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## Functional Analysis Identifies Glycine Max Genes Involved in Defense to Heterodera Glycines

Prachi D. Matsye

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Functional analysis identifies *Glycine max* genes involved in defense to *Heterodera*  
*glycines*

By

Prachi D. Matsye

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

Mississippi State, Mississippi

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2013

Functional analysis identifies *Glycine max* genes involved in defense to *Heterodera*  
*glycines*

By

Prachi D. Matsye

Approved:

---

Vincent Klink  
Assistant Professor of Biological  
Sciences  
(Director of Dissertation)

---

Gary W. Lawrence  
Associate Professor of Biochemistry,  
Molecular Biology, Entomology, and  
Plant Pathology  
(Committee Member)

---

Karen Coats  
Professor of Biological Sciences  
(Committee Member)

---

Giselle T. Munn  
Associate Professor of Biological  
Sciences  
(Committee Member)

---

Gary N Ervin  
Professor and Graduate Coordinator of  
Biological Sciences  
(Committee Member)

---

R. Gregory Dunaway  
Professor and Interim Dean  
College of Arts & Sciences

Name: Prachi D. Matsye

Date of Degree: August 17, 2013

Institution: Mississippi State University

Major Field: Biological Sciences

Major Professor: Dr. Vincent Klink

Title of Study: Functional analysis identifies *Glycine max* genes involved in defense to *Heterodera glycines*

Pages in Study: 241

Candidate for Degree of Doctor of Philosophy

The infection of plants by *Heterodera glycines*, commonly known as soybean cyst nematode (SCN), is a serious agricultural problem of worldwide extent. Meanwhile, it provides an excellent experimental model to study basic aspects of how cells function, in particular, during biotic challenge. *Heterodera glycines* challenges plant cells by initiating, developing and sustaining an interaction that results in the formation of a nurse cell from which the nematode derives nourishment. The presented experiments examine (1) how a cell can be de-differentiated and reprogrammed to perform a much different biological role and (2) how a cell's immune responses can be engaged or suppressed to accomplish that goal.

The observation of alpha soluble N-ethylmaleimide-sensitive factor attachment protein (alpha-SNAP) expression, its location within the *rhg1* locus and known involvement in the vesicular transport machinery relating to defense made it a strong candidate for further functional analysis. Functional studies demonstrated that overexpression of alpha-SNAP in the susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> genotype that lacks its expression results in the partial suppression of *H. glycines* infection. This

indicated that the vesicles could be delivering cargo to the site of infection to engage a defense response.

High levels of expression of a cell wall modifying gene called xyloglucan endotransglycosylase also occur during defense. XTHs associate with vesicles, act in the apoplast outside of the cell, and have a well-known function in cell wall restructuring. These observations indicated that alterations in the cell wall composition of nurse cells could be important for the successful defense response. Overexpression of a *G. max* xyloglucan endotransglycosylase (Gm-XTH) in the susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> genotype resulted in a significant negative effect on *H. glycines* as well as *R. reniformis* parasitism. The results, including preliminary experiments on components of the vesicle transport system, identify a potent mechanism employed by plants to defend themselves from two types of plant-parasitic nematodes.

## DEDICATION

This dissertation dedicated to my parents, Dilipkumar and Archana Matsye, my sister Manasi Matsye, my dear grandmother Mai Matsye, and my beloved husband Ranjit Kumar.

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## CHAPTER I

### BACKGROUND

Plant-parasitic nematodes that are sedentary and endoparasitic in nature establish a nurse cell that acts as their niche during parasitism. They are a group of pathogens causing 0.1-0.125 trillion dollars in damage, worldwide, annually (Sasser and Freckman, 1987; Chitwood, 2003). Thus, these plant-parasitic nematode pathogens represent a significant challenge to agriculture. Meanwhile, the interaction between the plant and nematode represents an excellent experimental model to study basic aspects of cell function with particular concern about cellular response to biotic challenge. The presented experiments examine (1) how a cell can be de-differentiated and reprogrammed to perform a much different biological role and (2) how the cellular immune responses can be engaged or suppressed to accomplish that goal. These questions are central to studies of pathology and are not limited to plants.

Successful parasitism by plant-parasitic nematodes occurs in all land plant lineages including bryophytes (Dixon, 1908), ferns (Bird and DiGennaro 2012), gymnosperms (Cobb, 1930), angiosperms (dicots [Cobb 1890] and monocots [Cobb 1893]) and even multicellular algae (Barton et al. 1892). These observations indicate that a common and ancient mechanism is in place that regulates the process of infection. Plant-parasitic nematodes that are sedentary and endoparasitic in nature accomplish parasitism through the use of genes that were transferred horizontally from bacteria that

effect nurse cell formation (Atkinson and Harris, 1989; Smant et al. 1998; Lambert et al. 1999; De Boer et al. 1999, 2002; Robertson et al. 1999; Bekal et al. 2003; Gao et al. 2001, 2003; Huang et al. 2005, 2006; Bakhietia et al. 2007, 2008; Sindhu et al. 2009; Lee et al. 2010; Haegeman et al. 2011; Hamamouch et al. 2011). The details of how these plant-parasitic nematodes accomplish parasitism are poorly understood with only a few cell wall degrading genes and an auxin efflux carrier inhibiting gene being shown to perform specific roles. However, the plant has the ability to defend itself from parasitism (Zimmerman, 1897) and this portion of the interaction between plants and nematodes is the focus of the experiments presented here.

The *Glycine max-Heterodera glycines* experimental pathosystem was chosen for a number of reasons as described in Chapter II. *H. glycines* (soybean cyst nematode [SCN]) cause more damage to soybeans than the combined effect of the rest of its pathogens (Wrather et al. 2006). Furthermore, soybeans are the top export crop of the U.S., allowing basic information learned in this system to be applied directly to solving a serious agricultural problem. The progress made in the presented experiments owes much to the foresight of the United States Department of Agriculture that began making collections of natural accessions of *G. max* as early as 1898 (Morse et al. 1927). The work resulted in the collection of approximately 8,000 *G. max* accessions by the time *H. glycines* arrived to the U.S. from China in 1954 (Winstead et al. 1955), becoming an invasive species. There are currently over 20,000 accessions of *G. max* that have been screened for their ability to resist infection by *H. glycines*. The collections were central to the genetic mapping work that identified the recessive *rhg1*, *rhg2*, *rhg3* loci (Caldwell et al. 1960) and the dominant *Rhg4* (Matson and Williams, 1965) and *Rhg5* (Rao Arelli, 1994) loci.

The collections were also important to the generation of a significant catalog of cytological and ultrastructural research made from the 1950s through the 1990s (Ross, 1958; Endo, 1965, 1991; Kim et al. 1987; Mahalingham and Skorupska. 1996). The cell biology approach used for the presented studies relied on the availability of the germplasm, the cytological and ultrastructural work, and the genetic mapping investigations to study the process of susceptibility and defense. The experiments presented here, however, focused on the defense.

Very specific cell biological features become evident during an incompatible interaction between *G. max* and *H. glycines*, leading to a resistant reaction. These features include an extensive accumulation of cisternae and vesicles (Endo, 1965, 1991; Kim et al. 1987; Mahalingham and Skorupska. 1996). These observations indicated that the vesicular transport system, including the Golgi apparatus and endoplasmic reticulum, are probably important to the process. Furthermore, vesicles are well known to deliver cargo to the site of infection in other plant pathosystems, performing important roles in defense (Collins et al. 2003). This led to the hypothesis that the vesicular transport system and membrane fusion machinery is important for defense in the *G. max-H. glycines* pathosystem. The major components of the vesicular transport machinery are highly conserved; the protein components are found in diverse organisms including yeast, insects, human, plants and free-living nematodes (Novick et al. 1980; Clary et al. 1990; Hata et al. 1993; Collins et al. 2003; Babcock et al. 2004). To provide molecular evidence that these genes are actively transcribed during the defense response, the cells undergoing both compatible (susceptible) and incompatible (defense) responses were physically collected and their RNA expression profiles compared to each other as well as a control

cell population that represented the developmental starting point for both compatible and incompatible reactions. As presented in chapter III, the comparative genomics analyses resulted in the identification of an alpha soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein ( $\alpha$ -SNAP) gene that was expressed at all studied time points only during the defense response in multiple *G. max* genotypes (Matsye et al. 2011). The  $\alpha$ -SNAP gene was located in the most important defense locus, *rhg1* (Matsye et al. 2011), that was fine mapped to a position within approximately 59 kb on chromosome 18 (Kim et al. 2010).

As presented in Chapter IV, to functionally test the genes, an  $\alpha$ -SNAP allele was isolated from a resistant genotype and expressed to high levels (overexpressed) in a genotype that normally, lacks the accumulation of cisternae and vesicles while undergoing a compatible (susceptible) reaction. These genetically engineered roots that would otherwise be compatible, exhibited a partial suppression of infection. While the plants exhibited only a 50% reduction in *H. glycines* pathogenicity, the results were consistent with the hypothesis that the vesicular transport and membrane fusion machinery performed an important role in defense. Subsequent work confirmed the involvement of  $\alpha$ -SNAP in the process (Cook et al. 2012).

The identification of  $\alpha$ -SNAP as a gene performing some role in defense provided a molecular target to further study the system. These targeted investigations demonstrated that involvement of other proteins associated with the vesicle transport machinery are important to defense. The vesicle transport machinery is known to process and deliver enzymes that modify the cell wall and deliver them to the site of activity (Yokoyama and Nishitani, 2001). One of these proteins is xyloglucan endotransglycosylase/hydrolase

(XTH) which is known to modify hemicellulose enzymatically (Fry et al. 1992). Using the same approach for the work employed to investigate  $\alpha$ -SNAP, overexpression experiments presented in Chapter V demonstrate that XTH also has a significant negative influence on the ability of *H. glycines* as well as *R. reniformis* to parasitize *G. max*. The involvement of other components of the vesicular transport having the same negative impact on parasitism as compared to experimental controls provides the identification of the vesicular transport and membrane fusion apparatus as a plant defense mechanism against the two types of plant-parasitic nematodes. The work functionally links the vesicle transport and membrane fusion protein machinery with the biology of several plant diseases (Collins et al. 2003). Notably, the conserved nature of the genes demonstrate commonality between plant defense and several human diseases, including Alzheimer's disease (Furuya et al. 2012; Mukaetova-Ladinska et al. 2013), enteropathogenic and enterohaemorrhagic *Escherichia coli* pathogenicity (Thanabalasuriar et al. 2012), Huntington's disease (Rush et al. 2012), Parkinson's disease (Shin et al. 2008), among others.

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## CHAPTER II

### ENGINEERED SOYBEAN CYST NEMATODE RESISTANCE<sup>1</sup>

#### 2.1 Abstract

The parasitism of plants by plant-parasitic nematodes that are sedentary and endoparasitic in nature is accomplished in three successive steps by the initiation, development and maintenance of a nurse cell from which it feeds. The initiation of nurse cell formation, followed by its development and maintenance is essential for the survival of the nematode. In contrast, removal of the nematode from this interaction results in the collapse of the nurse cell. These outcomes indicate that the sustained interaction of root cell and nematode is a coordinated process and essential for each of their survival. This chapter focuses in on one of the most important plant-parasitic nematodes, the soybean cyst nematode (SCN), *Heterodera glycines*, describing how basic science is being used in solving this significant agricultural problem.

#### 2.2 Introduction

A variety of plant-parasitic nematodes, including the soybean cyst nematode (SCN), elicit the initiation, development, and maintenance of a specialized nurse cell

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<sup>1</sup> Most of the content of this chapter has been adapted from the book chapter: Klink VP, Matsye PD, Lawrence GW, Lawrence KS. 2012. Engineered soybean cyst nematode resistance. Soybean - A Review / Book 1", ISBN 980-953-307-542-1

from which they derive their nutriment (**Fig. 2.1**). Remarkably, during parasitism by SCN, the nurse cell survives the apparently significant resource drain on the root cell that would be expected to impact normal physiological processes of the cell detrimentally. This outcome indicates that the nematode has developed a well-tuned apparatus to ensure that the root cell does not collapse and die during parasitism. In contrast, in the soybean-SCN pathosystem, the nurse cell and sometimes the surrounding cells are the sites of the defense response to the parasite (Ross, 1958; Endo 1965). Therefore, plants have in place a mechanism to overcome the influence of the activities of the nematode. Identifying the factor(s) is of utmost importance in developing resistance to the plant-parasitic nematodes.

### **2.3 History**

Documented accounts reveal that soybean has been in cultivation for thousands of years (Hymowitz, 1970), beginning in Asia perhaps as early as 3,500 B.C. (Liu et al. 1997). While the natural range of soybean is East Asia, after thousands of years of cultivation a true understanding of its native range is complicated at best. However, the extensive range of wild soybean and obvious differences in its growth habit indicates that while environmental cues may be responsible for changes in soybean and plant growth habit in general (Garner et al. 1930; Chapin and Shaver, 1985; Day et al. 1999), genetic variation that exists in wild populations is of significant benefit to agriculture for production purposes and developing resistance to its many pathogens. This assessment is particularly true for soybean and its most significant pathogen, SCN, as many ecological collections have resulted in the identification of naturally occurring resistance (Ross,

1958; Ross and Brim 1957; Epps and Hartwig 1972; Concibido et al. 2004; Ma et al. 2006; Li et al. 2011; Matsye et al. 2012).

While knowledge of soybean's cultivation is long and extensive, scientific information on its dominant pathogen, the SCN, began with its description (Ichinohe, 1952). However, reports going back as early as the 1880's (Noel 1992) and late 1930s (Ichinohe, 1961) reveal knowledge of the nematode and appreciation of its pathogenic capacity. The SCN is a devastating pathogen that causes approximately 7-10% production loss, worldwide, annually and suppresses seed yield more than any other single soybean pathogen (Wrather et al. 1995, 2001a, 2001b, 2003; Pratt and Wrather 1998; Wrather and Koenning, 2006). In contrast, in some fields, as much as a 15% loss in yield has been observed with no visible signs of disease on soybean (Wang et al. 2003). Observations such as these could complicate SCN management since the disease can occur without knowledge of it being present in a particular field.

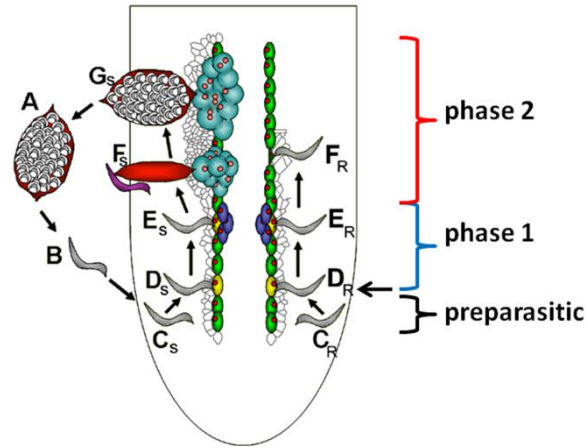


Figure 2.1 SCN life cycle during susceptible and resistant reactions

A, cysts with eggs (white). B, second stage pre-infective juveniles (pi-J2) (gray) migrate toward the root. **SUSCEPTIBLE REACTION:** C<sub>s</sub>, the infective-J2 (i-J2) nematodes (light gray) burrow in and migrate toward the stele. D<sub>s</sub>, feeding site selection (yellow). E<sub>s</sub>, p-J2 nematodes molt into J3, then J4. During this time, the original feeding site is incorporating adjacent cells (purple) via cell wall degradation events. Meanwhile, the male discontinues feeding at the end of its J3 stage. F<sub>s</sub>, After maturation, the male and female nematodes copulate. G<sub>s</sub>, the female at ~30 dpi. **RESISTANT REACTION:** C<sub>R</sub>, Like the susceptible reaction, the infective-J2 (i-J2) nematodes (dark gray) burrow into the root and migrate toward the root stele. D<sub>R</sub>, feeding site selection by the parasitic J2 (p-J2). E<sub>R</sub>, the syncytium begins to develop. F<sub>R</sub>, the syncytium has collapsed resulting in nematode mortality. The right panel shows the initiation phase of infection (black arrow). Phase 1 is the development period. Phase 2 is the maintenance period (adapted from Klink et al. 2013).

The near perfect overlap of the agricultural range of soybean with the distribution of SCN, infection creates a scenario where there is a high probability of a widespread and significant effect on yield. Conservative reports have shown that there is approximately 1.1-1.5 billion dollars in agronomic losses, annually, worldwide (Wrather et al. 2001a). The overlapping distribution of SCN with that of soybean production was not always the case. Historically, SCN was not found in the U.S., or likely even North America or the New World. That situation changed when the SCN was first identified in the U.S. in North Carolina in 1954 by Winstead et al. and the first report was published a year later

(Winstead et al. 1955). Unfortunately for agriculture, SCN is readily transmissible as evidenced by its identification in localities as far away as Mississippi by 1957 (Spears, 1957). The SCN now is a registered invasive species in the U.S. Notably, in the U.S., SCN causes more agricultural loss to soybean than the rest of its pathogens combined (Wrather et al. 2001b; Wrather and Koenning, 2006). Making the problem worse for agriculture is the genetic diversity of the SCN (Golden et al. 1970; Riggs and Schmitt, 1988, 1991; Niblack et al. 2002). The nematode is a species complex originally subdivided into four races (Golden et al. 1970) which later was expanded into 16 races (Riggs and Schmitt, 1988) that have been reorganized, further subdivided, and reclassified into distinct populations (Niblack et al. 2002; Niblack and Riggs, 2004). The term population was designated since genetically pure clones are impossible to obtain in the sexually reproducing SCN system (Niblack et al. 2002). The classification scheme of Niblack et al. (2002) is based on the varying ability of SCN populations to infect a panel of 7 soybean genotypes that can resist infection to varying levels. It is noted that some of these designated populations are “strains” that are maintained in the greenhouse and genetically purified through hundreds of generations of single cyst descent (Niblack et al. 1993). Therefore, the genetic background in these “strains” may not resemble the original field-extracted population as allelic forms of the parasitism genes would likely be lost through this purification process.

The genetic diversity found in SCN (Golden et al. 1970; Riggs and Schmitt, 1988, 1991; Niblack et al. 2002; Bekal et al. 2003) likely aids in its ability to infect and reproduce on plants other than soybean. Thus, from an ecological standpoint, SCN could pose a threat to plants that grow outside of production areas. This potential problem

would be exacerbated if those plant species are listed as endangered or threatened species or are a significant component of the ecological community. A number of studies have shown that the SCN reproduces on at least, but certainly is not limited to, 97 legume and 63 non-legume hosts (Epps and Chambers, 1958; Riggs and Hamblen 1962, 1966a, b) and new SCN hosts are determined on a regular basis (Creech and Johnson, 2006). It has been many years since species range tests have been performed for SCN so it is likely that these lists of hosts are not comprehensive. This virulence capability of SCN poses a problem in terms of its management since SCN populations could be maintained by weedy plants that grow or overwinter in fallow fields or along the boundaries of acreage that is in production. In addition to these problems, SCN does not even have to reproduce in the plant to still cause damage to the plant. While the genetic diversity of both soybean and SCN may appear to complicate an understanding of the process of infection and the development of resistant cultivars, the natural variation in both the germplasm of soybean (Doyle et al. 1999) and SCN (Bekal et al. 2003, 2008) presents many opportunities to understand the basic machinery of infection of the SCN and the genotype-specific nuances that regulate both susceptibility and defense. These features make the soybean-SCN pathosystem an extremely valuable experimental model (Barker et al. 1993; Opperman et al. 1998; Niblack et al. 2006; Klink et al. 2010a).

## **2.4 Methods to control SCN infection**

Historically, SCN has been managed through a combination of chemical control, cropping systems, biological control and the identification and use of resistant germplasm. Chemical control for pathogens using methyl bromide first occurred in France (Roskopf et al. 2005; Chen and Dickson, 1996) and had subsequently been used



for decades for both pre- and post planting nematode control. However, methyl bromide is a chemical that was phased out of use in 2005 in the U.S. (Rosskopf et al. 2005) because it was classified as a Class 1 (Group VI) stratospheric ozone depletor by the Environmental Protection Agency. Because of the loss of this major control agent for the SCN, even in developing countries by the year 2015 (Rosskopf et al. 2005), it was important to identify other strategies that could be included in the SCN management plan. Biocontrol measures that include bacteria, fungi or even their proteins are feasible (Kim and Riggs 1991, 1995; Chen and Dickson, 1996; Meyer and Heuttel, 1996; Meyer and Meyer, 1996; Timper et al. 1999; Liu et al. 2001). Other control methods that had already been used extensively for decades include the time honored crop rotation strategy. This strategy has reduced SCN populations below damaging levels (Francl and Dropkin, 1986; Sasser and Uzzell Jr. 1991; Koenning et al. 1993). Rotating with nonhosts over a 2-3 year period mitigated the undesirable levels of SCN in the field (Ross, 1962; Francl and Dropkin, 1986). Other cropping systems that have had success in SCN control are the use of blending, resistant cultivars and cropping sequence, among others (Niblack and Chen, 2004). While successful, a problem with cropping strategies is that the interval is not long enough to compete with the 9 year cycle that cysts can remain dormant, but viable in production fields (Inagaki and Tsutsumi, 1971). With these strategies in place it is possible to develop a tightly managed regime, incorporating some or all of these technologies and principles to mitigate SCN damage. Lastly, genetic engineering has begun to take root as a potential method to generate resistance (Steeves et al. 2006; McLean et al. 2007; Klink et al. 2009a; Matsye et al. 2012). However, for genetic engineering to be successful, candidate genes must first be identified. The identification

of these genes has happened through a series of RNA gene expression studies, employing soybean germplasm that exhibits resistance to SCN.

## **2.5 Available resistant germplasm**

Once SCN was identified in the U.S. (Winstead et al. 1955), a very large need existed to determine if soybean germplasm existed that could resist infection. The vast and expansive range of soybean (Morse, 1927; Bernard et al. 1987) and visually obvious variations in growth form in its various ecological habitats provided the possibility that germplasm that was resistant to SCN existed in its wild populations. Established in 1898, the development of a substantial and publically available seed bank was initiated that is maintained by the USDA-National Plant Germplasm System (USDA-NPGS) (Morse, 1927; Bernard et al. 1987). It now contains approximately 20,000 varieties (accessions) with each accession classified as a plant introduction (PI) through a numbering system. Many of the 7,867 PIs that were already available by 1944, just 10 years before the identification of SCN in the U.S., had been collected in trips to China, Japan, India and Korea in a small window of time between 1924 and 1932 (Bernard et al. 1987). The public availability of the germplasm allowed it to be used in a series of trials to determine if any of the available accessions was resistant to SCN. A number of accessions were determined to be resistant to SCN through two large trials that studied about 5,700 accessions (Ross and Brim, 1957; Ross, 1958; Epps and Hartwig, 1972). Research on these accessions resulted in the identification of approximately 118 sources of resistance (Concibido et al. 2004). However, only approximately seven sources are used for cultivar development in the U.S (Shannon et al. 2004). These accessions include the *G. max* PIs known as Peking (*G. max*<sub>[Peking]</sub>) and *G. max*<sub>[PI 88788]</sub>. Currently, *G. max*<sub>[Peking]</sub> and *G.*

*max*<sub>[PI 88788]</sub> resistant germplasm is present in >97% of all commercial cultivars in the U.S. (Concibido et al. 2004). In addition to these PIs, hundreds of additional accessions that can resist SCN infection have been identified in China (Ma et al. 2006; Li et al. 2011). These banks of germplasm provide an important and substantial genetic resource for understanding the process of parasitism in soybean at the cellular level. This is important to understand because the infection of soybean involves very specific cell types that react in very specific ways to SCN parasitism.

## **2.6 Cytological reaction during resistance**

The SCN can remain viable in the soil in eggs ensheathed within the carcass of the dead mother (cyst wall) for up to 9 years (Inagaki and Tsutsumi, 1971). However, the devastating interaction of the SCN with soybean begins when it burrows into the root through the epidermal and cortical cells. This has been shown both by cytological studies and gene expression studies of time points collected before the formation of syncytia (Alkharouf et al. 2006; Klink et al. 2009b). The interaction continues through the initiation and subsequent formation a multinucleate nurse cell known as a syncytium from pericycle or neighboring cells (Ross, 1958, Endo, 1964, 1991). The formation of the syncytium is likely to be a very coordinated process, occurring through the injection and subsequent activity of nematode parasitism proteins (Bekal et al. 2003; Atkinson and Harris, 1989; Smant et al. 1998; Lambert et al. 1999; De Boer et al. 1999, 2002; Wang et al. 1999, 2001; Gao et al. 2001, 2003). These substances likely orchestrate successive waves of interference of the root cell's normal physiological processes and initiate various cell wall dissolving events (Bekal et al. 2003; Atkinson and Harris, 1989; Smant et al. 1998; Lambert et al. 1999; De Boer et al. 1999, 2002; Wang et al. 1999, 2001; Gao

et al. 2001, 2003). The parasitism process merges approximately 200-250 root cells into a common cytoplasm containing as many nuclei, the definition of a syncytium (Jones and Northcote, 1972; Jones, 1981). Additional nematode activities alter the plant cell's physiology (Klink et al. 2005, 2007a; Ithal et al. 2007a). The activities benefit the nematode during the sedentary period of its life cycle as they feed and mature (Edens et al. 1995; Hermeister et al. 1998; Mahalingam et al. 1999; Vaghchhipawala et al. 2001; Alkharouf et al. 2006; Klink et al. 2005, 2007a; Ithal et al. 2007a; Matsye et al. 2011).

Cytological studies of the SCN infection process (**Fig. 2.1**) have shown that the cellular response of soybean to SCN infection can be divided into an earlier phase (phase 1) and a later phase (phase 2) (Ross, 1958; Endo, 1964, 1991; Riggs and Gipson, 1973; Kim et al. 1987; Mahalingam and Skorupska, 1996). Phase 1 and 2 span the periods including the initiation, development and maintenance of the syncytium (**Fig. 2.1**). These observations are not unique to SCN since similar observations have also been made for the cyst nematode *Rotylechulus reniformis* (Robinson et al. 1997), indicating that a basic level of conservation may exist for the process of defense at the site of infection while genotype-specific gene activities also exist (Klink et al. 2011a; Matsye et al. 2011, 2012).

During phase 1, the cellular reactions leading to susceptibility or defense appear the same at the cytological level. The cellular events occurring during the earlier stages of syncytium development include hypertrophy, the dissolution of cell walls, the development of dense cytoplasm, an enlargement of nuclei and an increase in endoplasmic reticulum (ER) and ribosome content (Endo, 1965, 1991; Riggs and Gipson, 1973; Kim et al. 1987; Mahalingam and Skorupska, 1996). The enlargement of nuclei and increase in ER and ribosome content indicate an increase in gene expression and

protein synthesis accompanies the activity of the nematode within the parasitized cells. Therefore, it is likely that the plant cell is being programmed to make specific materials to benefit the nutritional needs of the nematode. It is known that plant parasitic nematodes lack the ability to make materials such as sterols (Chitwood and Lusby, 1991). Therefore, altering the metabolism of the parasitized cell probably would involve the induction of metabolic activity that relates to these processes. Cell fate mapping experiments have demonstrated that metabolism occurs during these stages of parasitism and some of it relates to enhanced plant sterol production (Klink et al. 2011a; Matsye et al. 2011).

After these earlier events, the cytology of susceptibility and defense become apparent and is referred to as phase 2. Phase 2 of the susceptible reaction is characterized by hypertrophy of nuclei and nucleoli. This process is accompanied by the reduction and dissolution of the vacuole. The reduction and dissolution of the vacuole suggests important events or structural features involved in membrane fusion and/or maintenance are perturbed. This topic will be described in a later section. Other cellular events that have been identified during the susceptible reaction include cell expansion as it incorporates and fuses with adjacent cells (Endo and Veech, 1970; Gipson et al. 1971; Jones and Northcote, 1972; Riggs and Gipson, 1973; Jones, 1981). Additional activities include the proliferation of cytoplasmic organelles.

In contrast, the cellular aspects of the defense responses occurring during phase 2 depend on the soybean genotype being infected. Information that has been generated through a number of cytological studies have resulted in the development of a system that divides the PIs into cohorts having similar cytological reactions that is based on the

cellular characteristics associated with how SCN responds during resistance (Colgrove and Niblack, 2008). Currently, the PIs have been categorized into those genotypes having the *G. max*<sub>[Peking]</sub> and *G. max*<sub>[PI 88788]</sub>-types of defense responses (Colgrove and Niblack, 2008). Much more work in this area of research is required for a comprehensive understanding of the different forms of the defense response. Such knowledge would allow the commonalities of the cytological features to be correlated with the molecular events that are occurring in the parasitized cell types. By doing so, it would allow for the identification of genes that always correlate to resistance, regardless of the cytology or genotype of soybean. It would be likely that these genes are central to all forms of the defense response (Klink et al. 2011a; Matsye et al. 2011, 2012). Among the characteristics that define these cohorts, the *G. max*<sub>[Peking]</sub>-type of defense includes the development of a necrotic layer that surrounds the head of the nematode (Endo, 1991; Mahalinghan and Skorupska, 1996). This process is followed by necrosis of the initial cell that the nematode had parasitized. In contrast, in the *G. max*<sub>[PI 88788]</sub>-type of defense response, the necrotic layer that surrounds the head of the nematode is lacking and the initial cell that the nematode parasitized first experiences necrosis (Endo, 1991; Mahalinghan and Skorupska, 1999). In addition to these cytological characteristics found in the *G. max*<sub>[Peking]</sub> and *G. max*<sub>[PI 88788]</sub>-types of defense responses is the presence or absence of cell wall appositions (CWAs). CWAs are structures defined as physical and chemical barriers that are designed to prevent cell penetration (Aist, 1976; Schmelzer, 2002; An et al. 2006a,b; Hardham et al. 2008). CWAs have been found and studied in other plant-organism pathosystems (Collins et al. 2003; Assaad et al. 2004; Kalde et al. 2007). However, CWAs are not a defining characteristic of all types of defense responses

in soybean. CWAs have been found in the *G. max*<sub>[Peking]</sub>-type of resistant reaction and are found in the *G. max*<sub>[PI 437654]</sub> genotype (Mahalinghan and Skorupska, 1996). This makes the placement of *G. max*<sub>[PI 437654]</sub> in the *G. max*<sub>[Peking]</sub> cohort logical (Colgrove and Niblack, 2008). In contrast, CWAs are lacking in *G. max*<sub>[PI 88788]</sub>. More work is required in the understanding the role(s) that CWAs play, if any, during defense of soybean to SCN. However, the significance and role of CWAs during defense were first demonstrated by Collins et al. (2003), and followed by additional studies performed by Assaad et al. (2004) and Kalde et al. (2007). In those studies, it was shown at the molecular level that CWA formation involves the vesicular transport machinery protein component known as syntaxin. This was a striking discovery since the process of vesicular transport is a conserved cellular process. The syntaxin gene was first identified in animal systems (Inoue et al. 1992; Bennett et al. 1992) and through a number studies performed in animal and model genetic systems, it was shown that syntaxin interacts with other proteins to accomplish specific cellular functions. Unfortunately, while the role of syntaxin in plant defense has been studied (Collins et al. 2003; Assaad et al. 2004; Kalde et al. 2007), the examination of other components of the vesicular transport machinery has received little attention. Until very recently (Matsye et al. 2012), no information existed on how these proteins function or interact with syntaxin during the defense of plants to pathogens. The demonstration that syntaxin plays a role in the defense of plants to pathogens, implicates that other proteins that interact directly or indirectly with syntaxin probably may be involved in the process. A genetic pathway, involving *PEN1*, the  $\beta$ -glycosyl hydrolase *PEN2*, and the ABC transporter *PEN3* results in transportation of proteins that deliver antimicrobial compounds across the cell membrane to sites where

a fungal pathogen is attempting to enter (Collins et al. 2003, Lipka et al. 2005; Stein et al. 2006). Other proteins that interact directly with syntaxin have been studied in other experimental systems and include the ATPase known as N-ethylmaleimide-sensitive factor attachment protein (NSF) (Malhotra et al. 1988), the soluble N-ethylmaleimide-sensitive factor attachment recceptor protein (SNARE) complex and synaptosomal-associated protein 25 (SNAP25) (Oyler et al. 1989), the soluble N-ethylmaleimide-sensitive factor attachment protein  $\alpha$ -SNAP) (Weidman et al. 1989; Clary et al. 1990; Collins et al. 2003; Assaad et al. 2004; Kalde et al. 2007), among other proteins. Since these numerous studies have shown very specifically how the protein complex is assembled, it was then possible to determine how specific components of the CWA assembly process that are present during defense of soybean to SCN function (Matsye et al. 2012). However, even though CWAs are lacking in genotypes like *G. max*<sub>[PI 88788]</sub>, it does not mean that the proteins are not involved in defense through related activities. Membrane fusion has been shown to play a role in defense through a process known as autophagy. Autophagy is a process known in plants to play crucial roles in defense (Patel and Dinesh-Kumar, 2008; Hofius et al. 2009; Lenz et al. 2011; Lai et al. 2011). This knowledge has allowed for a targeted approach in understanding the protein machinery that is involved in defense (Matsye et al. 2012).

## 2.7 Genomics-based studies of SCN

A number of “omics” studies in the soybean-SCN pathosystem have been performed to understand both plant and nematode gene expression at the organismal level. Many of the gene expression studies that relied on the microarray technology were modeled after earlier experiments that were performed in the model plant *Arabidopsis*



*thaliana* that were infected with the beet cyst nematode, *Heterodera schachtii* (Puthoff et al. 2003). Studying SCN in *A. thaliana* is complicated by the fact that it is a nonhost to SCN infection so studies investigating the susceptible reaction elicited by SCN in *A. thaliana* cannot be done until suitable mutants or susceptible ecotypes are identified. A number of microarray studies using whole infected soybean roots as a source for the RNA samples have identified genes that are expressed during a susceptible reaction (Alkharouf et al. 2006; Ithal et al. 2007; Klink et al. 2007b).

The parasitism of soybean by SCN begins with and is sustained through the injection of materials that are synthesized in subventral and esophageal glands into the root cell. Identifying the genes involved in parasitism would likely occur through collecting the cytoplasm of the cells composing the subventral and esophageal glands. It was hypothesized that these genes would be important for the events of parasitism and would be involved in altering the metabolic processes of the soybean to benefit the nematode. The experiments were performed by microaspirating the cytoplasm of the gland cells, constructing cDNA libraries and sequencing the genes, allowing for downstream bioinformatics analyses to help elucidate what the genes could actually be (Smant et al. 1998). The experiments were then repeated for the SCN, identifying a number of putative parasitism genes (Wang et al. 1999, 2001, Gao et al. 2001, 2003). With the development of the Affymetrix® Soybean GeneChip, it was possible to examine the expression of thousands of SCN genes simultaneously. This was made possible because 7,539 *H. glycines* probe sets representing 7,431 transcripts (genes) were printed onto the array. One analysis examined the expression of SCN genes that were expressed specifically during infection of the *G. max*<sub>[Williams 82/PI 518671]</sub> genotype that lacks

a functional defense response (Elling et al. 2009). This means that gene expression occurring during a susceptible reaction was monitored. The work examined the expression of previously identified (Wang et al. 1999, 2001; Gao et al. 2001, 2003) and analyzed (Alkharouf et al. 2007) putative parasitism genes (Bakheita et al. 2007). The remaining genes that were fabricated onto the array were not a focus of the analysis. The experiments confirmed the expression pattern of dozens of putative parasitism genes (Bakheita et al. 2007). A gap in the knowledge was that the experiments were not designed to determine what genes were expressed as the nematode experienced a resistant reaction in a soybean genotype that was capable of a defense reaction. This information would be important because it would provide knowledge on the metabolic pathways that may be sensitive to genetically-based control measures. That gap in knowledge was filled in experiments that performed population-specific analyses of gene expression, comparing the susceptible and resistant reactions experienced by SCN as they infected the *G. max*<sub>[Peking/PI 548402]</sub> genotype that has a functional defense response to some populations of SCN (Klink et al. 2007a). Thus, from the experiments of Ithal et al. (2007a) and Klink et al. (2007a), specific knowledge of gene expression occurring in genotypes both lacking and having functional resistance genes was obtained. It is noted that additional gene expression profiling experiments have also been performed (Alkharouf et al. 2007). In earlier studies, Alkharouf et al. (2007) annotated all of the SCN genes that were available in Genbank and compared them to the genetic model free living nematode, *Caenorhabditis elegans*. The advantage of these comparisons was that the genome of *C. elegans* had been sequenced (*C. elegans* Sequencing Consortium, 1998), allowing for a substantial annotation process to be executed. In addition, there was

a massive amount of functional data obtained through genetic and reverse genetic experiments (Fire et al. 1998; Piano et al. 2000; Kamath et al. 2003; Sonnichsen et al. 2005) that was available for essentially every gene in the genome housed in the *C. elegans* database at <http://www.wormbase.org>. Since the genomic sequence of *C. elegans* is known, it is possible to find highly conserved and related genes in SCN. The working hypothesis was that if the genes in *C. elegans* and SCN are nearly identical in primary sequence, it would be likely that they have similar function. If the genes have similar function, for example an essential function for survival in *C. elegans*, knocking out that gene in SCN would probably result in lethality of the nematode. The annotation of the SCN genes was driven by a homology criterion whereby the SCN genes were pooled into six bins referred to as Groups 1-6 (Alkharouf et al. 2007). The six bins were based on the level of homology the sequence had to *C. elegans* genes. Group 1 had the highest level of homology and Group 6 had the lowest level. For example, Group 1 had *E*-values between 0 and  $1E-100$ ; Group 2 had *E*-values between  $1E-100$  and  $1E-80$ ; Group 3 had *E*-values between  $1E-80$  and  $1E-60$ ; Group 4 had *E*-values between  $1E-60$  and  $1E-40$ ; Group 5 has *E*-values between  $1E-40$  and  $1E-20$  while Group 6 has *E*-values  $> 1E-20$  (Alkharouf et al. 2007). The gene annotation process resulted in taking the nearly 8,334 conserved genes between *H. glycines* and *C. elegans* and identifying 1,508 that have been shown to have lethal phenotypes/phenocopies in *C. elegans* (Alkharouf et al. 2007). The research then was poised to test the function of the 1,508 genes, but it was an unmanageable number of genes. To narrow down the 1,508 genes to a manageable number for functional studies, the genes were further annotated (Alkharouf et al. 2007). To do this annotation procedure, firstly, a pool of 150 highly conserved, Group 1, *H. glycines* homologs of

genes having lethal mutant phenotypes or phenocopies from the free living nematode *C. elegans* were identified from the pool of 1,508 genes that were fabricated onto the Affymetrix® microarray. Secondly, it was determined that of those 150 genes on the Affymetrix® soybean GeneChip, a subset of 131 genes could have their expression monitored during the parasitic phase of their life cycle. Thirdly, a microarray analyses identified a core set of 32 genes with induced expression occurring during the parasitic stages of infection. The identification of 32 genes that had known expression during the parasitic stages of infection provided a small, but feasible, core set of genes that could be targeted in RNAi-based, reverse genetic screens (**Table 2.1**).

## **2.8 Reverse genetic screens to identify essential SCN genes**

Unlike *C. elegans*, SCN is not an ideal system for genetic studies because of its obligate endoparasitic life cycle. However, from information learned in *C. elegans*, gene function can be studied by an mRNA nuclease process called RNA interference (RNAi) (Fire et al. 1998). Through this process, a specific mRNA is targeted through a ribonucleoprotein complex for degradation (Hammond et al. 2001; Caudy et al. 2003). The challenge then became demonstrating whether RNAi was functional and reliable in the SCN since the approach does not work in some organisms. However, there are two demonstrated ways that RNAi-based experiments can be performed for SCN, allowing gene function experiments to be performed through the a reverse genetic manner allowed by the RNAi technology.

The first demonstration of RNAi in SCN accomplished the experiment by taking cDNAs for the gene of interest, synthesizing double stranded RNA (dsRNA) *in vitro* and soaking the nematodes in the dsRNA cocktail (Urwin et al. 2002; Alkharouf et al. 2007).

Urwin et al. (2007) examined how the SCN actin gene could be knocked down in its expression. The experiments resembled those performed in *C. elegans* whereby soaking the nematodes in dsRNA resulted in a phenocopy of the normal phenotype generated by the hypomorphic null mutant (Fire et al. 1998; Timmons et al. 2001). Similar experiments that relied on an extensive, but simple, gene annotation pipeline that identified 1,508 candidate genes (Alkharouf et al. 2007) used the cloned genes to synthesize dsRNA from *H. glycines* homologs of small ribosomal protein 3a. Experiments that soaked the SCN with dsRNA resulted in nematode mortality that was demonstrated by vital fluorescent dyes and a phenocopy where the nematodes appeared stiff (Alkharouf et al. 2007). Therefore, RNAi would work in the SCN system. The experiments were then taken a step further in experiments that used the RNAi-soaked nematodes to infect soybean plants to see if the nematodes were impaired in their ability to parasitize soybean. Modeled after the earlier experiments of Urwin et al. (2002), in experiments that used this approach for parasitism genes, it was shown that SCN infection could be suppressed (Alkharouf et al. 2007; Bakheita et al. 2007). The problem with these experiments, from a nematode biocontrol perspective, is that it would be virtually impossible to synthesize, apply and deliver enough dsRNA to nematodes that are living in the environment to obtain a positive effect even though crude dsRNA extracts can be used (Tenllado et al. 2003). Other problems would be whether the dsRNA remained residually in the soil. Therefore, a second method would be needed that could express the genes as dsRNA in soybean, allowing greater control over the delivery of the dsRNA to SCN.

The second way to perform RNAi experiments for SCN control would be to express the genes in transgenic soybean roots, allowing the nematodes to feed on the

genetically engineered roots. The hypothesis is that if the SCN was able to ingest the double stranded RNA manufactured in the plant cells through its stylet in high enough concentrations and if the RNAi metabolic process occurred in SCN, there was a chance that nematode development could be controlled. Prior experiments already demonstrated that the RNAi pathway functioned in SCN (Huang et al. 2006, Alkharouf et al. 2007). The original experiments that performed host-mediated expression of SCN genes as inverted tandemly duplicated copies for RNAi control in soybean to examine SCN biology were done by Steeves et al. (2006), examining the major sperm protein. Huang et al. (2006) demonstrated the same effect for root knot nematode in the model plant *A. thaliana* so the approach would have broad applicability for nematode control. The experiments were followed by Klink et al. (2009a) that identified many genes from microarray studies that would serve as candidates for RNAi control during parasitism.

Table 2.1 Annotation of the Affymetrix ® soybean GeneChip in relation to gene pathway analyses.

ProbeSetID	Afx H.g. Accn	Best C.e. Hit	Brief ID
HgAffx.13360.1.S1_at	CB374622	pes-9	Yeast hypothetical 52.9 KD protein
HgAffx.10986.1.S1_at	CA939315	gpd-3	NULL
HgAffx.19591.1.S1_at	CB278666	cpf-1	cleavage stimulation factor like
HgAffx.16755.1.S1_at	CA939427	phb-1	NULL
HgAffx.6532.1.S1_at	CK350603	hsp-6	heat shock 70 protein
HgAffx.19651.1.S1_at	CD748666	phb-2	Prohibitin
HgAffx.17567.1.S1_at	CB825030	ben-1	tubulin
HgAffx.20551.1.S1_at	CB281634	E02H1.1	rRNA methyltransferase
HgAffx.19055.1.S1_at	CB826041	T21B10.2	enolase
HgAffx.24001.2.S1_at	CK351582	ftt-2	14-3-3 protein
HgAffx.22771.2.S1_at	BI749139	uaf-1	NULL
HgAffx.21332.1.S1_at	BI748882	daf-21	heat shock protein (HSP90) guanine nucleotide-binding protein
HgAffx.10691.1.S1_at	CD748651	K04D7.1	t-complex protein 1
HgAffx.10821.1.S1_at	CB935592	T21B10.7	inorganic pyrophosphatase
HgAffx.13633.1.S1_at	CD748017	pyp-1	Ribosomal protein S3a homolog
HgAffx.20969.1.S1_at	CB379877	rps-1	Elongation factor Tu family
HgAffx.24120.1.S1_at	CB935135	eft-2	casein kinase I
HgAffx.22597.1.S1_at	CB826306	kin-19	ATP citrate lyase
HgAffx.11150.1.S1_at	CB378957	D1005.1	transketolase
HgAffx.17961.1.S1_at	CB281421	F01G10.1	actin
HgAffx.18740.2.S1_at	CA940055	act-4	malate dehydrogenase
HgAffx.14431.1.S1_at	CB935363	mdh-1	NULL
HgAffx.19636.2.S1_at	CA939544	rps-4	NULL
HgAffx.18811.1.S1_at	CA940369	F43G9.5	glucose-6-phosphate isomerase
HgAffx.5490.1.S1_at	CD747934	gpi-1	cathepsin-like protease
HgAffx.22868.1.S1_at	BG310682	cpl-1	NULL
HgAffx.13283.1.S1_at	CD748764	K10D6.2	NULL
HgAffx.17866.1.S1_at	CB824474	M03C11.7	NULL
HgAffx.20065.1.S1_at	AF318605	hsp-1	HSP-1 heat shock 70kd protein A
HgAffx.15252.1.S1_at	CK348813	rho-1	p21 ras-related rho (RhoA)
HgAffx.16942.1.S1_at	CK349264	ruvb-2	NULL
HgAffx.23555.2.S1_at	CD748675	Y54E10BR.6	NULL

The problem with the transgenic approach is that soybean is a difficult to genetically engineer. However, strategies whereby composite plants (Collier et al. 2005) that are chimeras having nontransformed aerial stocks having transgenic root stocks can be readily made in soybean. The simplicity of the approach is evident because the transgenic plants can be made in non-axenic conditions with the use of fluorescent reporter (Klink et al. 2008) (**Fig. 2.2**). The development of vectors that work in soybean (Klink et al. 2009a; Ibrahim et al. 2010; Matsye et al. 2012) have made the experiments possible. Further improvements whereby the plant expression vectors are Gateway®-compatible (Klink et al. 2009a; Ibrahim et al. 2010; Matsye et al. 2012) allows for semi-large reverse genetic screens to be performed. In such experiments, SCN homologs of the small ribosomal protein 3a (Hg-rps-3a) and Hg-rps-4, synaptobrevin (Hg-snb-1) and a spliceosomal SR protein (Hg-spk-1) were tested for functionality in host mediated expression, RNAi-based studies (Klink et al. 2009a). After 8 days of infection, the experiments demonstrated that 81–93% fewer females developed on transgenic roots containing the genes engineered as tandem inverted repeats. Those experiments resulted in lethality for SCN feeding on plants that were expressing the genes as duplicated, tandem-inverted repeats (Klink et al. 2009a). The same outcome was shown for root knot nematode in soybean using the same plant expression vector system (Ibrahim et al. 2010). These observations demonstrated that broad spectrum resistance for plant-parasitic nematodes in soybean was probable.



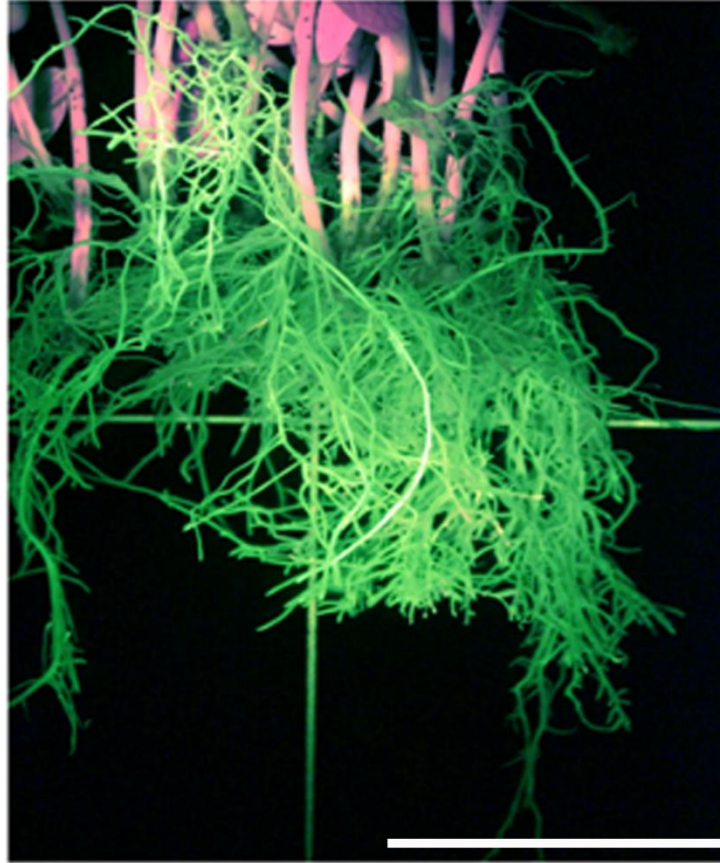


Figure 2.2 Soybean plants with transgenic roots

The transgenic soybean roots are expressing the enhanced green fluorescent protein (eGFP) (Haseloff et al. 1997) found in the pRAP vectors (Klink et al. 2009a; Matsye et al. 2012). Bar = 10 cm.

## 2.9 Proteomic studies of SCN

The prior experiments have discussed gene expression in SCN at the RNA level. These experiments are technologically simplistic to perform, because of major advances in sequencing and detection technologies. However, in these experiments using hybridization to study gene expression, little to no information is obtained as to how much protein is actually synthesized from the RNA or modifications that are known to exist on the protein molecules. Recently, the proteome of SCN was investigated (Chen et

al. 2011), resulting in a reference map of protein expression. These experiments add to the already extensive gene expression databases that are available for SCN (Alkharouf et al. 2007; Ithal et al. 2007a; Klink et al. 2009a). The advantage of the proteomic studies is that it allows for the identification of the relative amounts of the studied proteins to be known. This is in contrast to microarray-based experiments where only different levels of expression can be inferred, but their absolute amounts are not known. Experiments were performed using LC-MS/MS on pre-infective J2 SCN. The nematodes were highly pure samples since they had not yet infected soybean roots. The experiments were able to discern 803 spots on 2-D gels (Chen et al. 2011). Of those spots, 426 proteins were identified (Chen et al. 2011). Gene Ontology analyses allowed for the identification of a number of different functional groups, including secreted proteins that may act during parasitism (Chen et al. 2011). While it is likely that the protein list is not comprehensive, the work provides a solid foundation for future work to examine the proteome of SCN and compare with the gene expression studies based on the RNA.

## **2.10 Soybean gene expression**

To understand how soybean was reacting to infection, it was going to be imperative to develop ways to monitor gene expression during infection. Unlike the model system, *A. thaliana*, where a number of gene expression microarrays existed (Mussig et al. 2002; Tao et al. 2003) no commercially available microarrays were in place for soybean. The fabrication of soybean microarrays from cDNAs isolated from uninfected and SCN-infected tissues resulted in the identification of genes that are expressed during parasitism by SCN (Alkharouf et al. 2006). Subsequently, after the availability of the Affymetrix soybean GeneChip, a number of gene expression studies

have been performed on whole infected soybean roots (Ithal et al. 2007b; Klink et al. 2007b). Some of the studies focused in on expression occurring during the susceptible reaction (Alkharouf et al. 2006; Ithal et al. 2007b; Klink et al. 2007b). These studies resulted in the identification of genes that are highly expressed during the susceptible reaction. Alkharouf et al. (2006) performed experiments that examined the preparasitic stages of infection of the compatible reaction. The experiments identified defense-related genes such as Kunitz trypsin inhibitor (KTI), germin, peroxidase, phospholipase D, 12-oxyphytodienoate reductase (OPR), pathogenesis related-1 (PR1), phospholipase C, lipoxygenase, WRKY6 transcription factor and calmodulin. The experiments demonstrated that multiple defense pathways were induced even early (by 6 hours post infection) during the compatible reaction. This is important to note because the time point at which the sample was collected occurred before the nematode initiated the formation of the syncytium. Therefore, soybean was responding in important ways to the presence of the nematode within its root tissues. Similar lists of genes were identified by Ithal et al. (2007b), demonstrating a commonality of expression even though the experiments used different soybean genotypes and populations of SCN. Unfortunately, since only the susceptible reaction was studied, it was unclear whether the expressed genes were specific to the susceptible reaction or would also be differentially expressed in roots if they were undergoing a resistant reaction. This knowledge is important to identify actively expressed genes that relate specifically to defense.

To distinguish between expression of genes during the susceptible and resistant reactions, an experiment was performed whereby both susceptible and resistant reactions could be obtained in the same soybean genotype (*G. max*<sub>[Peking/PI 548402]</sub>) (Klink et al.

2007b). The importance of the experimental design was that it allowed gene expression that pertained specifically to the susceptible or resistant reaction to be identified. Thus, differences in plant genotype could not introduce error into the experiment. The experiments were set up whereby the *G. max*<sub>[Peking/PI 548402]</sub> genotype was infected with one of two SCN populations that would result in a susceptible or a resistant reaction. Another important feature of the experiment was that the gene expression that occurred as *G. max*<sub>[Peking/PI 548402]</sub>, a genotype with functional resistance genes, failed in its effort to defend itself from SCN would be identified. The *G. max*<sub>[Peking/PI 548402]</sub> genotype was infected with *H. glycines*<sub>[NL1-RHg/HG-type 7]</sub> (originally called race 3) that developed a resistant reaction and *H. glycines*<sub>[TN8/HG-type 1.3.6.7]</sub> (originally called race 14) that developed a susceptible reaction (Klink et al. 2007b). The experiments revealed induced levels of some genes during different points of the susceptible reaction as compared to the resistant reaction. Some of the genes that were induced in their expression during the susceptible reaction at 12 hours post infection (hpi) were an expansin, peroxidase, plasma membrane intrinsic protein 1C (PIP1C), germin-like protein (GER) 1, beta-Ig-H3 domain-containing protein and chorismate mutase (Klink et al. 2007b). Genes induced during the susceptible reaction at 3 dpi included 4-coumarateCoA ligase family protein, expansin, LTP1, transketolase and a cytochrome P450 (Klink et al. 2007b). Related experiments showing genes that were induced specifically during a susceptible reaction at 8 dpi included 4-coumarate CoA ligase family protein, peroxidase, expansin, matrix metalloproteinase, matrixin family protein and a lipid transfer protein (LTP) (Klink et al. 2007b). All of these proteins were suppressed in their activity during the resistant reaction. However, the problem with the vast amounts of data that was being generated at

the time was the difficulty in obtaining a meaningful annotation that would provide an understanding of the global events occurring in the sample types.

## 2.11 Improvements in annotation

The described experiments resulted in the generation of a massive amount of gene expression data and gene lists for the 38,099 genes fabricated on the Affymetrix® soybean GeneChip. Annotated gene lists for soybean genes are very useful because no information is lost from the analysis (**Table 2.1**). However, the gene lists do not provide higher order knowledge of how the many genes are functioning during a process under study. It is possible that various metabolic pathways that pertain to a specific process could be identified if the data could be organized into a higher order structure. Since the aforementioned work was done in soybean, often considered a non-model organism, it was difficult to translate the information into gene pathway analyses applications in a manner that would reveal how the gene expression is orchestrated during the process under study. However, an investigation that had been done in *A. thaliana* infected with *Pseudomonas syringae* pv. tomato did show how useful the higher order gene expression knowledge could be in allowing for a visualization of the switch in metabolism from housekeeping to pathogen defense during infection (Scheidler et al. 2001). The development and presentation of gene pathway information, a procedure that merged the Kyoto Encyclopedia of Genes and Genomes (KEGG) framework (Goto et al. 1997) with the gene expression data was accomplished through the development of a computer application called Pathway Analysis and Integrated Coloring of Experiments (PAICE) (Hosseini et al. 2010; Klink et al. 2011a). This allowed for obtaining higher order cell fate mapping studies to be performed (Klink et al. 2011a; Matsye et al. 2011). Moreover, the

sequencing of the soybean genome (Schmutz et al. 2010) made transcriptional mapping experiments that relate to resistance loci possible (Matsye et al. 2011).

## 2.12 Genomics of the syncytium

While strides were being made in obtaining a deep analysis of the physiological processes occurring in whole infected roots, the greater challenge was to identify gene expression that occurred within the syncytium because it required either drawing the cytoplasm out of the syncytium or developing a way to physically isolate the cells. The original studies attempted to determine gene expression in nematode nurse cells (Bird et al. 1994; Wilson et al. 1994). The hypothesis was that by extracting the cytoplasm of the cells that are specifically undergoing the parasitism, it would be possible to determine the gene expression that pertains specifically to parasitism. However, it is noted that gene expression in the cells surrounding the syncytium probably plays some role in the maintenance and development of the susceptible and resistant reactions. This approach to isolate the cytoplasm (Bird et al. 1994; Wilson et al. 1994) would be more challenging for syncytia because it is virtually impossible to determine what cells are infected by SCN. Therefore, instead of collecting the cytoplasm, it was necessary to collect the cells through their physical isolation.

The physical isolation of syncytia undergoing a susceptible reaction to the SCN was first described by Klink et al. (2005). The study collected syncytia by a procedure called laser microdissection (Isenberg et al. 1976; Meier-Ruge et al. 1976; Emmert-Buck et al. 1996) (**Fig. 2.3**). The experiments obtained RNA of suitable quality for making cDNA libraries, cloning and sequencing full length genes greater than 1,000 base pairs, making probes for *in situ* hybridization and quantitative PCR (qPCR) and

immunocytochemistry which would allow for the visualization of gene expression inside of the infected cells (Klink et al. 2005). These results made it possible to study gene expression occurring within the syncytium at the genome-wide level.

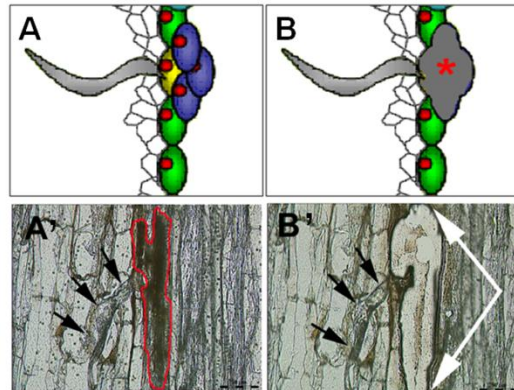


Figure 2.3 Laser microdissection (LM) of nematode feeding sites

**A**, cartoon of a nematode (gray) parasitizing a pericycle cell (yellow) that previously was uninfected (green). The parasitism process is resulting in the incorporation of neighboring cells by dissolving their cell walls, forming a syncytium. **B**, the syncytium (asterisk) was collected after LM. **A'**, an actual root used for LM. The black arrows point to a nematode infecting a root cell (red outline). **B'**, white arrows point to the feeding cell that was collected by LM.

Genomics approaches to syncytium biology resulted in a series of investigations that have focused in on gene expression that occurs during a susceptible reaction in the syncytium (Klink et al. 2005, 2007a, 2009b, 2010a, 2010b, c, 2011a; Ithal et al. 2007a; Matsye et al. 2011, 2012; Kandoth et al. 2011). In these studies, a number of genes were identified. However, to understand gene expression as it specifically pertains to defense, it was necessary to study the cells undergoing the defense response. The main obstacle in performing studies with this goal in mind was determining whether the cells undergoing the defense response were already dead at the time of cell collection. The prediction is that cells that were dead would have already halted their physiological processes that

pertained to defense and also may not provide RNA of suitable quality for microarray studies. However, it was unlikely that the cells progressing through the earlier stages of defense were dead (**Fig. 2.1**) since the EM studies revealed very specific progression of cellular architecture during the defense response, suggesting that the cells had to be alive to progress through this developmental process (Endo, 1965, 1991; Kim et al. 1987). The initial collection of syncytia undergoing the developmental process that leads to their eventual collapse and death were then performed (Klink et al. 2007a). These experiments demonstrated that the cells would be a suitable source for RNA collection and genomics-based analyses. The first set of experiments using laser microdissected cells undergoing an incompatible reaction for genomics studies determined that the expression of lipoxygenase (LOX), arabinogalactan-protein (AGP18), annexin, a thioesterase family protein heat shock protein (HSP) 70 and superoxidase dismutase (SOD) (Klink et al. 2007a). Many of the genes have very well-known roles in plant defense. Subsequent experiments examined more time points occurring during the defense response, spanning phase 1 and phase 2 (Klink et al. 2009b; Klink et al. 2010b, c). The experiments identified a number a genes that were very highly expressed during the resistant reaction, specifically within the syncytium. In contrast, a number of genes were very highly suppressed (>1,000 fold) during the resistant reaction (Klink et al. 2009b; Klink et al. 2010b, c). The experiments were repeated later by Kandoth et al. (2011) in the *G. max*<sub>[PI 209322]</sub> genetic background that either has or lacks the *rhg1* resistance locus. During this time, studies were also performed that examined and compared multiple forms of the resistant reaction that were found in the *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> genotypes (Klink et al. 2009b; Klink et al. 2010b, c; Matsye et al. 2011). These studies



were important because the *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> genotypes are well known to undergo different forms of the resistant reaction at the cellular level (Endo, 1991; Kim et al. 1987; Mahalingham and Skorupska, 1996). The *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> PIs are also important genotypes to obtain knowledge from because they are the source of >97% of the resistance germplasm used in commercial breeding programs (Concibido et al. 2004). In some of the earlier studies (Klink et al. 2009b), a number of genes were identified that were induced preferentially in their expression during the resistant reaction. The genes included lipoxygenase, S-adenosylmethionine synthetase, a dnaK domain-containing protein, GRF2 GENERAL REGULATORY FACTOR 2, ACT7 (actin 7), major latex protein-related protein, xyloglucan endotransglucosylase/hydrolase protein 26, cytochrome P450 monooxygenase CYP93D1, pyruvate dehydrogenase E1 beta subunit isoform 2, nitrate transporter (NTP2), endo-1,4-beta-glucanase that were all expressed preferentially between 100 to 383-fold higher in syncytia undergoing the defense response as compared to syncytia undergoing the early stages of a susceptible reaction (Klink et al. 2009b). Additional experiments aided by Illumina® deep sequencing technology which is a sequence by synthesis procedure much like quantitative PCR, but for every gene in the genome simultaneously, identified genes that were expressed only in syncytia undergoing the defense response (Matsye et al. 2011). Some of the genes were expressed at all times during the defense response. Importantly, the Illumina® deep sequencing technology revealed that some of the transcripts that are genes known to be important in defense responses represented between 1 and 17% of the sequenced tags from RNA isolated from the syncytia undergoing the defense response (**Table 2.2**) (Matsye et al. 2011). The knowledge gained

from these gene expression experiments was then used to select candidate genes whose function during infection could be tested. The cross-comparison of data obtained from the Illumina® sequencing platform with the Affymetrix® microarrays determined the genes within the *rhg1* locus that were expressed specifically during defense (Matsye et al. 2011). Experimentation of these genes in functional tests determined that some of these genes play a role in defense to SCN (Matsye et al. 2012). It was shown that one gene, an  $\alpha$ -SNAP allele isolated from the resistant *G. max*<sub>[Peking/PI 548402]</sub>, provided resistance when genetically engineered into the susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> (Matsye et al. 2012). Gene expression and functional studies will be further expanded on in a subsequent section.

Table 2.2 Group 1 SCN genes expressed during parasitism and used in RNAi studies (Klink et al. 2011b).

Category	total probe sets	percent
<b>Affymetrix® soybean GeneChip® probe sets (PS)</b>	<b>38,099</b>	<b>N/A</b>
<b>PS with matches to <i>Arabidopsis thaliana</i> accessions</b>	<b>23,583</b>	<b>62%</b>
<b>PS with enzyme commission (E.C.) numbers</b>	<b>9,717</b>	<b>29%</b>
<b>PS matching both <i>A. thaliana</i> accessions and having E.C. numbers</b>	<b>4,156</b>	<b>11%</b>
<b>PS with chromosomal coordinates</b>	<b>31,188</b>	<b>82%</b>

N/A, not applicable because that is the total number of probe sets.

### 2.13 Soybean resistance clusters

The major SCN resistance trait, *rhg1*, was first identified by Caldwell et al. (1960). In and around the same time, four other major loci, the recessive *rhg2*, *rhg3* (Caldwell et al. 1960) and the dominant *Rhg4* (Matson and Williams, 1965) and *Rhg5*

(Matson and Williams, 1965) have been identified. In all, there are approximately 61 QTLs that associate with resistance to SCN (Concibido et al. 1994). Many of the details of the numerous mapping studies can be found in a review by Concibido et al. (2004). Of all of the loci that associate with resistance to SCN, the best studied is *rhg1*. It is a major resistance locus and has been fine mapped to a region defined in a span of approximately 611,794 nucleotides between the molecular markers ss107914244 and Satt038 on chromosome 18 (Concibido et al. 1994; Mudge et al. 1997; Cregan et al. 1999a, b; Hyten et al. 2010). It is important to note that the *rhg1* loci found in the different genotypes that exhibit resistance are not the same (Cregan et al. 1999b; Brucker et al. 2005; Matsye et al. 2012). For example, due to the variation in how soybean responds to infection by the SCN, the *rhg1* resistance allele in *G. max*<sub>[PI 88788]</sub> is designated *rhg1-b* (Kim et al. 2010). Work by Kim et al. (2010) has resulted in the fine-mapping of the *rhg1-b* locus to within a region of approximately 67 kb. This development was important for the SCN research field because the locus contains approximately 9 genes. However, work in understanding the biological nature of the genes within the locus was not the focus of the Kim et al. (2010) study because the investigation was a genetic mapping analysis. Other resistance loci that are not as well mapped, such as *Rhg4* (Matson and Williams, 1965), while providing resistance, account for about a 30% of the resistance of soybean to SCN. In addition to this feature, it functions only against certain populations of SCN.

#### **2.14 Gene expression found during defense at the *rhg1* locus**

Knowing how and when genes are expressed in syncytia specifically during defense would likely provide knowledge of the genes that regulate or contribute to the process. Matsye et al. (2012) demonstrated in complimentary studies, that an amino acid

transporter (AAT) (Glyma18g02580) and an  $\alpha$ -soluble NSF attachment protein ( $\alpha$ -SNAP) (Glyma18g02590) found in the *rhg1* locus, undergo expression specifically in syncytia undergoing defense in both the *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> genotypes (Matsye et al. 2011). What was notable about the analysis was that AAT and  $\alpha$ -SNAP were shown to be expressed throughout the defense response in experiments that sampled time points at 3, 6 and 9 days post infection (dpi), spanning phase 1 and 2 (Matsye et al. 2011). A third gene within the *rhg1* locus that is annotated as a wound inducible protein (accession number: CF808381) was also expressed only in syncytia undergoing defense (Matsye et al. 2011). However, its expression was detected only in *G. max*<sub>[PI 88788]</sub> at the 3 and 6 dpi time points (Matsye et al. 2011). This observation further strengthens the observed differences for the *G. max*<sub>[PI 88788]</sub> and *G. max*<sub>[Peking/PI 548402]</sub> defense responses to SCN. Notably, the AAT,  $\alpha$ -SNAP and wound inducible genes did not appear to be expressed in syncytia undergoing the susceptible reaction. This difference in expression that was occurring between the resistant and susceptible reaction suggested the possibility, that the genes may be involved in the defense response. However, this could only be determined in functional studies that tested how the gene acted during infection (Matsye et al. 2012).

## 2.15 Genetic engineering as a solution for SCN

A number of approaches like conventional breeding programs have been shown for decades to generate resistance to SCN (Brim and Ross, 1966). The resistant cultivars have been shown to result in savings of hundreds of millions of dollars (Bradley and Duffy, 1982). One drawback of conventional breeding programs is that along with the resistance genes that are bred in, a number of genes are also introgressed that could have

undesirable characteristics. This is especially a problem when desirable traits are tightly linked to the undesirable traits. To circumvent this problem, it is possible to genetically engineer in genes of interest. A number of strategies that have been described in this chapter have shown promise in disrupting the soybean-SCN interaction. As noted earlier, RNAi of nematode parasitism genes has been shown in the *Arabidopsis thaliana*-*Meloidogyne* sp. system to perturb giant cell formation (Huang et al. 2006). This was also shown to work in the soybean-SCN pathosystem (Steeves et al. 2006). Later work that identified highly conserved SCN genes that were expressed during parasitism could be knocked down by RNAi and suppress infection (Klink et al. 2009a; Li et al. 2010). Due to the duplicated nature of the soybean genome (Doyle et al. 1999; Schmutz et al. 2010), RNAi studies of soybean genes may be met with complications and may require methodologies that can knock down entire gene families (Alvarez et al. 2006).

Another procedure to modulate resistance gene expression involves the engineering of soybean genes as overexpression constructs (Matsye et al. 2012). Genes that are highly expressed during a resistant reaction, identified in accessions of little agronomic value, can be expressed to high levels in a soybean genotype that is normally susceptible, but of great economic value. The hypothesis is that if the gene is important in the defense response, the overexpression of that gene in a genotype that is normally susceptible would result in suppressed nematode infection. Such a result was obtained by Matsye et al. (2012) with the overexpression of a naturally occurring truncated allele of an  $\alpha$ -SNAP gene. When the  $\alpha$ -SNAP gene that was identified in the *G. max*<sub>[Peking/PI 548402]</sub> accession was overexpressed in the normally susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> genotype, nematode infection was suppressed (**Fig. 2.4**). The experiments demonstrated

the efficacy of the approach, opening up the possibility for large scale reverse genetic screens since the plasmid vectors used to engineer the genes into soybean through the hairy root procedure (Tepfer, 1984) were designed with an enhanced green fluorescent reporter (eGFP) (Klink et al. 2008; Ibrahim et al. 2010) using the Gateway® technology for both RNAi and overexpression studies (Klink et al. 2009a; Matsye et al. 2012).

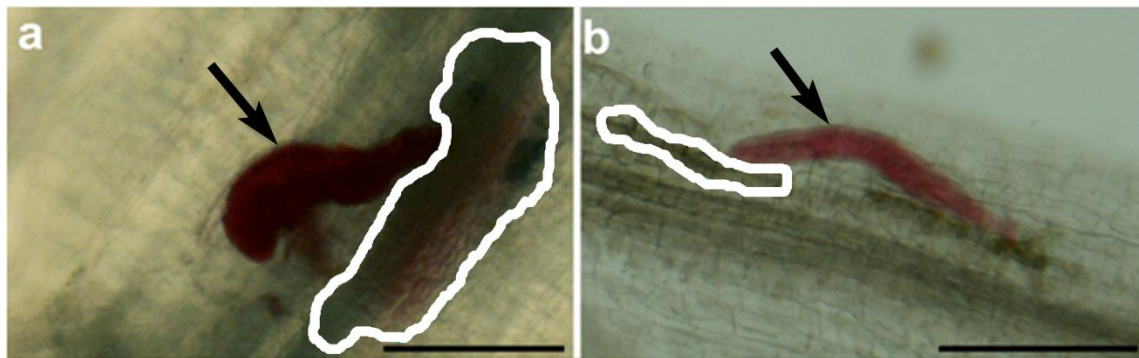


Figure 2.4 An overexpressed gene affects nematode development.

**a**, nematode, stained with acid fuchsin for visualization, developing in an experimental control plant. The boundary of the nematode feeding site is encircled in white. **b**, nematode failing to develop in a plant overexpressing a gene identified in the gene expression studies of the syncytium.

## 2.16 Conclusion

The soybean-SCN pathosystem has been under study for over 60 years. Through a massive amount of basic studies involving agricultural production practices, genetics, genomics and genetic engineering, solutions to the chronic and global SCN problem are emerging. The difficulty of studying the system has been met with many improvements in technology that are allowing for basic features of the pathosystem to be exploited so that agricultural practices and economic returns are improved. The basic knowledge gained in this system can now be applied as a model for understanding other recalcitrant

pathogens affecting soybean to obtain a comprehensive understanding of infection and defense.

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## CHAPTER III

### MAPPING CELL FATE DECISIONS THAT OCCUR DURING SOYBEAN DEFENSE RESPONSES<sup>2</sup>

#### 3.1 Abstract

The soybean defense response to the soybean cyst nematode was used as a model to map at cellular resolution its genotype-defined cell fate decisions occurring during its resistant reactions. The defense responses occur at the site of infection, a nurse cell known as the syncytium. Two major genotype-defined defense responses exist, the *G. max*<sub>[Peking]</sub>- and *G. max*<sub>[PI 88788]</sub>-types. Resistance in *G. max*<sub>[Peking]</sub> is potent and rapid, accompanied by the formation of cell wall appositions (CWAs), structures known to perform important defense roles. In contrast, defense occurs by a potent but more prolonged reaction in *G. max*<sub>[PI 88788]</sub>, lacking CWAs. Comparative transcriptomic analyses with confirmation by Illumina® deep sequencing were organized through a custom-developed application, Pathway Analysis and Integrated Coloring of Experiments (PAICE) that presents gene expression of these cytologically and developmentally distinct defense responses using the Kyoto Encyclopedia of Genes and Genomes (KEGG)

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<sup>2</sup> Most of the content of this chapter has been adapted from the journal article: Matsye PD, Kumar R, Hosseini P, Jones CM, Tremblay A, Alkharouf NW, Matthews BF, Klink VP. 2011. Mapping cell fate decisions that occur during soybean defense responses. *Plant Molecular Biology* 77: 513-528

framework. The analyses resulted in the generation of 1,643 PAICE pathways, allowing better understanding of gene activity across all chromosomes. Analyses of the *rhg1* resistance locus, defined within a 67 kb region of DNA demonstrate expression of an amino acid transporter and an  $\alpha$ -soluble NSF attachment protein gene specifically in syncytia undergoing their defense responses.

### 3.2 Introduction

The dominant pathogen of *Glycine max* (L.) Merr. (soybean) is the parasitic nematode *Heterodera glycines* Ichinohe (soybean cyst nematode [SCN]), an invasive species first observed in the U.S. in 1954 (Winstead et al. 1955). The SCN reproduces on at least 97 legume and 63 non-legume hosts (Epps and Chambers, 1958; Riggs and Hamblen, 1962, 1966a, b) with new hosts determined on a regular basis (Creech et al. 2006). SCN causes 7-10% reduction in production, worldwide. SCN causes more economic damage than the rest of its pathogens combined (Wrather et al. 2006), resulting in about \$1.5 billion in losses annually in the U.S. alone. Approximately 20,000 publically available collections of *G. max*, classified as plant introductions (PIs), are maintained through the USDA National Plant Germplasm System (USDA-NPGS). This seed bank, including many natural collections, is a resource that has been screened to identify *G. max* germplasm that can resist *H. glycines* infection. Through screening studies, two major groups of PIs each composed of a few *G. max* genotypes have been shown to exhibit specific, but contrasting ways to combat *H. glycines* (Ross and Brim, 1957; Ross, 1958; reviewed in Riggs, 1992). Defense occurs at the site of infection, a nurse cell known as a syncytium (**Fig. 3.1**). The cellular response of *G. max*<sub>[Peking]</sub> to SCN has been determined (Ross, 1958) and other genotypes including PI 89772, PI 90763 and



partially PI 437654 (Mahalingam and Skorpska, 1996) have been found to defend against SCN in a similar manner. The *G. max*<sub>[PI 88788]</sub> genotype was identified from a second screen (Epps and Hartwig, 1972) with PI 209332, PI 548316 and partially PI 437654 (Mahalingam and Skorpska, 1996) having similar cytological features occurring during their defense responses.

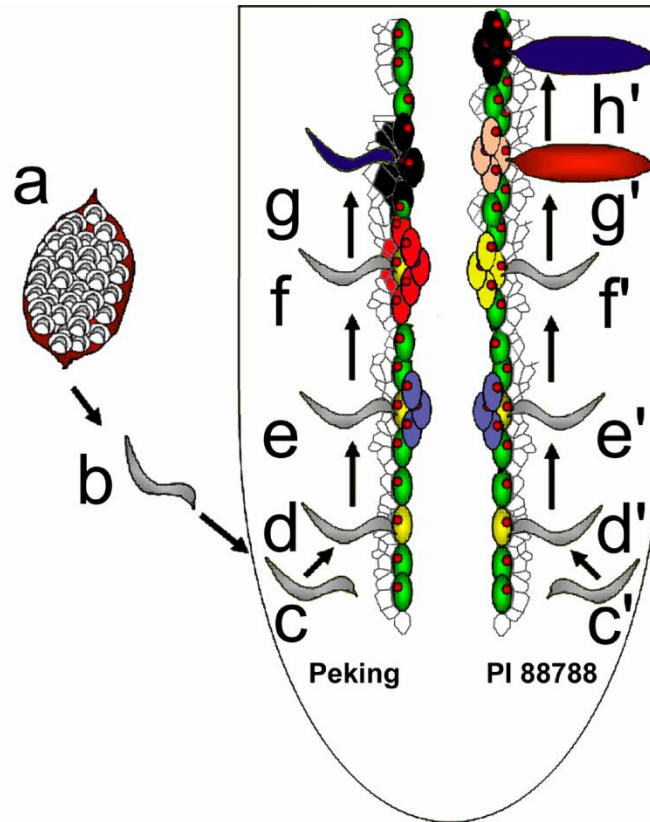


Figure 3.1 *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> resistant reactions

The phase 1 period of the defense response is represented in **Figs. 3.1. c, c'** through **Figs. 3.1. e, e'**. The phase 2 period of the defense response is represented in **Figs. 3.1. f, f'** through **Fig. 3.1.h'**. **Fig. 3.1.a**, cyst containing eggs. **Fig. 3.1.b**, pre-infective J<sub>2</sub> (pi-J<sub>2</sub>) nematodes (gray) migrate toward the root. **Fig. 3.1.c, c'**, the infective J<sub>2</sub> (i-J<sub>2</sub>) nematodes burrow into the root and migrate toward the root stele, **Fig. 3.1.d, d'**, the parasitic J<sub>2</sub> (p-J<sub>2</sub>) typically selects a pericycle (green cells) or neighboring cell as the feeding site initial (FS<sub>i</sub>) (yellow cell). This cell is used to initiate the formation of the syncytium. The earlier stages of syncytium development (between 1.1. an 4 dpi) are similar between *G. max*<sub>[Peking]</sub> and *G. max*<sub>[PI 88788]</sub>-type of resistant reactions. **Fig. 3.1.e'**, in *G. max*<sub>[Peking]</sub>-type, the neighboring cells (purple) are incorporated into the syncytium at 3 dpi. **Fig. 3.1.e'**, in *G. max*<sub>[PI 88788]</sub>, the neighboring cells (purple) are incorporated into the syncytium at 3 dpi. **Fig. 3.1.f**, in *G. max*<sub>[Peking]</sub>, a rapid and potent resistant reaction occurs by the formation of a necrotic region that surrounds the syncytium (red layer of cells surrounding the yellow FS<sub>i</sub>) by 4 dpi. **Fig. 3.1.f'**, in *G. max*<sub>[PI 88788]</sub>, a prolonged but potent resistant reaction at the syncytium (pink cells) is not yet evident at the cytological level at 4 dpi. **Fig. 3.1.g**, in *G. max*<sub>[Peking]</sub>, degradation of the syncytium (black cells) is engaged that is accompanied by the mortality of the SCN at the p-J<sub>2</sub> stage (purple nematode). **Fig. 3.1.g'**, In contrast, in *G. max*<sub>[PI 88788]</sub>, the syncytium (pink cells) continues to develop until 5 dpi. The SCN feeding from the syncytium continues to develop, molting into J<sub>3</sub>s (red nematode). **Fig. 3.1.h'**, in *G. max*<sub>[PI 88788]</sub>, the entire syncytium collapses (black cells) and the SCN dies at the J<sub>3</sub> or J<sub>4</sub> stage (purple nematode). (The timing of stages is adapted from Endo, 1965; Riggs et al. 1973; Lauritis et al. 1983; Kim et al. 1987).

Numerous studies have investigated the soybean defense responses to SCN. The *G. max*<sub>[Peking]</sub> defense response is potent and rapid because most nematodes die early during parasitism at the parasitic second stage juvenile (p-J<sub>2</sub>) stage (Colgrove and

Niblack, 2008). The *G. max*<sub>[Peking]</sub>-type of defense response is evident at the cellular level at 4 days post infection (dpi), involves necrosis of the cells that surround the head of the nematode and separates the syncytium from the cells that surround it (Endo 1964, 1965; Riggs et al, 1973; Kim et al, 1987; Kim and Riggs 1992). Another defining feature of the *G. max*<sub>[Peking]</sub>-type of defense response is the presence of cell wall appositions (CWAs), structures defined as physical and chemical barriers to cell penetration (Aist et al. 1976, Schmelzer, 2002; Hardham et al. 2007). In contrast, the *G. max*<sub>[PI 88788]</sub> defense response is potent but prolonged as the nematodes die at the J3 or J4 stages (Acido et al. 1984; Kim et al. 1987; Colgrove and Niblack, 2008). In contrast to the *G. max*<sub>[Peking]</sub>-type of defense, the *G. max*<sub>[PI 88788]</sub>-type of response lacks the development of a necrotic layer that surrounds the head of the nematode (Kim et al. 1987). The initial stages of the *G. max*<sub>[PI 88788]</sub>-type of defense response involves extensive accumulation of cisternae and rough ER that is accompanied by nuclear degeneration within the syncytium by 5 dpi (Kim et al. 1987). The *G. max*<sub>[PI 88788]</sub>-type of defense response lacks thickened cell walls or appositions.

The genetic basis underlying defense to SCN resulted in the identification of the major recessive (*rhg1*, *rhg2* and *rhg3*) (Caldwell et al. 1960), and dominant (*Rhg4*) (Matson and Williams 1965) and *Rhg5* (Rao-Arelli 1994) loci. Of these, the *rhg1* locus is currently the best understood since it has been defined in a region spanning approximately 611,794 nucleotides on chromosome 18 (Concibido et al. 1994; Mudge et al, 1997; Cregan et al. 1999; Hyten et al. 2010). Allelic variants are known to exist between different soybean genotypes harboring *rhg1* (Brucker et al. 2005; Kim et al. 2010). Furthermore, fine mapping efforts in the *G. max*<sub>[PI 88788]</sub> background has allowed

the locus to be narrowed down to within a region of approximately 67 kb (Kim et al. 2010).

While mapping efforts have made large contributions to understanding resistance, recent evidence has shown the value in applying gene expression to compliment mapping efforts in plants with complex duplicated genomes (Bancroft et al. 2011). The availability of the *G. max* genome (Schmutz et al. 2010) allows for similar expression mapping to be performed. However, expression studies typically examine differentially expressed genes whereby expression is measured in both a control and experimental sample and relative levels of expression are compared under various statistical parameters. The problem with the differential expression approach is that genes that have expression in one sample type and lack expression in a second sample type are discarded because statistical analyses cannot be done when expression is lacking in one of the two samples (**Fig. 3.2**).

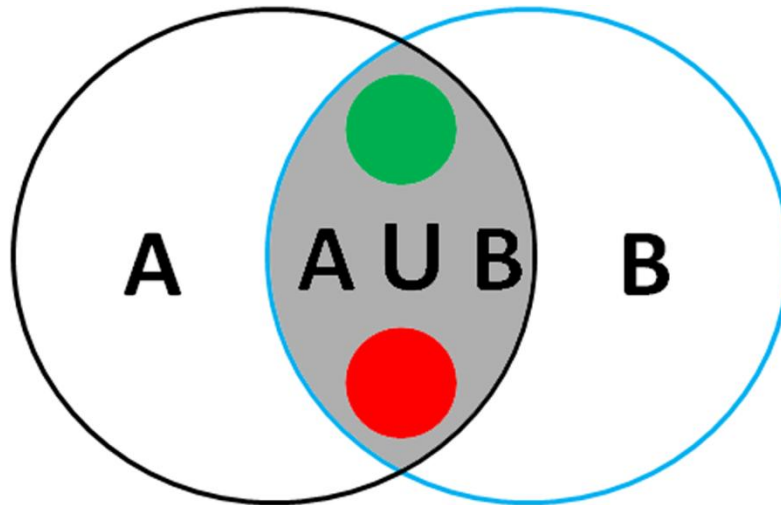


Figure 3.2 Detection call methodology

A and B represent genes with measured detection from two different cell types. The center gray region, which represents the union of A and B having the same pool of genes, including the green pool (induced genes) and red pool (suppressed genes), are genes that can be analyzed by differential expression analyses because they are expressed in both sample types. The gray region, lacking the red and green pools are the genes expressed in both sample types but do not exhibit statistically significant differences in expression between A and B. The genes of the white region of the A pool do not meet the statistical criteria of differential expression studies and would be discarded because they are expressed in only one sample type. Likewise, the genes in the white region of pool B pool are those that are discarded in differential expression studies. The white pools of genes are the focus of the detection call methodology

Detection call methodology (DCM) makes possible the cross-comparison of gene activity measured in one sample type to a second sample type where activity is not measured. Therefore, it is possible to identify and analyze genes with expression that is limited to one cell type. The important concept to recognize is that this pool of expressed genes could represent gene activity that defines a specialized cell type such as a syncytium proceeding through a series developmental events that culminates in a terminal phenotype such as cell death. Therefore, it could be imagined that an undifferentiated cell type like pericycle would lack expression of genes involved in

programmed cell death where they would be found to be expressed in a syncytium undergoing the terminal steps of resistance. Thus, DCM in concert with differential expression analyses could provide a broader and more comprehensive analysis of gene expression in specialized cell types such as syncytia undergoing defense.

The analysis presented here compares gene expression occurring during the potent and rapid defense response found in *G. max*<sub>[Peking/PI 548402]</sub> to the potent but prolonged process found in *G. max*<sub>[PI 88788]</sub>. Expression is presented graphically using a custom-developed KEGG application called Pathway Analysis and Integrated Coloring of Experiments (PAICE) (Hosseini et al. unpublished). Comparative analyses of transcriptional activity in these cytologically and developmentally distinct defense responses are used to determine gene expression in relation to the sequenced genome of *G. max*<sub>[Williams 82/PI 518671]</sub>, identifying the chromosomal coordinates of the expressed genes. Further analyses place the expressed genes in relationship to an important resistance locus, *rhg1*, defined within a 67 kb region of DNA on chromosome 18 between the markers BARCSOYSSR\_18\_0090 and BARCSOYSSR\_18\_0094 (Kim et al. 2010). Gene expression of a subset of 1,000 genes is confirmed by Illumina® deep sequencing.

### **3.3 Materials and methods**

#### **3.3.1 Plant and nematode procurement**

The materials and methods pertaining to *H. glycines* populations, *G. max* genotypes, experimental procedures and data analysis methods are published (Klink et al. 2005, 2007, 2009, Alkharouf et al. 2006). The *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> stocks were originally obtained from the USDA-NPGS ([http://www.ars-grin.gov/npgs/acc/acc\\_queries.html](http://www.ars-grin.gov/npgs/acc/acc_queries.html)). The *H. glycines* NL1-RHg population used in the

studies is race 3, HG-type 7 (*H. glycines*<sub>[NLI-RHg/HG-type 7]</sub>) (Klink et al. 2009, 2010a). The *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> genotypes were used in the experiments to obtain defense responses by the use of *H. glycines*<sub>[NLI-RHg/HG-type 7]</sub>. The *H. glycines*<sub>[TN8/HG-type 1.3.6.7]</sub> (race 14) population was used to obtain susceptible reactions (Klink et al. 2007, 2009, 2010a). Seedlings were grown according to Klink et al. (2007, 2009). Prior to infection, the nematodes were diluted to a final concentration of 2,000 pi-J2/ml and one ml of nematode stock was added to each root of each plant. The roots, including the mock-infected control samples, were washed after one day to remove nematodes that had not penetrated the roots. Infected roots were grown for 3, 6 or 9 dpi. Maximally infected lateral roots were harvested for analyses. The process was subsequently repeated twice, providing three independent sets of samples for each genotype.

### 3.3.2 LCM and microarray hybridization

Slides were prepared according to Klink et al. (2005, 2007, 2009, 2010a). LCM was performed on a Leica® ASLMD microscope® (Leica®). Serial sections of approximately 100 syncytia were used to obtain the RNA for the studies for each replicate. Over 100 ng of RNA per replicate was obtained for the studies. RNA purification was done with the PicoPure RNA Isolation kit, (Molecular Devices®). A DNase treatment was added, just before the second column wash, using DNafree® (Ambion®). RNA quality and yield were determined using the RNA 6000 Pico Assay® (Agilent Technologies®) using the Agilent 2100 Bioanalyzer® according to the manufacturer's instructions. Both probe preparation and hybridization procedures on the GeneChip® Soybean Genome Array (Affymetrix®) were performed according to their guidelines.

### 3.3.3 Data analysis

All microarray hybridizations were performed at the Laboratory of Molecular Technology, SAIC-Frederick, National Cancer Institute at Frederick, Frederick, MD 21701, USA. Local normalization was used. The measurement of the presence or absence of transcripts by particular probe set on a single array was determined using the Bioconductor implementation of the standard Affymetrix® DCM according to Klink et al. (2010b). In summary, the DCM consists of four steps: (1) removal of saturated probes, (2) calculation of discrimination scores, (3) *p*-value calculation using the Wilcoxon's rank test, and (4) the detection (present/marginal/absent). Ultimately, the algorithm determines if the presence of a probe set's transcript is provably different from zero (present [P]), uncertain (marginal [M]), or not provably different from zero (absent [A]). A probe set was considered present only if it measured expression on all three replicate microarrays corresponding to that condition. To be considered absent, the probe set had to lack detection on all three replicates for a given condition. A description of the supplemental files is provided. Microarray gene expression has been confirmed using the Illumina® Genome Analyzer II® (Illumina®) at the USDA-ARS Beltsville, MD according to the manufacturer's protocols. Data is maintained at the Soybean Genomics and Microarray Database (Alkharouf and Matthews, 2004).

### 3.3.4 Gene pathway analyses

The PAICE software (Paice\_v2\_90.jar) (Hosseini et al. unpublished) (<http://sourceforge.net/projects/paice/>) was developed for the pathway analyses. The PAICE software visualizes pathways according to Kyoto Encyclopedia of Genes and Genomes (KEGG) ([http://www.genome.jp/kegg/catalog/org\\_list.html](http://www.genome.jp/kegg/catalog/org_list.html)) from Affymetrix®



gene expression data. There are 38,099 probe sets on The Affymetrix® soybean GeneChip®. As of June 2011, 9,717 probe sets (29%) have reference pathway enzyme commission (E.C.) numbers. There are 23,583 probe sets with matches to *Arabidopsis thaliana* accessions (62%). The number of probe sets matching both *A. thaliana* accessions and having E.C. numbers is 4,156 (11%). The PAICE pathway analysis was performed according to Klink et al. (2011) using a modified version for data obtained through the DCM. Data supplemental to each table and figure and GO terms (Harris et al. 2004) are provided. The seven supplemental datasets (**Supplemental Datasets 1-7**) can be found at the website:

<http://dl.dropbox.com/u/428435/DCM%20PATHWAYS.zip>.

### 3.3.5 Chromosomal map coordinates

The Genbank accessions of probe sets on the Affymetrix® soybean GeneChip® (**Supplemental table 1**) were queried against the sequences *G. max*<sub>[Williams 82/PI 518671]</sub> (**Supplemental table 2**) (Schmutz et al. 2010) genome at <http://www.phytozome.org/>. The queries were performed in the *Glycine max* database using the Blast option. Once the chromosomal map coordinates were obtained, the coordinates were queried into <http://www.soybase.org>, allowing for the identification of the microarray-identified genes and their chromosomal map coordinates to physical map positions in relation to the genetic positions of the resistance loci.

## 3.4 Results

### 3.4.1 Intergenotype analyses identify genes that are expressed in a genotype-dependent manner

Detection call methodology (DCM) was used to compare the *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> defense responses using the Affymetrix® GeneChip fabricated with 38,099 soybean probe sets. Comparisons were made at the 3 dpi time point syncytia (**Fig. 3.3.a; Supplemental table 3**), 6 dpi (**Fig. 3.3.b; Supplemental table 4**) and 9 dpi time points (**Fig. 3.3.c; Supplemental table 5**). The analyses of the combined data from the 3, 6 and 9 dpi time points demonstrated that *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> gene expression was different from syncytia undergoing the susceptible reaction (**Fig. 3.3.d; Supplemental table 6**). Expression was confirmed using Illumina® deep sequencing platform (**Table 3.1.; Supplemental table 7**).

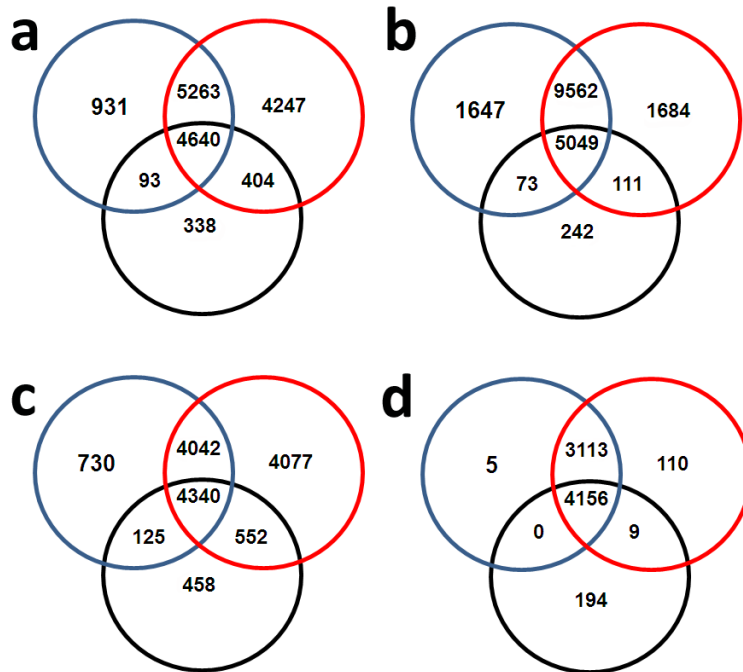


Figure 3.3 Intergenotype analyses

Represented by Venn diagrams depicting the four comparative analyses made between the *G. max*<sub>[Peking/PI 548402]</sub> resistant syncytium (blue circle), *G. max*<sub>[PI 88788]</sub> resistant syncytium (red circle) and *G. max*<sub>[Peking/PI 548402]</sub> susceptible time course (black circle). The blue ring represents the *G. max*<sub>[Peking/PI 548402]</sub> resistant syncytium pools of genes. The red ring represents the *G. max*<sub>[PI 88788]</sub> resistant syncytium pool of genes. The black ring represents the *G. max*<sub>[Peking/PI 548402]</sub> susceptible syncytium time course pool of genes. **Fig. 3.3.a**, 3 dpi *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> resistant syncytium samples compared to *G. max*<sub>[Peking/PI 548402]</sub> susceptible syncytium time course pool of genes. **Fig. 3.3.b**, 6 dpi *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> resistant syncytium samples compared to *G. max*<sub>[Peking/PI 548402]</sub> susceptible syncytium time course pool of genes. **Fig. 3.3.c**, 9 dpi *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> resistant syncytium sample pool compared to *G. max*<sub>[Peking/PI 548402]</sub> susceptible syncytium pool of genes. **Fig. 3.3.d**, combined 3, 6 and 9 dpi *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> resistant syncytium sample pool compared to *G. max*<sub>[Peking/PI 548402]</sub> susceptible syncytium time course pool of genes.

Table 3.1 Illumina® deep sequencing experiment for the *G. max*<sub>[P188788]</sub> 9 dpi resistant reaction time point.

AFFYMETRI X probe set	chromosomal location	Illumina: % of tags*	Gene Annotation	KEGG annotation
Gma.3940.1.S1_at	Glyma13g06450.1	17.3967	unknown	
Gma.16471.1.S1_at	Glyma17g07250.1	11.66824	XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6 (XTR6)	2.4.1.207
Gma.6290.1.S1_at	Glyma14g07460.1	9.966236	BOTRYTIS-INDUCED KINASE1 (BIK1)	-
GmaAffx.21298.1.S1_at	Glyma13g25020.1	9.796265	dentin phosphoryn protein	-
GmaAffx.93619.1.S1_s_at	Glyma12g33530.1	7.118084	fasciclin-like arabinogalactan protein 9.2	-
Gma.10919.2.S1_s_at	Glyma03g35180.2	6.697751	zinc finger (AN1-like) family protein	-
GmaAffx.88992.1.A1_at	Glyma20g05560.1	5.64807	conserved hypothetical	-
GmaAffx.11803.1.A1_at	Glyma11g11240.1	3.805958	eukaryotic initiation factor 4B-like	-
GmaAffx.87597.1.S1_at	Glyma10g07450.1	3.126077	Phloem protein 2-A15 (ATPP2-A15)	-
Gma.13476.1.A1_at	Glyma08g03130.1	1.846705	hua enhancer 2 (HEN2) RNA helicase IN	-
GmaAffx.34450.1.S1_at	Glyma05g05290.1	1.529733	Bax inhibitor-like protein	-
Gma.9307.1.S1_at	Glyma12g35990.1	1.442451	Bax inhibitor-like protein	-
GmaAffx.22691.S1_at	Glyma02g11060.1	1.120886	AP2/EREBP-Mediated Defense Pathway	K09287
Gma.10632.1.S1_a_at	Glyma04g43160.1	1.070354	ubiquitin-fold modifier 1-like (Ufm1)	K12162
GmaAffx.90320.1.S1_s_at	Glyma17g23870.1	0.856743	conserved hypothetical	-
GmaAffx.89786.1.S1_at	Glyma13g12070.1	0.542068	conserved hypothetical	-
GmaAffx.13321.S1_at	Glyma11g01070.1	0.537474	predicted protein	-
GmaAffx.65693.2.S1_s_at	Glyma09g33650.1	0.39966	Phosphoenolpyruvate carboxykinase	-
Gma.12624.1.S1_at	Glyma13g39120.1	0.383582	predicted protein	-

Table 3.1 (continued)

GmaAffx.74918.1.S1_at	Glyma02g41490.1	0.298597	APK1A ( <i>Arabidopsis</i> protein kinase 1A)	-
GmaAffx.89726.1.A1_s_at	Glyma13g42330.1	0.243471	LIPOXYGENASE 1 (LOX1)	1.13.11.12
GmaAffx.53904.1.S1_at	Glyma18g48350.1	0.218205	Peptidyl-prolyl cis-trans isomerase	-
GmaAffx.90263.1.S1_s_at	Glyma07g00900.2	0.192939	Seed lipoxygenase	1.13.11.12
GmaAffx.86757.1.S1_at	Glyma02g25950.1	0.179158	Bet v I allergen family protein	-
GmaAffx.82344.1.S1_at	Glyma12g34570.2	0.156189	Sali3-2	-
Gma.11106.2.S1_at	Glyma06g02990.1	0.142408	MADS-box protein	K09264
GmaAffx.89861.1.A1_at	Glyma20g27940.1	0.126329	Ubiquitin C variant	-
Gma.6533.1.S1_at	Glyma17g37400.1	0.124032	Cysteine proteinase precursor	3.4.22.-
Gma.4999.1.S1_s_at	Glyma08g46240.1	0.117142	Wound-induced basic protein	-
GmaAffx.60419.1.S1_x_at	Glyma17g23900.1	0.110251	no match	-
GmaAffx.35639.1.A1_at	Glyma01g35480.1	0.107954	no match	-
Gma.3881.1.S1_s_at	Glyma02g40290.2	0.105657	Trans-cinnamate 4-monooxygenase	1.14.13.11
GmaAffx.89225.1.S1_s_at	Glyma17g02260.1	0.105657	Diamine oxidase	-
Gma.2590.1.A1_s_at	Glyma10g35870.2	0.101064	predicted protein	-
Gma.443.1.S1_at	Glyma08g18130.1	0.091876	Myb, DNA-binding; Glycosyl transferase, group 1	2.4.1.142
Gma.6664.2.S1_at	Glyma19g37000.1	0.091876	Nodulin-26	K09873
Gma.11247.1.S1_at	Glyma08g41060.1	0.089579	ATHVA22C	-
GmaAffx.89113.1.S1_x_at	Glyma03g34310.1	0.089579	Nodulin-26	K09873
Gma.5947.1.S1_s_at	Glyma09g04530.1	0.084985	pathogenesis-related class 10 protein SPE-16	-
GmaAffx.1301.92.S1_s_at	Glyma13g12020.1	0.080391	cytochrome P450 monooxygenase	-

Table 3.1 (continued)

Gma.1034.4.S1_s_at	Glyma17g18800.1	0.073501	caffeoyl-CoA O-methyltransferase	-
GmaAffx.92561.1.S1_s_at	Glyma16g07750.1	0.068907	predicted protein	-
Gma.4189.1.S1_at	Glyma17g01720.1	0.059719	Cationic peroxidase 2	1.11.1.7
GmaAffx.81963.1.S1_at	Glyma16g19560.1	0.059719	Phototropin	-
Gma.1079.1.S1_s_at	Glyma08g11480.1	0.059719	adenosylhomocysteinase	-
GmaAffx.89772.14.A1_s_at	Glyma17g03350.1	0.057422	PR10-like protein	-
GmaAffx.34785.9.S1_at	Glyma13g11930.1	0.057422	conserved hypothetical	-
Gma.15478.2.S1_at	Glyma17g04340.1	0.057422	S-ADENOSYLMETHIONINE SYNTHETASE 2 (SAM-2)	-
GmaAffx.90331.1.S1_s_at	Glyma08g24760.1	0.055126	Bet v I family protein	-
Gma.16887.2.S1_at	Glyma04g08200.1	0.055126	proteasome subunit beta type-6	3.4.25.1
GmaAffx.89703.1.A1_at	Glyma12g07780.3	0.050532	L-ascorbate peroxidase, cytosolic	-
GmaAffx.92028.1.S1_at	Glyma10g01080.1	0.050532	RAD5-DNA repair	-
GmaAffx.29710.1.S1_at	Glyma18g53470.1	0.048235	RAD23-DNA repair	K10839
Gma.11179.1.S1_s_at	Glyma03g32850.1	0.048235	HEAT SHOCK COGNATE PROTEIN 70-1 (HSC70-1)	K03283
GmaAffx.92063.1.S1_s_at	Glyma17g01500.1	0.048235	Aspartic proteinase 1	3.4.23.40
Gma.17595.1.S1_s_at	Glyma18g44850.1	0.045938	UBIQUITIN CONJUGATING ENZYME 8 (UBC8)	-
GmaAffx.93164.1.S1_s_at	Glyma18g39690.1	0.045938	Type 2 metallothionein	-
Gma.2133.1.S1_at	Glyma10g30110.1	0.045938	alcohol acyl-transferases	-
Gma.756.2.S1_s_at	Glyma15g08300.1	0.043641	Auxin-repressed protein	-
GmaAffx.1301.58.S1_s_at	Glyma15g19580.1	0.043641	Cysteine protease	-
Gma.1634.1.S1_at	Glyma12g10150.2	0.043641	DnaJ Seed maturation protein PM37	-

Table 3.1 (continued)

Gma.8449.1.S1_s_at	Glyma11g01240.1	0.043641	4-coumarate:CoA ligase isoenzyme 3	6.2.1.12
GmaAffx.83146.1.S1_s_at	Glyma17g35720.1	0.043641	cysteine proteinase precursor	-
Gma.2655.2.S1_a_at	Glyma12g16560.1	0.041344	IAA-LEUCINE RESISTANT3 (ILR3) bHLH transcription factor	-
GmaAffx.15940.1.S1_at	Glyma17g14750.1	0.041344	beta-fructosidase (BFRUCT3)	3.2.1.26
Gma.11179.3.S1_x_at	Glyma19g35560.1	0.039047	heat shock protein 70 (HSP70)	K03283
GmaAffx.89705.1.S1_s_at	Glyma04g01130.2	0.039047	ERD14	-
GmaAffx.91487.1.S1_x_at	Glyma05g11630.1	0.039047	elongation factor 1-alpha	-
GmaAffx.89781.1.S1_s_at	Glyma12g00390.1	0.039047	Patellin-3; SEC14 homolog	-
GmaAffx.25068.1.A1_s_at	Glyma02g47960.1	0.039047	Chlorophyll a-b binding protein 8	-
GmaAffx.42055.1.S1_at	Glyma17g13510.1	0.039047	Esterase/lipase/thioesterase	-
Gma.10990.2.S1_x_at	Glyma11g11900.1	0.03675	fructose-bisphosphate aldolase-phosphate shunt	4.1.2.13
Gma.5129.1.S1_at	Glyma11g18320.1	0.03675	pyridine nucleotide-disulphide oxidoreductase family protein	-
GmaAffx.87207.1.S1_at	Glyma06g19890.1	0.03675	ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)	-
Gma.2892.2.S1_at	Glyma06g08380.1	0.03675	26S proteasome regulatory particle non-ATPase subunit6	K03036
Gma.17525.1.S1_at	Glyma18g08220.1	0.034454	HEAT SHOCK PROTEIN 81-2 (HSP81-2)	K04079
GmaAffx.78601.1.S1_s_at	Glyma12g08040.1	0.034454	aquaporin PIP2,2	-
Gma.10969.1.S1_a_at	Glyma13g42340.1	0.034454	LIPOXYGENASE 1 (LOX1)	1.13.1.12
Gma.10988.3.S1_x_at	Glyma14g09440.1	0.034454	cysteine proteinase precursor	3.4.22.-
Gma.17594.2.S1_at	Glyma10g31590.1	0.034454	ARABIDOPSIS THALIANA METHIONINE GAMMA-LYASE (ATMGL)	4.4.1.11
Gma.11130.2.S1_at	Glyma12g29510.2	0.032157	PLASMA MEMBRANE INTRINSIC PROTEIN 2;4 (PIP2;4)	K09872
GmaAffx.90275.1.S1_at	Glyma08g13130.1	0.032157	conserved hypothetical	-

Table 3.1 (continued)

GmaAffx.89946 .1.S1_x_at	Glyma16g 28590.1	0.03 2157	no match	-
GmaAffx.93348 .1.S1_at	Glyma15g 40860.1	0.03 2157	elongation factor 1	3.6.5. 3
GmaAffx.84025 .1.S1_at	Glyma01g 41460.1	0.03 2157	polycomb group protein EMF2	-
Gma.8141.1.A1 _at	Glyma09g 33680.1	0.03 2157	sugar transporter/spinster transmembrane protein	-
Gma.186.1.S1_a _t	Glyma15g 32800.1	0.02 986	SOS3-INTERACTING PROTEIN 3 (SIP3)	-
GmaAffx.1301. 131.A1_x_at	Glyma05g 25810.1	0.02 986	Photosystem II light harvesting complex gene 1.4	K089 12
Gma.17610.1.S 1_x_at	Glyma15g 03050.1	0.02 986	LIPOXYGENASE 1 (LOX1)	1.13. 11.12
GmaAffx.93032 .1.S1_s_at	Glyma03g 28410.2	0.02 986	Ferredoxin-dependent glutamate synthase	1.4.7. 1
Gma.3893.1.S1 _at	Glyma08g 05610.2	0.02 986	receptor for activated protein kinase C (RACK1)	-
Gma.11037.1.S 1_at	Glyma16g 04940.1	0.02 986	GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE A SUBUNIT (GAPA)	1.2.1. 13
Gma.7112.1.S1 _a_at	Glyma16g 21590.2	0.02 986	RNA polymerase I specific transcription initiation factor	-
Gma.2266.1.S1 _s_at	Glyma10g 06600.1	0.02 986	cell wall-associated hydrolase-homologous to bacterial proteins	-
GmaAffx.90998 .1.S1_s_at	Glyma05g 24110.1	0.02 986	elongation factor 1A	-
GmaAffx.497.1. S1_at	Glyma13g 19330.1	0.02 7563	Heat shock cognate 70 kDa protein 1	K032 83
GmaAffx.82418 .1.S1_x_at	Glyma20g 34880.1	0.02 7563	DAG putative plastid developmental protein	-
Gma.2313.1.S1 _s_at	Glyma10g 38760.1	0.02 7563	ATP sulfurylase 2 (APS3)	2.7.7. 4
GmaAffx.50338 .1.S1_at	Glyma10g 39450.1	0.02 7563	haloacid dehalogenase-like hydrolase family protein	-
Gma.5451.1.S1 _at	Glyma02g 15190.1	0.02 7563	RNA-binding protein 47B	-
* percent of Illumina® tags for the 1,000 Affymetrix® probe sets				



### 3.4.2 Time point analyses identify genes that pertain to defense

Experiments were then designed to determine gene expression that is common to *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> syncytia as they undergo their respective defense responses. Experimental data are presented here only from genes that are present in all replicates for a particular cell type of both genotypes (*G. max*<sub>[Peking/PI 548402 + PI 88788]</sub>) undergoing their defense responses (**Fig. 3.4**). The analyses of the 3 dpi time point demonstrate that *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> syncytia undergo gene expression that is different from both pericycle and the surrounding cells as well as syncytia undergoing the susceptible reaction (**Fig. 3.4.a; Supplemental table 8**). The analyses were followed by examining expression occurring at 6 dpi (**Fig. 3.4.b; Supplemental table 9**) and 9 dpi (**Fig. 3.4.c; Supplemental table 10**). Combining data from the 3, 6 and 9 dpi time points demonstrate a core set of constitutively and perhaps uniquely active genes in *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> syncytia undergoing their defense responses (**Fig. 3.4.d; Supplemental table 11**). Analyses presented in Figure 4d show that 1,787 probe sets, representing ~5% of the total array, measure expression specifically in *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> syncytia throughout defense.

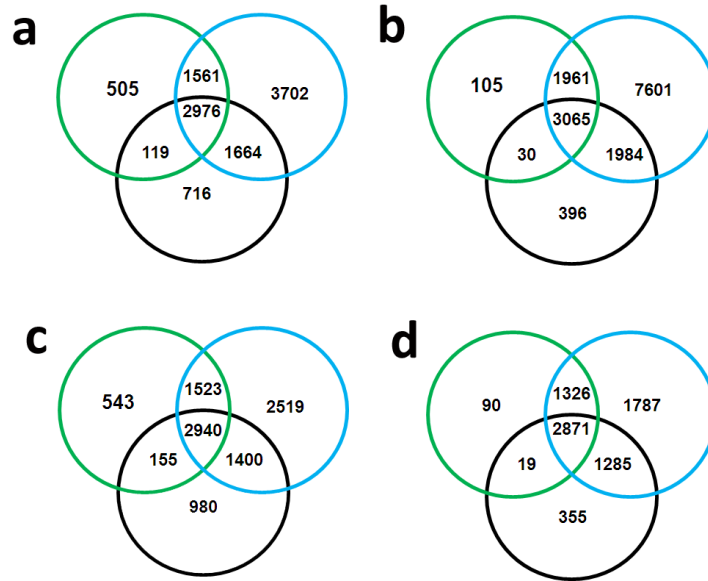


Figure 3.4 Time point analyses

Represented by Venn diagrams depicting the four comparative analyses made between the *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> pericycle and surrounding cells, the *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> defense response and the *G. max*<sub>[Peking/PI 548402]</sub> susceptible reaction. The green ring represents the *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> syncytium and surrounding cells pool of genes. The light blue ring represents the *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> syncytium defense response pool of genes. The black ring represents the *G. max*<sub>[Peking/PI 548402]</sub> susceptible syncytium pool of genes during a time course of infection. **Fig. 3.4.a**, Comparative analysis of 3 dpi *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> resistant reaction to *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> pericycle and surrounding cells and *G. max*<sub>[Peking/PI 548402]</sub> susceptible reaction sample pool of genes. **Fig. 3.4.b**, Comparative analysis of 6 dpi *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> defense response to *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> pericycle and surrounding cells and *G. max*<sub>[Peking/PI 548402]</sub> susceptible reaction sample pool of genes. **Fig. 3.4.c**, Comparative analysis of 9 dpi *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> defense response to *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> pericycle and surrounding cells and *G. max*<sub>[Peking/PI 548402]</sub> susceptible reaction sample pool. **Fig. 3.4.d**, Comparative analysis of 3, 6 and 9 dpi *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> defense response to *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> pericycle and surrounding cells and *G. max*<sub>[Peking/PI 548402]</sub> susceptible reaction sample pool.

### 3.4.3 Intergenotype PAICE analyses reveal genotype-specific metabolic pathway activity

PAICE (Hosseini et al. unpublished) was developed to place the expressed genes into their metabolic context (**Table 3.2**).

Table 3.2 PAICE pathway count for the intergenotype analyses accompanying Figure 3.3.

COMPARISON	Peking	PI 88788	susceptible	common	TOTAL
<b>pericycle</b>	<b>45</b>	<b>133</b>	<b>N/A</b>	<b>121</b>	<b>299</b>
<b>3 dpi</b>	<b>38</b>	<b>99</b>	<b>15</b>	<b>90</b>	<b>242</b>
<b>6 dpi</b>	<b>50</b>	<b>48</b>	<b>13</b>	<b>94</b>	<b>205</b>
<b>9 dpi</b>	<b>33</b>	<b>92</b>	<b>24</b>	<b>90</b>	<b><u>239</u></b>
TOTAL					<b>985</b>

The pathways that are identified in the resistant reactions of *G. max*<sub>[Peking/PI 548402]</sub>, *G. max*<sub>[PI 88788]</sub>, the susceptible reaction and those found in all three cell types for pericycle (**Supplemental dataset 1**), 3 dpi (**Supplemental dataset 2**) 6 dpi (**Supplemental dataset 3**) and 9 dpi comparisons (**Supplemental dataset 4**) are provided. The analyses reveal that while commonalities exist in gene activity for hundreds of pathways between *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub>, specific alterations in expression exist that accompany the distinct forms of their genotype-defined defense responses (**Fig. 3.5**).

## Intergenotype PAICE analysis of sphingolipid metabolism

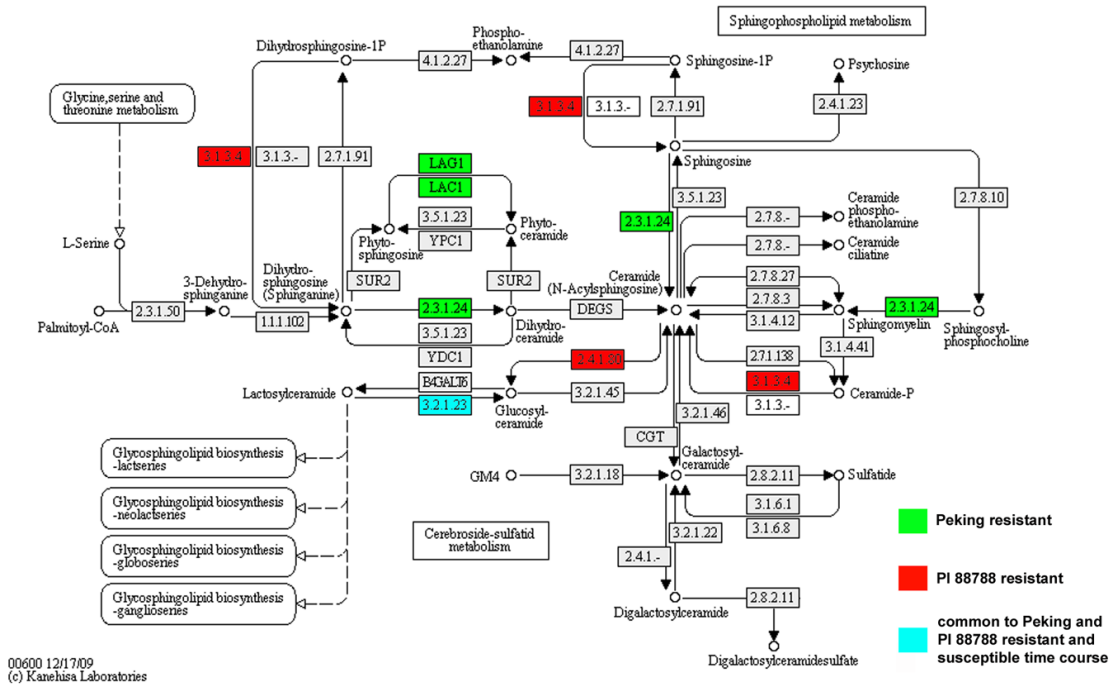


Figure 3.5 Intergenotype PAICE analysis of sphingolipid metabolism, map 00600

Probe sets detecting expression for Peking resistant reaction at 9 dpi (green); PI 88788 resistant reaction at 9 dpi (red); Peking resistant reaction at 9 dpi, PI 88788 resistant reaction at 9 dpi and the susceptible time course (blue). Note: no expression was found for sphingolipid metabolism that was limited only to the susceptible time course.

### 3.4.4 Time point PAICE pathway analyses identify syncytium-specific expression

PAICE was then used to analyze the pooled transcript data obtained from *G. max*<sub>[Peking/PI 548402 + PI 88788]</sub> syncytia undergoing their defense responses at 3, 6 and 9 dpi to both pericycle and their surrounding cells and syncytia undergoing the susceptible reaction.

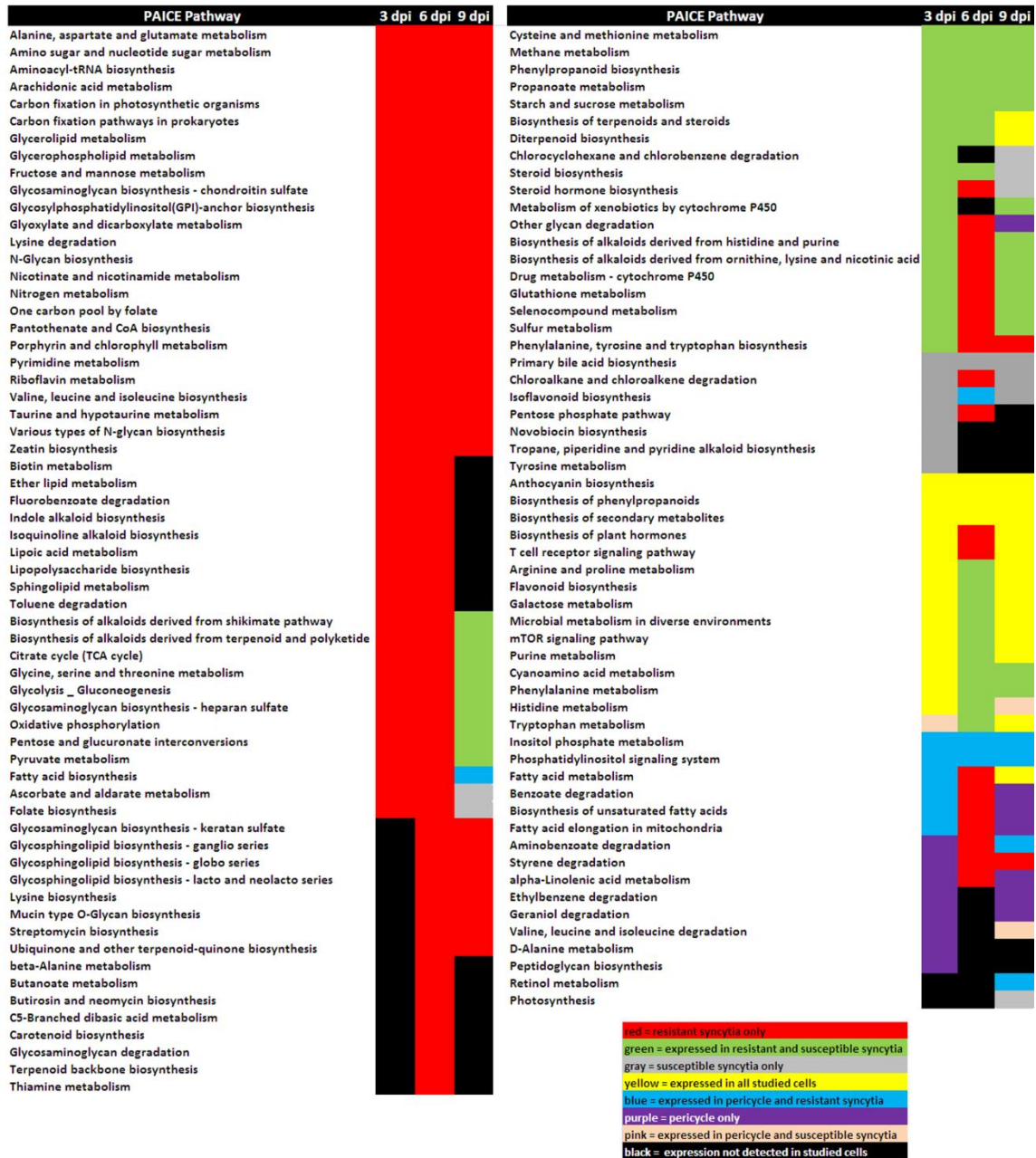


Figure 3.6 PAICE pathway analyses

Pathway activity is shown for 3, 6 and 9 dpi time points. red = resistant syncytia only, green = expressed in resistant and susceptible syncytia, gray = susceptible syncytia only, yellow = expressed in all cell types, blue = expressed in pericycle and resistant syncytia, purple = pericycle only, pink = expressed in pericycle and susceptible syncytia, black = expression not detected in sample.

The time point analyses resulted in the generation of a total of 658 PAICE pathways (**Table 3.3**). Removing duplicate pathways occurring in the different time points and cell types resulted in the identification of 119 pathways with gene expression activity (**Fig. 3.6**).

Table 3.3 PAICE pathway count for the time point analyses accompanying Figure 3.4.

COMPARISON	susceptible syncytia	resistant syncytia	pericycle	common	TOTAL
<b>3 dpi</b>	<b>41</b>	<b>86</b>	<b>29</b>	<b>69</b>	<b>225</b>
<b>6 dpi</b>	<b>22</b>	<b>108</b>	<b>6</b>	<b>71</b>	<b>207</b>
<b>9 dpi</b>	<b>49</b>	<b>78</b>	<b>28</b>	<b>71</b>	<b>226</b>
<b>TOTAL</b>					<b>658</b>

#### 3.4.5 Time point analyses identify genes found at the *rhg1* locus that are expressed in the syncytium during defense

The time point PAICE analyses are designed to visualize metabolic activity for all genes exhibiting expression. Gene activity at the *rhg1* locus can be measured by the 112 probe sets fabricated onto the array that represent the locus (**Supplemental table 12**). Expression studies show that 18 of the 112 probe sets spanning the *rhg1* locus are measuring expression in at least one sample at the studied time points (**Fig. 3.7**). The gene lists for the 3 dpi (**Supplemental dataset 5**) 6 dpi (**Supplemental dataset 6**) and 9 dpi time points (**Supplemental dataset 7**) are provided. In sum, two adjacent genes within the 67 kb *rhg1* region had expression only in syncytia undergoing defense and at all time points as revealed by the experimental conditions (**Fig. 3.7**).

## Gene expression occurring at the *rhg1* locus during the resistant reaction in both Peking and PI 88788

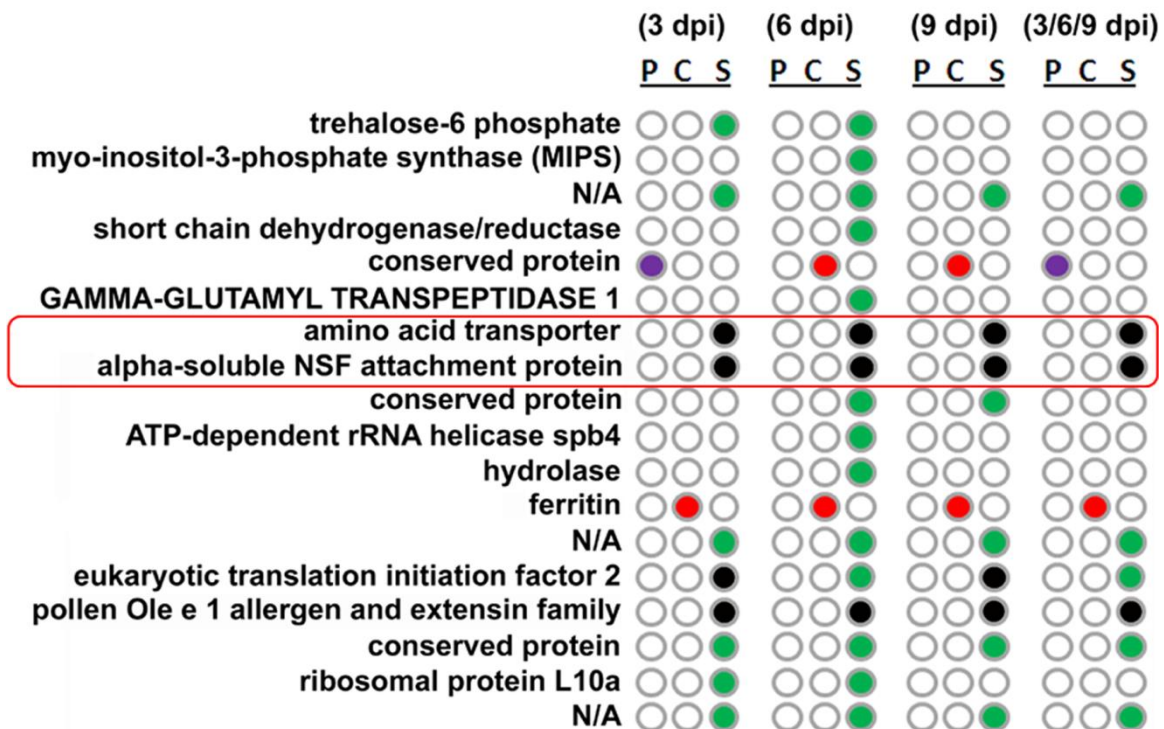


Figure 3.7 Time point analyses of the *rhg1* locus

Expression is presented in relation to map positions of Affymetrix® probe sets and genes at the locus with gene activity as demonstrated. The list represents probe sets that have chromosomal coordinates on chromosome 18 in the region of *rhg1* and also have expression data. P, pericycle and surrounding cells; C, common; S, syncytium. Purple, pericycle; red, expressed in pericycle and syncytium; green, syncytium; black, uniquely expressed in syncytia undergoing a defense response as compared to pericycle and syncytia undergoing a susceptible reaction. Duplicate probe sets having identical gene expression were consolidated. The red box represents the only genes within the 67 kb region (Kim et al. 2010) between the markers BARCSOYSSR\_18\_0090 and BARCSOYSSR\_18\_0094 that had any expression.

### 3.5 Discussion

An analysis of gene expression of soybean germplasm obtained originally from ecological collections was used to show how natural genetic variation is a useful tool in understanding defense at cellular resolution. The study generated a map of cell fate

decisions as soybean was undergoing infection leading to either a susceptible reaction or a successful defense response to SCN infection. The analysis presented here was accomplished by examining gene expression occurring at the site of infection, the syncytium. Notably, the genes focused on did not meet the statistical cut-off parameters in differential expression studies and were, therefore, discarded from further analysis (Klink et al. 2007, 2009). This outcome occurred because the probe sets measured expression in samples isolated from one cell type but lacked the measurement of expression in other cell types, making statistical comparisons impossible for differential expression studies. Thus work was built off the premise that cell-type specific expression is a hallmark of cellular identity, especially during specialized processes such as defense to pathogens. These analyses demonstrated that there is a basic conserved expression program in place that is likely to be common to all soybean genotypes undergoing defense to SCN (Klink et al. 2011). It is on this conserved gene expression platform that genotype-specific expression is organized and orchestrated during defense to SCN (Klink et al. 2011). This expression is what governs the different cellular features that are present during the *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> forms of the resistant reaction. Identifying differences in gene expression at the cellular level is not unexpected and is consistent with single cell type gene expression studies done in other experimental systems undergoing a developmental process (Benfey et al. 2003; Chiang and Melton, 2007; Guo et al. 2011; Tang et al. 2011).

### **3.5.1 Detection calls confirmed by Illumina® deep sequencing**

While the DCM provides a statistical output, it is difficult to determine what the relative quantities of a transcript are. It was revealed through Illumina® deep sequencing



that some of these genes can be represented by a fairly large percentage of the transcripts in a sample type. For example, the Affymetrix probe set Gma.3940.1.S1\_at, whose sequence is Glyma13g06450.1, is an unknown gene that does not appear to be conserved with other organisms. However, it represented over 17 percent of the transcripts in the 9 dpi *G. max*<sub>[PI 88788]</sub> sample isolated from cells undergoing resistance. The XYLOGLUCAN ENDOTTRANSGLYCOSYLASE 6 (XTR6) gene was represented by over 11% of the transcripts. The XTRs modify the plant cell wall xyloglucan-cellulose framework and by doing so, modulate strength and expansion. This activity appears to occur at the point of formation of secondary cell walls through restructuring of the primary cell walls (Bourquin et al. 2002). Very little is understood about XTRs and defense. However, the reinforcement of cell walls is a component of defense of soybean to SCN (Mahalingam and Skorpska, 1996). Another gene, BOTRYTIS-INDUCED KINASE1 (BIK1) that constituted almost 10 % of the Illumina®-identified transcripts was originally identified as a defense gene (Veronese et al. 2006). BIK1 is activated within minutes after infection of *Arabidopsis thaliana* by *Botrytis cinerea* (Veronese et al. 2006; Laluk et al, [in press]). BIK1 was shown to play essential roles in plant growth, ethylene signaling and pathogen activated molecular patterning (PAMP) during defense. The BIK1 protein localizes to cell membranes, and it was suggested that it may act early during the interaction between the plant and pathogen (Veronese et al. 2006). Of note, the defense requirement of BIK1 functions in relation to salicylic acid levels. The addition of the Illumina® sequencing thus places the Affymetrix® detection data into an expression context since relative amounts of transcripts that are present within a sample are obtained.

What is clear from the Illumina® experiments is the accuracy of the Affymetrix® detection calls.

### 3.5.2 Time point PAICE analyses

The time point PAICE analyses revealed gene activity that occurred specifically during the resistant reaction. These experiments demonstrated the activity of many pathways at 3 dpi, a period when the soybean alters its gene expression leading to visible signs of the defense response (Ross, 1958; Endo, 1964, 1965; Endo and Veech 1970; Gipson et al 1971; Jones and Northcote, 1972; Riggs et al. 1973; Kim et al. 1987; Kim and Riggs 1992; Mahalingam and Skorpska, 1996; Klink et al. 2007, 2009, 2010). However, the expression of a pathway in cells that will undergo defense was never limited to only the 3 dpi syncytium samples. In some cases gene expression was measured in syncytia undergoing defense for the earlier 3 and 6 dpi time points while lacking any expression at the 9 dpi time point. In these cases, the analyses revealed the expression of lipoic acid metabolism that is part of a nonenzymatic antioxidant system in plant cells (Pérez-López et al. 2010) and processes leading to lipid production (Baud et al. 2007). The pathways for indole alkaloid biosynthesis (Onkokesung et al. 2010; Hanssen et al. 2011) and isoquinoline alkaloid biosynthesis (Facchini et al. 1996; Holková et al. 2010), having roles in defense, were also observed to be active. The pathway involved in the biosynthesis of glycosphingolipids was observed to be active. Glycosphingolipids are a class of sphingolipids of which there are greater than 168 in *A. thaliana* (Markham and Jaworski, 2007) and perform roles in cell death. Other active pathways included the biosynthesis of alkaloids from the shikimate pathway (Zulak et al. 2008). The shikimate pathway provides metabolites for the production of

phenylpropanoids that are metabolized into substances that perform defense roles. The pathway involved in the biosynthesis of alkaloids from terpenoid and polyketides that are jasmonate regulated (Menke et al. 1999; Montiel et al. 2011) and fatty acid biosynthesis (Savchenko et al. 2010; Gao et al. 2011; Meldau et al. 2011) were also active.

Some pathways appeared to be active at all points during the defense response, including increased activity of components of the arachidonic acid pathway (Klink et al. 2011). The direct link of arachidonic acid metabolism to  $\alpha$ -linoleic acid metabolism, shown to be differentially expressed during defense to SCN (Klink et al. 2011) provides additional support to the metabolic pathway leading to the synthesis of methyl jasmonate possibly being involved in *G. max* defense to *H. glycines* (Klink et al. 2007, 2009, 2010a). Notably, genetic data in *Zea mays* has already linked the involvement of JA signaling during its defense of the plant parasitic nematode *Meloidogyne incognita* (Gao et al. 2008). Arachidonic acid functions in defense by triggering programmed cell death (Bostoc et al. 1981, 1986). Arachidonic acid metabolism is active through genes leading to the synthesis of hydroxyepoxyeicosadienoic acid and tetrahydrofuran diols. This observation is important because experiments in other plant-pathogen systems demonstrate that furans are an important component of plant defense responses, working efficiently on inhibiting larva development of insects (Rodriguez-Saona et al. 2000). Other gene pathways that are active at the 3, 6 and 9 dpi time points include N-glycan biosynthesis (reviewed in Pattison and Amtmann, 2009) and nicotinate and nicotinamide metabolism (Steppuhn et al. 2004) perform roles in defense. The analyses also identified glycerolipid metabolism (Kachroo et al. 2004; Chaturvedi et al. 2008; Xia et al. 2010),

glyoxylate and dicarboxylate metabolism (Emmerlich et al. 2003) and zeatin biosynthesis (Smigocki et al. 1993; Gális et al. 2004), pathways known to perform roles in defense.

### **3.5.3 Gene activity at the *rhg1* locus**

An expression mapping analysis focused in on *rhg1*. In the time point analyses, 18 probe sets representing 18 different genes, detected expression in one or more cell types. Of those 18 probe sets, only two map within the 67 kb *rhg1* region between the markers BARCSOYSSR\_18\_0090 and BARCSOYSSR\_18\_0094 (Kim et al. 2010). The identified amino acid transporter and the  $\alpha$ -SNAP probe sets measured expression at all of the time points (3, 6 and 9 dpi) throughout the defense response.  $\alpha$ -SNAP, through vacuolar sorting would be considered to play a role in defense involving autophagy (Liu et al. 2005; Hofius et al. 2009). In contrast, no clear role has been determined for the amino acid transporter and defense. The lack of detection for CBL-interacting protein kinase, a conserved unknown gene, a speckle-type POZ protein-related gene, a conserved unknown gene, a cys-rich domain protein gene, an elicitor inducible protein gene and an unknown gene in pericycle and their surrounding cell samples and samples isolated from syncytia undergoing defense or the susceptible reactions are noted. However, the expression presented here only reflects what was observed under our experimental conditions.

### **3.5.4 PAICE analyses link the cytological events pertaining to resistance to genes present at the *rhg1* locus**

From the studies that combined the expression data from the two genotypes, it appears that methyl jasmonate activity may be a part of a pathway that leads to transcriptional activation of genes involved in defense of soybean to the SCN. Previous

reports have shown JA activity to be important in the resistance of plants to parasitic nematodes (Gao et al. 2008), supporting the transcriptomic work in *G. max* (Klink et al. 2007, 2009, 2010a). While JA activates defense pathways to many pathogens, upstream events including arachidonic acid metabolism supply metabolic products through the  $\alpha$ -linoleic acid metabolic pathway that leads to the synthesis of 12-oxo-phytodienoic acid (OPDA) and methyl jasmonate. As already discussed, many pathways involved in lipid metabolism are observed to be expressed only in samples isolated from syncytia undergoing defense. Some of the downstream events, obvious through cytological examination of roots undergoing the defense response, include lignification and suberization that stain readily with safranin as demonstrated by the original studies of Ross (1958) and re-examined later (Klink et al. 2009, 2010a, 2011). The synthesis of lignin and suberin is mediated through the activity of the phenylpropanoid pathway and shown to be induced in syncytia undergoing defense (Klink et al. 2007, 2009). Phenylpropanoid metabolites are involved in defense, providing a physical barrier to infection. One of the earliest structural features identified as providing a barrier against infection by some pathogens are CWAs. CWAs are present during the defense of plants to fungi (Aist et al. 1976) and the plant parasitic nematode *H. glycines* in *G. max*<sub>[Peking]</sub> and *G. max*<sub>[PI 437654]</sub>, but not *G. max*<sub>[PI 88788]</sub> (Kim et al. 1987; Mahalingham and Skorupska 1996). The formation of CWAs is linked to the aggregation of subcellular components at the infection site, a process that is dependent on the polarization of actin at the site of infection. The induced transcriptional activity of actin is observed in syncytia undergoing defense in *G. max*<sub>[Peking/PI 548402]</sub> (Klink et al. 2007, 2009), a soybean genotype known to have CWAs. The work of Böhlenius et al. (2010) implicates vesicular transport

in CWA formation during infection of barley by *Blumeria graminis*. The experiments Böhlenius et al. (2010) relate increased production of phenylpropanoids to their delivery at localized sites in the cell at the cell wall. In related experiments, RNAi of the phenylpropanoid pathway components for monolignol biosynthesis that includes phenylalanine ammonia lyase (PAL), caffeic acid O-methyltransferase (CAOMT), caffeoyl-CoA methyltransferase (CCoAMT), and cinnamyl alcohol dehydrogenase (CAD), key components of lignin synthesis, results in super-susceptibility of wheat leaf tissues to an appropriate pathogen, *B. graminis* f. sp. tritici (Bgt) (Bhuiyan et al. 2009). All of these components have been shown induced during the defense response (Klink et al. 2007, 2009). The RNAi treatment also resulted in compromised penetration defense to a non-appropriate pathogen, *B. graminis* f. sp. Hordei (Bhuiyan et al. 2009). These observations are not surprising since CWAs are composed of materials such as lignin, pectin, suberin and chitin that are synthesized through the phenylpropanoid pathway. It is suggested that the methyl units synthesized through S-adenosylmethionine synthetase activity in the epidermal cells at the site of infection are metabolized into CWAs (Bhuiyan et al. 2007). A link between the synthesis of the CWA component lignin and methyl units has been made. *Arabidopsis thaliana* S-methionine synthetase mutants, although appearing identical to wild type, have a 22% decrease in lignin (Shen et al. 2002). The enzyme S-methionine synthetase is found to be highly induced in syncytia undergoing a defense response in *G. max*<sub>[Peking/PI 548402]</sub> (Klink et al. 2009). Other proteins known to compose CWAs include hydroxyproline-rich glycoproteins (HRGPs) and peroxidases. The analysis of the *rhg1* region identified an extensin protein that is expressed specifically at all stages of the defense response. However, the extensin lies

outside of the 67 kb *rhg1* region as defined by Kim et al. (2010). The synthesis, deposition and assembly of extensin appear to be accompanied by localized release of reactive oxygen species (ROS) including H<sub>2</sub>O<sub>2</sub>. The release of H<sub>2</sub>O<sub>2</sub> can drive the cross-linking of proteins like extensin, directly intoxicate the pathogen and/or drive the defense response in neighboring cells (Aist, 1976; Bradley et al. 1992; Levine et al. 1994; McLusky et al. 1999; Hueckelhoven et al. 1999; Mellersh et al. 2002). The activity of genes involved in H<sub>2</sub>O<sub>2</sub> production is observed in syncytia undergoing a defense response (Klink et al. 2007). Many of these observations suggest altered cell wall composition being important in defense.

### **3.6 Conclusion**

The experiments show that a vast amount of relevant gene expression data, typically representing between 5-20% of the genes in the soybean genome, is discarded from cell-type specific differential expression studies. Analyzing biological processes occurring in homogeneous cell types will require a modified approach that can examine the unique gene expression profiles of the different cell types and different genotypes. As shown by the Illumina® deep sequencing data, the Affymetrix® DCM profiling is accurate and should be used as an additional complementary measure of gene activity during any biological process under study. The advantage of the quantitative Illumina® methodology, in the absence of differential expression knowledge, is that relative expression of these genes can be obtained, providing a measure of the activity of the gene in a specific cell type. The ability to map the expression to resistance loci in a genotype-dependent manner should allow for a better understanding of expression nuances that

define the cellular strategies employed by the different genotypes as well as generalized expression features as they combat SCN.

### **3.7 Supplemental Material**

The supplementary material of this chapter can be found in the online version of this article (<http://dx.doi.org/10.1007/s11103-011-9828-3>).



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CHAPTER IV  
THE EXPRESSION OF A NATURALLY OCCURRING, TRUNCATED ALLELE OF  
AN  $\alpha$ -SNAP GENE SUPPRESSES PLANT PARASITIC  
NEMATODE INFECTION<sup>3</sup>

**4.1 Abstract**

Transcriptional mapping experiments of the major soybean cyst nematode resistance locus, *rhg1*, identified expression of the vesicular transport machinery component,  $\alpha$ -soluble NSF attachment protein ( $\alpha$ -SNAP), occurring during defense. Sequencing the  $\alpha$ -SNAP coding regions from the resistant genotypes *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 437654]</sub> revealed they are identical, but differ from the susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> by the presence of several single nucleotide polymorphisms (SNPs). Using *G. max*<sub>[Williams 82/PI 518671]</sub> as a reference, a G $\rightarrow$ T<sup>2,822</sup> transversion in the genomic DNA sequence at a functional splice site of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele produced an additional 17 nucleotides of mRNA sequence that contains an in-frame stop codon caused by a downstream G $\rightarrow$ A<sup>2,832</sup> transition. The *G. max*<sub>[Peking/PI 548402]</sub> genotype has cell wall

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<sup>3</sup> Most of the content of this chapter has been adapted from the journal article: Matsye PD, Lawrence GW, Youssef RM, Kim K-H, Matthews BF, Klink VP. 2012. The expression of a naturally occurring, truncated allele of an  $\alpha$ -SNAP gene suppresses plant parasitic nematode infection. *Plant Molecular Biology* 80(2):131-55

appositions (CWAs), structures identified as forming as part of a defense response by the activity of the vesicular transport machinery. In contrast, the 17 nt  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> mRNA motif is not found in *G. max*<sup>[PI 88788]</sup> that exhibits defense to *H. glycines*, but lack CWAs. The  $\alpha$ -SNAP<sup>[PI 88788]</sup> promoter contains sequence elements that are nearly identical to the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele, but differs from the *G. max*<sup>[Williams 82/PI 518671]</sup> ortholog. Overexpressing the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele in the susceptible *G. max*<sup>[Williams 82/PI 518671]</sup> genotype suppressed *H. glycines* infection. The experiments indicate a role for the vesicular transport machinery during infection of soybean by the soybean cyst nematode. However, increased *GmEREBP1*, *PR1*, *PR2*, *PR5* gene activity but suppressed *PR3* expression accompanied the overexpression of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele prior to infection.

## 4.2 Introduction

A number of plant parasitic nematodes establish a nurse cell that acts as their niche during parasitism. One of the most important is the soybean cyst nematode (SCN), *Heterodera glycines*, a registered invasive species in the U.S. that is responsible for approximately 1.5 billion dollars in agronomic losses world-wide, annually (Wrather et al. 2001). The SCN accomplishes its devastating parasitic interaction with soybean by burrowing into the root and subsequently initiating the formation a multinucleate nurse cell known as a syncytium (Ross, 1958; Endo, 1964). This process is coordinated, likely occurring through the activity of nematode parasitism proteins orchestrating successive waves of cell wall dissolving events (Atkinson and Harris, 1989; Smant et al. 1999; Lambert et al. 1999; De Boer et al. 1999, 2002; Bekal et al. 2003). The process merges 200-250 cells (Jones and Northcote, 1972; Jones, 1981). Additional activities elicited by

the nematode alter the plant cell's physiology, benefiting the nematode during the sedentary period of its life cycle (Edens et al. 1995; Hermsmeier et al. 1998; Mahalingam et al. 1999; Vaghchhipawala et al. 2001; Klink et al. 2007).

Natural resistance to SCN has been identified through the partial screening of the USDA soybean seed bank containing approximately 20,000 publically available plant introductions (PIs) (Ross and Brim, 1957; Ross, 1958; Epps and Hartwig, 1972). From those screens the *G. max* PIs known as Peking (*G. max*<sub>[Peking]</sub>) and *G. max*<sub>[PI 88788]</sub>, whose resistance germplasm now is present in >97% of all commercial cultivars in the U.S. (Concibido et al. 2004), were identified. Hundreds of additional accessions that can resist SCN infection have been identified in China (Ma et al. 2006; Li et al. 2011). These banks of germplasm provide an important and substantial genetic resource for understanding the process of parasitism in soybean at the cellular level.

A number of cytological studies have shown that the cellular response of soybean to SCN can be divided into an earlier phase (phase 1) and a later phase (phase 2) (Ross, 1958; Endo, 1964, 1965, 1991; Riggs et al. 1973; Kim et al. 1987; Mahalingam and Skorpska, 1996). As judged by cytology, the steps in the parasitism process and thus the underlying molecular events may exhibit some level of conservation because similar observations have been made by Robinson et al. (1997) for the nematode *Rotylechulus reniformis*. During phase 1, the cellular reactions leading to susceptibility or defense appear the same and include the dissolution of cell walls, hypertrophy, an enlargement of nuclei, the development of dense cytoplasm and an increase in ER and ribosome content (Endo, 1964, 1965; Riggs et al. 1973; Kim et al. 1987; Kim and Riggs, 1992; Mahalingam and Skorpska, 1996). During phase 2, the susceptible reaction and defense

responses appear different. Phase 2 of the susceptible reaction is characterized by hypertrophy of nuclei and nucleoli. This process is accompanied by the proliferation of cytoplasmic organelles, reduction and dissolution of the vacuole and cell expansion as it incorporates and fuses with adjacent cells (Endo and Veech 1970; Gipson et al 1971; Jones and Northcote, 1972; Riggs et al. 1973; Jones, 1981). The cellular aspects of the defense responses occurring during phase 2 vary and are dependent on the soybean genotype. Due to the cellular characteristics associated with how SCN responds during resistance, the PIs have been categorized into those genotypes having the *G. max*<sub>[Peking]</sub> and *G. max*<sub>[PI 88788]</sub>-types of defense responses (Colgrove and Niblack, 2008). Among these characteristics, the *G. max*<sub>[Peking]</sub>-type of defense includes the development of a necrotic layer that surrounds the head of the nematode (Kim et al. 1987; Endo, 1991). In contrast, in the *G. max*<sub>[PI 88788]</sub>-type of defense response, the necrotic layer that surrounds the head of the nematode is lacking (Kim et al. 1987; Endo, 1991).

Another salient feature of defense is the presence of cell wall appositions (CWAs) that develop during the *G. max*<sub>[Peking]</sub>-type of resistant reaction. CWAs are structures defined as physical and chemical barriers to cell penetration (Aist et al. 1976, Schmelzer, 2002; An et al. 2006a, b; Hardham et al. 2008). CWAs are also observed in the *G. max*<sub>[PI 437654]</sub> genotype (Mahalingam and Skorpska, 1996), making its placement in the *G. max*<sub>[Peking]</sub> cohort logical (Colgrove and Niblack et al. 2008). As demonstrated by Collins et al. (2003), Assaad et al. (2004) and Kalde et al. (2007), CWA formation involves the vesicular transport machinery component syntaxin. Syntaxin was first identified in animal systems (Inoue et al. 1992; Bennett et al. 1992). While the role of syntaxin in plant defense has been studied, the examination of other components of the vesicular

transport machinery such as the soluble N-ethylmaleimide-sensitive factor attachment protein  $\alpha$ -SNAP) (Weidman et al. 1989; Clary et al, 1990; Collins et al. 2003; Assaad et al. 2004; Kalde et al. 2007), the ATPase known as N-ethylmaleimide-sensitive factor (NSF) (Malhotra et al. 1988), the soluble N-ethylmaleimide-sensitive factor attachment receptor protein (SNARE) complex and synaptosomal-associated protein 25 (SNAP25) (Oyler et al. 1989) among other proteins have not. From this knowledge, specific components of the CWA assembly process that are present during defense of soybean to SCN can be inferred. This knowledge then allows for a targeted approach in understanding the protein machinery that may be involved in defense.

Identifying gene expression that pertains to defense, locally at the site of infection, has aided a targeted approach in understanding the cellular process. The original study that physically isolated syncytia for molecular studies in soybean was performed in the susceptible genotype *G. max*<sub>[Kent/PI 548586]</sub> using a procedure known as laser microdissection (Klink et al. 2005). To study nurse cell metabolism occurring during susceptibility and defense, syncytia were collected from *G. max*<sub>[Peking/PI 548402]</sub> (Klink et al. 2007, 2009a) and *G. max*<sub>[PI 88788]</sub> (Klink et al. 2010a). These genotypes have functional defense genes. Susceptible reactions have also been obtained in these studies because SCN races (Golden et al. 1970) now further classified as populations (Niblack et al. 2002) are available that can accomplish a susceptible reaction in genotypes with functional defense genes. These studies were complimented by Ithal et al. (2007) who investigated syncytium development in the susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> genotype that lacks a functional defense response and Kandoth et al. (2011) that compared two different genotypes having or lacking the *G. max*<sub>[PI 209332]</sub> *rhg1* resistance background.

The mRNA was extracted from the collected cells and gene expression was studied by the Affymetrix® microarray technology (Klink et al. 2007, 2009a, 2010a, b, 2011a, b; Ithal et al. 2007; Matsye et al. 2011; Kandoth et al. 2011) and confirmed by Illumina® deep sequencing (Matsye et al. 2011). The analyses were complimented by custom transcriptional mapping experiments and gene pathway studies (Klink et al. 2009a, 2010a, b, 2011a, b; Matsye et al. 2011).

Matsye et al. (2011) performed a focused analysis of gene expression at the major SCN resistance locus, *rhg1*, first identified by Caldwell et al. (1960). That work was done because *rhg1* had been fine mapped to a region defined in a span of approximately 611,794 nucleotides between the molecular markers ss107914244 and Satt038 on chromosome 18 (Concibido et al. 1994; Mudge et al, 1997; Cregan et al. 1999a; Hyten et al. 2010). It is noted, however, that allelic variants of *rhg1* exist between the different soybean genotypes (Cregan et al. 1999b; Brucker et al. 2005). Due to the variation in how soybean responds to infection by the SCN, the *rhg1* resistance allele in *G. max*<sub>[PI 88788]</sub> is designated *rhg1-b* (Kim et al. 2010). Kim et al. (2010) have since fine-mapped the *rhg1-b* locus down to a region of approximately 67 kb. However, work in understanding the biological nature of the genes within the locus was not the focus of the study. In complimentary studies it was shown that two genes within the newly defined *rhg1* locus, an amino acid transporter (AAT) (Glyma18g02580) and an  $\alpha$ -soluble NSF attachment protein ( $\alpha$ -SNAP) (Glyma18g02590), undergo expression specifically in syncytia undergoing defense in both the *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> genotypes (Matsye et al. 2011). Furthermore, AAT and  $\alpha$ -SNAP appear to be expressed throughout the defense response in experiments that sampled time points at 3, 6 and 9 days post

infection (dpi) that span phase 1 and 2 (Matsye et al. 2011). In contrast, the AAT and  $\alpha$ -SNAP genes did not appear to be expressed during the susceptible reaction. Functional experiments were beyond the scope of the analysis. Resequencing the  $\alpha$ -SNAP (Glyma18g02590) cDNAs in the *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[Williams 82/PI 518671]</sub> genotypes revealed structural variations that merited further investigation in functional tests. The analysis presented here functionally characterizes the  $\alpha$ -SNAP allele found in the *G. max*<sub>[Peking/PI 548402]</sub> genotype ( $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup>).

### 4.3 Materials and Methods

#### 4.3.1 pRAP15 plasmid construction for overexpression studies

The pRAP15 vector (13,796 nucleotides [nt] in length) (**Fig. 4.1**) is identical in backbone to the previously published pRAP17 vector that was designed for RNAi studies (Klink et al. 2009b). The pRAP15 vector differs from the pRAP17 vector by having a single Gateway® (Invitrogen, Carlsbad, CA)-compatible *attR1-ccdB-attR2* cassette whose expression is driven by the figwort mosaic virus sub-genomic transcript (FMV-*sgt*) promoter (Bhattacharyya et al. 2002). The cassette is designed to drive the overexpression of full length genes. Prior studies have shown that the FMV-*sgt* promoter in the pKSF3 vector backbone exhibits strong, constitutive root overexpression throughout the entire course of *H. glycines* infection (Klink et al. 2008, 2009b). The pRAP15 vector was developed for *Agrobacterium rhizogenes*-mediated root genetic engineering experiments (Tepfer 1984).



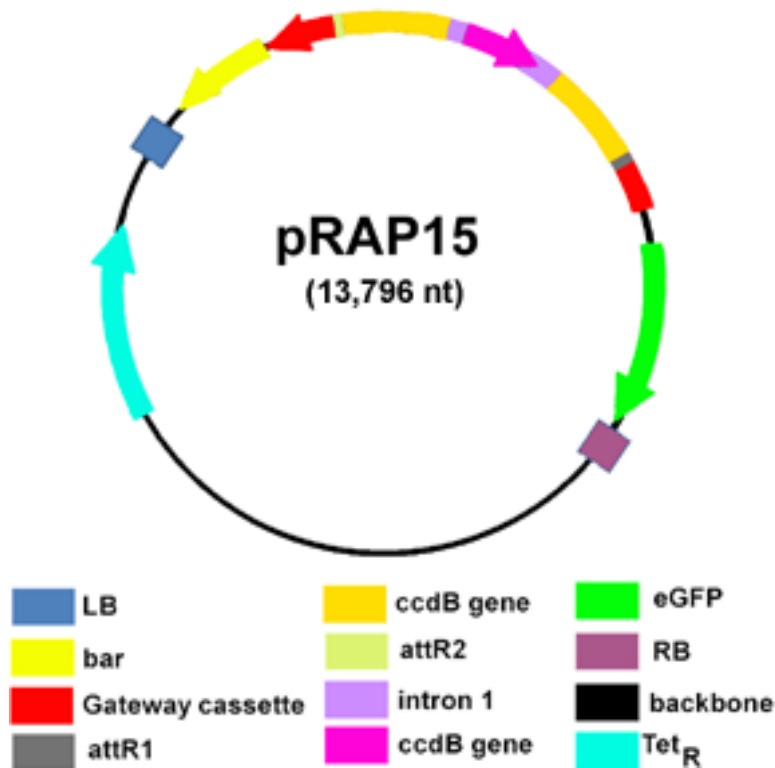


Figure 4.1 The pRAP15 vector

Legend, Tet<sub>R</sub>, tetracycline resistance gene (red); LB, left border; bar, Basta® resistance gene (yellow); t35S, 35S terminator; ccdB, lethality gene (Bernard and Couturier, 1991; Salmon et al. 1994); CmR, chloramphenicol resistance gene (red); intron 1; FMV sgt, figwort mosaic virus sub-genomic transcript promoter (blue); eGFP, enhanced green fluorescent protein cassette containing the *roLD* promoter and the 35S terminator (green); RB, right border; attR1, LR bacteriophage  $\lambda$ -derived recombination site #1; attR2, LR bacteriophage  $\lambda$ -derived recombination site #2.

The Gateway®-compatible pRAP15 destination vector was engineered specifically for research involving the infection of *G. max* by *H. glycines* (SCN). The pRAP15 vector was engineered from pH7GWIWG2(II) (Functional Genomics Division of the Department of Plant Systems Biology [VIB, the Flanders Institute for Biotechnology, Ghent University]). The information required for engineering pRAP15 from the pH7GWIWG2(II) backbone was derived from Klink et al. (2009b). The pRAP15 vector has the tetracycline resistance gene (Tet<sub>R</sub>) for bacterial selection

engineered into a *BstEII* site that lies outside the left and right borders. The pRAP15 vector contains an enhanced green fluorescent protein (eGFP) (Haseloff et al. 1997) gene driven by the *roID* root promoter (White et al. 1985; Elmayan and Tepfer 1995). The eGFP gene product is a visual beacon for screening transformed roots in non-axenic conditions (Collier et al. 2006) and works particularly well for studying *H. glycines* infection of *G. max* (Klink et al. 2008, 2009b; Ibrahim et al. 2011). The eGFP cassette was ligated into a *HindIII* site of pRAP15. The Gateway® compatible *attR1-ccdB-attR2* cassette was engineered into the pRAP15 vector between *SpeI* (5') and *XbaI* (3') sites. The inserted gene cassette is terminated by the cauliflower mosaic virus 35S terminator. The *attR* sites are LR bacteriophage  $\lambda$ -derived recombination sites. The *ccdB* gene (Bernard and Couturier 1991) is one selective agent for *E. coli* selection. The *attR* cassette is interrupted by a *ccdB* selectable marker gene that acts as an intron. Thus, engineering *G. max* genes into pRAP15 would result in a gene positioned in the correct orientation for overexpression.

#### 4.3.2 cDNA construction

RNA was extracted from *G. max* roots using the UltraClean® Plant RNA Isolation Kit (Mo Bio Laboratories®, Inc.; Carlsbad, CA) and treated with DNase I to remove genomic DNA. The cDNA was reversed transcribed from RNA using SuperScript First Strand Synthesis System for RT-PCR (Invitrogen®) with oligo d(T) as the primer according to protocol (Invitrogen®). Genomic DNA contamination was assessed by PCR by using beta-conglycinin primer pair (**Supplemental Table 1**) that amplify across an intron, thus yielding different sized DNA fragments based on the presence/absence of that intron. PCR reactions containing no template and reactions

using RNA processed in parallel but with no Superscript® reverse transcriptase also served as controls and produced no amplicon, proving no contaminating genomic DNA existed in the cDNA. PCR amplification of targeted genes was done using high fidelity Platinum® *taq* according to protocol (Invitrogen®). DNA for the PCR was dissociated for 10 min at 96° C, followed by PCR cycling and temperatures set for denaturation for 30 sec at 96° C, annealing for 60 sec at 55° C and extension for 30 sec at 72° C.

### 4.3.3 Vector construct pipeline

A vector construct pipeline based on Klink et al. (2009b) was designed for the study. For overexpression experiments using the pRAP15 vector, PCR primer pairs were designed to amplify  $\alpha$ -SNAP allele from the *G. max*<sub>[Peking/PI 548402]</sub> genotype ( $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup>) that has a functional defense response. Amplicons from the PCR reactions were gel purified in 1.0% agarose using the Qiagen® gel purification kit. The gel-purified amplicons were ligated into the directional pENTR/D-TOPO® vector and transformed into chemically competent *E. coli* strain One Shot TOP10 and selected on LB-kanamycin (50  $\mu$ g/ml) according to protocol (Invitrogen®). Colony PCR was used to confirm the presence of amplicons. Plasmids having amplicons were sequenced. DNA sequencing was used to identify amplicons having matches to their original Genbank accession. To generate the genetic engineering construct, the *G. max* amplicon that is in the pENTR/D-TOPO entry vector was shuttled into the pRAP15 destination vector by a LR clonase reaction according to protocol (Invitrogen®). The LR reaction contents were used in bacterial transformation experiments into the chemically competent One Shot TOP10 *E. coli* strain (Invitrogen®) and selected on LB-tetracycline (5  $\mu$ g/ml) according to protocol (Invitrogen®). Colony PCR was used to confirm the presence of the  $\alpha$ -

SNAP<sup>[Peking/PI 548402]</sup> allele. The pRAP15 vector, engineered with the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele was transformed into chemically competent *A. rhizogenes* strain K599 (K599) (Haas et al. 1995), a generous gift from Dr. Walter Ream, University of Oregon. The K599 transformations were performed using the freeze-thaw method (Hofgen and Willmitzer 1988). Selection was performed on LB-tetracycline (5  $\mu$ g/ml) according to Klink et al. (2008). Colony PCR was used to confirm the presence of the  $\alpha$ -SNAP and eGFP. The cloned genes were sequenced to determine that the full length gene was present and of correct in-frame sequence. The K599 colonies used for soybean transformation were tested for their root-inducing ability through partial colony PCR using primers designed against the root inducing (Ri) plasmid (**Supplemental Table 1**).

#### **4.3.4 Agrobacterium rhizogenes-mediated non-axenic transformation of *G. max***

A modified version of the non-axenic *G. max* transformation procedure (Klink et al. 2008, 2009b), originally performed by Tepfer (1984), was used for the experiments. Seeds of *G. max*<sup>[Williams 82/PI 518671]</sup> and *G. max*<sup>[Peking/PI 548402]</sup> were planted in pre-wetted sterilized sand, germinated and grown for 6 days at ambient greenhouse temperatures (~26-29°C). The plants were cut at the hypocotyl with a freshly unwrapped, clean and sterile razor in a Petri plate with the transformed *A. rhizogenes* containing the K599 (transformed with various constructs in the pRAP15 vector). The procedure ensured that bacterial infection occurred at the exact moment the plant was injured. The rootless plants (25 plants per beaker) were placed in 50 ml beakers containing K599 cultured in Murashige and Skoog (MS) media (Murashige and Skoog 1962), including vitamins (Duchefa Biochemie; The Netherlands) and 3.0 % sucrose, pH 5.7 (MS media). *G. max* underwent vacuum infiltration for 30 minutes. The vacuum then was removed slowly,

allowing the bacterial suspension to infiltrate the tissue. Cocultivation was performed overnight in MS media in a covered 67.3 X 40.1 X 26.4 cm plastic container (Rubbermaid® Revelations®; Rubbermaid Home Products; Fairlawn, OH) on a rotary shaker at 28° C. After an overnight cocultivation, the cut ends of *G. max* were placed individually 3-4 cm deep into fresh coarse vermiculite (The Schundler Company; Edison, NJ) in 50-cell flats. The plants were uncovered and incubated at 28° C for 1.5 days in an incubator (Heraeus Model B 6760, Thermo Electron Co.; Langenselbold, Germany) without light. The plants then were covered and grown at a distance of 20 cm from standard fluorescent cool white 4100K, 32 watt bulbs emitting 2,800 lumens (Sylvania®; Danvers, MA) for 5 days at ambient lab temperatures (~22° C). The plants then were uncovered and transferred to the greenhouse. The eGFP-expressing root primordia were usually evident 5 days after planting, demonstrating that the procedure was successful. The preparation of roots for the SCN infection studies began 17 days after cocultivation. Roots were identified by carefully dislodging the plant and root ball from its pot and inspecting them for the expression of eGFP. The eGFP expression was determined using the Dark Reader Spot Lamp (Clare Chemical Research; Dolores, CO). The remaining vermiculite was removed from these plants by washing the root ball in distilled, deionized water. The easily identified untransformed roots, evident by the lack of fluorescence, were excised from the plants. The resulting plants were chimeras (having transformed roots and untransformed aerial stocks). The chimeras were planted in a sterilized 50-50 mixture of a Freestone fine sandy loam (46.25 % sand, 46.50 % silt, and 7.25 % clay) and a sandy (93.00 % sand, 5.75 % silt, and 1.25 % clay) soil and allowed to recover for a week.

#### 4.3.5 Testing pRAP15 vector functionality

The functionality of the FMV-*sgt* promoter for overexpression experiments was first tested by PCR. For these experiments, cDNA was synthesized as described previously from uninfected and *H. glycines* infected *G. max* roots. PCR was performed using primers directed toward eGFP (**Supplemental Table 1**). The *ccdB* gene that is harbored within pRAP15 would be transcribed and translated, thus functioning as a negative control (Klink et al. 2009b). Overexpression of the constructs was then evaluated by quantitative real-time PCR (qPCR) (**Supplemental Table 1**). For the analysis, qPCR Taqman® 6-carboxyfluorescein (6-FAM) probes (MWG Operon; Birmingham, AL) were used. The 6-FAM probes have a maximum excitation at 495 nm and maximum emission at 520 nm. The quencher used in the qPCR reactions was the Black Hole Quencher (BHQ1) (MWG Operon), with maximum excitation at 534 nm. The qPCR conditions were a preincubation of 50° C for 2 min, followed by 95° C for 10 min. This was followed by alternating 95° C for 15 sec followed by 60° C for 1 min for 40 cycles. Assays were conducted for primers that produced a single amplicon. The qPCR reaction conditions included a 20 µl Taqman Gene Expression Master Mix (Applied Biosystems; Foster City, CA), 0.9 µl of µM forward primer, 0.9 µl of 100 µM reverse primer, 2 µl of 2.5 µM 6-FAM (MWG Operon®) probe and 4.4 µl of template DNA. The qPCR reactions were performed on an ABI 7300 (Applied Biosystems®). The qPCR differential expression tests were performed according to Livak and Schmittgen (2001).

The ability of the plant expression vector pRAP15 to overexpress protein was examined using the red fluorescent protein (RFP) as a reporter in *Allium cepa* (onion) bulb scale epidermal cells following the biolistic-mediated transformation method (Sheen

et al. 1995). The outer epidermal layer was peeled and the exposed surface was sterilized with 70% ETOH for 15 minutes and rinsed three times in sterile distilled water under sterile conditions inside a laminar flow hood. After conclusion of the surface sterilization procedure, the onion cells were placed in a Petri dish containing solid MS media containing 8 g/L agar. Two plasmid DNA constructs were purified from *Escherichia coli* cultures using the Miniprep kit (QIAGEN; Valencia, CA). The pRAP15 plasmid contains the gene encoding eGFP under control of *ro1D* promoter and has the *ccdB* gene within the Gateway® *attR* sites. The second construct, pRAP15 + RFP, contains the gene encoding RFP within the *attR* sites. Onion cells were transformed using the PDS-1000/He system (BioRad; Richmond, CA) which is a helium-driven particle accelerator with flying membrane for particle delivery that delivers 0.60 µm gold particles. Gold particles were coated with plasmid DNA according to the method of (Sanford et al. 1993). Briefly, 60 mg of gold was placed in Treff microtubes. The gold was vortexed and then soaked in 1 ml of 70% ETOH at room temperature for 10 min. Then the gold-coated particles were collected by centrifugation at 15,000 rpm for 15 min. The ETOH was decanted and the gold was washed 3 times with sterile, distilled H<sub>2</sub>O. Subsequently, 1,000 µl of 50% glycerol solution was added. A mixture of 30 µl of gold particles, 6 µl of DNA, 30 µl of 2.5 M CaCl<sub>2</sub> and 12 µl of 100 mM spermidine was used for bombardment. The gene gun was set to a 1 cm gap and 1 cm flying membrane distance. The target distance was 12 cm and onion cells were bombarded at 1,300 psi. After bombardment, the plates were kept in the dark in 22° C for 24 hr. The transformed roots were identified using a Zeiss 710 Confocal Laser Microscope (CFM) with filters for GFP, RFP and GFP + RFP.

Protein expression in *G. max* was tested by  $\beta$ -glucuronidase (GUS) in pRAP15 engineered with the *uidA* reporter gene (Klink et al. 2008; 2009b). A gene fusion between the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele and the *uidA* reporter gene was made to show the protein overexpression according to prior methodologies (Abel and Theologis, 1994; Sakamoto et al. 2009). GUS activity was revealed with the GUS stain (2 mM 5-bromo-4-chloro-3-indolyl glucuronide, 100 mM potassium phosphate buffer pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100) (Jefferson et al. 1987). The colorimetric reaction proceeded by immersion in GUS stain and subsequent vacuum infiltration with 500  $\mu$ l of GUS stain for 1 hour. Tissue was subsequently incubated at 37° C overnight to promote development of the GUS stain reaction. These two validation experiments demonstrated activity of both the *roID* promoter (driving eGFP expression) and FMV-*sgt* (driving GUS expression) in pRAP15.

#### 4.3.6 Plant and nematode procurement

Female *H. glycines*<sub>[NLI-Rhg/HG-type 7/race 3]</sub> were purified by sucrose flotation (Jenkins, 1964; Matthews et al. 2003). During this procedure, the females were crushed gently with a rubber stopper in a 7.5 cm diameter, 250  $\mu$ m sieves to release the eggs. The eggs flowed through the sieves into a small plastic tray. Debris smaller than the eggs was removed by washing them in a 25  $\mu$ m mesh sieves. The eggs were placed in a small plastic tray with one cm of water. The tray was covered with plastic wrap and placed on a rotary shaker at 25 rpm. After three days, the pi-J2s were separated from unhatched eggs by running them through a 41  $\mu$ m mesh cloth. The pi-J2s were concentrated by centrifugation in an IEC clinical centrifuge for 30 seconds at 1,720 rpm to a final



optimized concentration of 2,000 pi-J2s/ml. One ml of nematodes at a concentration of 2,000 J2s/ml per root system (per plant) was used for the experiments. This represented the inoculum. The nematodes were introduced to the soil and allowed to infect roots for thirty days in the greenhouse. Confirmation of infection in representative infected root samples was performed by the acid fuchsin staining procedure of Byrd et al. (1983). Female indices (FI) were calculated (see below).

#### **4.3.7 Female index**

At the end of the experiment, the roots were checked for eGFP expression. This procedure was done after extraction of nematodes from the soil, but prior to quantifying the number of nematodes. Roots having sectors failing to exhibit eGFP expression were noted and those replicates were discarded from further analyses. The decision was made because transformed roots having sectors lacking eGFP expression likely were untransformed, either reverting to their original genetic background or caused by some developmental event and could skew the outcome of the experiments. Roots having sectors failing to exhibit eGFP expression were noted in less than 5% of the replicates.

Females were collected from individual plants by gently massaging the roots with the index finger and thumb, dislodging the nematodes (Klink et al. 2009b). This procedure was done over nested 20 and 100-mesh sieves. Additionally, the soil was washed several times and the rinse water sieved to assure collection of all females. Females present in ~30 ml of water were washed into 150 ml beakers. The females were then poured evenly into a Buchner funnel system, on a 9 cm diameter S & S #8 Ruled filter paper (Schleicher and Schuell; Keene, NH) under constant vacuum. The filters were

stored in standard disposable Petri plates, wrapped in parafilm and stored at 4°C. The females were counted immediately under a dissecting microscope after collection.

The FI was calculated according to the original work of Golden et al. (1970) that has been further modified (Riggs and Schmitt, 1988, 1991; Niblack et al. 2002; Klink et al. 2009b). The FI is calculated as  $FI = (N_x/N_s) \times 100$ , where  $N_x$  is the average number of females on the test cultivar and  $N_s$  is the average number of females on the standard susceptible cultivar. Statistical error is not calculated as a part of the FI (Golden et al. 1970; Riggs and Schmitt, 1988, 1991; Niblack et al. 2002). A total of 10 nontransformed *G. max*<sub>[Williams 82/PI 518671]</sub> or *G. max*<sub>[Peking/PI 548402]</sub> plants infected with *H. glycines* for each experiment were compared to the pRAP15 controls. No differences in *H. glycines* infection and maturation capability were observed between the pRAP15 vector control and untransformed plants. Thus, the *G. max* roots engineered with pRAP15 behave like normal, untransformed roots. In our genetic engineering experiments,  $N_x$  was the pRAP15-transformed line that had the engineered  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele. In our experiments,  $N_s$  would be the pRAP15 control in their respective *G. max*<sub>[Williams 82/PI 518671]</sub> or *G. max*<sub>[Peking/PI 548402]</sub> genotypes. This procedure has been adopted by other labs using genetically engineered constructs in soybean to examine SCN biology (Steeves et al. 2007; McLean et al. 2007; Mazalei et al. 2007; Li et al. 2010; Melito et al. 2011). In the experiments of Golden et al. (1970), Riggs and Schmidt (1988, 1991), Kim et al. (1998) and Niblack et al. (2002) the FI is typically calculated from a total of 3-10 experimental and 3-10 control plants, each serving as a replicate and experimental replicates may or may not be performed. In the presented experiments, there were a total of 81  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup>-expressing *G. max*<sub>[Williams 82/PI 518671]</sub> plants that were infected with *H.*

*glycines* and used in the analysis. There were a total of 49 pRAP15 vector control *G. max*<sub>[Williams 82/PI 518671]</sub> plants that were infected with *H. glycines* and used in the analysis. The number of experimental plants used in the presented analysis exceeded other reported investigations employing genetically engineered soybean to examine nematode infection of soybean (Steeves et al. 2006; McLean et al. 2007; Mazarei et al. 2007; Li et al. 2010; Melito et al. 2010; Ibrahim et al. 2011). As a control, there were a total of 15 *G. max*<sub>[Peking/PI 548402]</sub> plants that were engineered with its own  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele. Because the pRAP15 control has the *ccdB* gene (**Fig. 4.1**), it also controls for non-specific effects of protein overexpression that does not pertain to *G. max* biology (Klink et al. 2009b; Ibrahim et al. 2011). Other controls, included engineering in soybean genes whose outcomes in experiments resulted in no alterations in infection capability (data not presented). While the FI does not calculate error as part of its accepted analysis procedure, a statistical analysis of the effects of the genetically engineered roots was done using the Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test,  $p < 0.05$ .

#### **4.3.8 Promoter bioinformatics**

The promoters of  $\alpha$ -SNAP were analyzed using the program: [The Plant Cis-acting Regulatory DNA Elements \(PLACE\)](#) database (Higo et al. 1999). PLACE has undergone several updates (<http://www.dna.affrc.go.jp/PLACE/signalscan.html>). The outputs were compared between the different soybean genotypes presented. PLACE allows for the identification of elements that are common and unique between the different soybean genotypes.

## 4.4 Results

### 4.4.1 Gene sequence characteristics of $\alpha$ -SNAP in *G. max*

The gene expression studies presented in Matsye et al. (2011) using the *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> genotypes determined that the only expressed genes in the *rhg1* locus were AAT (Glyma18g02580) and  $\alpha$ -SNAP (Glyma18g02590). The small number of genes exhibiting expression in the *rhg1* locus prompted cloning experiments with the aim of examining their structural elements. Comparative analyses of the cloned  $\alpha$ -SNAP alleles revealed that the cDNAs of the resistant genotypes *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 437654]</sub> are identical. Comparative analyses of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele to the reference *G. max*<sub>[Williams 82/PI 518671]</sub> genotype revealed its cDNA contains SNPs that change the amino acid composition of the polypeptide that could alter the functionality of the protein in reference to the human  $\alpha$ -SNAP that has been functionally characterized (Clary et al. 1990; Bennett et al. 1992). A comparative analysis of the N-terminal, central and C-terminal domains have revealed how the observed SNPs within the three domains in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele could affect its protein structure (**Table 4.1**).

The N-terminal domain of the human  $\alpha$ -SNAP protein occurs between amino acids 3 and 34. This stretch of amino acids is required for binding to the integral membrane protein syntaxin (Clary et al. 1990; Bennett et al. 1992), a protein involved in vesicular trafficking. While silent SNPs exist in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> N-terminal domain, no nonsynonymous SNPs have been observed within this region (**Table 4.1**).

Table 4.1 The SNPs found in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> and  $\alpha$ -SNAP<sup>[PI 437654]</sup> alleles in relation to the  $G. max$ <sup>[Williams 82/PI 518671]</sup> reference.

base in Peking (cDNA)*	AA position (Peking)	AA character in Williams 82	AA character in Peking
244 <sup>T→A</sup>	82 <sup>C→S</sup>	polar, uncharged	polar, uncharged
290 <sup>G→A</sup>	97 <sup>R→Q</sup>	positively charged	polar, uncharged
519 <sup>C→G</sup>	173 <sup>D→E</sup>	negatively charged	negatively charged
535 <sup>G→A</sup>	179 <sup>A→T</sup>	nonpolar, aliphatic	polar, uncharged
620 <sup>A→G</sup>	207 <sup>E→G</sup>	negatively charged	nonpolar, aliphatic
628 <sup>G→A</sup>	210 <sup>V→I</sup>	nonpolar, aliphatic	nonpolar, aliphatic
634 <sup>A→G</sup>	212 <sup>I→V</sup>	nonpolar, aliphatic	nonpolar, aliphatic
702 <sup>G→T</sup>	234 <sup>L→F</sup>	nonpolar, aliphatic	aromatic
709 <sup>G→T*</sup>	237 <sup>D→L</sup> (splice site mutant)	negatively charged	nonpolar, aliphatic
719 <sup>G→A*</sup>	240	n/a	STOP

The \* represents the nucleotide position that exists in the cDNA of the  $G. max$ <sup>[Peking/PI 548402]</sup> allele. The cDNA position of the  $G. max$ <sup>[Peking/PI 548402]</sup> allele (\*) is presented because  $G. max$ <sup>[Williams 82/PI 518671]</sup> lacks the mutated splice site that causes the extension of the mRNA found in the  $G. max$ <sup>[Peking/PI 548402]</sup> allele. The  $G. max$ <sup>[Williams 82/PI 518671]</sup> has that corresponding DNA sequence, but it is spliced out and, thus, not translated. The gray highlighted rows are the nucleotide substitutions that cause changes in the physical properties of the aa at that residue.

The central domain of human  $\alpha$ -SNAP is defined between aa 34 and 236 (Clary et al. 1990). The central domain has two regions predicted to be composed of coiled-coils (Clary et al. 1990). However, the domain lacks a well known role. The central domain of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allelic form contains 7 aa-altering SNPs in comparison to the  $G. max$ <sup>[Williams 82/PI 518671]</sup> reference (**Table 4.1**). Four of the SNPs could significantly affect the physical properties of the corresponding residue in  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup>. The position of an  $\alpha$ -SNAP<sup>97R→Q</sup> conversion observed in the  $G. max$ <sup>[Peking/PI 548402]</sup> allele correlates to a G→A transition occurring in the fifth  $\alpha$ -helix of this central domain that has been shown in mice to result in the hydrocephaly with hop gait (*hyh*) phenotype (Hong et al. 2004). The hypomorphic missense mutation, *hyh*, results in lethality because

it converts a highly conserved methionine at aa position 105 to isoleucine (Hong et al. 2004). The presence of the  $\alpha$ -SNAP<sup>[Peking/PI 548402] 97R→Q</sup> conversion, lying directly adjacent to and downstream from the residue creating  $\alpha$ -SNAP<sup>[hyh]</sup> in mouse, suggests the central domain of  $\alpha$ -SNAP performs an important functional role. The  $\alpha$ -SNAP<sup>[hyh]</sup> allele exhibits altered binding properties for both syntaxin and NSF (Rodríguez et al. 2011). Further upstream, a G→A transition in  $\alpha$ -SNAP<sup>[Drosophila]</sup> generates an alanine to threonine conversion at aa position 59 that is lethal in adults. This mutated  $\alpha$ -SNAP<sup>[Drosophila]</sup> protein doubles the SNARE complex to syntaxin binding ratio in heterozygotes and increases it 3.5 times in mutants homozygous for the mutation (Babcock et al. 2004). A second mutation that is a C→T transition in the central region results in a  $\alpha$ -SNAP<sup>[Drosophila] 168Q→STOP</sup> nonsense mutation that is lethal in embryos (Babcock et al. 2004). The  $\alpha$ -SNAP<sup>[Drosophila]</sup> mutation only slightly elevates the SNARE complex to syntaxin binding ratio (Babcock et al. 2004). Three other aa conversions are observed in the central domain of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele (**Table 4.1**). Firstly, a G→T<sup>2,815</sup> transversion was observed in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele (**Fig. 4.2**).

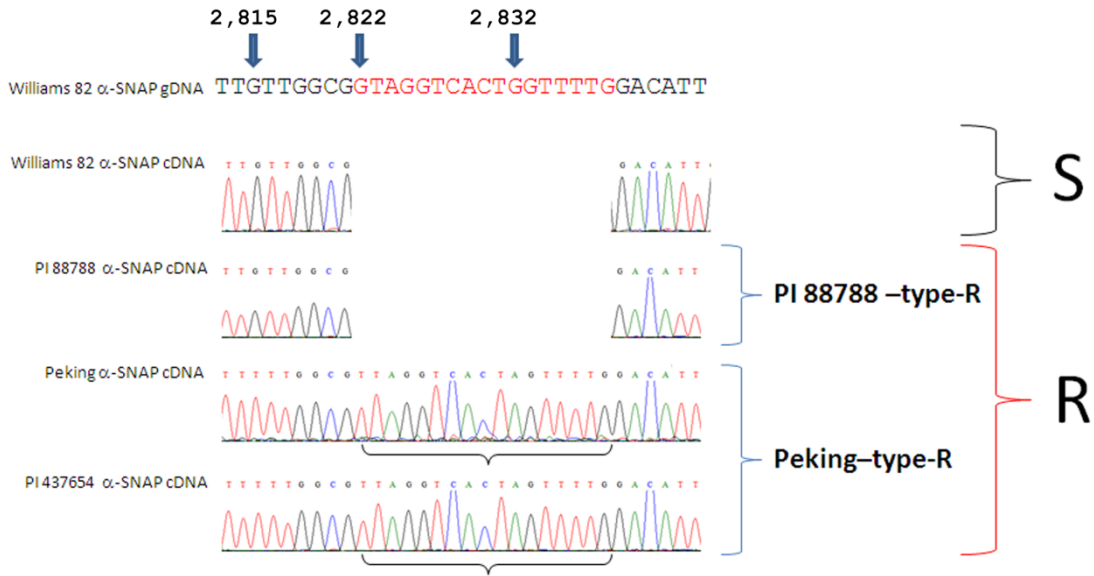


Figure 4.2 Sequence analysis of the C-terminal region of Gm- $\alpha$ -SNAP

The representative chromatograms are from sequenced *G. max*<sub>[Williams 82/ PI 518671]</sub>, Peking/PI 548402, PI 437654 and PI 88788 cDNA. The reference *G. max*<sub>[Williams 82/ PI 518671]</sub> genomic DNA sequence that was confirmed by sequencing is located above the chromatograms. The genomic positions of nucleotide positions having SNPs are presented in relation to the reference *G. max*<sub>[Williams 82/ PI 518671]</sub> sequence. The chromatograms show (1) the G→T<sup>2,815</sup> transversion; (2) a G→T<sup>2,822</sup> transversion that results in a 17 bp motif (black bracket in Peking/PI 548402 and PI 437654) caused by a defective intron splice site; (3) a premature termination codon due to a G→A<sup>2,832</sup> transition. The black bracket (S) represents the susceptible *G. max*<sub>[Williams 82/ PI 518671]</sub> genotype that lacks the three SNPs. The red bracket (R) represents the two resistant genotypes (Peking/PI 548402 and PI 437654) that have the G→T<sup>2,815</sup> transversion. The blue bracket (PI 88788-type R) represents the resistant genotype *G. max*<sub>[PI 88788]</sub> that is resistant to SCN, lacks the 17 nt sequence motif and lacks CWAs. The blue bracket (Peking-type-R) represents the Peking/PI 548402 and PI 437654 genotypes that are resistant to SCN, both have the 17 nt sequence motif, both have the G→T<sup>2,815</sup> transversion, have the 17 nt sequence motif and the premature stop codon caused by the a G→T<sup>2,822</sup> transversion.

That G→T<sup>2,815</sup> transversion converts the aliphatic hydrophobic leucine ( $\alpha$ -SNAP<sup>234L</sup>) found in *G. max*<sub>[Williams 82/PI 518671]</sub> to an aromatic hydrophobic phenylalanine in  $\alpha$ -SNAP<sub>[Peking/PI 548402]</sub><sup>234L→F</sup>. This aa lies just outside of the C-terminal domain of  $\alpha$ -SNAP. In the human synaptotagmin-II, a protein involved in vesicular transport and

botulinum neurotoxin binding (Jin et al. 2006), mutants converting the wild type phenylalanine to leucine or phenylalanine to alanine resulted in abolished binding to its botulinum neurotoxin target (Chai et al. 2006; Jin et al. 2006; Mukherjee et al. 2006; Strotmeier et al. 2012). This outcome occurs because the phenyl side chain that mediated the hydrophobic interaction with its target is disrupted (Strotmeier et al. 2012). Two other SNPs that affect the physical properties of the residue exist in the central domain (**Table 4.1**), but no experimental data on the residues is available in other biological systems to indicate a definitive role. The three other aa conversions do not alter the physical properties of the amino acid (**Table 4.1**).

The C-terminal domain of human  $\alpha$ -SNAP is composed of a coiled-coil domain between aa 236 and 295. This C-terminal domain has been shown to bind syntaxin and NSF (Clary et al. 1990; Barszczewski et al. 2008). NSF is a protein first identified by Malhotra et al. (1988) that promotes fusion of transport vesicles with cisternae of the Golgi stack. There are two SNPs in this domain that could impact the function of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele (**Table 4.1**). The first of those two SNPs in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele creates a 17 base pair motif that does not exist in the *G. max*<sup>[Williams 82/PI 518671]</sup> allelic form (**Fig. 4.2**). The 17 nt motif found in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele is created by a G $\rightarrow$ T<sup>2,822</sup> transversion in a functional splice site found in the *G. max*<sup>[Williams 82/PI 518671]</sup> reference sequence. This transversion results in a  $\alpha$ -SNAP<sup>[Peking/PI 548402] 237D $\rightarrow$ L</sup> conversion. The leucine in  $\alpha$ -SNAP<sup>[Peking/PI 548402] 237D $\rightarrow$ L</sup> is generated from the G $\rightarrow$ T<sup>2,822</sup> transversion and failed intron splicing, resulting in translation of the TTA codon. The aspartic acid in  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> occurs from the properly spliced mRNA and subsequent translation of the GAC codon. It would be expected that the 17 bp sequence would put



the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele out of frame, making it nonfunctional. However, further analysis of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> cDNA revealed that the translational reading frame was maintained because of a second SNP, characterized as a G $\rightarrow$ A<sup>2,832</sup> transition. That SNP creates an in-frame, premature termination codon 11 nt downstream in the 3' end of the 17 bp mRNA sequence motif (**Fig. 4.2**). It is known that premature, but functional translational termination codons in genes can have significant developmental consequences (Wang et al. 1995; McPherron et al. 1997; Babcock et al. 2004; Imai et al. 2006), even increasing their own transcript level. The resultant proteins can exhibit altered posttranslational modifications that have relevant biological activity and govern stress responses (Wang et al. 1995; McPherron et al. 1997; Imai et al. 2006; Ren et al. 2010; Li et al. 2011). In contrast to translational start sites in eukaryotes that have been shown in rare cases to deviate from the canonical ATG that encodes methionine (Zhang and Hinnebusch, 2011), the TAG codon does not encode for any known amino acid.

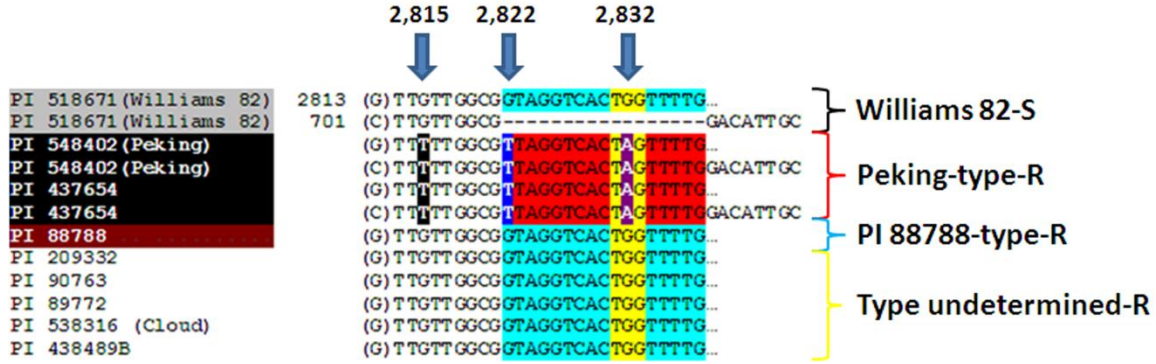


Figure 4.3 SNP analysis of *Glycine max*  $\alpha$ -SNAP (Gm  $\alpha$ -SNAP) in the susceptible genotype *G. max*<sub>[Williams 82/ PI 518671]</sub> as compared to the resistant genotypes Peking/ PI 548402, PI 88788, Cloud/PI 538316, PI 89772, PI 438489B, PI 90763 and PI 209332.

PI color scheme: gray, susceptible genotype; black, Peking-group where CWAs exist; maroon, PI 88788 group where CWAs are lacking. The remainder of the genotypes (white) has no data published on CWAs. (C), cDNA sequence for that genotype. (G), genomic sequence. In these cases, cDNA sequence can be inferred from the genomic DNA sequence. The numbering scheme is related to the  $\alpha$ -SNAP<sub>[Williams 82/PI 518671]</sub> because full genomic and/or cDNA sequence is not available for the other genotypes. (---), sequence lacking when the splicing site at position 2,822 is not altered; (#), SNP abolishing the exon-intron splice site; (\*) SNP causing the premature stop codon; (...), genomic sequence that is unalignable to cDNA sequence. A  $G \rightarrow T^{2,815}$  transversion (+), converts the aliphatic hydrophobic leucine to an aromatic hydrophobic phenylalanine. Note, the invariant GT at the exon-intron junction in the genomic sequence of  $\alpha$ -SNAP<sub>[Williams 82/PI 518671]</sub> is altered in the  $\alpha$ -SNAP<sub>[Peking/PI 548402]</sub> allele by a  $G \rightarrow T^{2,822}$  transversion, resulting in abolished splicing as confirmed by cDNA sequencing. There also is a premature termination codon due to a  $G \rightarrow A^{2,832}$  transition in the  $\alpha$ -SNAP<sub>[Peking/PI 548402]</sub> allele.

Thus, the in-frame TAG codon beginning at position 2,831 and ending at position 2,833 in  $\alpha$ -SNAP<sub>[Peking/PI 548402]</sub> must function as a stop codon. The presence of the extended mRNA sequence in the  $\alpha$ -SNAP<sub>[Peking/PI 548402]</sub> cDNA also excludes the possibility that some other alternate splicing event would eliminate this additional mRNA sequence that contains the premature stop codon. An  $\alpha$ -SNAP<sub>[Drosophila] 292R \rightarrow STOP</sub> conversion, representing the 3' terminus, is created by a  $C \rightarrow T$  transition that is lethal in 1<sup>st</sup>/2<sup>nd</sup> instars (Babcock et al. 2004). The  $\alpha$ -SNAP<sub>[Drosophila] 292R \rightarrow STOP</sub> mutation resulted in

a 3.5-fold increase in the SNARE complex:syntaxin binding ratio (Babcock et al. 2004). The resequencing of Gm- $\alpha$ -SNAP from several genotypes that exhibit resistance to SCN revealed the genotypes harboring the premature termination event (**Fig. 4.3**). The premature translational termination codon found in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele lies at the beginning of the C-terminal coiled-coil domain (**Fig. 4.4**). If the *G. max*  $\alpha$ -SNAP (Gm- $\alpha$ -SNAP) protein functions in a manner that is similar in human, the premature termination codon in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele would result in a truncated protein lacking the C-terminal coiled-coil domain. The truncated protein would lack both the second binding site for syntaxin and the only binding site for NSF (**Fig. 4.4**).

Examination of the *G. max*<sub>[Williams 82/PI 518671]</sub> genome showed the existence of at least 5  $\alpha$ -SNAP homologs. The  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> homologs are located on chromosomes 2 (Glyma02g42820), chromosome 11 (Glyma11g35820), chromosome 14 (Glyma14g05920), and chromosome 18 (Glyma18g02590), encoding 289 amino acid (aa) proteins while the  $\alpha$ -SNAP on chromosome 9 (Glyma09g41590) encodes a protein that is 293 aa (Schmutz et al. 2010). Only Glyma09g41590, located on chromosome 9, is expressed in both genotypes specifically in syncytia undergoing defense in *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub>, but identified only at the 6 dpi time point (Matsye et al. 2011). Functional studies of  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> were merited since the Glyma18g02590 allele found in *G. max*<sub>[Peking/PI 548402]</sub> is expressed throughout defense.

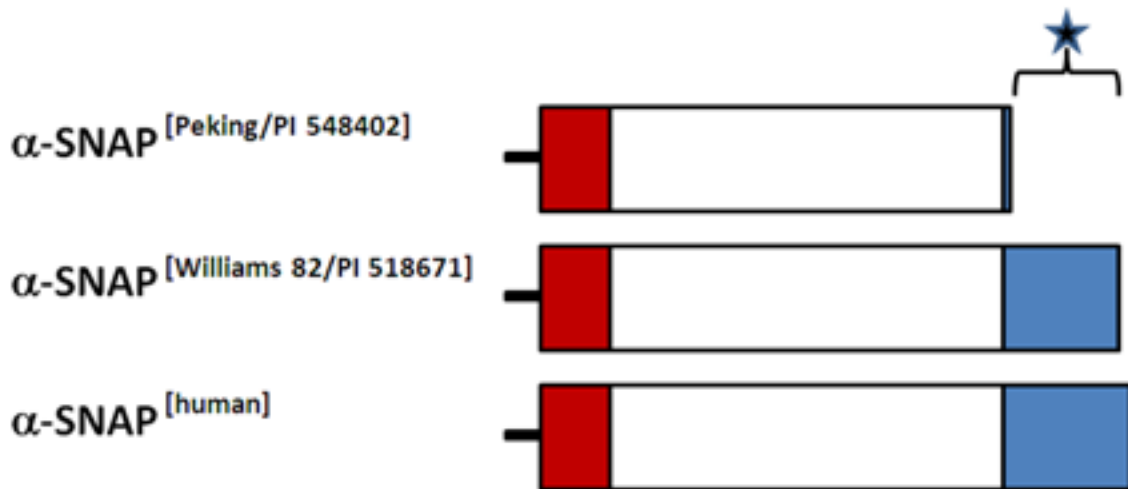


Figure 4.4 Diagram of  $\alpha$ -SNAP protein domains according to Clary et al. (1990)

Black box, N-terminal region lacking a functional domain. Maroon, N-terminal domain occurring between amino acids 3 and 34 of human  $\alpha$ -SNAP that is required for binding to syntaxin; white, the central region between aa 34 and 236 in human  $\alpha$ -SNAP lacking a well defined role, but having the *hyh* mutation in mouse that alters its binding to syntaxin; blue, the C-terminal domain having a coiled-coil domain between aa 236 and 295 that has been shown to bind syntaxin and *N*-ethylmaleimide-sensitive fusion (NSF) protein. Bracket and star, the deleted C-terminus in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele caused by the premature stop codon.

#### 4.4.2 Functional analyses

The pRAP15 vector has been designed for overexpression experiments (Fig. 4.5), allowing for the functional testing of genes. Overexpression experiments performed in onion root cells demonstrate the capability of pRAP15 to overexpress protein (Figs. 4.5.a-c). The outcome of the hairy root transformation procedure using the pRAP15 vector demonstrates that the *ro/D* promoter driving eGFP expression functions in soybean (Figs. 4.5.d-f). Overexpression of  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> mRNA in whole uninfected *G. max*<sub>[Williams 82/PI 518671]</sub> roots was confirmed by qPCR (Fig. 4.5.g). The ability of the pRAP15 vector to drive the overexpression of soybean genes has been tested using  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup>:*uidA* fusion protein (Fig. 4.5.h) according to the procedures of

Abel and Theologis (1994) and Sakamoto et al. (2009). These results confirm reports of the stability of the GUS protein is ~4 h to a few days in transgenic tissue (de Ruijter et al. 2003). Therefore, any overexpression observed in SCN-infected roots at the conclusion of the 30 day test period would likely be the result of sustained expression. Rapid expression (after 24 h post biolistic treatment) of transgenes in plant tissue for the pRAP15 vector engineered with RFP was already demonstrated (**Figs. 4.5.b, c**) and at the conclusion of SCN infection (**Fig. 4.5.h**). These experiments show that the pRAP15 vector would be suitable for the overexpression studies in soybean aimed at revealing the effect of the expression of the altered  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele during infection by SCN.

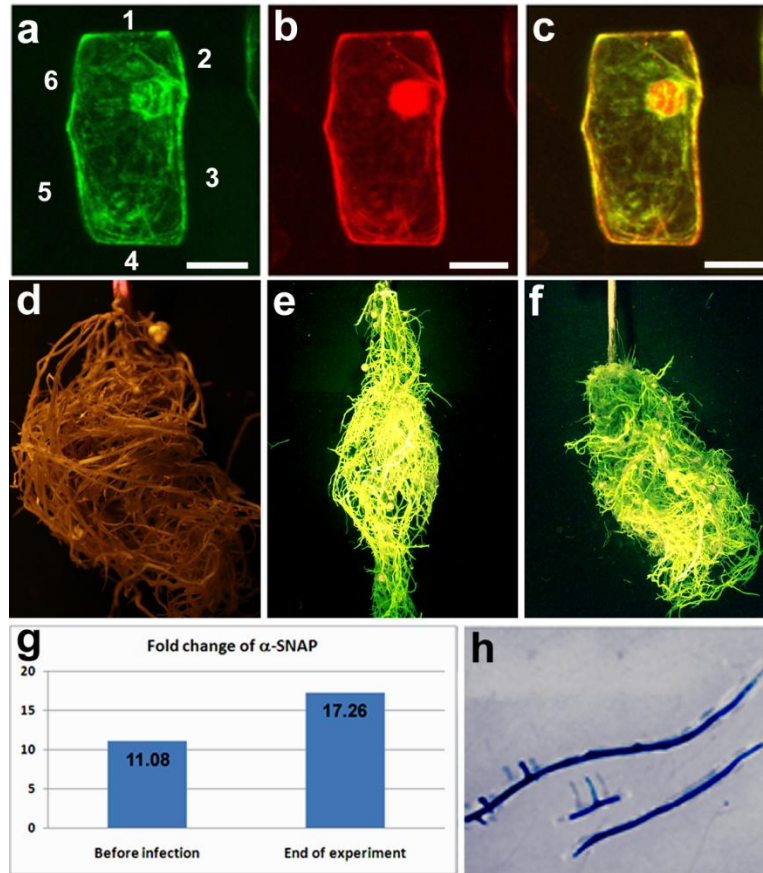


Figure 4.5 Functionality of the pRAP15 vector. a-c

the transformed *Allium cepa* (onion) cell having fluorescence caused by the engineered genes (\*) is surrounded by 6 cells (1-6) that are not transformed and lack fluorescence. (a) PRAP15 RFP expression driven by the FMV-*sgt* promoter in onion root cells. Bar = 40  $\mu$ m. (b) GFP reporter expression driven by the *roID* promoter in onion root cells. Bar = 40  $\mu$ m. (c) Merged image of b and c. Bar = 40  $\mu$ m. (d) An untransformed root of *G. max*<sub>[Williams 82/PI 518671]</sub>. (e) A transformed root of *G. max*<sub>[Williams 82/PI 518671]</sub> but lacking an engineered transgene (pRAP15 control). (f) A transformed root of *G. max*<sub>[Williams 82/PI 518671]</sub> with an engineered GUS transgene. (g) qPCR of  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> expressed in *G. max*<sub>[Williams 82/PI 518671]</sub>. X-axis represents the point in the experiment that the RNA samples were isolated. The y-axis represents the fold expression over and above the pRAP15 control. (h)  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup>:GUS expression in *G. max*<sub>[Williams 82/PI 518671]</sub> at the end of the experiment, revealing the presence of the protein at the termination of the experiment.

Experiments expressing gene fragments as dominant negatives in wild-type backgrounds have been shown to be a powerful tool to examine the functionality of the

vesicle fusion pathway components in plants (Geelen et al. 2002; Tyrrell et al. 2007). A similar approach is presented here for the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele. Since the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele lacks its well-defined C-terminal functional domain, its overexpression in a genotype having what would be considered a wild-type genetic background (*G. max*<sub>[Williams 82/PI 518671]</sub>) would fit the definition of a dominant negative experiment (Geelen et al. 2002; Tyrrell et al. 2007). In functional studies, the susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> roots possessing its endogenous  $\alpha$ -SNAP allele have been engineered to express the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele. Normally, the *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background yields a susceptible reaction to SCN infection (**Fig. 4.6**). The only genetic difference between the pRAP15 control and the *G. max*<sub>[Williams 82/PI 518671]</sub> experimental plants is the presence of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele. Infection of *G. max*<sub>[Williams 82/PI 518671]</sub> roots expressing the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele by SCN was then done, allowing infection to develop for 30 days. The results of the experiment show that overexpression of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele in *G. max*<sub>[Williams 82/PI 518671]</sub> roots partially suppresses the development of SCN infection (**Fig. 4.6**).

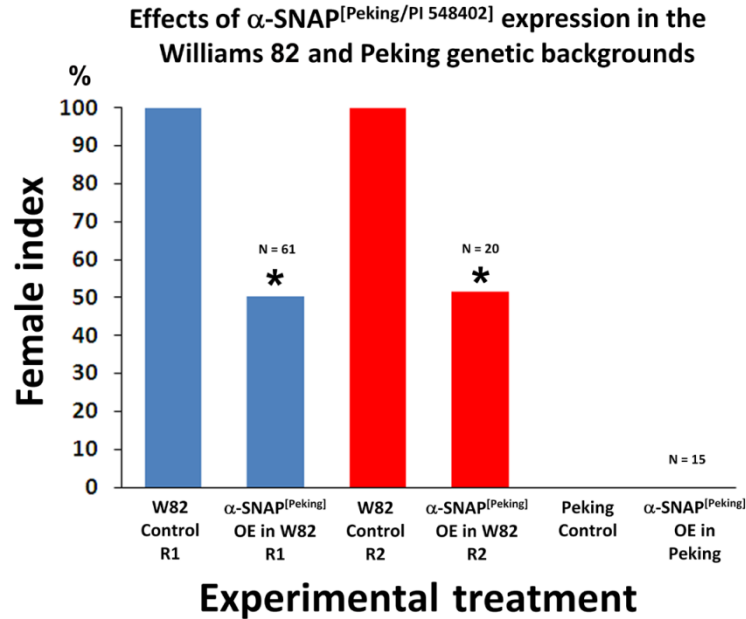


Figure 4.6 Female index (FI)

The FI was calculated for *G. max*<sub>[Williams 82/PI 518671]</sub> or *G. max*<sub>[Peking/PI 548402]</sub> plants genetically engineered to contain the control pRAP15 vector. This vector contains the *ccdB* gene that acts as a negative control gene (Klink et al. 2009b; Ibrahim et al. 2011). Other test plants were transformed with the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele. The FI of the pRAP15 control plants, calculated as a comparison to itself, is shown having a FI of 100% solely for comparative purposes. Legend: **bar 1**, (blue) W82 Control R1 represents the first control replicate where the pRAP15 vector lacking any  $\alpha$ -SNAP allele was engineered into the *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background, having on average 149 females; **bar 2**, (blue)  $\alpha$ -SNAP<sup>[Peking]</sup> OE in W82 R1 represents the first replicate of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele expressed in the *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background (N = 61 plants/independent lines); **bar 3**, (red) W82 Control R2 represents the second replicate of the control experiment where the pRAP15 vector lacking any  $\alpha$ -SNAP allele was engineered into the *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background (N = 23 plants/independent lines), **bar 4**, (red)  $\alpha$ -SNAP<sup>[Peking]</sup> OE in W82 R2 represents the second replicate of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele expressed in the *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background (N = 20 plants/independent lines); **bar 5**, Peking Control represents the control experiment where the pRAP15 vector lacking any  $\alpha$ -SNAP allele was engineered into the *G. max*<sub>[Peking/PI 548402]</sub> genetic background (N = 9 plants/independent lines); **bar 6**,  $\alpha$ -SNAP<sup>[Peking]</sup> OE in Peking represents the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele expressed in the *G. max*<sub>[Peking/PI 548402]</sub> genetic background (N = 15 plants/independent lines). Note, the *G. max*<sub>[Peking/PI 548402]</sub> genetic background is highly resistant to the SCN population used in the presented studies (Klink et al. 2007). Therefore, it is expected that the FI would be at or near zero. Statistically significant replicates ( $p < 0.05$ ) are denoted with (\*). The lack of statistical significance in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele expressed in *G. max*<sub>[Peking/PI 548402]</sub> genetic background experiment demonstrates that the construct had no effect on the normal defense response.



As shown previously (**Fig. 4.5.f**) *G. max*<sub>[Williams 82/PI 518671]</sub> roots expressing the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele had well-developed roots even at the end of the experiment. To demonstrate that the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele did not detrimentally affect how *G. max*<sub>[Peking/PI 548402]</sub> roots normally suppress infection by SCN, the *G. max*<sub>[Peking/PI 548402]</sub> genotype was transformed with its own  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele. These control experiments resemble those by Ren et al. (2010). As one of their controls in a series of experiments on osmotic stress, expressed the Landsberg erecta (Ler) allele of the *Response to ABA and Salt1 (RAS1)* gene back in the *A. thaliana*<sub>[Ler]</sub> genotype. The experiments revealed no additive or detrimental effect of the overexpressed *RAS1*<sup>[Ler]</sup> in the *A. thaliana* Ler genotype as it normally responds to stress (Ren et al. 2010). A similar approach was taken by Li et al. (2011) to understand fructose sensitivity in *A. thaliana* using a fructose-sensing quantitative trait locus 6 (QTL) (FSQ6) allele identified in a natural population. Therefore, the overexpression of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele in *G. max*<sub>[Peking/PI 548402]</sub> is an accepted approach to control for the expression experiments presented here. In the experiments presented here, overexpression of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele in *G. max*<sub>[Peking/PI 548402]</sub> had no obvious effect on root development at the beginning or end of the experiment. The expressed  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele in *G. max*<sub>[Peking/PI 548402]</sub> genotype also had no detrimental effect on its normal defense response (**Fig. 4.6**). The *G. max*<sub>[Peking/PI 548402]</sub> genotype has been shown to have a FI of 0-0.8% when infected by several different isolates of *H. glycines*<sub>[HG-type 7]</sub> (Niblack et al. 2002). We have observed a similar FI for *H. glycines*<sub>[NL1-Rhg/HG-type 7/race 3]</sub> in the *G. max*<sub>[Peking/PI 548402]</sub> genotype (Klink et al. 2009a). Therefore, the observation that female development did not occur in the pRAP15 controls or *G. max*<sub>[Peking/PI 548402]</sub> plants expressing the  $\alpha$ -

SNAP<sup>[Peking/PI 548402]</sup> allele were not unexpected. From the outcome of the experiments, it was concluded that the results obtained by the overexpression of  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> in the *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background is not due to a general toxicity to the root cells because root development is normal (**Fig. 4.5.f**). It is also concluded that the overexpression of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele back in the *G. max*<sub>[Peking/PI 548402]</sub> genetic background does not have deleterious effects on root development in ways that would alter its normal ability to defend itself from *H. glycines* infection. However, tests to reveal enhanced fortification of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup>-expressing *G. max*<sub>[Peking/PI 548402]</sub> roots were not performed.

#### **4.4.3 Quantitative PCR confirms the overexpression of the $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele alters gene expression**

Prior experiments in a number of systems examining  $\alpha$ -SNAP have determined that mutated alleles have the capacity to alter the expression of other genes, particularly those relating to vesicular transport (Clary et al. 1990; Hong et al. 2004; Babcock et al. 2004). The same observation has been made for naturally truncated alleles and various mutants of the vesicular transport machinery, suggesting that various feedback regulatory loops exist in maintaining the relative levels of the proteins. Other experiments that examined gene expression in the SCN-resistant, *rhg1* locus-containing *G. max*<sub>[PI 209332]</sub> genetic background as compared to an *rhg1*<sup>[PI 209332 (-/-)]</sup> genetic background lacking resistance revealed that thousands of genes are altered in their expression (Kandoth et al. 2011). Notably, the *rhg1* locus contains Gm- $\alpha$ -SNAP. Therefore, the overexpression of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele in *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background would be expected to alter the expression of other genes (Mazarei et al. 2007). These observations

show that the relationship of  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> to defense could be complex, involving enhanced expression of plant defense pathways. To test this, we performed qPCR of the *G. max* ethylene-responsive element-binding protein 1 (*GmEREBP1*) (Mazarei et al. 2007) and the pathogenesis-related (PR) genes *PR1* (Antoniw and Pierpoint, 1978), *PR2* (Kauffmann et al. 1987), *PR3* (Legrand et al., 1987) and *PR5* (Kauffmann et al., 1990) using ubiquitin (*UBQ3*) as a control. The experiments were repeated using the ribosomal *S21* gene as a control (Klink et al. 2005; Alkharouf et al. 2006) and show the same trends in expression. The experiments demonstrate that the overexpression of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele in *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background induces the expression of *GmEREBP1*, *PR1*, *PR2* and *PR5* prior to infection by SCN (Table 4.2). However, the suppressed expression of *PR3* prior to infection indicates a level of specificity for  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup>.

Table 4.2 The influence of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele on the expression of *GmEREBP1*, *PR1*, *PR2*, *PR3*, *PR5* in *G. max*<sub>[Williams 82/PI 518671]</sub> roots prior to SCN infection as assessed by qPCR.

Target	FC: S21 as control	FC: UBI3 as control
<i>EREBP 1</i>	17.12	6.09
<i>PR1</i>	11.64	4.14
<i>PR2</i>	15.69	12.87
<i>PR3</i>	-4.95	-6.25
<i>PR5</i>	6.78	5.56
<i>UBI3</i>	2.63	n/a
<i>S21</i>	n/a	-1.21

The experiments used the ribosomal *S21* gene as a control (column 1) (Klink et al. 2005; Alkharouf et al. 2006). The experiments were repeated using the ubiquitin (*UBQ3*) gene (column 2) as a control for the qPCR experiment. FC: fold change.

#### 4.4.4 Promoter bioinformatics

Functional studies in transgenic roots have shown that the prematurely truncated  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele can alter the ability of SCN to infect roots in a genotype that is otherwise susceptible to infection. This observation is in agreement with mapping analyses that have demonstrated there are genetic differences existing between the resistant genotypes harboring *rhg1* (Cregan et al. 1999b; Concibido et al, 2004; Brucker et al. 2005; Kim et al. 2010). However, gene sequencing experiments have shown that the protein coding region of the  $\alpha$ -SNAP allele from *G. max*<sup>[PI 88788]</sup> is the same as *G. max*<sup>[Williams 82/PI 518671]</sup>. This outcome would suggest that  $\alpha$ -SNAP is not the *RHG1* gene. The gene sequencing experiments did not exclude the possibility that alterations in promoter sequence existed that allow the  $\alpha$ -SNAP<sup>[PI 88788]</sup> allele to be regulated differently from  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup>, but similar to  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> at the level of transcription. This is important to note because the  $\alpha$ -SNAP<sup>[PI 88788]</sup> and  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> alleles appear to be expressed in a similar manner during SCN infection (Matsye et al. 2011). Specific promoter elements are known to be important for eliciting responses in soybean under pathogen attack or physiological stress (Park et al. 2004). The promoter sequences of the  $\alpha$ -SNAP<sup>[PI 88788]</sup> and  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> alleles were then obtained for comparative analyses with the  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> allele. Alignments of over 2,300 nucleotides of sequence show that the promoters of the  $\alpha$ -SNAP<sup>[PI 88788]</sup> and  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> alleles were nearly identical except for two nucleotide insertions that each were over 2,000 nt upstream from the translational start sites and a SNP approximately 1,500 bp upstream (**Supplemental Fig. 2**). In contrast, numerous SNPs, IN/DELS and possibly a microsatellite beginning at position -68 characterize the

differences between the  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> promoter sequence from both the  $\alpha$ -SNAP<sup>[PI 88788]</sup> and  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> alleles (**Supplemental Fig. 2**). These observations indicated that while the  $\alpha$ -SNAP alleles from the resistant genotypes would be nearly identical in their ability to bind transcription factors, they could be quite different from the  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> allele. The promoter sequences were then examined to see how those differences could affect the binding of transcription factors (TFs).

A bioinformatics-based promoter analysis was performed (**Supplemental Table 2**). Part of the analysis was designed to show that some of the TF binding sites are found only in the  $\alpha$ -SNAP<sup>[PI 88788]</sup> and  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> promoters (**Table 4.3**). Furthermore, some of these TF-binding sites are found at only one site in the two resistant genotypes under examination while others are found at relatively few or numerous sites along the promoter (**Table 4.3**). Analyses of the  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> promoter revealed that some TF-binding motifs are found at only one site while other motifs are found at relatively few or numerous sites along the promoter (**Table 4.4**). These observations indicate that the  $\alpha$ -SNAP alleles from the susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> and resistant *G. max*<sub>[PI 88788]</sub> genotypes, while having identical primary sequence in their protein coding regions, could be regulated differently. The observations also indicate the resistant genotypes, while having nearly identical promoter elements, differ from the susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> genotype.

Table 4.3 A bioinformatics-based promoter analysis showing the promoter binding sites that are unique to the  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> allele.

Factor or site name	binding sequence	strand	true position in W 82 $\alpha$ SNAP promoter	no. of occurrences at other positions in W 82 $\alpha$ SNAP promoter
ELRECOREPCRP1	TTGACC	(-)	-1106	0
WBBOXPCWRKY1	TTTGACY	(-)	-1106	0
VSF1PVGRP18	GCTCCGTTG	(-)	-282	0
TBOXATGAPB	ACTTTG	(-)	-1173	1
MYBCOREATCYCB1	AACGG	(+)	-281	1
TATABOX2	TATAAAT	(+)	-630	3
MYB2CONSENSUSAT	YAACKG	(+)	-282	3
WBOXNTERF3	TGACY	(-)	-1106	4
WBOXATNPR1	TTGAC	(-)	-1105	4
MARTBOX	TTWTWTTWTT	(-)	-626	8
MYBCORE	CNGTTR	(-)	-282	8
WRKY71OS	TGAC	(-)	-1105	9
POLASIG1	AATAAA	(+)	-626	12
TATABOX5	TTATTT	(-)	-627	15
CAATBOX1	CAAT	(-)	-1405	30

Only elements unique to the different genotypes are shown. The symbols used in addition to A, G, C, or T nucleotides are: (1) **B**: C, G or T; (2) **D**: A, G or T; (3) **H**: A, C or T; (4) **K**: G or T; (5) **M**: A or C; (6) **N**: A, C, G or T; (7) **R**: A or G; (8) **S**: C or G; (9) **V**: A, C or G; (10) **W**: A or T; (11) **Y**: C or T. Legend, element, the name of the element from the application A Database of Plant Cis-acting Regulatory DNA Elements (PLACE); <http://www.dna.affrc.go.jp/PLACE/signalscan.html> (Higo et al. 1999). Factor name, PLACE transcription factor naming convention; binding sequence, the transcription factor recognition sequence; true position, relative location in the promoter sequence with position 1 being distal from the translational start site; strand, sense (Watson) or antisense (Crick) strand; no. of occurrences (common or unique between the different genotypes) at other positions in the promoter.

Table 4.4 A bioinformatics-based promoter analysis showing the promoter binding sites that are unique to the  $\alpha$ -SNAP<sup>[Peking/ PI 548402]</sup> and  $\alpha$ -SNAP<sup>[PI 88788]</sup> genotypes.

Peking and PI 88788 factor	binding sequence	strand	true position in Peking promoter	true position in PI88788 promoter	no. of occurrence at other positions in Peking $\alpha$ SNAP promoter	no. of occurrence at other positions in PI88788 $\alpha$ SNAP promoter
DRE1COREZMRAB17	ACCGAGA	(-)	-431	-431	0	0
CPBCSPOR	TATTAG	(+)	-1183	-1183	2	2
NODCON1GM	AAAGAT	(-)	-187	-187	3	3
OSE1ROOTNODULE	AAAGAT	(-)	-187	-187	3	3
E2FCONSENSUS	WTTSSCSS	(-)	-2057	-2057	3	3
PYRIMIDINEBOXOSRAM Y1A	CCTTTT	(-)	-285	-285	4	4
CCAATBOX1	CCAAT	(+)	-2054	-2054	7	7
	TTWTWTTW					
MARTBOX	TT	(+)	-2189	-2188	8	8
SEF4MOTIFGM7S	RTTTTTR	(+)	-1342	-1342	9	9
POLASIG1	AATAAA	(-)	-1345	-1345	12	12
TATABOX5	TTATTT	(+)	-1344	-1344	15	15
POLASIG3	AATAAT	(+)	-1421	-1421	16	16
POLLEN1LELAT52	AGAAA	(+)	-1179	-1179	19	19
POLLEN1LELAT52	AGAAA	(+)	-477	-477	19	19
POLLEN1LELAT52	AGAAA	(-)	-184	-184	19	19
CAATBOX1	CAAT	(+)	-2053	-2053	30	30
DOFCOREZM	AAAG	(-)	-1117	-1117	36	36
DOFCOREZM	AAAG	(+)	-475	-475	36	36
DOFCOREZM	AAAG	(+)	-284	-284	36	36
DOFCOREZM	AAAG	(-)	-185	-185	36	36

The same parameters are used in this analysis as was used in Table 4.3.

#### 4.5 Discussion

Recent whole genome sequencing efforts (1000 Genomes Project Consortium, 2010; Bancroft et al. 2011; Xu et al. 2012) support the hypothesis that naturally occurring, truncated alleles of genes may play important biological roles. The ability of these naturally occurring, truncated alleles to have important biological activity has been

demonstrated (Wang et al. 1995; McPherron et al. 1997; Ren et al. 2010; Li et al. 2011). This property makes them an invaluable resource for understanding the biology of complex traits found in natural populations. The experiments presented here were designed to determine, directly, if a defense role exists for a naturally occurring truncated allele of  $\alpha$ -SNAP in soybean. Importantly, the gene was identified through quantitative sequencing of RNA samples isolated from specialized cells involved in a plant-plant parasitic nematode interaction (Matsye et al. 2011). The functional experiments show that the overexpression of a naturally occurring, truncated form of  $\alpha$ -SNAP in an otherwise susceptible genotype impairs infection. More broadly, the results may indicate a mechanism employed by plants, particularly plants with complex and duplicated genomes, to regulate niche development of a pathogen in natural populations. According to the functional and sequence data presented here, Gm- $\alpha$ -SNAP (Glyma18g02590) could contribute to the resistance effect that has been mapped to the *rhg1* locus. However, sequencing data indicates that the *rhg1* locus is complex. Therefore, the altered  $\alpha$ -SNAP may only contribute to the overall *rhg1* effect.

#### **4.5.1 Soybean as an experimental system for plant-parasitic nematode research**

Soybean presents many experimental advantages in understanding the relationship between complex and duplicated genomes and defense to plant parasitic nematodes. First, soybean has been shown to have a complex genome (Doyle et al. 1999; Schmutz et al. 2010). Secondly, approximately 118 genetic sources of SCN resistance exist (Rao-Arelli et al. 1997; Concibido et al. 2004; Shannon et al. 2004) with hundreds of more accessions identified in China (Ma et al. 2006; Li et al. 2011). Currently, the vast majority of commercial varieties in the U.S. (> 97%) obtain their resistance germplasm from the *G.*



*max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> genotypes (Diers et al. 1999; Concibido et al. 2004). The use of these genotypes has influenced the relative amount of basic research done on these genotypes, resulting in the generation of a substantial base of genetic, cytological and gene expression knowledge in understanding defense to SCN. Lastly, genetically distinct SCN races (Golden et al. 1970; Riggs and Schmitt, 1988, 1991) that can be further divided and reclassified into populations (Niblack et al. 2002) based on their virulence provide the capability of obtaining both susceptible and resistant outcomes in the identical soybean genetic background (Mahalingam and Skorpska, 1996; Klink et al. 2007, 2009a, 2011; Matsye et al. 2011). Therefore, no influence of plant genotype can be introduced in experiments examining and comparing gene expression during susceptibility and defense. These features of the soybean-SCN pathosystem make it an ideal experimental model (Barker et al. 1993; Opperman and Bird, 1998; Niblack et al. 2006).

#### **4.5.2 Characteristics of $\alpha$ -SNAP relate to its functional role in defense**

The observation of  $\alpha$ -SNAP expression (Matsye et al. 2011), its location within the *rhg1* locus (Kim et al. 2010) and known involvement in processes relating to defense (Collins et al. 2003) made it a reasonable candidate for bioinformatics data mining to determine how its expression could relate to SCN pathogenicity. Many experiments have been performed in several model systems such as yeast (Novick et al. 1980); human (Clary et al. 1990), mouse (Hong et al. 2004) and *Drosophila* (Babcock et al. 2004) to determine the function of  $\alpha$ -SNAP. Much of the earliest work on  $\alpha$ -SNAP was done in the *Saccharomyces cerevisiae* mutant *sec17*, (Novick et al. 1980), revealing that it plays a role in the fusion of vesicles (Kaiser and Schekman, 1990). The conserved nature of the

gene was realized when the  $\alpha$ -SNAP gene was identified in human as serving a central role in intracellular membrane fusion (Clary et al. 1990). Membrane fusion engages in important roles in the growth of cells, hormonal release, exocytosis, neurotransmission and autophagy (Clary et al. 1990; Peter et al. 1998; Ishihara et al. 2001). Membrane fusion also is a process performing crucial roles in plant defense (Collins et al. 2003; Kalde et al. 2007; Kwon et al. 2008; Pajonk et al. 2008; Meyer et al. 2009). The  $\alpha$ -SNAP<sup>[human]</sup> protein is 295 aa in length and has three functional domains (Clary et al. 1990). Notably, an N-terminal coiled coil domain occurs between amino acids (aa) 3 and 34. There are two coiled coil regions in the central domain that occur between aa 113 and 195. There is another coiled coil region between aa 236 and 274 in the C-terminal domain (Barnard et al. 1996). In addition to the involvement of  $\alpha$ -SNAP, membrane fusion and vesicular trafficking are processes including the protein syntaxin, the NSF ATPase and the SNARE protein complex. This is important to note because the  $\alpha$ -SNAP protein has an N-terminal syntaxin binding domain and a C-terminal domain that binds both syntaxin and NSF (Clary et al. 1990) and the process of membrane fusion and vesicular transport is dependent on specific interactions occurring between these proteins at specific sites along the protein.

#### **4.5.3 The identification of allelic variants of Gm- $\alpha$ -SNAP**

The application of the Illumina® deep sequencing technology to understanding syncytium biology (Matsye et al. 2011) has allowed for the identification of allelic variations in gene structure. During the resequencing of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele, SNPs that would both structurally alter and prematurely truncate the protein were observed. These SNPs did not exist in the reference  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> allele that

is identical in sequence to its ortholog found in the resistant *G. max*<sup>[PI 88788]</sup> genotype. This observation provided support to the work of Cregan et al. (1999b) and Brucker et al. (2005) that the *rhg1* loci of different soybean genotypes are structurally different. Naturally occurring, truncated forms of proteins having dominant negative functions have been identified (Nakabeppu and Nathans, 1991; Wang et al. 1995; McPherron et al. 1997) and have important biological functions that deviate from their normal role(s). A comparison of a number of soybean genotypes that can resist infection by SCN demonstrated that the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele is also present in *G. max*<sup>[PI 437654]</sup>, but not in the other tested genotypes. This would suggest that the Gm- $\alpha$ -SNAP gene located within the *rhg1* locus is not the *RHGI* gene. However, sequencing of the  $\alpha$ -SNAP<sup>[PI 88788]</sup> promoter identified structural characteristics that are not found in its ortholog in *G. max*<sup>[Williams 82/PI 518671]</sup> which is consistent with the work of Cregan et al. (1999b) and Brucker et al. (2005). This observation differentiates the  $\alpha$ -SNAP<sup>[PI 88788]</sup> allele from its ortholog found in *G. max*<sup>[Williams 82/PI 518671]</sup>, possibly at a functional level.

#### 4.5.4 The relation of $\alpha$ -SNAP to defense

Defense to pathogens can be pre- or post-invasive (Lipka et al. 2005). A pre-invasive defense strategy has been shown to be based on the expression and activity of a vesicular transport machinery component syntaxin identified as the *penetration1* (*pen1*) mutation (Collins et al. 2003; Lipka et al. 2005; Kwon et al. 2008). In the pre-invasive defense strategy, the cell interacting directly with the pathogen survives (Kwon et al. 2008). Very little information exists on this form of defense in relation to plants and parasitic nematodes. However, it is clear that such a strategy would be highly beneficial

to the plant. In contrast, the post-invasive defense strategy appears to relate more to SCN since the attacked cell ultimately dies (Ross, 1958; Endo, 1965).

A classification scheme of soybean's defense to SCN has been proposed, based partially on the cellular features found in the root cells undergoing an incompatible reaction (Niblack et al. 2008). The cellular features include the presence of CWAs in the Peking-type of defense response and their absence in the PI 88788-type (Kim et al. 1987; Endo, 1991). CWAs are structures whose development involves vesicle dynamics, delivering materials to the site of infection. The organization of the CWAs has been shown to be influenced by *pen1* (Collins et al. 2003; Assaad et al. 2004), further implicating vesicular transport in its assembly since syntaxin binds  $\alpha$ -SNAP (Clary et al. 1990; Barszczewski et al. 2008; Rodriguez et al. 2011) and its dynamics have been shown to be influenced by specific structural alterations that include premature termination (Babcock et al. 2004). In experiments examining the CWAs in the roots of *Asplenium* (fern), Leroux et al. (2011) demonstrated the presence of pectic homogalacturonan, xyloglucan, mannan and cellulose. Callose has also been reported to be associated with CWAs in soybean under attack by the basidiomycete *Phakopsora pachyrhizi* (Asian soybean rust) (Hoefle et al. 2009). The metabolic pathways involved in the synthesis of these substances have been identified by KEGG pathway analyses of syncytia undergoing defense (Klink et al. 2010a, 2011a; Matsye et al. 2011). Therefore, CWAs would relate to  $\alpha$ -SNAP functionality through the ability of vesicles to deliver materials to the site of infection. Moreover, Trujillo et al. (2004) have demonstrated  $H_2O_2$  at CWAs in wheat infected with *Blumeria graminis*. Thus, it is likely that these compounds are undergoing extensive cross-linking at the site of infection during defense.

However, the lack of CWAs in some soybean genotypes that undergo defense does not mean that the vesicular machinery is not involved in some manner. At this time, it is unclear if CWAs are found only in genotypes with the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele. The limited data currently makes that view possible.

If  $\alpha$ -SNAP is important to the defense process in the absence of CWAs, then it likely accomplishes the same task by related processes or performs other cellular defense roles. The  $\alpha$ -SNAP homolog in *S. cerevisiae*, Sec17p, and other vesicular components are also involved in autophagy (Ishihara et al. 2001; Furuta et al. 2010). Autophagy is a process known in plants to play crucial roles in defense (Patel and Dinesh-Kumar 2008; Hofius et al. 2009; Lenz et al. 2011; Lai et al. 2011). Components of the autophagy machinery have been identified in the defense responses of soybean to SCN (Klink et al. 2007, 2009a, 2010a, 2011a; Matsye et al. 2011; Kandoth et al. 2011). Therefore, the transcriptional analyses appear to support the hypothesis that it is the expression of the  $\alpha$ -SNAP gene and not the allelic form that may be important for defense (Matsye et al. 2011). Additional experiments are needed to demonstrate whether this is true of Gm- $\alpha$ -SNAP. While there is very little information on the role(s) that  $\alpha$ -SNAPs play in plant development, antisense knockdown of the *Solanum tuberosum* (St)  $\alpha$ -SNAP (StSNAP) resulted in transgenic plants having altered morphological features (Bachem et al. 2000). In contrast to Bachem et al. (2000), the *G. max*<sub>[Williams 82/PI 518671]</sub> roots expressing the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele appeared normal in morphology and visually apparent health both prior to infection and at the end of the experiment (**Fig. 4.5**). This growth feature was surprising since it occurred even though the gene expression experiments revealed that *PRI*, *PR2*, *PR5* and *GmEREBP1* genes were more highly expressed. Elevated

activity of defense genes can be accompanied by deleterious plant growth (Rate et al. 1999). In contrast, suppressed *PR3* activity was observed when the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele was expressed in the roots of the *G. max*<sub>[Williams 82/PI 518671]</sub> genotype. This experiment indicates that PR gene activity was under specific regulation in *G. max*<sub>[Williams 82/PI 518671]</sub> roots expressing the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele and was not generically upregulated. In the experiments of Matsye et al. (2011), it was shown that Gm- $\alpha$ -SNAP was absent in syncytia undergoing a susceptible reaction. This suggests that reduced levels of  $\alpha$ -SNAP cause specific alterations in normal development that may benefit the formation of the syncytium.

#### **4.5.5 Functional experiments reveal the $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele partially suppresses *H. glycines* infection**

The overexpression of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele in the susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> genotype resulted in a partial suppression of SCN infection. This observation indicates a biological role for  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> that previously was not known. In contrast, the overexpression of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele back in the *G. max*<sub>[Peking/PI 548402]</sub> genetic background from which it was originally isolated had no antagonizing effect on its known functional defense response to SCN (Ross et al. 1958; Endo, 1965). This observation is consistent with other experiments that have expressed an allelic form of a gene back in its same genetic background as a control, showing the gene does not detrimentally affect how the plant normally responds to the experimental condition under examination (Ren et al. 2010; Li et al. 2011). The experiments presented here appear to indicate  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> can disrupt the interaction between soybean and SCN, yielding approximately a 50% reduction in infection. The *rhg1* locus that is

responsible for partial resistance has been shown to control the variation for resistance while controlling for a number of different SCN populations (Webb et al. 1995; Concibido et al. 1996, 1997).

#### **4.5.6 Understanding a $\alpha$ -SNAP role in defense through investigations involving its binding partners**

Since vesicular trafficking involves interacting partners, it is possible to gain insight into the role(s) that Gm- $\alpha$ -SNAP may have by examining the function(s) of its interacting partners like syntaxin, NSF and SNAP25. In *A. thaliana*, the  $\alpha$ -SNAP binding partner syntaxin *PEN1* was identified in genetic screens aimed to determine the genes that underlie resistance to *Blumeria graminis* f. sp. hordei (Collins et al. 2003). The *pen1* mutant interrupted the defense to pathogens at the cell wall, a process involving the formation of CWAs (Collins et al. 2003). Similar observations were made for syntaxin in the *Nicotiana benthamiana*-*Pseudomonas syringae* pv. *tabaci*-pathosystem whereby its knock-down in RNAi experiments resulted in reduced resistance (Kalde et al. 2007). A genetic pathway, involving *PEN1*, the  $\beta$ -glycosyl hydrolase *PEN2* and the ABC transporter *PEN3* transports and delivers antimicrobial compounds across the cell membrane to sites where the fungus is attempting to enter (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006). In the *G. max*-*H. glycines* pathosystem presented here, the syntaxin binding partner,  $\alpha$ -SNAP, appears to not be expressed in syncytia undergoing a susceptible reaction (Matsye et al. 2011). These observations resemble those made by Collins et al. (2003) and Kalde et al. (2007) whereby depletion of syntaxin function results in susceptibility.

In addition to the expression of the  $\alpha$ -SNAP gene, a number of studies have been done showing how structural alterations affect the binding properties of the protein. Based on experiments of  $\alpha$ -SNAP<sup>[human]</sup> (Clary et al. 1990), the premature termination of  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> translation would eliminate the C-terminal binding site for syntaxin and the only binding site for NSF. However, an  $\alpha$ -SNAP<sup>[Peking/PI 548402] 97R→Q</sup> conversion in its central domain resembles a mutant identified by Hong et al. (2004) known as hydrocephaly with hop gait (*hyh*). By studying the  $\alpha$ -SNAP<sup>[hyh]</sup> mutant, Rodriguez et al. (2011) demonstrated it has a greater binding potential for syntaxin than wild type  $\alpha$ -SNAP. This altered binding happens because NSF is less efficient in releasing  $\alpha$ -SNAP<sup>[hyh]</sup>. To rescue the exocytosis-blocking effect of  $\alpha$ -SNAP<sup>[hyh]</sup>, higher concentrations of NSF were shown to be required. In *G. max*<sup>[Peking/PI 548402]</sup>, there is an  $\alpha$ -SNAP<sup>97R→Q</sup> conversion that lies directly adjacent to and downstream from the residue creating the hypomorphic  $\alpha$ -SNAP<sup>[hyh]</sup> missense mutation. These observations demonstrate that the C-terminal  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> truncation that would normally abolish syntaxin binding could have that binding capability restored by the  $\alpha$ -SNAP<sup>97R→Q</sup> conversion. However, syntaxin binding would occur in a structurally altered manner.

Altered  $\alpha$ -SNAP activity is also observed in the *Drosophila* hypomorphic  $\alpha$ -SNAP<sup>59A→T</sup> and  $\alpha$ -SNAP<sup>168Q→STOP</sup> SNARE binding domain mutant proteins (Babcock et al. 2004). Abolishing the only binding site for NSF, as would be the case for the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele, would have a well understood consequence in its relation to SNARE. The ability to disassemble complexes of *cis*-SNAREs that form from target membrane fusion to a transport vesicle is mediated by NSF (Winter et al. 2009). However, what is known about NSF is that its binding to the SNARE complex is not



direct. NSF binding to the SNARE complex is mediated by three copies of  $\alpha$ -SNAP (Hayashi et al. 1995; McMahon and Südhof, 1995). Thus, eliminating the C-terminal domain of  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> that has the only NSF binding site would result in the abolishment of NSF binding to *cis*-SNAREs (Barszczewski et al. 2008; Winter et al. 2009). The altered  $\alpha$ -SNAP prevents the disassembly of *cis*-SNAREs that form from target membrane fusion to a transport vesicle. These structural features that are found in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele do not necessarily mean the protein would have no function. Naturally truncated alleles of genes have important biological function (Ren et al. 2010; Li et al. 2011). As shown for  $\alpha$ -SNAP, the result can be due to altered binding characteristics (Clary et al. 1990; Babcock et al. 2004; Barszczewski et al. 2008; Rodriguez et al. 2011).

Through the use of a C-terminal domain  $\alpha$ -SNAP<sup>294L→A</sup> mutant protein, Barszczewski et al. (2008) have shown that in the absence of NSF activity,  $\alpha$ -SNAP potently inhibits membrane fusion. This inhibition occurs by its binding to free syntaxin 1. By binding in this manner,  $\alpha$ -SNAP directly inhibits its SNARE function in membrane fusion. While the  $\alpha$ -SNAP<sup>[PI 88788]</sup> allele contains this highly conserved leucine residue, it is absent in the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele due to the truncated nature of the protein. Thus, in complementing the work of Clary et al. (1990), the work of Barszczewski et al. (2008) clearly demonstrates how the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele would have altered functionality by promoting its binding to syntaxin. It is unclear whether the syntaxin binding of  $\alpha$ -SNAP<sup>294L→A</sup> protein is mediated through its N-terminal or C-terminal binding site. This is important to note because the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele would lack its C-terminal syntaxin binding site due to the combination of the G→T<sup>2,822</sup> transversion

and  $G \rightarrow A^{2,832}$  transition. As demonstrated in the work of Rodriguez et al. (2011), the  $\alpha$ -SNAP<sup>97R→Q</sup> conversion in *G. max*<sub>[Peking/PI 548402]</sub> could restore syntaxin binding, but in a structurally unrelated manner. These observations demonstrate that the central domain of  $\alpha$ -SNAP performs an important functional role and may account for the different forms of the defense response observed in *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> that would be determined through experimental testing.

#### **4.5.7 The rate of membrane fusion is governed by the biophysical properties of its components**

The rates at which vesicle membranes fuse are a property that is well-documented (Holroyd et al. 1999; Sørensen et al. 2002; Martens et al. 2007; Chicka et al. 2008; Mohrmann et al. 2010) and involve  $\alpha$ -SNAP (Xu et al. 1999; Graham and Burgoyne. 2000; Swanton et al. 2000). In these animal systems, the fusion process is governed by the types of proteins and ions composing the vesicles (Geppert et al. 1994; Goda and Stevens, 1994). Therefore, altering the structure of the vesicle transport machinery affects the rate at which it functions. This principle has been observed in *A. thaliana*. While *A. thaliana* normally makes CWAs as it defends against *B. graminis* f. sp. hordei, *pen1* delays the formation of CWAs for 2 hours (Assaad et al. 2004). This means inhibiting vesicular transport through an altered genetic structure of one of its components does not necessarily abolish the function of the process, but can affect its rate of activity. Altered timing of the cellular events leading to defense to SCN in the different *G. max* genotypes is known (Kim et al. 1987; Endo 1991; Mahalingam and Skorpska, 1996). The *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 437654]</sub> genotypes have the most rapid defense responses to SCN infection, initiating between 2 and 4 dpi (Endo 1991; Mahalingam and Skorpska,

1996). Both genotypes have CWAs (Endo 1991; Mahalingam and Skorpiska, 1996) and as shown here, have the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele. In contrast, the defense response in *G. max*<sup>[PI 88788]</sup> genotype is slower, evident by 5 dpi, and lacks CWAs (Kim et al. 1987; Endo, 1991). The  $\alpha$ -SNAP<sup>[PI 88788]</sup> allelic form is also expressed during defense (Matsye et al. 2011). It would be expected that structural alterations in the protein components could greatly affect the thermodynamics of vesicular fusion during plant defense. However, almost no information exists on this process. The presence of small vesicles in cells undergoing an incompatible reaction has been observed during the defense of soybean to SCN (Endo 1991).

#### **4.5.8 Promoter bioinformatics of the $\alpha$ -SNAP alleles reveals structural differences**

Experiments designed to measure gene expression occurring during infection by virulent or avirulent SCN populations have been done in the identical soybean genotype (Matsye et al. 2011). The experiments identified that  $\alpha$ -SNAP was expressed at all time points during a resistant reaction. In such experiments where a single soybean genotype is infected by virulent or avirulent SCN populations, the same suite of promoter elements is engaged differently during susceptible or resistant reactions. This likely occurs because of genetic differences in the different SCN populations (Bekal et al. 2003; 2008) or differences in their ability to express genes (Klink et al. 2009c).

In contrast, the identification of structural differences existing between the  $\alpha$ -SNAP<sup>[PI 88788]</sup> and the  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> promoters would show how the regulation of gene expression could also play a role in defense. The presented bioinformatics results demonstrate how subtle differences in promoter sequence

composition could have an impact on TF binding capability between the  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> allele and its orthologs found in the resistant *G. max*<sub>[PI 88788]</sub> and *G. max*<sub>[Peking/PI 548402]</sub> genotypes. Functional studies in soybean have demonstrated how such differences in promoter sequence composition can affect defense through variations in gene expression (Park et al. 2004). To examine this, the promoter sequences of  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> and  $\alpha$ -SNAP<sup>[PI 88788]</sup> and their homolog in the susceptible *G. max*<sub>[Williams 82/PI 518671]</sub> genotype were compared. The  $\alpha$ -SNAP promoters from the resistant genotypes had many TF binding motifs in common with *G. max*<sub>[Williams 82/PI 518671]</sub> genotype. However, the numerous SNPs that exist between the resistant and susceptible genotypes generated TF binding site diversity. A number of TF binding motifs were found only in the resistant genotypes. Notably, the DRE1COREZMRAB17, PYRIMIDINEBOXOSRAMY1A, SEF4MOTIFGM7S and four DOFCOREZM are involved in binding of the Dof-type of TF. The Dof TF was first identified in *Zea mays* (Yanagisawa and Izui, 1993) and is involved in many basic aspects of plant metabolism, including defense (Zhang et al. 1995; Chen et al. 1996). The DRE1COREZMRAB17 TF binding motif was found only once and only in the promoter sequences of  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> and  $\alpha$ -SNAP<sup>[PI 88788]</sup>. In contrast, a number of TF binding motifs were found in locations in the  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> promoter that did not exist in the resistant genotypes. Notably, several TF binding motifs are involved in WRKY-mediated transcriptional repression. The TF binding motifs include those for ELRECOREPCR1, WBOXCWRKY, WBOXNTERF3, WBOXATNPR1 and WRKY710S. The ELRECOREPCR1 and WBOXCWRKY binding motifs that are variations on the same sequence at the same site are found only once and only in the  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> promoter.

#### 4.5.9 Potential for non-specific effects caused by the overexpression of the $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele

Gene overexpression can activate the transcription of defense genes. For example, the overexpression of *GmEREBP1* in soybean affects the expression of proteins associated with defense activities, while in some cases enhancing its susceptibility to SCN (Mazarei et al. 2007). Repeating those experiments in *G. max*<sup>[Williams 82/PI 518671]</sup> roots transformed with either with the pRAP15 control vector or roots expressing the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele resulted in induced expression of *GmEREBP1*, the salicylic acid regulated gene *PR1*, the ethylene responsive *PR2* and the SA-responsive gene *PR5* prior to infection. In contrast, the activity of the ethylene and jasmonic acid responsive gene *PR3*, a family of proteins exhibiting chitinase activity, was suppressed prior to infection. The experiments demonstrate that there is altered gene expression in the *G. max*<sup>[Williams 82/PI 518671]</sup> roots expressing the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele prior to infection. However, in contrast to the outcome obtained in Mazarei et al. (2007) the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup>-expressing *G. max*<sup>[Williams 82/PI 518671]</sup> roots suppressed SCN infection. Thus, the altered expression of *GmEREBP1*, *PR1*, *PR2*, *PR3* and *PR5* may not relate to the outcome of the experiment in any way that is relevant to SCN infection. However, it cannot be entirely ruled out as contributing to the observed suppression of infection. Experiments to separate  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> overexpression from defense gene activation have not been attempted. Experiments have shown that the dominant gain-of-function *Arabidopsis* mutant, *accelerated cell death 6 (acd6)*, exhibits induced expression of *PR1* and has elevated defenses that result in patches of both dead and enlarged cells (Rate et al. 1999). ACD-like genes are suppressed during the soybean defense response (Klink et al. 2009). Thus, the significant cross talk between signaling, metabolism and

structural protein activity can complicate the understanding of how each function during defense (Pieterse and Van Loon, 2004). Nonetheless, the results presented here suggest the possibility that the suppressed infection is a direct result of the introduced  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele in the *G. max*<sup>[Williams 82/PI 518671]</sup> roots. Clearly, the transcriptional regulation of defense genes is specific in roots overexpressing the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele since *PR3* activity is suppressed. In contrast, Collins et al. (2003) demonstrated that the absence of expression of the vesicular fusion component syntaxin was important for susceptibility to a pathogen. This observation is consistent with the observations of Matsye et al. (2011) that demonstrated the lack of expression of  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> in *G. max*<sup>[Peking/PI 548402]</sup> roots undergoing a susceptible reaction to the virulent *H. glycines*<sup>[TN8/HG-type 1.3.6.7/race 14]</sup>.

The results presented here, showing differences in coding sequence that are overlain on additional heterogeneity of promoter elements in Gm- $\alpha$ -SNAP, demonstrate the complexities in understanding the defense response of soybean to SCN. In soybean, a single genotype infected by two different SCN populations differing in their virulence can accomplish a compatible or incompatible reaction. In contrast, a single SCN population can experience either a compatible or incompatible reaction in a soybean genotype-dependent manner. These observations reinforce how a complex and duplicated genome like soybean (Doyle et al. 1999; Schmutz et al. 2010) can be used as a valuable tool to study basic aspects of niche establishment, development and maintenance by a pathogen with that knowledge also having direct and potentially very beneficial agricultural impact.

#### **4.6 Supplemental Material**

The supplementary material of this chapter can be found in the online version (<http://dx.doi.org/10.1007/s11103-012-9932-z>).

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## CHAPTER V

### XYLOGLUCAN ENDOTRANSGLYCOSYLASE/HYDROLASE INTERFERES WITH PLANT PATHOGEN INFECTION

#### 5.1 Abstract

The plant cell wall is an encasement capable of structural reorganization that occurs through enzymatic modification. Among the many known roles for cell walls are defense from pathogen infection, indicating the importance of precise spatial and temporal expression patterns of its regulating enzymes during the process. A *Glycine max* xyloglucan endotransglycosylase/hydrolase (Gm-XTH43) is highly induced, specifically in nurse cells formed by *Heterodera glycines*, during defense. Phylogenetic analyses of the XTH gene families extracted from the sequenced genomes of *G. max*, *Physcomitrella patens* (bryophyte), *Selaginella moellendorffii* (lycoperd), *Oryza sativa* (monocot), *Arabidopsis thaliana* (genetic model dicot), *Populus trichocarpa* (genetic model woody dicot) and RNA sequence data from green algae have been done. Gm-XTH43 resides within a complex locus on chromosome 17 having 6 adjacent paralogs. The duplicated nature of the locus does not appear to be the product of localized copy number variation. The overexpression of Gm-XTH43 in *G. max*<sup>[Williams 82/PI 518671]</sup> roots that normally lack a successful defense response results in a significant reduction in the ability of *Heterodera glycines* and *Rotylenchulus reniformis* to parasitize. These results indicated that the ability of *G. max* to metabolize its cell wall was important for defense, even when



parasitized by nematodes that would normally elicit a compatible interaction. The results highlight the complex metabolic events occurring during Gm-XTH-mediated defense.

## **5.2 Introduction**

The cell wall is an ancient structure, predating the colonization of land by plants. It is composed of numerous synthesized and deposited materials whose organization is dynamic and governed enzymatically (Aspinall et al. 1969; Bauer et al. 1973; Fry et al. 1992; Popper and Fry, 2003; Popper 2008; Popper and Tuohy, 2010). Part of the function of the cell wall is to respond to and reorganize as a consequence of a variety of abiotic and biotic stresses, making knowledge of its metabolism important in relation to many factors including climate change (Bacic et al. 1988; O'Neill et al. 1990; Johnson et al. 1993a, b; Carpita and Gibeau, 1993; Ridley et al. 2001; Sørensen et al. 2010; Pechanova et al. 2010). One of the most important biotic stresses whose infection capability becomes altered with abiotic change comes from plant-parasitic nematodes (Johnson et al. 1993a, b). Successful nematode infection occurs in all land plant lineages including bryophytes (Dixon, 1908), ferns (Bird and DiGennaro 2012), gymnosperms (Cobb, 1930), angiosperms (dicots [Cobb 1890] and monocots [Cobb 1893]) and even multicellular algae (Barton et al. 1892). These observations indicate that a common and ancient circuitry is in place that regulates the process of infection.

Parasitism by sedentary endoparasitic nematodes is reliant on the formation of a nurse cell from which the pathogen feeds (Ross, 1958; Bird, 1961; Balasubramanian and Rangaswami 1962; Endo, 1964; Chitwood and Lusby 1991). The nurse cell forms by one of two major developmental routes. One route results in the formation of a single giant cell that develops through hyperplasia (Bird 1961). These giant cells can be arranged

adjacently as clusters. The second route results in the formation of a syncytium that develops through cell wall degradation, merging the cytoplasm of 200-250 root cells (Endo, 1964; Jones and Northcote, 1972; Jones, 1981). Genes transferred horizontally from bacteria to a branch of plant-parasitic nematodes effect nurse cell formation (Atkinson and Harris, 1989; Smant et al. 1998; Lambert et al. 1999; De Boer et al. 1999, 2002; Robertson et al. 1999; Bekal et al. 2003; Gao et al. 2001, 2003; Huang et al. 2005; Huang et al. 2006; Bakhetia et al. 2007, 2008; Sindhu et al. 2009; Lee et al. 2010; Haegeman et al. 2011; Hamamouch et al. 2011). These genes resemble endo-1,4- $\beta$ -glucanase, cellulase, xylanase, arabinose, polygalacturonidase, pectate lyase, expansin and invertases (Abad et al. 2008). These observations indicate that the cell wall is passive during the process of parasitism, only acted on by the secreted nematode enzymes as they successfully parasitize plants (Smant et al. 1998; Yan et al. 1998; Abad et al. 2008; Hamamouch et al. 2011). However, altered plant gene expression does occur within the nurse cell during a compatible plant-nematode interaction, (reviewed in Gheysen and Fenoll, 2002; Jammes et al. 2005).

*Glycine max*, has become an important model for studying plant-nematode interaction in this regard because thousands of accessions are available for study and it can undergo compatible and incompatible interactions with giant cell and syncytium-forming plant-parasitic nematodes (Morse, 1927; Ichinohe, 1952; Ross, 1958; Rebois et al. 1970; Epps and Hartwig, 1972; Bernard et al. 1987; Atkinson and Harris, 1989; Kirkpatrick and May 1989; Barker et al. 1993; Robbins et al. 1994; Rao-Arelli, 1994; Opperman and Bird, 1998; Ma et al. 2006; Niblack et al. 2006; Matsye et al. 2011, 2012; Cook et al. 2012; Liu et al. 2012). Therefore, the plant cell can interfere with the

deployment and engagement of these injected materials by apparently transducing signals in some manner as an effective defense response as shown in both *Lycopersicon esculentum* and *G. max* (Smith et al. 1944; Ross and Brim 1957; Ross 1958; Endo, 1965, 1991; Endo and Veech 1970; Gipson et al. 1971; Riggs et al. 1973; Kim et al. 1987, 1998; Kim and Riggs, 1992; Mahalingham and Skorupska, 1996; Milligan et al. 1998). The defense response is a localized reaction that occurs at the site of nematode parasitism (Ross et al. 1958). Thus, studying the cells that are in direct contact with the nematode provides a high probability of identifying the gene expression that pertains to the plant-organism interaction although long distance signaling is also likely to be occurring. For example, by infecting *G. max*<sub>[Peking/PI 548402]</sub> with two different populations of *H. glycines*, compatible and incompatible interactions have been obtained (Klink et al. 2007, 2009a). This approach eliminates any analytical artifacts that could be caused by differences in plant genotype (Klink et al. 2011; Matsye et al. 2011). The analyses identified thousands of differentially expressed genes that associate with both the compatible and incompatible interaction (Klink et al. 2007, 2009a). Kandoth et al. (2011) repeated the experiments by examining genotypes that vary in the composition of a resistance locus known as *rhg1*.

A problem with these studies is that thousands of additional genes are discarded by the parameters of the differential expression analysis procedure. The premise of differential expression analyses is that the gene has to be expressed to measurable levels in both control and experimental samples so they can be compared in a statistically meaningful manner. However, since single cell types are under examination, it is likely that gene expression could be active in one cell type and inactive in another. Therefore,

genes active in one cell type (i.e. experimental-cells undergoing defense) and inactive in another (i.e. control-pericycle and surrounding cells) would be inadvertently discarded. Detection call methodology (DCM) is an analytical procedure whereby genes exhibiting these types of expression profiles would be identified (Birnbaum et al. 2003; Seo et al. 2004; McClintick and Edenberg 2006; Reme et al. 2008). A re-analysis of the gene expression data obtained from nurse cells confirmed that thousands of additional genes are active only during the incompatible interaction (Klink et al. 2010b; Matsye et al. 2011). Furthermore, by employing Illumina® deep sequencing, it became evident that some of those genes are also expressed at very high relative levels and map to actual resistance loci (Matsye et al. 2011). While the large number of identified genes is problematic for functional studies, the Illumina®-based analyses of the microarray-identified genes allowed them to be narrowed down to relatively few that were expressed at levels greater than 1% of the expressed sequence tags (Matsye et al. 2011). Of these genes, a *G. max* xyloglucan endotransglycosylase/hydrolase homolog (Gm-XTH) related to *A. thaliana* XTH (At-XTH23/XTR6) has been shown to be the most highly expressed gene (> 11 % of the tags) having a known function (Matsye et al. 2011). This observation indicated that precise alterations in the cell wall composition of nurse cells are important for the successful defense response and these alterations, if mediated by XTH, would be significantly different from those reported previously for endo-1,4- $\beta$ -glucanase (EGase) (Goellner et al. 2001; Matthews et al. 2013) because of their different enzymatic activities (Fry et al. 1992; Loopstra et al. 1998). The high levels of Gm-XTH43 expression in the cells specifically undergoing defense (Matsye et al. 2011) indicate a specialized and central role in resistance, rather than a passive role played by the cell

wall. Notably, plant-parasitic nematodes appear to lack XTH-like genes (Abad et al. 2008), making xyloglucan metabolism by the plant a good defense strategy.

XTHs (EC 2.4.1.207) are ancient genes found in all land plants and mounting evidence indicates their presence and activity predates the colonization of land (Fry et al. 1992, 2008; Van Sandt et al. 2006, 2007; reviewed in Eklof and Brumer, 2010). There are 33 XTHs in the genome of *A. thaliana* and approximately one-third of them exist as clusters of 2-4 genes (Blanc et al. 2000; Rose et al. 2002). XTH is different from, but distantly related to, EGase (EC 3.2.1.4) (Smith and Fry, 1991; Farkas et al. 1992; Fry et al. 1992; Nishitani and Tominaga, 1992; Nishitani 1997). Among EGase functions are cellulose synthesis and cleavage of nonsubstituted and noncrystalline 1,4- $\beta$ -linked glucan chains that compose microfibrils (Smith and Fry, 1991; Farkas et al. 1992; Fry et al. 1992; Nishitani and Tominaga, 1992; Nishitani 1997; Molhøj et al. 2001; Master et al. 2004). In contrast, XTH functions by cutting and rejoining xyloglucan chains that interconnect adjacent microfibrils (Fry et al. 1992). Furthermore, EGase lacks activity against xyloglucans (Molhøj et al. 2001; Master et al. 2004). Therefore, XTH differs from EGase in its specificity for xyloglucan, although additional substrates acted on only at very low efficiencies by XTH have been identified (Fry et al. 1992; Matsumoto et al. 1997; reviewed in Eklof and Brumer, 2010). XTHs are involved in cell wall restructuring through two means. Firstly, XTHs reversibly or irreversibly loosen cell walls (Fry et al. 1992). This process permits the expansion of cells. Secondly, cell wall restructuring can also happen through the integration of newly synthesized xyloglucans (Fry et al. 1992). In meristems, integration of xyloglucan into the cell wall accompanies a process called vacuolation in which cell expansion is accompanied by maintenance of the thickness of

the cell wall (de Vries, 1885; Whaley and Mollenhauer. 1963, Mollenhauer and Whaley, 1963; Matile and Moor, 1968; Rose et al. 2002). XTH gene expression becomes highly induced in rapidly elongating tissues and their expression is regulated by auxin (Potter and Fry 1993; Pritchard et al. 1993; Nishitani et al. 1996; Xu et al. 1996). Furthermore, XTHs associate with vesicles, indicating regulated trafficking as it is transported to its site of activity (Yokoyama and Nishitani, 2001a; Albert et al. 2004). This association resembles that identified for glycosylphosphatidylinositol (GPI)-anchored EGase (Brummell et al. 1997). Vesicles are associated with defense to *H. glycines* and the membrane fusion and vesicular trafficking gene, alpha-soluble NSF attachment protein ( $\alpha$ -SNAP), is involved in this process (Ross 1958; Endo, 1965, 1991; Endo and Veech 1970; Novick et al. 1980; Kim et al. 1987, 1998; Clary et al. 1990; Mahalingham and Skorupska, 1996; Matsye et al. 2012; Cook et al. 2012). These observations may explain the presence of cell wall appositions, membranous structures known to be involved in the defense response, for certain *G. max* genotypes undergoing defense to plant-parasitic nematodes (Aist, 1976; Kim et al. 1987, 1998; Mahalingham and Skorupska, 1996). Furthermore, the movement of XTH within the cell is regulated differently during the cell cycle (Yokoyama and Nishitani 2001a). During interphase, XTH is secreted into the apoplast which is mediated by the endoplasmic reticulum (ER)-Golgi apparatus (Yokoyama and Nishitani 2001a). In contrast, XTH localizes to the phragmoplast and cell plate during cytokinesis as vesicles deliver the enzyme (Yokoyama and Nishitani 2001a). Xyloglucan and other cell wall components can also be internalized (Baluska et al. 2005). Furthermore, XTH expression correlates with lysigenous processes leading to aerenchyma formation in the cortical tissues of flooded roots in *Zea mays* (Peschke and

Sachs, 1994; Saab and Sachs, 1995, 1996). The process of cell death occurring during the process of lysigeny is long known in plant biology, but its mechanism has only recently started to become understood at the molecular level (Karsten, 1857; Mühlenbock et al. 2007). Furthermore, promoter element analyses have provided some evidence linking lysigeny and nurse cell development (Tucker et al. 2011). The defense response was not studied in those analyses (Tucker et al. 2011).

While much information is available for the enzymatic activity and expression pattern of XTH, almost no functional information is available for its involvement in plant-organism interactions and/or defense. However, overexpression of XTH in *Populus* sp., results in the initial shortening of xyloglucan chain length which is later unaffected by the higher levels of XTH as the cells mature, maintaining a well-defined cell boundary (Nishikubo et al. 2011). This work provided mechanistic insight into how plants can use XTH to limit cellular expansion. Nishikubo et al. (2011) discussed that the xyloglucan chain length rendered the cell wall matrix inaccessible to enzymatic degradation. Preventing access of nematode-synthesized cell wall degrading enzymes would be a simple way to prevent cellular expansion and nurse cell development. These observations, along with those recently reported for the involvement of  $\alpha$ -SNAP in defense; indicate that Gm-XTH could be part of a genetic program leading to the restriction of the boundary of the nurse cell and their subsequent collapse during the defense response (Matsye et al. 2012; Cook et al. 2012). These observations are in agreement with Ross (1958) in which the cell boundary is well-delimited during defense to *H. glycines* and the observed sustained expression of Gm-XTH during its defense response to *H. glycines* (Matsye et al. 2011).

In the analysis presented here, functional experiments in the form of Gm-XTH overexpression lead to a suppression of nematode parasitism in an otherwise susceptible *G. max* genotype. The resistance obtained by Gm-XTH overexpression is not limited to *H. glycines* since *R. reniformis* infection is also detrimentally affected, indicating a common mechanism.

### 5.3 Materials and Methods

#### 5.3.1 Details

The phylogenetic tree was made in CLUSTALW (<http://www.genome.jp/tools-bin/clustalw>) in default, producing both unweighted pair group method with arithmetic mean (UPGMA) and neighbor joining (N-J) unrooted phylogenetic trees with branch lengths. The signal peptide was predicted using the SignalP-4.1 prediction server (<http://www.cbs.dtu.dk/services/SignalP/>) on default (Petersen et al. 2011). *N*-glycosylation sites were predicted using *N*-GlycoSite (Zhang et al. 2005) ([http://www.hiv.lanl.gov/cgi-bin/GLYCOSITE/glycosite\\_main.cgi](http://www.hiv.lanl.gov/cgi-bin/GLYCOSITE/glycosite_main.cgi)) set on default. Phosphorylation sites were predicted using KinasePhos 2.0 (Wong et al. 2.0) set on default (<http://kinasephos.mbc.nctu.edu>). Disulfide bridges were predicted using DiANNA 1.1 Web Server (<http://clavius.bc.edu/~clotelab/DiANNA/>) (Ferre and Clote, 2005) set on default. The mapping position of Satt082 was obtained at Soybase (<http://soybase.org/>) (Cregan et al. 1999). The sequence variation data of Gm-XTH was obtained from McHale et al. (2012). XTH gene sequences used on the phylogenetic analyses were obtained from *Physcomitrella patens*, *Selaginella moellendorffii*, *Oryza sativa*, *Glycine max*, *Arabidopsis thaliana* and *Populus trichocarpa* at <http://phytozome.net/> (Schmutz et al. 2010).



### 5.3.2 Vector construct and pipeline

The pRAP15 (overexpression) and pRAP17 (RNAi) vectors were used to alter gene expression (Klink et al. 2009b; Matsye et al. 2012). PCR primer pairs (**Supplemental Table 1**) were used to amplify the gene of interest (GOI). Amplicons were gel purified in 1.0% agarose using the Qiagen® gel purification kit, ligated into the directional pENTR/D-TOPO® vector and transformed into chemically competent *E. coli* strain One Shot TOP10. Chemical selection was on LB-kanamycin (50 µg/ml) according to protocol (Invitrogen®). Amplicon sequencing confirmed the sequence matched its original Genbank accession. The *G. max* amplicon was shuttled into the pRAP15 or pRAP17 destination vector by a LR clonase reaction according to protocol (Invitrogen®). The pRAP15 vector, engineered with the GOI was transformed into chemically competent *A. rhizogenes* strain K599 (K599) (Haas et al. 1995) using the freeze-thaw method (Hofgen and Willmitzer 1988). Selection was performed on LB-tetracycline (5 µg/ml) according to Klink et al. (2008).

### 5.3.3 Genetic transformations

Many of the transformation experiments were performed in *G. max*<sub>[Williams 82/PI 518671]</sub> (Bernard and Cremeens, 1988) because it lacks a functional defense response to *H. glycines* (Atkinson and Harris, 1989), *Rotylenchulus reniformis* (Robbins et al. 1994) and *Meloidogyne incognita* (Kirkpatrick and May 1989) and has a sequenced genome (Schmutz et al. 2010) that would be useful for later deep sequencing work. The *H. glycines* and *R. reniformis* -resistant *G. max*<sub>[Peking/PI 548402]</sub> (Ross, 1958;) was used in described experiments. The *H. glycines* resistant *G. max*<sub>[PI PI 88788]</sub> (Epps and Hartwig, 1972) and *G. max*<sub>[PI 437654]</sub> (Rao-Arelli, 1994) were used in described experiments.

The non-axenic *G. max* transformation was used for the experiments (Matsye et al. 2012). The chimeras were planted in a sterilized 50-50 mixture of a Freestone fine sandy loam (46.25 % sand, 46.50 % silt, and 7.25 % clay) and a sandy (93.00 % sand, 5.75 % silt, and 1.25 % clay) soil and allowed to recover for a week (Matsye et al. 2012). At the end of the experiment, the roots were checked for eGFP expression. This procedure was done to screen out plants that may have developed untransformed roots during the infection period.

#### **5.3.4 Quantitative real-time PCR (qPCR)**

The qPCR procedure was performed according to Matsye et al. (2012) using qPCR Taqman® 6-carboxyfluorescein (6-FAM) probes (MWG Operon; Birmingham, AL). The quencher was the Black Hole Quencer (BHQ1) (MWG Operon). The qPCR reaction conditions included a 20 µl Taqman Gene Expression Master Mix (Applied Biosystems; Foster City, CA), 0.9 µl of µM forward primer, 0.9 µl of 100 µM reverse primer, 2 µl of 2.5 µM 6-FAM (MWG Operon®) probe and 4.4 µl of template DNA. The qPCR reactions were performed on an ABI 7300 (Applied Biosystems®). The qPCR conditions included a preincubation of 50° C for 2 min, followed by 95° C for 10 min. This was followed by alternating 95° C for 15 sec followed by 60° C for 1 min for 40 cycles. The qPCR differential expression tests were performed according to Livak and Schmittgen (2001).

#### **5.3.5 Nematode analysis**

Female *H. glycines*<sub>[NL1-Rhg/HG-type 7/race 3]</sub> were purified by sucrose flotation (Jenkins, 1964; Matthews et al. 2003). The pi-J2s were concentrated by centrifugation in

an IEC clinical centrifuge for 30 seconds at 1,720 rpm to a final optimized concentration of 2,000 pi-J2s/ml. Each root was inoculated with one ml of nematodes at a concentration of 2,000 J2s/ml per root system (per plant). The nematodes were introduced to the soil and allowed to infect roots for thirty days in the greenhouse. Confirmation of infection in representative infected root samples was performed by the acid fuchsin staining procedure of Byrd et al. (1983). Female indices (FI) were calculated after collecting females over nested 20 and 100-mesh sieves. Additionally, the soil was washed several times and the rinse water sieved to assure collection of all females. Females present in ~30 ml of water were washed into 150 ml beakers. The females were then poured evenly into a Buchner funnel system, on a 9 cm diameter S & S #8 Ruled filter paper (Schleicher and Schuell; Keene, NH) under constant vacuum. The filters were stored in standard disposable Petri plates, wrapped in parafilm and stored at 4°C. The females were counted immediately under a dissecting microscope after collection. The FI was calculated according to the original work of Golden et al. (1970) that has been further modified (Riggs and Schmitt, 1988, 1991; Niblack et al. 2002; Klink et al. 2009b). The FI is calculated as  $FI = (N_x/N_s) \times 100$ , where  $N_x$  is the average number of females on the test cultivar and  $N_s$  is the average number of females on the standard susceptible cultivar. In our genetic engineering experiments,  $N_x$  was the pRAP15-transformed line that had the engineered GOI. In the experiments presented here,  $N_s$  would be the pRAP15 control in their respective *G. max*<sub>[Williams 82/PI 518671]</sub> or *G. max*<sub>[Peking/PI 548402]</sub> genotypes. Because the pRAP15 control has the *ccdB* gene, it also controls for non-specific effects of protein overexpression that does not pertain to *G. max* biology (Klink et al. 2009b; Ibrahim et al. 2010; Matsye et al. 2012; Matthews et al. 2013). FI were also calculated as a function of

root mass. A statistical analysis of the effects of the genetically engineered roots was done using the Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test,  $p < 0.05$ .

*Rotylenchulus reniformis* were extracted from plant samples according to the procedures of Lawrence et al. (2005). Soil and roots were submerged in tap water and agitated by hand to loosen the soil from the roots. *R. reniformis* were brought into suspension by hand agitation followed by gravity sieving and sucrose floatation (specific gravity = 1.13). The chimeras were planted in a sterilized 50-50 mixture of a Freestone fine sandy loam (46.25 % sand, 46.50 % silt, and 7.25 % clay) and a sandy (93.00 % sand, 5.75 % silt, and 1.25 % clay) soil and allowed to recover for a week according to the procedures of Matsye et al. (2012). The chimeras were then infected with *R. reniformis* according to the procedures of Lawrence et al. (2005). *R. reniformis* were allowed to infect for 50 days post infection (dpi) when the nematodes were extracted and the reproductive index (RI) was calculated as the number of eggs + vermiform nematodes at test termination (Pf)/initial inoculation level (PI). A statistical analysis of the effects of the genetically engineered roots was done using the Mann–Whitney–Wilcoxon (MWW) Rank-Sum Test,  $p < 0.05$ .

## **5.4 Results**

### **5.4.1 Gm-XTH**

Cytological studies have shown that an extensive accumulation of vesicles occurs during an incompatible reaction between *G. max* and *H. glycines* (Ross 1958; Endo, 1965, 1991; Endo and Veech 1970; Kim et al. 1987, 1998; Mahalingham and Skorupska, 1996). Vesicles are known to deliver cargo to sites of activity. The vesicular transport and membrane fusion machinery is composed of, in part, the highly conserved  $\alpha$ -SNAP

protein (Novick et al. 1980, Clary et al. 1990; Hong et al. 2004; Babcock et al. 2004).  $\alpha$ -SNAP performs well known and crucial roles in plant defense, including incompatible interactions occurring between *G. max* and *H. glycines* (Collins et al. 2003; Kalde et al. 2007; Kwon et al. 2008; Pajonk et al. 2008; Meyer et al. 2009; Matsye et al. 2011, 2012; Cook et al. 2012). An analysis of the role of genes identified in nurse cell gene expression experiments and functionally studied in *G. max* show many of them are associated directly or indirectly with vesicles or membranes (**Supplemental Table 2**) (Matsye et al. 2012; Cook et al. 2012; Liu et al. 2012; Matthews et al. 2013). Thus, defense to plant-parasitic nematodes like *H. glycines*, may occur through the coalescence of several metabolic processes at the site of infection. This led to the hypothesis that highly expressed genes shown to have associations with vesicles and/or membranes were important for defense to plant-parasitic nematodes that are sedentary and endoparasitic in nature. Quantitative exon sequencing analyses identified genes expressed to high levels specifically in syncytia undergoing the defense response (Matsye et al. 2011). One of those genes was related to a xyloglucan endotransglycosylase (XET)-related (XTR) homolog 6 (Gm-XTR6) (*Arabidopsis* Genome Initiative 2000). Gm-XTR6 (Glyma17g07250.1) represented over 11 % of the expressed tags (Matsye et al. 2011). XTR has undergone reclassification and are now referred to as xyloglucan endotransglucosylase/hydrolase (XTH) to reflect their xyloglucan endotransglucosylase (XET) and xyloglucan endohydrolase (XEH) activities (Rose et al. 2002). The expression of XTHs has been shown to associate with a number of processes in different plants requiring reorganization of the cell wall (**Supplemental Table 3**). Some of these processes involve plant-organism interactions (**Supplemental Table 3**). XTHs are

elicitor inducible and their expression can be regulated by hormones (**Supplemental Table 3**). While Gm-XTH is a gene that is highly expressed during defense to *H. glycines*, its function had yet to be determined (Matsye et al. 2011). In addition, XTH associates with membranes (Yokoyama and Nishitani, 2001a). These observations, and the availability of the *G. max* genome, prompted and permitted a bioinformatics analysis of soybean XTH (Schmutz et al. 2010).

A bioinformatics analysis of *G. max* XTH is presented (**Supplemental Tables 4 and 5**). There have been 71 XTH-like genes annotated in the *G. max* genome (Schmutz et al. 2010) (**Supplemental Table 4**). Analysis of the XTH genes presented here resulted in some of the annotated XTH accessions having no identified gene (**Supplemental Table 4**). Furthermore, some of the XTH genes were represented by alternate transcripts (**Supplemental Table 4**). Two genes appeared to represent pseudogenes, characterized by premature stop codons (**Supplemental Table 4**). Several Phytozome-annotated Gm-XTH sequences had no accompanying transcript or protein. The total number of XTH genes identified after these gene properties were accommodated for was 53 (**Fig. 5.1; Supplemental Table 5**). Phytozome has an option whereby gene family counts can be obtained without further bioinformatics analyses. Thus, the difference in total number of XTH genes presented here and those in Eklof and Brumer (2010) could be that the gene sequences presented here were isolated and examined. A phylogenetic analysis was performed to demonstrate graphically the relationships between the different Gm-XTH paralogs (**Supplemental Figure 1**). An analysis was then done that examined *G. max* in relation to other organisms having sequenced genomes including the genetic model, *Arabidopsis thaliana*, the genetic woody model *Populus trichocarpa*, a model monocot

*Oryza sativa*, the model lycopod *Selaginella moellendorffii* and the model bryophyte *Physcomitrella patens*. The analysis demonstrated the XTH genes fit into the three group model originally proposed by Campbell and Braam (1999), although the rooting of Group 2 was well within Group 1 (**Supplemental Figure 2**).

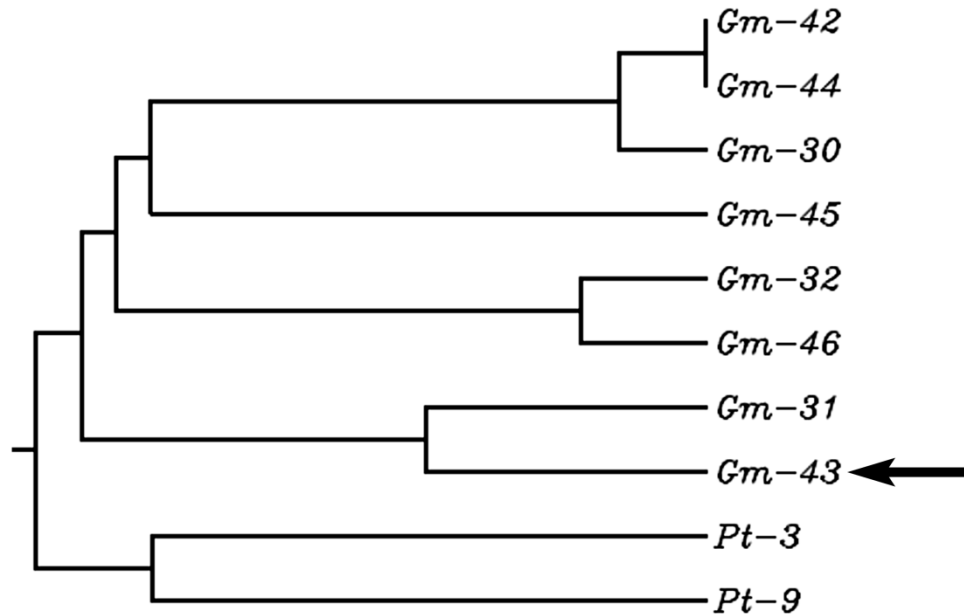


Figure 5.1 Unweighted pair group method with arithmetic mean (UPGMA) XTH phylogenetic comparison on default settings of CLUSTALW

Inset from Supplemental Figure 2. The *G. max* (Gm) Group 2 XTH genes composing parts of the chromosome 13 and chromosome 17 are shown in relation to two XTH sequences of *Populus trichocarpa* (Pt). Protein sequences were obtained from [www.http//Phytozome.net](http://Phytozome.net). For details see **Supplemental Figure 2**.

Protein blast searches of the Gm-XTH proteins identified the *A. thaliana* homologs (**Supplemental Table 5**). The *G. max* XTH gene shown to be highly induced during the defense response to *H. glycines* was most closely related to the annotated *A. thaliana* XTR6 (*Arabidopsis* Genome Initiative 2000; Matsye et al. 2011). The XTR genes named in the *Arabidopsis* Genome Initiative (2000) underwent a reclassification to

reflect more accurately the biochemical activity of the members of the gene family (Rose et al. 2002). Thus, *A. thaliana* XTR6 is XTH23. A numbering scheme for the *G. max* XTH genes is presented that is based on chromosome position rather than gene similarity (**Supplemental Table 5**). The Gm-XTH identified in Matsye et al. (2011) is Glyma17g07250.1, designated as Gm-XTH43 (**Supplemental Table 5**). An analysis of chromosome 17 identified 5 other closely related paralogous Gm-XTH genes flanking the Glyma17g07250.1 sequence (**Supplemental Figures 3 and 4**). The 6 total Gm-XTH paralogs clustered on chromosome 17 are flanked 5' by Glyma17g07210.1 that encodes NADH dehydrogenase I chain G and 3' by Glyma17g07290.3 encoding a calmodulin binding protein-like gene (**Supplemental Figure 3**). Gm-XTH paralogs homologous to At-XTH23 are found elsewhere in the genome on chromosome 13 (Glyma13g01120.1) (**Supplemental Table 5**). Like chromosome 17, chromosome 13 has a cluster of 4 tandem arranged At-XTH23-like genes with a 5<sup>th</sup> paralog located slightly upstream of Glyma13g01110.1. Phylogenetic analyses show the relationships of the chromosome 13 and chromosome 17 paralogs (**Supplemental Figure 1**). Gm-XTH43 is significantly different from, but distantly related to EGases (Fry et al. 1992; Matsumoto et al. 1997; reviewed in Eklof and Brumer, 2010) (**Supplemental Figure 5**). In addition to the numerous XTH paralogs, there also exists genotype-specific sequence variation as observed by McHale et al. (2012) (**Supplemental Table 6**). Notably, of the XTH paralogs found in the chromosome 17 gene cluster (**Supplemental Figure 3**), only Gm-XTR43 has been shown to exhibit genotype-specific sequence variation in the form of an AT motif repeated 24.5 times on average (**Supplemental Table 6**). A number of post-translational modifications have been predicted and/or determined for XTHs and are



examined (Okazawa et al. 1993; Campbell and Braam, 1998; Henriksson et al. 2003; Kallas et al. 2005; Van Sandt et al. 2006; Genovesi et al. 2008; Maris et al. 2009; Stratilová et al. 2010). XTHs are known to have an N-terminal signal peptide, allowing for secretion into the apoplast (Yokoyama and Nishitani, 2001a). Using the SignalP 4.1 Server, the first 22 amino acids (aa) of Gm-XTH43 are predicted to compose the signal peptide (**Supplemental Figure 6**). There is also the DEIDFEFLG catalytic sequence motif that allows the protein to function both as a hydrolase and transferase (Okazawa et al. 1993; Campbell and Braam 1998). XTH proteins can be *N*-glycosylated (Campbell and Braam, 1998; Henriksson et al. 2003; Kallas et al. 2005; Van Sandt et al. 2006; Genovesi et al. 2008; Maris et al. 2009; Stratilová et al. 2010). Positions 106 and 232 are predicted to be *N*-linked glycosylation sites. Disulfide bridges exist in XTHs and are important for their function (Campbell and Braam, 1998; Van Sandt et al. 2006). Disulfide bridges are predicted for Gm-XTH43 bridging aa positions 19-284 and 220-229. There are 8 predicted phosphorylation sites, 4 serine (aa positions 120, 231, 243, 239), 2 threonine (aa positions 95, 159) and 2 tyrosine (aa positions 66, 131).

#### **5.4.2 Functional analysis of Gm-XTH43**

Prior analyses indicated that loci exhibiting copy number variation performed a role in *G. max* defense to *H. glycines* (Matsye et al. 2012; Cook et al. 2012). Analyses of different genotypes that would determine this were not performed. However, functional studies were done to determine whether the high levels of Gm-XTH43 expression observed in the nurse cells undergoing defense and the duplicated nature of the chromosome 17 XTH locus reflected a role in plant defense (Matsye et al. 2011). Overexpression has been shown to be a valuable tool in determining the role of XTH

genes, providing methodological support for the approach (Nishikubo et al. 2011). The overexpression of Gm-XTH43 can be accomplished in the roots with no obvious influence on their growth or development (**Supplemental Figure 7**). This observation resembled those made by Nishikubo et al. (2011) in *Populus* sp. whereby only subtle changes in morphology were observed in plants overexpressing XTH. Gene overexpression was done to recapitulate the transcriptional activity observed in experiments that identified Gm-XTH43 during the defense response to *H. glycines* (Matsye et al. 2011). Overexpression of Gm-XTH43 was performed in *G. max*<sub>[Williams 82/PI 518671]</sub> which lacks a functional defense response to *H. glycines*, as well as *Rotylenchulus reniformis* and other plant-parasitic nematodes (Bernard and Cromeens, 1988; Atkinson and Harris, 1989; Kirkpatrick and May 1989; Robbins et al. 1994). Infection of plants carrying transgenic roots for Gm-XTH43 resulted in a significant negative effect on parasitism by the syncytium-forming *H. glycines* and *R. reniformis* (**Fig. 5.2**). As a control, parasitism was not affected by the expression of genes known to not be involved in defense (**Fig. 5.2**). The results demonstrated that Gm-XTH43 antagonized the process of compatibility that is normally found in *G. max*<sub>[Williams 82/PI 518671]</sub>. The only other overexpressed XTH gene to show a developmental consequence has been studied in *Populus* sp. (Pt-XTH16) (Nishikubo et al. 2011). However, Pt-XTH16 resides in Group 1 that are more distantly related to Gm-XTH43 (Group 2) (**Supplemental Figure 8**).

## Overexpression analysis

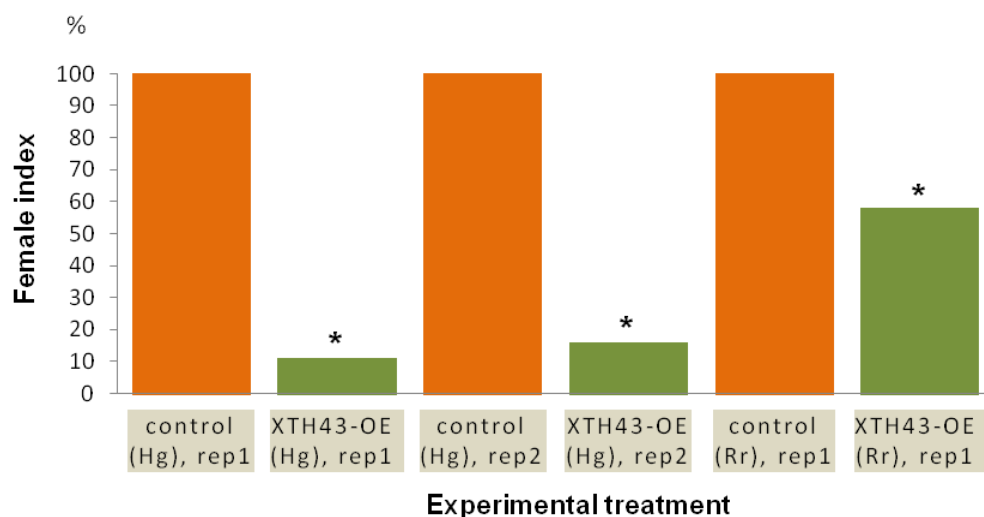


Figure 5.2 Overexpression (OE) analysis

The Female Index (FI) of the pRAP15 control plants, having a FI of 100%, is calculated as a comparison to itself and shown solely for comparative purposes. **Legend:** **bar1**, control (Hg) rep1, represents the control replicate 1 where the pRAP15 vector lacking any XTH43 allele was engineered into the *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background, plants (n=11) were infected with *H. glycines* <sub>[NL1-Rhg/HG-type 7/race 3]</sub>; **bar2**, XTH43-OE (Hg) rep1, represents the treatment replicate 1 where XTH43 allele is overexpressed in *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background, plants (N=40) were infected with *H. glycines* <sub>[NL1-Rhg/HG-type 7/race 3]</sub>; **bar3**, control (Hg) rep2, represents the control replicate 2 where the pRAP15 vector lacking any XTH43 allele was engineered into the *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background, plants (N=25) were infected with *H. glycines* <sub>[NL1-Rhg/HG-type 7/race 3]</sub>; **bar4**, XTH43-OE (Hg) rep2, represents the treatment replicate 2 where XTH43 allele is overexpressed in *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background, plants (N=34) were infected with *H. glycines* <sub>[NL1-Rhg/HG-type 7/race 3]</sub>; **bar5**, control (Rr) rep1, represents the control replicate 1 where the pRAP15 vector lacking any XTH43 allele was engineered into the *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background, plants (N=22) were infected with *R. reniformis*; **bar6**, XTH43-OE (Rr), rep1, represents the treatment replicate 1 where XTH43 allele is overexpressed in *G. max*<sub>[Williams 82/PI 518671]</sub> genetic background, plants (N=22) were infected with *R. reniformis*. (\*) = statistically significant (P value < 0.05).

### 5.4.3 The gene expression of defense

The most important mapped genes that provide resistance to *H. glycines* reside in the *rhg1* and *Rhg4* loci (Caldwell et al. 1960; Matson and Williams, 1965). Recent

studies in *G. max* have identified the genes that compose the *H. glycines* resistance loci, *rhg1* and *Rhg4* (Kim et al. 2010; Matsye et al. 2011, 2012; Cook et al. 2012; Liu et al. 2012). Transcriptional mapping studies of the *rhg1* locus identified an amino acid transporter (AAT) and an  $\alpha$ -SNAP that are expressed regardless of genotype and time point studied, while a wound inducible protein (WIP) is expressed only in genotypes having the *G. max*<sub>[PI 88788]</sub> type of defense response (Kim et al. 2010; Matsye et al. 2011). Each of the three genes contributes to defense (Matsye et al. 2012; Cook et al. 2012). The *Rhg4* gene is a serine hydroxymethyltransferase that plays a role in photorespiration in the mitochondrion and mitigates abiotic and biotic stress (Woo, 1979; Moreno et al. 2005; Liu et al. 2012). While *rhg1* and *Rhg4* require each other for resistance (Concibido et al. 2004), it is unclear how this would happen since the genes serve two vastly different roles in the cell (Woo, 1979; Novick et al. 1980; Li and Bush 1991; Concibido et al. 1997, 2004). Experiments using qPCR were performed to determine if Gm-XTH43 expression affects the activity of genes pertaining to *rhg1* and *Rhg4*-mediated defense (**Supplemental Table 7**). Furthermore, the expression of pathogenesis-related (PR) genes is known to accompany *H. glycines* infection and is a valuable tool to gain insight into basic metabolic processes during defense (Mazarei et al. 2007). These genes include the salicylic acid regulated PR1 (Antoniw and Pierpoint 1978), the ethylene responsive PR2 (Kauffmann et al. 1987), the ethylene and jasmonic acid responsive PR3 (Legrand et al. 1987) and the SA-responsive PR5 (Kauffmann et al. 1990) defense genes. Prior experiments examining their activity in roots overexpressing the *rhg1* locus gene,  $\alpha$ -SNAP, demonstrated induction of PR1, PR2 and PR5 (Matsye et al. 2012). In contrast, PR3 expression was suppressed (Matsye et al. 2012). Quantitative

experiments using qPCR reveal how Gm-XTH43 overexpression influenced the relative amounts of these transcripts (**Supplemental Table 7**). While these experiments provide insight into gene expression during Gm-XTH43-mediated defense, it was likely to be only a small picture of the transcriptional activity that is actually occurring.

## 5.5 Discussion

The cell wall is a structure that has aided plants in colonizing land and defending itself from biotic and abiotic stress (Bacic et al. 1988; O'Neill et al. 1990; Carpita and Gibeaut, 1993; Ridley et al. 2001; Sørensen et al. 2010). Part of this adaptive advantage of the cell wall is its ability to be remodeled as its enclosed cell undergoes normal developmental processes and encounters stress. A major biotic stress for plants is plant-parasitic nematodes, global in their distribution and capable of successfully infecting all lineages of land plants and multicellular algae (Cobb 1890; Barton et al. 1892; Cobb 1893; Dixon, 1908; Cobb, 1930; Bird and DiGennaro 2012). Successful infection by sedentary endoparasitic nematodes involves the formation of a nurse cell in the host, a process that is accompanied by a reduction in host fitness which presents a significant challenge to agriculture (Sasser and Freckman 1987; Chitwood 2003). While this ubiquitous class of plant-organism interactions presents a significant challenge for agriculture, it represents an important model for biological analysis because unlike other plant pathogens the sedentary endoparasitic nematodes induce the formation of a well-delimited niche that is amenable to analysis at the cellular level. In this regard, *G. max* has emerged as an important model to study plant-parasitic nematode biology because it is one of the most important world crops, is polyploid like most agricultural plants, can be used as a genetic system and is able to undergo both compatible and incompatible

interactions for some of the most devastating syncytium and giant cell forming nematodes (Ichinohe 1952; Ross, 1958; Rebois et al. 1970; Epps and Hartwig, 1972; Atkinson and Harris, 1989; Kirkpatrick and May 1989; Barker et al. 1993; Robbins et al. 1994; Rao-Arelli, 1994; Opperman and Bird, 1998; Niblack et al. 2006; Wood et al. 2009; Klink et al. 2010; Matsye et al. 2011, 2012; Li et al. 2011; Cook et al. 2012; Liu et al. 2012). Furthermore, *G. max* can also be used as a model for the effect that climate change has on infection because changes in water availability and temperature influence the positioning and development of nurse cells (Hamblen et al. 1972; Johnson et al. 1993a, b; Anand et al. 1995).

### **5.5.1 XTH and defense**

The cell wall is composed of a number of carbohydrate and proteinaceous molecules, long known to be acted on and modified by enzymes of plant or non-plant origin (Newcombe 1899; Cleveland 1923; Trager 1932). Several studies have shown that a number of plant-parasitic nematodes have an unprecedented capability to target the cell wall during infection (Smant et al. 1998; Yan et al. 1998; Abad et al. 2008; Hamamouch et al. 2011). Furthermore, effectors released by these nematodes can directly target and presumably inactivate cell wall metabolizing enzymes such as EGase (Hamamouch et al. 2011). EGases have a well understood role in normal cell wall metabolism and processes induced by nematode parasitism (Byrne et al. 1975; Hayashi et al. 1984; Hayashi and Maclachlan 1984; Smant et al. 1998; Yan et al. 1998; Bakheta et al. 2007, 2008; Hamamouch et al. 2011; Matthews et al. 2013). However, cell wall degradation engaged through cellulase activity is well documented in numerous plant and other non-nematode organisms as they are used to reorganize the cell wall for developmental purposes and

provide a carbon source for dietary needs (Newcombe 1899; Cleveland 1923; Trager 1932). EGases function by cleaving 1,4- $\beta$ -glucosyl linkages and their expression is dependent on supraoptimal auxin concentrations (Byrne et al. 1975; Hayashi et al. 1984; Hayashi and Maclachlan 1984). EGases have been isolated specifically from the subventral esophageal glands of *Globodera rostochiensis* and *H. glycines*, indicating the existence of a complex interaction occurring at the interface of infection (Smant et al. 1998; Abad et al. 2008). Furthermore, *H. glycines* have 4 EGases and their targeted disruption by RNAi impedes the ability of the nematode to parasitize (Yan et al. 1998; Bakhetia et al. 2007, 2008). Subsequently, an *H. schachtii* effector called 30C02 has been shown to bind to *A. thaliana* EGase (Hamamouch et al. 2011). Therefore, the nematode appears to be deactivating a plant EGase while injecting its own enzymatic form for a predetermined role in parasitism. Furthermore, the overexpression of the *H. schachtii* 30C02 effector in *A. thaliana* increased parasitism by 50% while its targeted disruption through host-mediated RNA interference decreased parasitism by 75% (Hamamouch et al. 2011). In contrast, overexpression of *A. thaliana* EGase (*At4g16260*) reduced parasitism by 22-38% (Hamamouch et al. 2011). These observations provided evidence that EGase activity may be part of a general mechanism occurring during plant-nematode interactions to promote parasitism. However, the low reduction in parasitism by *A. thaliana* EGase overexpression in *A. thaliana* indicated that it may not be central to the process. Studying the defense response of *A. thaliana* to *H. schachtii* is complicated because *A. thaliana* does not exhibit a defense response and thus may not be an ideal model. In contrast, *G. max* EGases have been shown to be differentially expressed and induced over 100-fold during the defense response to *H. glycines* (Klink et al. 2009a).

While this means that EGase expression is occurring in both uninfected and infected cells undergoing defense, its transcriptional activity is at higher levels in the cells undergoing defense. This observation led to the hypothesis that high levels of EGase expression were important for defense in *G. max*. The overexpression of *G. max* EGase (Glyma08g02610) resulted in a reduction in parasitism by 65% (Matthews et al. 2013). The incomplete resistance observed in *H. glycines*-infected roots overexpressing *G. max* EGase indicated that other enzymes, restricted in their expression to the cells undergoing the defense response, may be even more central to the process. In addition, the targeting of genes like EGase by nematode effectors indicated that it was imperative that the plant express other genes that would circumvent the activities of the nematode secretion. The expression characteristics identified for Gm-XTH43 made it a prime candidate in this regard, especially since XTH-like genes have not been identified in plant-parasitic nematodes (Abad et al. 2008; Matsye et al. 2011).

A majority of the hemicellulose component of the cell walls of all land plants is xyloglucan, molecules that bind tightly to cellulose microfibrils (Aspinall et al. 1969; Bauer et al. 1973; Keegstra et al. 1973; Wilder and Albersheim 1973). The hydrogen bonding of xyloglucans form links between the adjacent microfibrils that coil around the cell (Valent and Albersheim, 1974; Hayashi et al., 1987, 1994a, 1994b; Hayashi, 1989; Fry, 1989a, b; McCann et al., 1990; Fry et al. 1992; Passioura and Fry, 1992). The xyloglucan molecules can be loosely arranged, allowing growth and expansion and access to enzymes. In contrast, the xyloglucan molecules can be tightly arranged, preventing access to enzymes (Nishikubo et al. 2011). Thus, the re-organization of the cell wall in accomplishing defense would likely require a number of enzymes acting on



the different types of chemical crosslinking occurring on and between the cell wall molecules. One of the most highly expressed genes occurring during defense to *H. glycines* was related to a xyloglucan endotransglycosylase (XET), named XET-related (XTR) (*Arabidopsis* Genome Initiative 2000). The specific paralog was XTR6 (Matsye et al. 2011). Subsequently, XTRs were reclassified as xyloglucan endotransglucosylase/hydrolase (XTH) to reflect their xyloglucan endotransglucosylase (XET) and xyloglucan endohydrolase (XEH) activities (Rose et al. 2002). The effect that Gm-XTH43 overexpression has on *H. glycines* and *R. reniformis* infection is consistent with the hypothesis that its observed high levels of transcriptional activity indicated a role in plant defense. Meanwhile, control experiments revealed the specificity of the effect that Gm-XTH43 overexpression had on *H. glycines* and *R. reniformis* parasitism. These observations also indicated many genes would be altered in their expression as a consequence of Gm-XTH43 overexpression due to the transcriptional activity and delivery mechanism required to process and transport it to its site of function. However, while XTHs have well understood biological roles (Fry et al. 1992; Rose et al. 2002; Fauré et al. 2006), it has been unclear how high levels of the cell wall loosening activities of XTH would relate to the observed inhibition of nurse cell wall boundary expansion observed during infection (Ross, 1958; Endo, 1965). Recent work in *Populus* sp. overexpressing XTH may explain how the sedentary endoparasitic nematodes fail to expand the boundary of the feeding site during infection. In *Populus* sp., the overexpression of XTH genes is accompanied by subtle influences in plant growth. The subtle influence on growth was shown to be caused by a slight reduction in stem elongation that was accompanied by wood fibers that were 4-8% narrower and vessel

elements that were 6-12% wider (Nishikubo et al. 2011). This influence on growth was also time-dependent (Nishikubo et al. 2011). Therefore, contrasting, but highly regulated control mechanism(s) are in place that governs the deployment of XTH and its functionality. Furthermore, the xyloglucan molecules that compose hemicellulose can be loosely or tightly arranged and this property of the cell wall composition was measured in *Populus* sp., engineered to overexpress XTH (Nishikubo et al. 2011). XTH-overexpressing *Populus* sp. resulted in altered loosely vs. tightly bound xyloglucan content (Nishikubo et al. 2011). In the overexpressing lines, the tightly bound xyloglucan fraction in primary-walled xylem was increased up to 3-fold (from 68% to 90%) as the loosely bound fraction was decreased (Nishikubo et al. 2011). Furthermore, xyloglucan immunolabeling demonstrated a two-fold increase in xyloglucan in meristematic cells of the cambial zone with most of the label being present in the radial walls and lacking in the tangential walls. The label became less detectable and finally undetectable as the cells matured (Nishikubo et al. 2011). The tightly bound form of the XTH-generated xyloglucan is inaccessible to enzymes (Vissenberg et al. 2005; Nishikubo et al. 2011). This facet of XTH activity is important with regard to defense to syncytium forming plant-parasitic nematodes. If nematode cell wall targeting effectors are involved in susceptibility, the observations of Nishikubo et al. (2011) may explain why nematode is not successful during the Gm-XTH43-engineered defense response. The result demonstrates how the precise and localized expression of Gm-XTH43 is critical in the ability of the cell to overcome the activity of *H. glycines* and *R. reniformis*. Furthermore, XTHs appear to be one of the few major classes of cell wall modifying enzymes lacking in the repertoire of plant-parasitic nematodes (Abad et al. 2008).

### 5.5.2 Gm-XTH43 overexpression affects root transcriptional activity during defense

Prior experiments examining gene expression of the *rhg1* gene,  $\alpha$ -SNAP, identified induced expression of *PR1*, *PR2* and *PR5* while *PR3* was suppressed (Matsye et al. 2012). In the experiments presented here, Gm-XTH43 overexpression did not affect the activity of the *rhg1* gene,  $\alpha$ -SNAP. However, Gm-XTH43 overexpression was accompanied by slightly suppressed expression of the *rhg1* components AAT and WIP. These results were not anticipated. However, 10 copies of each gene have been observed to exist in tandem so any of them may or may not be expressed or they each could be expressed to low levels (Cook et al. 2012). Similar to roots overexpressing  $\alpha$ -SNAP (Matsye et al. 2012), roots overexpressing Gm-XTH43 resulted in induction of the salicylic acid regulated *PR1*. Salicylic acid levels have been shown to influence nematode infection in *A. thaliana* (Wubben et al. 2008). The induced *PR1* expression in roots overexpressing Gm-XTH43 was accompanied by induced levels of the ethylene and jasmonic acid (JA) responsive *PR3*. Mutants of 9-lipoxygenase (9-LOX), known as *ZmLOX3*, are increased in their susceptibility to the giant cell forming *M. incognita* (Gao et al. 2008). This observation indicated the involvement of ethylene and JA in defense to *H. glycines*. However, like roots overexpressing  $\alpha$ -SNAP (Matsye et al. 2012), roots overexpressing Gm-XTH43 had suppressed levels of the ethylene responsive *PR2* gene activity. The effect that Gm-XTH43 overexpression had on the activity of these genes indicated the defense response would be accompanied by globally altered transcription, consistent with prior gene expression experiments of the syncytium undergoing defense (Klink et al. 2007, 2009a, 2010a, 2010b, 2011; Matsye et al. 2011; Kandoth et al. 2011).

Localized alterations in transcription in nurse cells undergoing defense have been revealed by quantitative exon sequencing analyses mediated through Illumina® deep sequencing (Matsye et al. 2011). The experiments demonstrated the value of the deep sequencing in that it could take the expression data obtained through microarray studies, which does not provide information on relative levels of expression, and determine those genes that are expressed to their highest relative levels in different cell types. The experiments were expanded here by determining the genes that are expressed in roots that are normally susceptible to *H. glycines* infection, but instead have become resistant due to the introduction of the Gm-XTH43 gene.

### **5.5.3 Gene copy number, its variation and relation to defense**

Gm-XTH43 is a member of a gene cluster with each paralog related most closely to At-XTH23. Thus, it appears that gene duplication has occurred at some point in the evolutionary history of the locus. Gene amplification is well known to occur and some of the earliest examples are for mitochondrial genes where gene copy number variation can occur within an individual and even within different tissue types with part of the copy number variation relating directly to physiological activity (Bibb et al. 1981; Williams 1986; Rodgers and Bendich 1987; Zhang et al. 1990; Lusching et al. 1993). Copy number variation is also important for a variety of developmental and disease states (Pinkel et al. 1998; Dixon et al. 1998; Kuang et al. 2004; Aitman et al. 2006; Redon et al. 2006; Stranger et al. 2007; Perry et al. 2007; Kuang et al. 2008; Sudmant et al. 2010; Cooper et al. 2011). Locally amplified genes also relate to *G. max* and its defense to *H. glycines*, found at the *rhg1* locus (Cook et al. 2012). Furthermore, transcriptional mapping experiments, revealing gene activity at the *rhg1* locus of *G. max* undergoing defense to

*H. glycines*, identified active  $\alpha$ -SNAP and AAT transcription throughout the defense response while a wound inducible protein (WIP) was only active in *G. max*<sub>[PI 88788]</sub> early during its resistant reaction (Matsye et al. 2011). In experiments examining a single population of *H. glycines* infecting *G. max*<sub>[PI 88788]</sub>, this transcriptional activity (Matsye et al. 2011) correlated to copy number variation for  $\alpha$ -SNAP, AAT and WIP at the *rhg1-b* locus (Cook et al. 2012). However, even in genotypes having numerous copies of genes relating to a process, the duplicated genes may not always perform a functional role in all individuals in a population or all populations, but can positively or negatively affect gene activity (McCarroll et al. 2006; Stranger et al. 2007). *G. max*<sub>[PI 88788]</sub>, where *rhg1-b* was determined, is well known to become successfully infected by several different populations of *H. glycines* that are widespread in their distribution as well as different plant-parasitic nematodes (Niblack et al. 2002). This phenomenon is not restricted to *G. max*<sub>[PI 88788]</sub>. Thus, in these cases, other genes are likely to be playing important roles in defense.

The experiments presented here show that high levels of Gm-XTH43 expression are important for defense to two different plant-parasitic nematodes in a genotype lacking apparent *rhg1* function. However, it is not yet clear if its overexpression in *G. max*<sub>[Williams 82/PI 518671]</sub> would inhibit parasitism to all populations of *H. glycines* or untested plant-parasitic nematodes. The experiments, having a phylogenetic basis with reference to the selected nematodes, indicate a potentially broad role that could extend beyond plant-parasitic nematodes. In addition to the amplification of the chromosome 17 XTH locus, Gm-XTH43 has been shown to have sequence variation among different genotypes (Cregan et al. 1999; McHale et al. 2012). It is unclear if this sequence variation is

important for defense. These sequence variants do not map near to the *H. glycines* resistance QTL found on chromosome 17, SCN16-1, using Satt082 (Schuster et al. 2001). Furthermore, the overexpressed XTH43 gene isolated from *G. max*<sub>[Peking/PI 548402]</sub> is identical in primary sequence to the recipient *G. max*<sub>[Williams 82/PI 518671]</sub> genotype in which the genetic engineering experiments were done, therefore, how the gene becomes transcriptionally active is important.

The experimental design of Matsye et al. (2011) used the *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> genotypes to obtain both a susceptible or resistant reaction by using virulent and avirulent *H. glycines* populations. A caveat in the experimental design is that *G. max*<sub>[Peking/PI 548402]</sub> and *G. max*<sub>[PI 88788]</sub> harbor functional resistance genes. The experiments presented here show that by expressing a gene that is normally silent during a susceptible reaction, even in a genotype harboring resistance genes, one can obtain a potent defense response in a genotype like *G. max*<sub>[Williams 82/PI 518671]</sub> that lacks a defense response. This outcome is not limited to Gm-XTH43 since a large screen of candidate genes also negatively or positively affected parasitism, but to a lesser extent than presented here (Matthews et al. 2013). The experiments indicate that somehow the expression of genes like Gm-XTH43 is being targeted for their suppression, resulting in a compatible interaction even in genotypes with functional defense genes (Matsye et al. 2011). Knowledge of this mechanism is important for understanding the plant-parasitic nematode biology, but perhaps more broadly for understanding the link between cell wall modification and plant defense.

## 5.6 Supplemental Material

The supplementary material of this chapter can be found at the following link;

<https://dl.dropbox.com/u/28481758/CH4-SUPP.zip>

## 5.7 References

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## CHAPTER VI

### CONCLUSION

The experiments presented here have identified genes that are involved in the defense response of *G. max* to *H. glycines*. The identified genes are known to perform roles in very well-known biological processes. Thus, the experiments may have identified a mechanism by which plants can defend themselves from plant-parasitic nematodes that form a nurse cell during the development of the infection. Furthermore, it appears that the defense mechanism may be broad since additional experiments have shown a negative impact also on *R. reniformis*.

Prior to the work presented here, no information existed in the *G. max* system with regard to any soybean gene having a negative impact on *H. glycines* infection. However, numerous variety trials which tested the ability of natural *G. max* collections to resist *H. glycines* infection have been performed since the 1950s, shortly after the arrival of *H. glycines* to the U.S. (Winstead et al. 1955; Ross and Brim, 1957; Ross, 1958; Epps and Hartwig, 1972). The outcome of these test experiments were the identification of *rhg1*, *rhg2*, *rhg3* (Caldwell et al. 1960) and the dominant *Rhg4* (Matson and Williams, 1965) and *Rhg5* (Rao Arelli, 1994). This work was accompanied by cytological studies of resistant reactions (Endo, 1965; Riggs et al. 1973; Acido et al. 1984; Kim et al. 1987; Kim and Riggs, 1992; Mahalingham and Skorupska HT. 1996) Meanwhile, a large body of genetic mapping and fine mapping data was accumulating (Concibido et al. 1994,

2004; Mudge et al. 1997; Cregan et al. 1999; Kim et al. 2010). Furthermore, the availability of advanced gene expression testing platforms through high density microarrays and deep sequencing were allowing gene expression to be tested in genetically less-tractable systems like *G. max* (Brenner et al. 2000). Further improvements in cell collection techniques through laser microdissection (Isenberg et al. 1976; Meier-Ruge et al. 1976; Emmert-Buck et al. 1996) have allowed for the investigation of gene expression occurring specifically in the infected cells at the genomic level (Klink et al. 2005, 2007, 2009, 2011; Matsye et al. 2011), revolutionizing biological analyses of plant-plant-parasitic nematode interaction studies.

In the study presented here, the *G. max* defense response to *H. glycines* was used as a model to map cellular resolution of its genotype-defined cell fate decisions occurring during its resistant reactions. The analysis compared gene expression occurring during the potent and rapid defense response found in *G. max*<sub>[Peking/PI 548402]</sub> to the potent but prolonged process found in *G. max*<sub>[PI 88788]</sub>. Detection call methodology (DCM) made possible the cross-comparison of gene activity measured in one sample type to a second sample type where activity is not measured. Therefore, it was possible to identify and analyze genes with expression that is limited to one cell type. Comparative transcriptomic analyses with confirmation by Illumina® deep sequencing were done. The analysis demonstrated that while large differences in gene expression were evident when directly comparing the different individual time points of the two genotypes, the expression profiles when comparing the entire defense response were very similar. Furthermore, the gene expression data were organized through PAICE that presents gene expression using the KEGG framework. Further, analyses of the *rhg1* resistance locus demonstrated



expression of an  $\alpha$ -SNAP gene specifically in syncytia undergoing defense responses (Matsye et al. 2011).  $\alpha$ -SNAP is well known to play a role in vesicle transport and membrane fusion. Vesicle transport and membrane fusion perform important roles in the growth of cells, hormonal release, exocytosis, neurotransmission, autophagy and plant defense (Clary et al. 1990; Peter et al. 1998; Ishihara et al. 2001; Collins et al. 2003; Kalde et al. 2007; Kwon et al. 2008; Pajonk et al. 2008; Meyer et al. 2009). The *G. max*<sup>[Peking/PI 548402]</sup> genotype has cell wall appositions (CWAs), defined as physical and chemical barriers to cell penetration and structures identified as forming a part of a defense response by the activity of the vesicular transport machinery (Aist. 1976, Schmelzer. 2002, An et al. 2006, Hardham et al. 2008). Our studies involving the overexpression of the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele in the susceptible *G. max*<sup>[Williams 82/PI 518671]</sup> genotype resulted in a partial suppression of SCN infection (Matsye et al. 2012). These experiments indicate a role of the vesicular transport machinery during infection of *G. max* by *H. glycines*.

Sequencing the  $\alpha$ -SNAP coding regions from the resistant genotypes *G. max*<sup>[Peking/PI 548402]</sup> and *G. max*<sup>[PI 437654]</sup> revealed they are identical, but differ from the susceptible *G. max*<sup>[Williams 82/PI 518671]</sup> by the presence of several single nucleotide polymorphisms (SNPs). Using *G. max*<sup>[Williams 82/PI 518671]</sup> as a reference,  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele exhibits an additional 17 nucleotides of mRNA sequence that contains an in-frame stop codon. In contrast, the 17 nt  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> mRNA motif is not found in *G. max*<sup>[PI 88788]</sup> that exhibits defense to *H. glycines*, but lacks the CWAs. The  $\alpha$ -SNAP<sup>[PI 88788]</sup> promoter contains sequence elements that are nearly identical to the  $\alpha$ -SNAP<sup>[Peking/PI 548402]</sup> allele, but differs from the *G. max*<sup>[Williams 82/PI 518671]</sup> ortholog. The

presented bioinformatics results demonstrate how subtle differences in promoter sequence composition could have an impact on transcription factor binding capability between the  $\alpha$ -SNAP<sup>[Williams 82/PI 518671]</sup> allele and its orthologs found in the resistant *G. max*<sup>[PI 88788]</sup> and *G. max*<sup>[Peking/PI 548402]</sup> genotypes (Matsye et al. 2012). Functional studies that would reveal if these differences in promoter elements influence expression were not attempted and remain an area for future study. This work would require the development of gene expression vectors that could accomplish the work, something that is currently underway.

Furthermore, the Illumina® deep sequencing showed that a xyloglucan endotransglycosylase gene (Gm-XTH43) was represented by over 11% of the transcripts in nurse cells undergoing defense (Matsye et al. 2011). This observation indicated that alterations in the cell wall composition of nurse cells might be important for the successful defense response as XTHs are known to be involved in cell wall modifications. Gm-XTH43 resides within a complex locus on chromosome 17 having 6 adjacent paralogs. Functional experiments done here in the form of Gm-XTH43 overexpression led to a significant suppression of nematode parasitism in an otherwise susceptible *G. max* genotype. The resistance obtained by Gm-XTH43 overexpression was not limited to *H. glycines* since *R. reniformis* infection was also detrimentally affected, indicating a common mechanism of defense. The overexpressed Gm-XTH43 cDNA isolated from *G. max*<sup>[Peking/PI 548402]</sup> is identical in primary sequence to the recipient *G. max*<sup>[Williams 82/PI 518671]</sup> genotype in which the genetic engineering experiments were done. Therefore, it appears that the transcriptional activation of the gene during defense is important. The placement of gene expression data onto the sequenced *G. max* genome in

relation to mapped resistance loci followed by the functional analyses allowed for the development of a model of the *G. max* defense mechanism from *H. glycines* infection

(Fig. 6.1)

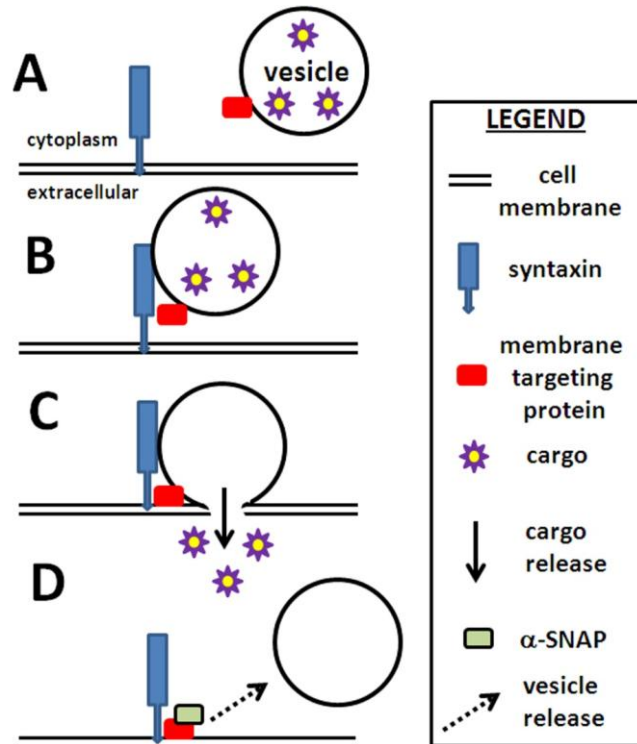


Figure 6.1 Vesicle delivery, membrane fusion and release back into the endosome cycle

**A**, A cell membrane has proteins that are involved in the docking of vesicles and their subsequent fusion, resulting in the delivery of cargo. The process involves many proteins including  $\alpha$ -SNAP and syntaxin. Vesicles have membrane targeting proteins such as synaptotagmin and synaptogamin that facilitate the fusion process. **B**, After activation of the SNAP (Soluble NSF Attachment Protein) REceptor (SNARE) acceptor complex, vesicles dock. **C**, After docking, the multiprotein complex undergoes a process called triggering which brings the vesicle in close opposition to cell membrane, followed by fusion which releases cargo into the apoplast. Cargo could be proteins like xyloglucan endotransglycosylase/hydrolase (XTH) which modify the cell wall. **D**. Through the activity of  $\alpha$ -SNAP and other proteins, the vesicle is bound then released from the cell membrane. Figure adapted from (Jahn and Fasshauer, 2012).

In summary, the basic knowledge gained in this system demonstrates the involvement of homologous proteins that are present in all land plants and their algal progenitors in the defense of *G. max* to *H. glycines*. The identification of  $\alpha$ -SNAP as it relates to the defense of *G. max* to *H. glycines* indicated the involvement of the vesicular transport and membrane fusion machinery functioning in this process. The demonstration that  $\alpha$ -SNAP is involved in this process provides insight into the defense mechanism of plants. Furthermore, the demonstration that the vesicular transport and membrane fusion machinery is involved in defense to *H. glycines* makes knowledge generated in other systems, including human disease, relevant in understanding the *G. max*-*H. glycines* pathosystem (Shin et al. 2008; Jahn and Fasshauer, 2012; Furuya et al. 2012; Thanabalasuriar et al. 2012; Rush et al. 2012; Mukaetova-Ladinska et al. 2013). Furthermore, knowledge generated in this pathosystem can now be applied as a model for understanding other recalcitrant pathogens affecting soybean and other crops. Part of this work has already been realized in experiments examining *R. reniformis*. The work is also allowing for the design of future experiments that extend beyond plant-parasitic nematodes so that a comprehensive understanding of biotic challenge can be generated.

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