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Engineered Soybean Cyst Nematode Resistance

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1. Introduction

A variety of plant parasitic nematodes (PPNs), including the soybean cyst nematode (SCN), elicit the initiation, development and maintenance of a specialized nurse cell from which they derive their nutriment (Figure 1). Remarkably, during parasitism by the PPN, the nurse cell survives the apparently significant resource drain on the root cell that would be expected to detrimentally impact normal physiological processes of the cell. 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 PPNs.

1.2. History

Documented accounts reveal that soybean has been in cultivation for thousands of years (Hymowitz et al. 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 Allard 1930; Chapin III et al. 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 and Brim, 1957; Ross, 1958; Epps and Hartwig, 1972; Concibido et al. 2004; Ma et al. 2006; Li et al. 2011; Matsye et al. 2012).
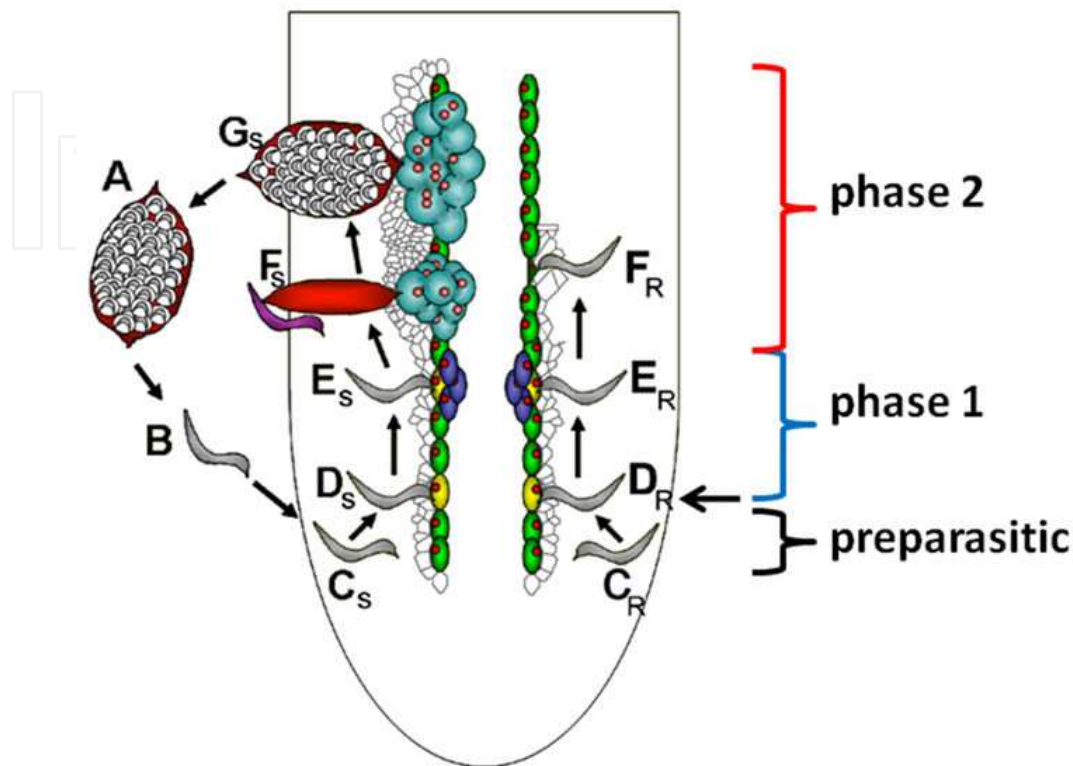

Figure 1. SCN life cycle during susceptible and resistant reactions Fig. 1A, cysts with eggs (white). Fig. 1B, second stage pre-infective juveniles (pi-J2) (gray) migrate toward the root. SUSCEPTIBLE REACTION: Fig. 1C$_s$, the infective-J2 (i-J2) nematodes (light gray) burrow in and migrate toward the stele. Fig. 1D$_s$, feeding site selection (yellow). Fig 1E$_s$, 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. Fig. 1F$_s$, After maturation, the male and female nematodes copulate. Fig. 1G$_s$, the female at ~30 dpi. RESISTANT REACTION: Fig. 1C$_R$, Like the susceptible reaction, the infective-J2 (i-J2) nematodes (dark gray) burrow into the root and migrate toward the root stele. Fig. 1D$_R$, feeding site selection by the parasitic J2 (p-J2). Fig 1E$_R$, the syncytium begins to develop. Fig. 1F$_R$, 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. 2011b).

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, b, 2003, 2006; Pratt and Wrather, 1998). 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.

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. 2001). 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 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 only a few years later 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. 2001, 2006). Making the problem worse for agriculture is the genetic diversity of the SCN (Golden et al. 1970; Riggs and Schmidt 1988; 1991; Niblack et al. 2002; Bekal et al. 2008). SCN research has determined that the nematode is a species complex originally subdivided into four races (Golden et al. 1970) which later was expanded into 16 races (Riggs and Schmidt 1988, 1991) 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 since 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 Schmidt 1988; 1991; Niblack et al. 2002; Bekal et al. 2008) 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 exasperated 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 et al. 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 (Creech et al. 2006). 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 and Bird, 1998; Niblack et al. 2006; Klink et al. 2010a).

1.3. 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 (Schneider et al. 2003; Rosskopf et al. 2005) and had subsequently been used for decades for both pre- and post plant nematode control. However, methyl bromide is a chemical that has been phased out of use in 2005 in the U.S. (Schneider et al. 2003; Rosskopf et al. 2005) because it has been classi-fied 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 bac-teria, fungi or even their proteins are feasible (Chen and Dickson, 1996; Kim and Riggs, 1991, 1995; Liu and Chen, 2001; Meyer and Huettel, 1996; Meyer and Meyer, 1996; Timper et al., 1999). 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, 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 viable, but dormant in production fields (Inagake 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 with potential as a 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, it is first required that candidate genes be identified. The identification of these genes has happened through a series of RNA gene expression studies, employing soy-bean germplasm that exhibits resistance to SCN.

1.3.1. Available resistant germplasm

Once SCN was identified in the U.S. (Winstead et al. 1955), a very large need existed to deter-mine if soybean germplasm existed that could resist infection. The vast and expansive range of soybean (Morse, 1927) 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 has 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*$_{[Peking]}$) and *G. max*$_{[PI 88788]}$. Currently, *G. max*$_{[Peking]}$ and *G. max*$_{[PI 88788]}$ resistance 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.

1.4. 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 (Inagake 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, 1992). The formation of the syncytium is likely to be a very coordinated process, occurring through the injection and subsequent activity of nematode parasitism proteins (Atkinson and Harris, 1989; Smant et al. 1999; Lambert et al. 1999; De Boer et al. 1999, 2002; Wang et al. 1999, 2001, 2003; Gao et al. 2001, 2003; Bekal et al. 2003). These substances likely orchestrate successive waves of interference of the root cell's normal physiological processes and initiate various cell wall dissolving events (Atkinson and Harris, 1989; Smant et al. 1999; Wang et al. 1999, 2001, 2003; Lambert et al. 1999; De Boer et al. 1999, 2002; Gao et al. 2001, 2003; Bekal et al. 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. 2007). The activities benefit the nematode during the sedentary period of its life cycle as they feed and mature (Edens et al. 1995; Hermsmeier et al. 1998; Mahalingam et al. 1999; Vaghchhipawala et al. 2001; Klink et al. 2005, 2007a; Alkharouf et al. 2006; Ithal et al. 2007; Matsye et al. 2011).

Cytological studies of the SCN infection process (Figure 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, 1965, 1991; Riggs et al. 1973; Kim et al. 1987; Mahalingam and Skorpska, 1996). Phase 1 and 2 span the periods including the initiation, development and maintenance of the syncytium (Figure 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, 1964, 1965; Riggs et al. 1973; Kim et al. 1987; Kim and Riggs, 1992; Mahalingam and Skorpska, 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, 1990). 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 the metabolism that 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 et al. 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*$_{[Peking]}$ and *G. max*$_{[PI\ 88788]}$-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 re-

sistance, 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 $G.\ max_{[Peking]}$-type of defense includes the development of a necrotic layer that surrounds the head of the nematode (Kim et al. 1987; Endo, 1991). This process is followed by necrosis of the initial cell that the nematode had parasitized. In contrast, in the $G.\ max_{[PI\ 88788]}$-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 (Kim et al. 1987; Endo, 1991). In addition to these cytological characteristics found in the $G.\ max_{[Peking]}$ and $G.\ max_{[PI\ 88788]}$-types of defense responses are 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 et al. 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 $G.\ max_{[Peking]}$-type of resistant reaction and are found in the $G.\ max_{[PI\ 437654]}$ genotype (Mahalingam and Skorpska, 1996). This makes the placement of $G.\ max_{[PI\ 437654]}$ in the $G.\ max_{[Peking]}$ cohort logical (Colgrove and Niblack et al. 2008). In contrast, CWAs are lacking in $G.\ max_{[PI\ 88788]}$. 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, meaning it has been found in other organisms. 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 are also involved in the process of defense. A genetic pathway, involving *PEN1*, the β-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). 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 receptor protein (SNARE) complex and synaptosomal-associated protein 25 (SNAP25) (Oyler et al. 1989), the soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) (Weidman et al. 1989; Clary et al, 1990; Collins et al. 2003; Assaad

et al. 2004; Kalde et al. 2007), 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*[PI 88788], 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).

1.5. 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 schactii* (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. It is a costly life strategy since typically only about 10% of the nematodes ever make it to maturity in a fully susceptible genotype like *G. max*[Williams 82/PI 518671]. 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; 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*[Williams 82/PI 518671] genotype that lacks a functional defense response (Ithal et al. 2007). This means that gene expression occurring during a susceptible reaction was monitored. The work examined the expression of previously identified (Wang et al. 1999; Gao et al.

2001, 2003) and analyzed (Bakhetia et al. 2007) putative parasitism genes (Ithal 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 (Ithal 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*$_{[Peking/PI\ 548402]}$ genotype that has a functional defense response to some populations of SCN (Klink et al. 2007a). Thus, from the experiments of Ithal et al. (2007) 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 (Elling et al. 2009). 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 for those nematodes if the gene could be knocked out. 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 1*E*-100; Group 2 had *E*-values between 1*E*-100 and 1*E*-80; Group 3 had *E*-values between 1*E*-80 and 1*E*-60; Group 4 had *E*-values between 1*E*-60 and 1*E*-40; Group 5 has *E*-values between 1*E*-40 and 1*E*-20 while Group 6 has *E*-values > 1*E*-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 underwent further annotation procedures (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 1).

1.6. 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 and Atkinson, 2002; Alkharouf et al. 2007). Urwin et al. (2002) 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 (Bakhetia et al. 2007, 2008). 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.

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)
HgAffx.10691.1.S1_at	CD748651	K04D7.1	guanine nucleotide-binding protein
HgAffx.10821.1.S1_at	CB935592	T21B10.7	t-complex protein 1
HgAffx.13633.1.S1_at	CD748017	pyp-1	inorganic pyrophosphatase
HgAffx.20969.1.S1_at	CB379877	rps-1	Ribosomal protein S3a homolog
HgAffx.24120.1.S1_at	CB935135	eft-2	Elongation factor Tu family
HgAffx.22597.1.S1_at	CB826306	kin-19	casein kinase I
HgAffx.11150.1.S1_at	CB378957	D1005.1	ATP citrate lyase
HgAffx.17961.1.S1_at	CB281421	F01G10.1	transketolase
HgAffx.18740.2.S1_at	CA940055	act-4	actin
HgAffx.14431.1.S1_at	CB935363	mdh-1	malate dehydrogenase
HgAffx.19636.2.S1_at	CA939544	rps-4	NULL
HgAffx.18811.1.S1_at	CA940369	F43G9.5	NULL
HgAffx.5490.1.S1_at	CD747934	gpi-1	glucose-6-phosphate isomerase
HgAffx.22868.1.S1_at	BG310682	cpl-1	cathepsin-like protease
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

Table 1. Annotation of the Affymetrix ® soybean GeneChip in relation to gene pathway analyses.

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 (Urwin et al. 2002; 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 PPN 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.
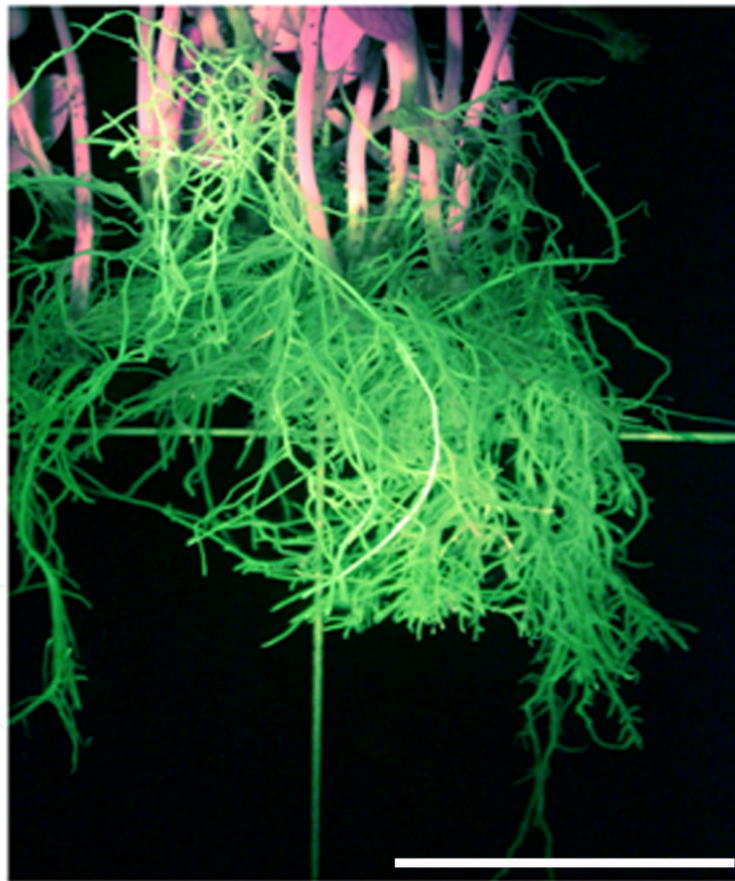

Figure 2. Soybean plants with transgenic roots. The transgenic soybean roots are expressing the enhanced green fluorescent protein (eGFP) (Haseloff et al. 1997) found in the pRAP vectors (Klink et al. 2009c; Matsye et al. 2012). Bar = 10 cm.

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 (Klink et al. 2008, 2009a). The simplicity of the approach is evident because the transgenic plants can be made in non-axenic conditions with the use of fluorescent reporter (Collier et al. 2005) (Figure 2). The development of vectors that work in soybean (Klink et al. 2008, 2009a; Ibrahim et al. 2011; 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. 2011; 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 tandemly duplicated 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. 2011). These observations demonstrated that broad spectrum resistance for PPNs in soybean was probable.

1.7. 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 (Ithal et al. 2007; Klink et al. 2007a, 2009a; Elling et al. 2009). 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. Chen et al. (2011) performed experiments 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.

1.8. 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. thali-*

ana, 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 (Klink et al. 2007b; Ithal et al. 2007). Some of the studies have focused in on expression occurring during the susceptible reaction (Alkharouf et al. 2006; Klink et al. 2007b; Ithal et al. 2007). These studies have 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. This meant that soybean was responding in important ways to the presence of the nematode within it s root tissues. Similar lists of genes were identified by Ithal et al. 2007, 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 would be 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*$_{[Peking/PI\ 548402]}$) (Klink et al. 2007b). The importance in the way the experiment was designed 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*$_{[Peking/PI\ 548402]}$ 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*$_{[Peking/PI\ 548402]}$, a genotype with functional resistance genes, failed in its effort to defend itself from SCN would be identified. The *G. max*$_{[Peking/PI\ 548402]}$ genotype was infected with *H. glycines*$_{[NL1-RHg/HG-type\ 7]}$ (originally called race 3) that obtained a resistant reaction and *H. glycines*$_{[TN8/HG-type\ 1.3.6.7]}$ (originally called race 14) that obtained 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 in obtaining a meaningful annotation that would provide an understanding of the global events occurring in the sample types.

1.9. 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 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 (Scheideler et al. 2001). The development and presentation of gene pathway information, a procedure that merged the Kyoto Encyclopedia of Genes and Genomes (KEGG) framework (http://www.genome.jp/kegg/ catalog/org_list.html) (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) (Paice_v2_90.jar) (http://sourceforge.net/projects/paice/) (Hosseini et al. unpublished; 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).

1.10. 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 would be to identify gene expression that occurred within the syncytium because it would require either drawing the cytoplasm out of the syncytium or a way to physically isolate the cells. The original studies that attempted to determine gene expression in nematode nurse cells was done by Bird et al. (1994) and 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, the collection of the cells would have to occur and it would have to be done 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) (Figure 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.
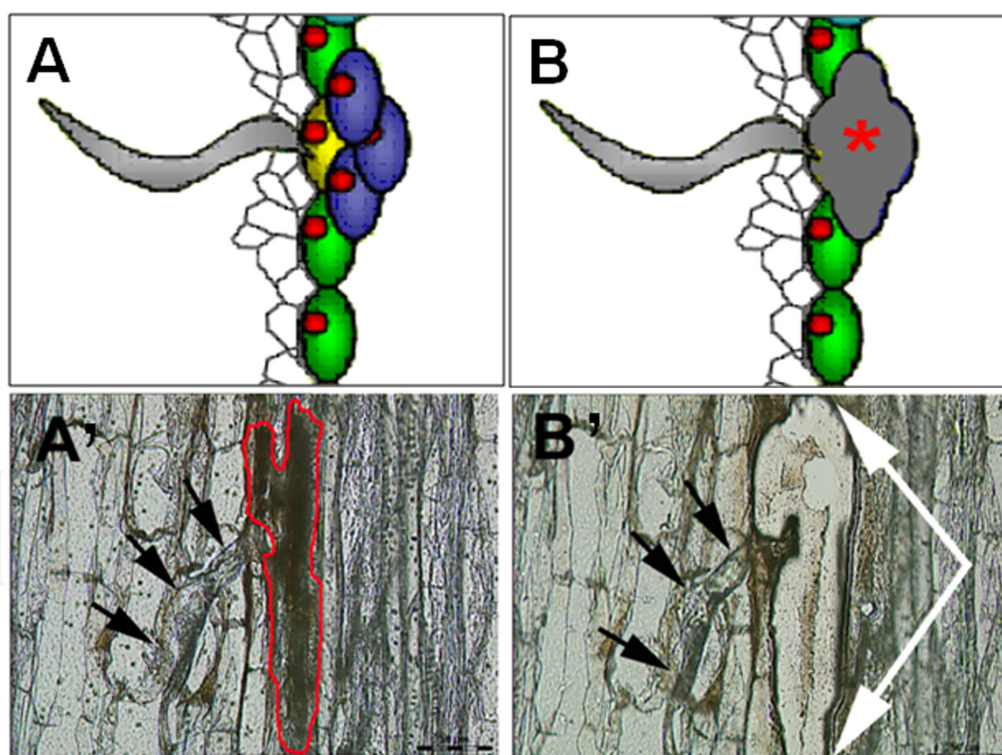

Figure 3. Laser microdissection (LM) of nematode feeding sites. A, cartoon of a nematode (gray) parasitizing a pericycle cell (yellow) that previously was uninfected (green). The parasitism process is resulting in the incorporation of neighboring cells by dissolving their cell walls, forming a syncytium. 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, 2010b, c, 2011a; Ithal et al. 2007; 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 would be required 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 (Figure 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; Kim et al. 1987; Endo, 1991). 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 to use 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, 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, 2010b, c). The experiments were repeated later by Kandoth et al. (2011) in the $G.\ max_{[PI\ 209322]}$ 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 $G.\ max_{[Peking/PI\ 548402]}$ and $G.\ max_{[PI\ 88788]}$ genotypes (Klink et al. 2009b, 2010b, c, 2011a; Matsye et al. 2011, 2012). These studies were important because the $G.\ max_{[Peking/PI\ 548402]}$ and $G.\ max_{[PI\ 88788]}$ genotypes are well known to undergo different forms of the resistant reaction at the cellular level (Kim et al. 1987; Endo, 1991; Mahalingham and Skorupska, 1996). The $G.\ max_{[Peking/PI\ 548402]}$ and $G.\ max_{[PI\ 88788]}$ 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-glucancase 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) (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 α-SNAP allele isolated from the resistant *G. max*[Peking/PI 548402], provided resistance when genetically engineered into the susceptible *G. max*[Williams 82/PI 518671] (Matsye et al. 2012). Gene expression and functional studies will be further expanded on in a subsequent section.

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

Table 2. Group 1 SCN genes expressed during parasitism and used in RNAi studies (Klink et al. 2009)

1.10.1. 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* (Rao Arelli 1994) have been identified. In all, there are approximately 61 QTLs that associate with resistance to SCN (Kim et al. 2010). 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; 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 in‐fection by the SCN, the *rhg1* resistance allele in *G. max*[PI 88788] 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 with‐in 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 resist‐ance, 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.

1.11. 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 trans‐porter (AAT) (Glyma18g02580) and an α soluble NSF attachment protein (α-SNAP) (Gly‐ma18g02590) found in the *rhg1* locus, undergo expression specifically in syncytia undergoing defense in both the *G. max*[Peking/PI 548402] and *G. max*[PI 88788] genotypes (Matsye et al. 2011). What was notable about the analysis was that AAT and α-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). The AAT and α-SNAP genes did not appear to be expressed in syncytia undergo‐ing the susceptible reaction. This difference in expression that was occurring between the resistant and susceptible reaction made it possible that the genes could be involved in the defense response. However, this would only be determined in functional studies that tested how the gene acted during infection (Matsye et al. 2012).

1.12. Genetic engineering as a solution for SCN

A number of approaches like conventional breeding programs have been shown for dec‐ades 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 undesira‐ble characteristics. This is especially a problem when is desirable traits are tightly linked to the undesirable traits. To circumvent this problem, it is possible to genetically engi‐neer 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 na‐

ture 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 gene expression in soybean to engineer resistance involves the engineering of soybean genes as overexpression constructs (Matsye et al. 2012). To do the studies, 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 α-SNAP gene. When the α-SNAP gene that was identified in the $G.\ max_{[Peking/PI\ 548402]}$ accession was overexpressed in the normally susceptible $G.\ max_{[Williams\ 82/PI\ 518671]}$ genotype, nematode infection was suppressed (Figure 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 et al. 1984) was designed with an enhanced green fluorescent reporter (eGFP) (Collins et al. 2005; Klink et al. 2008) was designed using the Gateway® technology for both RNAi and overexpression studies (Klink et al. 2009a; Matsye et al. 2012).
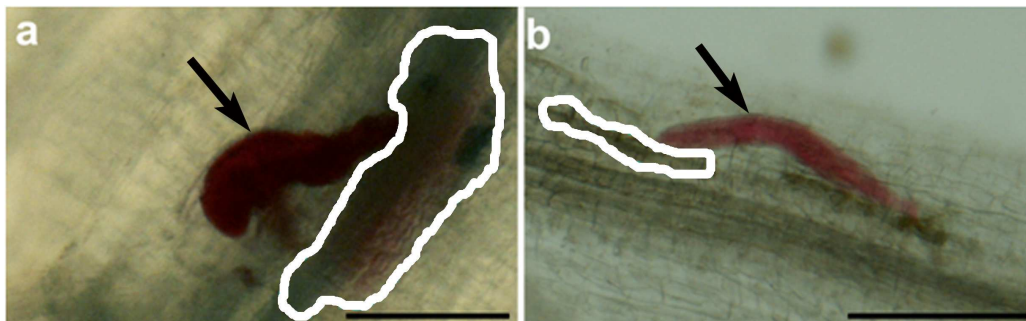

Figure 4. An overexpressed gene affects nematode development. A, a nematode, stained with acid fuchsin for visualization, developing in an experimental control plant. The boundary of the nematode feeding site is encircled in blue. B, a nematode failing to develop in a plant overexpressing a gene identified in the gene expression studies of the syncytium. The boundary of the nematode feeding site is encircled in blue.

2. Conclusion

The soybean-SCN pathosystem has been under study for over 60 years. Through a massive amount of basic studies involving agricultural production practices, 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 ap-

plied as a model for understanding other recalcitrant pathogens affecting soybean, to obtain a comprehensive understanding of infection and defense.

Acknowledgements

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