma02g35210	75.6465
SNAP-25-4	Glyma17g08450	162.969
Munc18-1	Glyma11g03230	18.4333
Synaptotagmin-3	Glyma10g35410	1.81509
VAMP-2	Glyma07g04740	24.9494
NSF-1	Glyma13g24850	12.1872

The roots with empty pRAP15 and treatments are shown in fig 3.1. The control

with empty vector has similar response in terms of root growth, nematode population and

maturity (Klink et al. 2009b; Matsye et al. 2012; Pant et al. 2014).
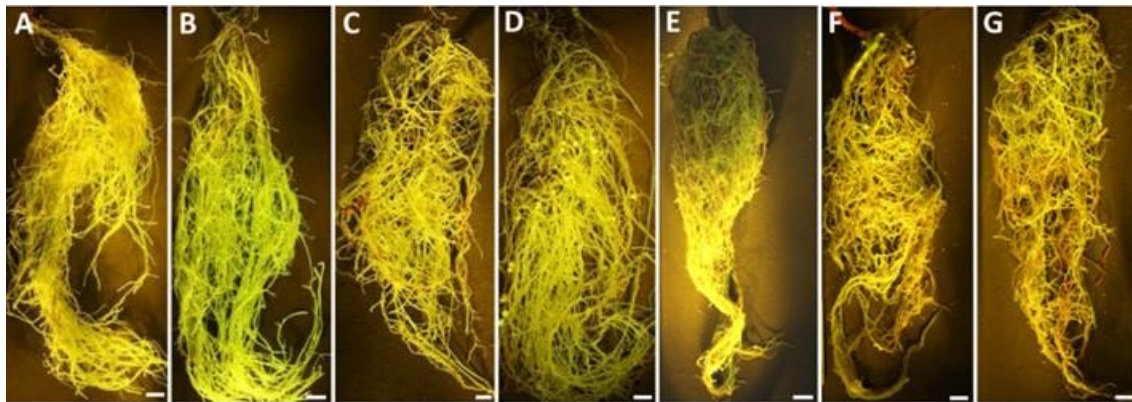

Figure 3.1   Genetically engineered roots *G. max* roots.

Note: A, pRAP15 control; B, SYP121-OE; C, SNAP25-4-OE; D, Munc-18-OE; E,
VAMP-2-OE; F, SYT-3-OE; G, NSF-1-OE. Bar = I cm.

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Table 3.2   Table of qPCR expression studies in roots overexpressing candidate genes

	PRIMER							
GENE TREATMENT	SNAP25	MUNC18	SYNAPTOBREVIN	SYNAPTOTAGMIN	NSF	SYNTAXIN 121	α-SNAP-5	SYNTAXIN 31
SNAP25-OE	5.076848	2.652682	8.159211	6.938819	3.760784	7.286889	2.355711	3.756111
MUNC18-OE	2.526263	2.313504	3.038441	(-)2.96583	2.569533	(-)4.66534	2.239826	1.319024
SYNAPTOBREVIN-OE	4.315266	4.188191	7.275957	1.698507	9.794547	5.290585	4.49627	2.203846
SYNAPTOTAGMIN-OE	9.187806	6.06209	4.224155	3.77379	6.406934	6.518199	3.452094	2.614552
NSF-OE	6.406832	3.823477	7.888125	2.038929	4.445287	1.704326	3.554219	2.908595
SYNTAXIN 121-OE	12.60012	4.02733	8.069313	9.292706	5.947093	3.491206	1.823704	2.791163
α-SNAP-5-OE	2.4233	4.5351	7.13523	1.38533	9.4996	1.90168	3.258832587	1.269692
SYNTAXIN 31-OE	3.46795	12.12833	9.065048	72.74945	14.50882	19.30272	4.508846	44.97

Table 3.3   Table of qPCR confirming RNAi of candidate genes

	PRIMER							
GENE TREATMENT	SNAP25	MUNC18	SYNAPTOBREVIN	SYNAPTOTAGMIN	NSF	SYNTAXIN 121	α-SNAP-5	SYNTAXIN 31
SNAP25-RNAi	(-)4.666746	(-)1.21374	(-)3.57638	(-)22.4352	(-)19.5229	(-)2.63114	(-)1.14166	(-)1.895317
MUNC18-RNAi	(-)8.25878	(-)1.31111	(-)4.79902	(-)18.2463	(-)14.6417	(-)4.73174	(-)1.72275	(-)1.39971
SYNAPTOBREVIN-RNAi	(-)4.68355	(-)2.26207	(-)9.26115	(-)8.18906	(-)24.3208	(-)1.64729	(-)2.899	(-)4.95298
SYNAPTOTAGMIN-RNAi	(-)3.09241	(-)2.1331	(-)7.32207	(-)2.98609	(-)14.3456	(-)2.78312	(-)1.32574	(-)1.32103
NSF-RNAi	(-)2.79199	(-)1.40285	(-)4.006	(-)37.9359	(-)4.64484	(-)1.51795	(-)1.5075	(-)1.7918
SYNTAXIN 121-RNAi	1.50546	(-)2.970764	(-)1.52392	(-)3.11917	(-)13.0236	(-)1.83726	(-)1.34311	(-)1.73909
α-SNAP-5-RNAi	(-)6.9783	(-)1.84306	(-)1.61593	(-)1.83486	(-)5.90495	(-)2.72963	(-)4.05098	(-)10.9651
SYNTAXIN 31-RNAi	(-)1.89532	(-)1.39971	(-)1.67314	(-)1.20737	(-)1.7918	(-)1.10708	(-)1.261886	(-)1.50263

OE FI and qPCR of all genes

The *G. max* [Williams 82/PI 518671] roots that were overexpressing core proteins of membrane fusion genes have resulted in suppression of females in susceptible cultivar *G. max* [Williams 82/PI 518671] (Fig 3.2-3.7). The process of OE of the candidate genes rescues *G. max* [Williams 82(PI518671)], rhg1^{-/-} from nematode parasitism making it resistant (Matsye et al. 2012, Pant et al. 2014). The qPCR analysis of these core proteins resulted their expression level in overexpressed *Glycine max* roots (Table 3.2). The value of OE is

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different for different genes. OE of Gm-α-SNAP induces the transcription of Gm-SYP38 (Pant et al. 2014). Similarly, the OE of the membrane fusion genes on this study induces transcription of other components of vesicular transport (Table 3.2). Higher level of genetic expression might have been regulated by various factors which needs further study to demonstrate this concept. These roots then were infected with SCN. The plants were allowed to undergo the process of infection for 30 days. Some representative plants were collected at 3 and 6 dpi for RNA isolation. This RNA was used in later studies that assayed gene expression during the course of the resistant reaction which normally reaches its conclusion by 6 dpi (Endo, 1965). For the rest of the plants, at the end of 30 days, the cysts were extracted from control roots and roots overexpressing core membrane fusion genes and analyzed for the effect they had on parasitism. Each test has three replicates; Replicate1 (R1), Replicate 2 (R2) and Replicate 3 (R3). Each replicate have 15 plants with genetically engineered roots. Control in each OE has 15 plants with engineered empty pRAP15 which is similar to *G. max* [Williams 82(PI518671)] in roots growth, nematode infection and maturation without any genetic effect (Matsye et al. 2012, Pant et al. 2014).

The FI analysis of Gm-SYP121 demonstrates there is 48.5-65.88 % reduction of the female population across all replicates relative to the control population (Fig 3.2). This data is supported by the qPCR which results 3.49 fold expression of Gm-SYP121 in transgenic roots of *G. max* [Williams 82(PI518671)] with Gm-SYP121 OE compared to control using formula as stated in materials and methods (Table 3.2).

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Figure 3.2   The female index (FI) of *G. max* [Williams 82(PI518671)] overexpressing Gm-
              SYP121.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3.
(* Statistically significant P value < 0.05).

The analysis of FI indicates there is reduction of nematode population from 54.26-62.96% in transgenic roots of *G. max* [Williams 82(PI518671)] with Gm-SNAP25-4 OE across all replicates compared to control population in *G. max* [Williams 82(PI518671)] (Fig 3.3). The reduction of FI indicates some genetic effects in roots which is supported by qPCR study that shows 5.07 fold increase in SNAP-25-4 expression in transgenic roots with SNAP25-4 OE compared to control (Table 3.2).

Figure 3.3   The female index (FI) of *G. max* [Williams 82(PI518671)] overexpressing Gm-
              SNAP-25-4.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3   (* Statistically significant P value < 0.05).

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The FI analysis of transgenic roots with Gm-Munc18-1 OE in *G. max* [Williams 82/PI 518671] shows there is a reduction of 65.29-70.2% nematode population across all the replicates compared to the control population (Fig 3.4). The qPCR studies of *G. max* [Williams 82/PI 518671] engineered to overexpress Munc18-1 showed 2.31 fold overexpression of Munc18-1 compared to control which supports results from FI analysis (Table 3.2).

Figure 3.4   The female index (FI) of *G. max* [Williams 82(PI518671)] overexpressing Gm-Munc18-1-OE.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3.
(* Statistically significant P value < 0.05).

The FI analysis demonstrates that VAMP-2 OE in *G. max* [Williams 82/PI 518671] reduced nematode population by 49.18-71.43% compared to control population in *G. max* [Williams 82/PI 518671] (Fig 3.5). The qPCR results shows VAMP-2 is overexpressed 7.27 fold in transgenic roots of *G. max* [Williams 82/PI 518671] with VAMP-2 OE compared to control (Table 3.2).
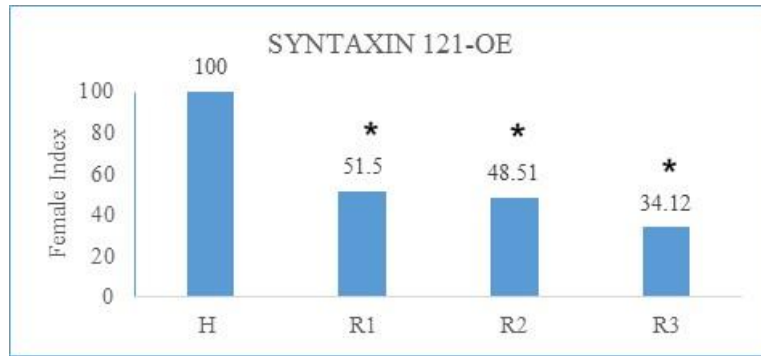
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Figure 3.5   The female index (FI) of *G. max* [Williams 82(PI518671)] overexpressing Gm-VAMP-2.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3.
 (* Statistically significant P value < 0.05).

The qPCR expression analysis in *G. max* [Williams 82/PI 518671] with SYT-3 OE results

3.77 fold induced expression of SYT-3 compared to control (Table 3.2). The nematode

population was suppressed 51.3-76.48% across the replicates in *G. max* [Williams 82/PI 518671]

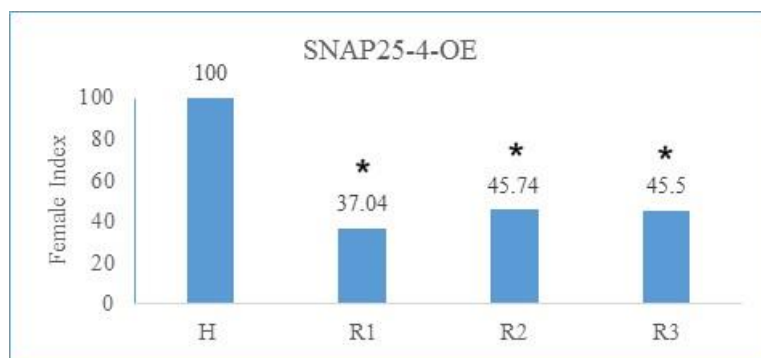with SYT-3 OE relative to nematode population in control roots (Fig 3.6).

Figure 3.6   The female index (FI) of *G. max* [Williams 82(PI518671)] overexpressing Gm-SYT-3.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3.
 (* Statistically significant P value < 0.05).

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The qPCR results shows NSF-1 is overexpressed 4.44 fold in *G. max* [Williams 82/PI 518671] roots with NSF-1 OE compared to control (Table 3.2). The FI analysis shows OE of NSF-1 in *G. max* [Williams 82(PI518671)] reduced nematode population by 59.64-77.32% across the replicates compared to nematode population in control roots (Fig 3.7).
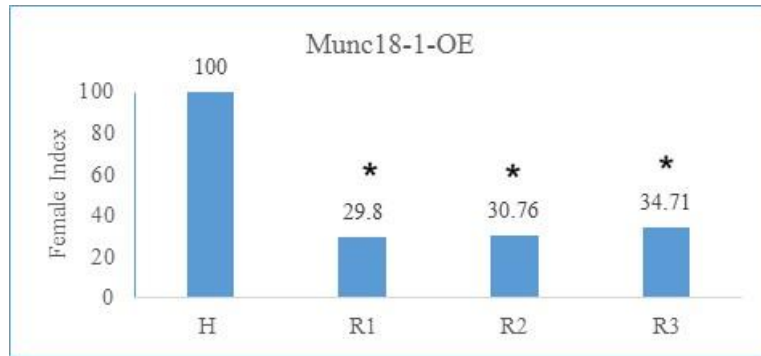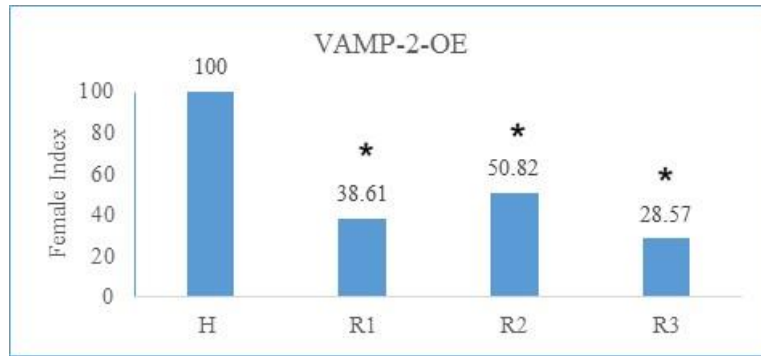

Figure 3.7   The female index (FI) of *G. max* [Williams 82(PI518671)] overexpressing Gm-NSF-1.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3.
(* Statistically significant P value < 0.05).

RNAi FI and qPCR of all genes

The approach used for roots genetically engineered to suppress nematode population through the RNAi in resistance cultivar *G. max* [Peking/PI 548402] was similar with OE studies. The gene expression studies of core components in transgenic roots of resistant cultivar *G. max* [Peking/PI 548402] with RNAi showed reduced level of gene expression for each specific gene that were targeted and other components of membrane fusion genes (Table 3.3). The resistant cultivar *G. max* [Peking/PI 548402] with rhg1$^{+/+}$ is turned susceptible by RNAi of core membrane fusion genes (Klink et al. 2009b; Pant et al. 2014). The reduced expression shows negative values for the genes tested in roots with the RNAi effect. These selected plant roots with eGFP expression were infected with

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SCN to further test their resistance ability towards infection. Roots were collected at 3 and 6 dpi to isolate RNA which is further used in gene expression studies during resistance reaction which reaches its conclusion by 6 dpi as in OE studies (Endo, 1965). The plants were allowed to undergo the process of infection for 30 days. For the rest of the plants, at the end of 30 days, the cysts were extracted from control roots and roots overexpressing core membrane fusion genes and analyzed for the effect they had on parasitism. The RNAi of the core membrane fusion proteins resulted increase in nematode population (Fig 3.8- 3.13). The transgenic roots were tested in 3 separate replicates R_1, R2 and R_3 with 15 plants in each replicates. The control consists of 15 plants with empty pRAP17 without gene. The plants with empty pRAP17 is normal as *G. max* [Peking/PI 548402] in terms of root growth, nematode infection and maturation (Klink et al. 2009b; Matsye et al. 2012; Pant et al. 2014).

Gm-syntaxin 121-RNAi plants exhibit suppressed syntaxin 121 expression. The expression of SYP121 in resistance cultivar *G. max* [Peking/PI 548402] after RNAi was -1.83 fold compared to control (Table 3.3). The FI analysis in Gm-syntaxin 121-RNAi plants shows increase in nematode population by 80-380% across three replicates compared to control (Fig 3.8).

Figure 3.8   The female index (FI) of *G. max* [Peking/PI 548402] expressing an RNAi gene
               cassette for Gm-Syntaxin 121.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3.
(* Statistically significant P value < 0.05).

        The level of expression of Gm-SNAP-25-4 in RNAi *G. max* [Peking/PI 548402] is -4.66

fold compared to control *G. max* [Peking/PI 548402] (Table 3.3). The FI analysis in *G. max*

[Peking/PI 548402] with Gm-SNAP-25-4 RNAi resulted 63.63-242.79% increase in nematode

population across the replicates compared to control (Fig 3.9).

Figure 3.9   The female index (FI) of *G. max* [Peking/PI 548402] expressing an RNAi-
               generating gene cassette for Gm-SNAP-25-4.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3.
(* Statistically significant P value < 0.05).

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RNAi studies revealed Munc18-1 is overexpressed -1.31 fold in their RNAi lines compared to controls *G. max* [Peking/PI 548402] (Table 3.3). The *G. max* [Peking/PI 548402] that were confirmed to be undergoing RNAi for Munc18-1 were then infected with SCN which shows 46.66-80% increase in nematode population across the replicates compared to control (Fig 3.10).

Figure 3.10   The female index (FI) of *G. max* [Peking/PI 548402] expressing an RNAi-generating gene cassette for Gm-Munc18-1.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3.
(* Statistically significant P value < 0.05).

The RNAi of VAMP-2 in *G. max* [Peking/PI 548402] shows -9.26 fold expression as compared to control *G. max* [Peking/PI 548402] (Table 3.3). The *G. max* [Peking/PI 548402] that were confirmed to be undergoing RNAi for VAMP-2 increased nematode population by 24.96-425% across the replicates compared to control (Fig 3.11).
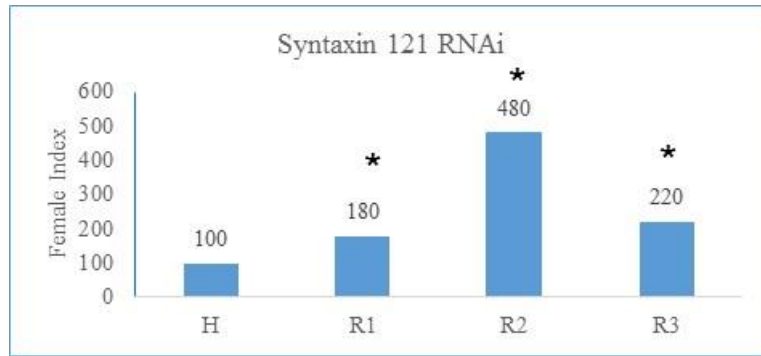
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Figure 3.11   The female index (FI) of *G. max* [Peking/PI 548402] expressing an RNAi-
               generating gene cassette for Gm-VAMP-2.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3.
(* Statistically significant P value < 0.05).

The qPCR analysis shows roots engineered with an SYT-3 RNAi have -2.98 fold

expression compared to control (Table 3.3). The *G. max* [Peking/PI 548402] that were

confirmed to be undergoing RNAi for SYT-3 were then infected with SCN, which shows

increase in nematode population by 20-22.22% across the replicates compared to control
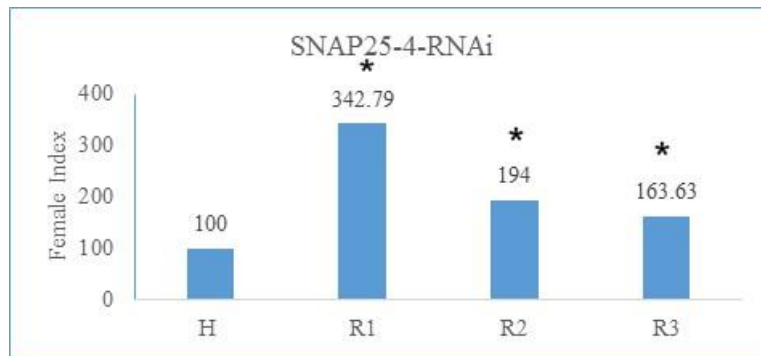
(Fig 3.11).

Figure 3.12   The female index (FI) of *G. max* [Peking/PI 548402] expressing an RNAi-
               generating gene cassette for Gm-SYT-3.

Note: H, control is set to a FI of 200. R1, replicate 1; R2, replicate 2; R3, replicate 3.
(* Statistically significant P value < 0.05).

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The qPCR analysis shows the RNAi of NSF-1 in *G. max* [Peking/PI 548402] has -4.64 fold expression as compared to respective controls *G. max* [Peking/PI 548402] (Table 3.2). The *G. max* [Peking/PI 548402] that were confirmed to be undergoing RNAi for NSF-1 increased nematode population by 33.33-50% across all replicates compared to control (Fig 3.13).
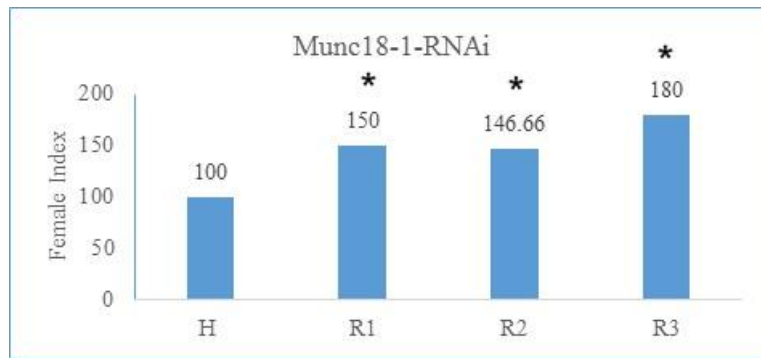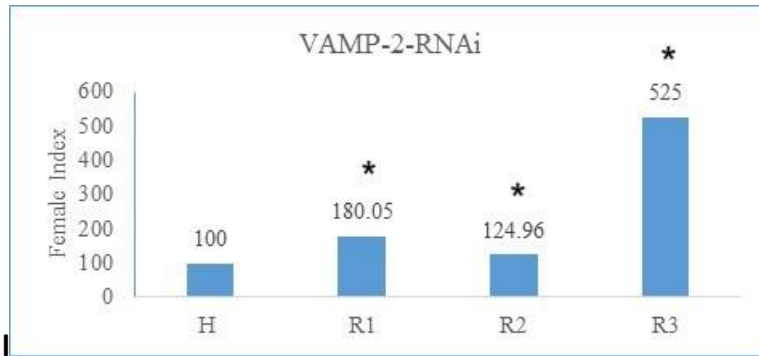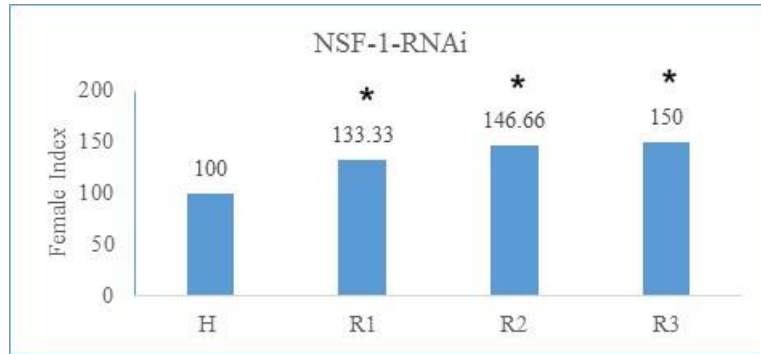

Figure 3.13   The female index (FI) of *G. max* [Peking/PI 548402] expressing an RNAi-generating gene cassette for Gm-NSF-1.

Note: H, control is set to a FI of 100. R1, replicate 1; R2, replicate 2; R3, replicate 3. (* Statistically significant P value < 0.05).

Quantitative Real Time PCR analysis of gene expression

The gene expression study of OE and RNAi of each tested gene cassette supported a role for those genes in resistance as demonstrated in the FI. As shown, the components of the membrane fusion genes syntaxin 121, SNAP-25, Munc18, VAMP, NSF and synaptotagmin are all overexpressed during the resistance reaction. In contrast, their suppressed gene activity is shown in the RNAi lines that resulted in engineered susceptibility. These results are consistent with prior studies that have shown the OE of α-SNAP (Matsye et al. 2012) and syntaxin 31 (Pant et al. 2014) suppresses nematode population. In an examination on the influence these genes have on each other, a matrix has been set up whereby the OE of syntaxin 121, SNAP-25-4, Munc18-1, VAMP-2,

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NSF-1, synaptotagmin-3 is examined for their influence on the other genes (syntaxin 121, SNAP-25-4, Munc18-1, VAMP-2, NSF-1, synaptotagmin-3) in the matrix (Table 3.2). The genes are examined further by examining how their expression is influenced by the α-SNAP resistance gene and its direct binding partner, syntaxin 31 (Matsye et al. 2011, 2012; Pant et al. 2014). The same matrix was examined for lines undergoing RNAi (Table 3.3).

CHAPTER IV

DISCUSSION

In the analysis presented here, soybean homologs of six important components of the vesicle transport machinery have been identified, cloned and tested in OE and RNAi experiments. These experiments were undertaken because of prior observations of the importance of α-SNAP in resistance of soybean to the SCN (Matsye et al. 2012). Furthermore, experiments supporting that observation were made by Pant et al. (2014), showing that syntaxin 31 (Gm-SYP38) was also an important aspect of the resistant reaction that soybean has to SCN infection. In the experiments presented here, I expand on those observations through experiments examining soybean homologs of SYP121, SNAP-25-4. Munc18-1, VAMP-2, SYT-3 and NSF-1. The genetic engineering experiments involve the OE of the candidate gene in *G. max* [Williams 82(PI518671)], a genotype that is normally susceptible to SCN. In all cases, *G. max* [Williams 82(PI518671)] became resistant to SCN parasitism. In contrast, the genetic engineering experiments involving the expression in RNAi-generating cassette of the candidate gene in *G. max* [Peking/PI 548402], a genotype that is normally resistant to SCN became susceptible to SCN parasitism. The combination of these two results indicate that the gene functions specifically in resistance. In complementary studies, qPCR demonstrates that the candidate gene is overexpressed in genetically engineered lines designed to overexpress the gene. In contrast, the candidate gene is suppressed in its transcriptional activity in genetically

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engineered lines designed with the RNAi cassette. The combination of these results demonstrates specificity. Further complimentary qPCR studies demonstrate a level of coexpression occurring between the candidate genes, including the α-SNAP and syntaxin 31 that have been demonstrated in earlier studies (Matsye et al. 2011, 2012; Pant et al. 2014). These observations indicate a higher order level of genetic interaction.

Components of the membrane fusion apparatus function in resistance

Prior analysis of the major SCN resistance locus (rhg1) of soybean identified the presence of a homolog of α-SNAP (Matsye et al. 2011). Subsequent experiments revealed that the genetic engineering of *G. max* [Williams 82(PI518671)], a genotype that is normally susceptible to SCN, with a gene cassette designed to overexpress the gene resulted in engineered resistance (Matsye et al. 2012). This result indicated the importance of the vesicle transport pathway and membrane fusion process as playing an important role in the process. Building off of these studies, Pant et al. (2014) demonstrated that the OE of a soybean homolog of syntaxin 31 (Gm-SYP38) resulted in engineered resistance in *G. max* [Williams 82(PI518671)]. Complimentary studies that engineered in a genetic cassette to activate RNAi of Gm-SYP38, resulted in suppression of the transcriptional activity of the gene and engineered susceptibility in *G. max* [Peking/PI 548402] which is normally resistant to SCN. Syntaxin 31 is localized to the cis-Golgi and interacts directly with α-SNAP. Based off of these observations, it was hypothesized that the other core components of the vesicle transport pathway that function in membrane fusion would also be important to resistance. The results as observed above strengthen the role of core components as they play and interact to suppress the nematode population. The analysis of the other components of the membrane fusion apparatus began by identifying

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soybean homologs of syntaxin 121 which functions at the plasma membrane in other organisms. It was believed that an analysis of syntaxin 121 would provide important insights as to how these membrane fusion genes were functioning at the cell membrane. Homologs of SNAP-25, Munc18, VAMP, SYT and NSF were also identified. Several paralogs were identified for each gene, consistent with the duplicated nature of the soybean genome (Schmutz et al. 2010). Initially it was believed that the duplicated nature of the genome would complicate the analysis of the genes under study. In order to narrow the number of genes down, gene expression data obtained from soybean plants genetically engineered to overexpress SYP38 was examined (Pant et al. 2015). The engineering of *G. max*[Williams 82(PI518671)] with Gm-SYP38 results in engineered resistance and the gene expression data indicated that several of the candidate genes studied here were induced in their transcriptional activity in RNA samples isolated at 6 dpi. The candidate genes examined here were selected based off of that analysis. The candidate genes were then genetically isolated through molecular means and engineered into plants for OE and RNAi analyses.

Syntaxin 121 is a protein that functions at the plasma membrane in other biological systems. Some studies in plants have shown that the syntaxin homologue PEN1/ SYP121 in *A. thaliana* mediate resistance reaction to suppress activity of *Blumeria graminis* f. sp. *Hordei* (Collins et al. 2003). For vesicular transport their formation, delivery and fusion with the targeted membrane is important. Syntaxin, a component of SNARE complex, located at the target membrane, accomplishes screening and fusion of the desired vesicle at the transmembrane (Chen and Scheller, 2001). Presented here, the OE of Gm-SYP121 resulted reduction in female index as compared to

control analyses. The results indicated that the OE of Gm-SYP121 effectively suppresses parasitism by SCN. In complimentary studies, the engineering of an RNAi cassette for Gm-SYP121 had some effect in increasing the ability of SCN to parasitize *G. max* [Peking/PI 548402]. However, the effect was less pronounced than the OE analysis. During the course of the analysis it was observed that soybean has 6 different SYP121 paralogs that each may function in various cellular processes, including defense. Therefore, it is possible that the RNAi analysis is complicated by the different SYP121 homologs that could substitute for each other's function. To demonstrate that concept, genetic constructs that are capable of suppressing the transcriptional activity of all of the paralogs are needed.

SNAP-25 is a conserved hydrophilic protein present in the cytoplasmic side of the plasma membrane that functions in membrane fusion (Hanson et al. 19997a; Hodel, 1998). Presented here, the OE of Gm-SNAP25-4 resulted reduction in FI. In complimentary studies, the engineering of an RNAi cassette for Gm-SNAP25-4 had some effect in increasing the ability of SCN population in *G. max* [Peking/PI 548402]. However, the effect was less pronounced than the Gm-SNAP-25 OE analysis. During the course of the analysis it was observed that soybean has 6 different SNAP-25 paralogs that each may function in various cellular processes, including defense. Therefore, it is possible that the RNAi analysis is complicated by the different SNAP-25 homologs that could substitute for each other's function.

Munc18 is a protein that functions in membrane fusion. The SNARE proteins bounded in membrane regulate intracellular fusion with the help of Sec1/Munc18 (SM) proteins (Park et al. 2012). SM proteins also play an important role in exocytosis through direct and indirect interaction with syntaxin proteins (Ciufo et al. 2005). Munc18

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interacts with the t-SNARE subunit syntaxin though the N peptide which induces SNARE formation and membrane fusion (Rathore et al. 2010). The mechanism behind role of N-peptide for this unit formation and mediated fusion remains unanswered (Rathore et al. 2010). Although OE of Munc18 has been found to involve in vesicular fusion process, some studies have demonstrated that their OE has no effect (Graham et al. 1997) or inhibitory effects on SNARE formation and neurotransmitter release (Schulze et al. 1994). Munc18 binds syntaxin with high affinity (Halachmi and Lev, 1996), which in animals induces neuronal exocytic SNARE units (SNAP-25, syntaxin 1 and VAMP2/3) and mediates membrane fusion (Shen et al. 2007; Weber et al. 1998; Jahn and Scheller, 2006). Various mutational studies in different organisms have resulted in loss of vesicular trafficking and lethality which makes SM proteins important in vesicular transport and fusion (Carr et al. 1999; Novick and Schekman, 1979, Harrison et al. 1994, Hata et al. 1993, Verhage et al. 2000, Schulze et al. 1994). Presented here, the OE of Gm-Munc18-1 resulted reduction in female index as compared to control analyses. The results indicated that the OE of Gm-Munc18-1 effectively suppresses parasitism by SCN. In complimentary studies, the engineering of an RNAi cassette for Gm-Munc18 had some effect in increasing the ability of SCN to parasitize *G. max* [Peking/PI 548402]. However, the effect was less pronounced than the OE analysis. During the course of the analysis it was observed that soybean has 6 different Munc18 paralogs that each may function in various cellular processes, including defense. Therefore, it is possible that the RNAi analysis is complicated by the different Munc18 homologs that could substitute for each other's function. Further analyses are required to demonstrate this concept.

Synaptotagmin is a Ca^{2+} sensor that binds syntaxin-SNAP-25 complex to promote fusion of dense core-vesicles (Popov and Poo, 1993; de Wit et al. 2009). The *A. thaliana* synaptotagmin (SYT1) is homologous with animal SYT7 and is usually located at plasma membrane and is ubiquitously expressed (Schapire et al. 2008). The SYT1 consists of functional C2 domain important for Ca^{2+} dependent membrane fusion at particular site (Fernandez-Chacon et al. 2001). These domains are highly conserved in all species (Popov and Poo, 1993). Functional disability of SYT1 has adverse effects as it reduces cell membrane integrity and survival (Schapire et al. 2008). Thus, these observations indicate an important role of SYT in membrane fusion. Presented here, the OE of Gm-SYT-3 resulted reduction in female index as compared to control analyses. The results indicated that the OE of Gm-SYT-3 effectively suppresses parasitism by SCN. In complimentary studies, the engineering of an RNAi cassette for Gm-SYT-3 had some effect in increasing the ability of SCN to parasitize *G. max* [Peking/PI 548402]. However, the effect was less pronounced than the OE analysis. During the course of the analysis it was observed that soybean has 7 different SYT paralogs that each may function in various cellular processes, including defense. Therefore, it is possible that the RNAi analysis is complicated by the different SYT homologs that could substitute for each other's function. Further analyses are required to demonstrate this concept.

VAMP also known as v-SNARE, plays an important role in vesicular fusion (Chapman et al. 1994; Edelmann et al. 1995; Kwon et al. 2008a; 2008b, Walter et al. 2010, Jahn and Fasshauer, 2012). The PEN1 syntaxin (SYP121) interacts with SNAP33 and VAMP721/722 *in vitro* and *in vivo* for molecular docking (Jahn and Scheller 2006; Kwon et al. 2008a). SYP 121 binds to SNAP33, VAMP721 and VAMP722 to form

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ternary complex (Kwon et al. 2008a). Since the mutants of SNAP 33 or VAMP721 with VAMP722 resulted in death, interaction of SNAP33 and VAMP721/722 with cell membrane are essential in plant physiological functions (Kwon et al. 2008a, b). Presented here, the OE of Gm-VAMP-2 resulted reduction in female index as compared to control analyses. The results indicated that the OE of Gm-VAMP-2 effectively suppresses parasitism by SCN. In complimentary studies, the engineering of an RNAi cassette for Gm-VAMP-2 had some effect in increasing the ability of SCN to parasitize *G. max* [Peking/PI 548402]. However, the effect was less pronounced than the OE analysis. During the course of the analysis it was observed that soybean has 2 different VAMP paralogs that each may function in various cellular processes, including defense. Therefore, it is possible that the RNAi analysis is complicated by the different VAMP homologs that could substitute for each other's function. Further analyses are required to demonstrate this concept.

NSF is a protein that functions in membrane fusion. For the successful vesicular fusion SM proteins coordinate with SNARE complex (Sudhof and Rothman, 2009). As the vesicle approaches the target membrane, fusion with target membrane is mediated by clasp binding of zippering SNARE complex of v-SNARE and t-SNARE by SM proteins (Sudhof and Rothman, 2009). The v-SNARE consists of synaptotagmin (Schiavo et al. 1997; Sanderfoot et al. 2001) and t-SNARE consists of syntaxin and SNAP-25 (Galli et al. 1995; Jahn and Fasshauer, 2012). In animals, SNAP-25 is located at presynaptic plasma membrane and consists of two SNARE motifs linked by palmitoylated linker domains (Jahn and Fasshauer, 2012) that helps in vesicular fusion through zippering action (Hanson et al. 19997a; Sudhof and Rothman, 2009; Jahn and Fasshauer, 2012).

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NSF and α SNAP then complete the membrane fusion process. When NSF is down regulated the binding of syntaxin to SNARE complex can be inhibited by α-SNAP, ceasing exocytosis. However, this process cannot stop if syntaxin is already bound to the SNARE complex (Barszczewski et al. 2008). The interaction of NSF and α-SNAP needs to be normal for proper exocytosis (Barszczewski et al. 2008). The vesicular fusion event, operates within a millisecond, and is induced due to calcium influx (Monck et al. 1996). NSF plays important roles in vesicular transport between ER and Golgi complex (Beckers et al. 1989) and also in fusion of endosomal vesicles (Diaz et al. 1989; Rodriguez et al. 1994). Presented here, the OE of Gm-NSF-1 resulted reduction in female index as compared to control analyses. The results indicated that the OE of Gm-NSF-1 effectively suppresses parasitism by SCN. In complimentary studies, the engineering of an RNAi cassette for Gm-NSF-1 had some effect in increasing the ability of SCN to parasitize *G. max* [Peking/PI 548402]. However, the effect was less pronounced than the OE analysis. During the course of the analysis it was observed that soybean has 2 different NSF paralogs that each may function in various cellular processes, including defense. Therefore, it is possible that the RNAi analysis is complicated by the different NSF homologs that could substitute for each other's function. Further analyses are required to demonstrate this concept.

Gene expression studies show that the OE of each of the membrane fusion components positively influences the expression of the other membrane fusion components, including the rhg1 resistance gene α-SNAP. In contrast, suppressing their activity results in the coordinated suppression of the other members of the membrane fusion machinery. The results here demonstrate that the expression of these genes may be

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under tight regulation. This result indicates that a tightly coordinated effort is important during the resistant reaction. Since each of these genes are known to function in secretion, it indicates that secretion is an important part of the defense reaction.

Conclusion

Plants as they are under constant attack by the biotic and abiotic stress try to overcome these stress through different strategies (Chisholm et al. 2006). The strategies includes the induction of various stress related genes and SAR (Glazebrook et al. 1997). However, plants become susceptible due to lack of induction of genotypes essential for resistance reaction. Soybean cyst nematode as being the major pest of soybean causes huge loss in production. The soybean cultivar *G. max* [Williams 82(PI518671)], rhg1$^{-/-}$ is susceptible to SCN due to lack of functional resistance against SCN infection (Bernard and Cremeens, 1998; Atkinson and Harris, 1989; Schmutz et al. 2010; Cook et al. 2012). Various approaches including field management strategies, use of biological agents and chemical such as nematicides have been tested however the success is very low. To overcome these problems we studied cellular approaches to sort actual genetic and physiological reaction going under susceptible and resistance reactions. The rhg1$^{-/-}$ locus in *G. max* [Williams 82(PI518671)] is rescued through transformation of membrane fusion genes which became resistant reducing nematode Population by confining their spread and parasitism (Matsye et al. 2012; Pant et al. 2014). In contrast RNAi of core components of membrane fusion genes in rhg$^{+/+}$ *G. max* [Peking/PI548402] increased nematode population (Klink et al. 2009a; Pant t al. 2014). The process of resistance examined here demonstrated that all of the core components of membrane fusion are important in the

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process. It has already been demonstrated that specific cargo, known to be transported by vesicles to the apoplast, function in soybean defense to SCN (Pant et al. 2014).

This observation is consistent with the observation that protein molecules (cargo) usually pass through ER to Golgi apparatus, where they are packed into vesicles by proteins, lipids and other components and directed towards plasma membrane for secretion (Sanderfoot et al. 2000). The major proteins used are soluble N-ethylmaleimide-sensitive factor (NSF) adaptor proteins (SNAPs) receptors (SNAREs) and their associated proteins (Sanderfoot et al. 2000). The SNARE complex is quite stable after it is formed and needs high concentrations of ATP for dissociation (Fasshauer et al. 1998). SNARE provide binding site for NSF, which has ATPase activity, and α-SNAP to separate the SNARE components (Sanderfoot et al. 2000). The results presented here show that the membrane fusion and vesicle transport machinery are a major component functioning in defense and that they are responsible for coordinated coexpression of these components. However, to better demonstrate this concept study of their homologs is necessary. The role and function of the vesicular components can further be studied through electron microscopy which helps to understand structure of each components, SNARE formation and regulation of vesicular fusion (Hanson et al. 1997b). Study of protein expression such as flag tagging of plants will help understand the expression of proteins at cellular level.

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