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Cell-Specific Studies of Soybean Resistance to Its Major Pathogen, the Soybean Cyst Nematode as Revealed by Laser Capture Microdissection, Gene Pathway Analyses and Functional Studies

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1. Introduction

The soybean cyst nematode, *Heterodera glycines*, is the major pathogen of soybean

Soybeans are hosts to a number of pests and pathogens. Of those numerous pathogens, the parasitic nematodes represent the major, ubiquitous, dominant and persistent problem for its cultivation, worldwide. Remarkably, soybeans are hosts to over 100 species of nematodes (reviewed in Sinclair and Backman, 1989). However, the most devastating pathogen of soybean is the soybean cyst nematode, *Heterodera glycines*. *Heterodera glycines* were first observed on soybean in Japan in 1881 (reviewed in Schmitt and Noel, 1984). The first scientific description of *H. glycines* was published in 1952 (Ichinohe, 1952). Up until this time there were no records of *H. glycines* in the New World. The combination of the growing agronomic status of soybean, the lack of agricultural practices to prevent the spread of any parasitic nematode and the biology of *H. glycines* set the stage for their rapid dispersal. *Heterodera glycines* were first identified in the U.S. in 1954 in North Carolina (Winstead et al. 1955). By 1957, *H. glycines* had been identified as far west as the state of Mississippi (reviewed in Riggs, 2004). Now *H. glycines* are nearly ubiquitous, found worldwide in 93.5% of the total acreage where soybean is cultivated (reviewed in Riggs 2004). In the U.S., reports rank *H. glycines* infection as causing more agronomic loss of soybean production than the rest of its pathogens combined (Wrather et al. 2006). *H. glycines* infection, itself, causes more damage to soybeans in the U.S. (~1.5 billion \$) than the entire value of some agricultural crops. These facts demonstrate the significant drain that *H. glycines* have on soybean production.

2. Life strategies of *H. glycines* augment its spread

The identification of *H. glycines* throughout regions cultivating soybeans, even in the presence of crop rotation practices, suggests that it can either survive for long periods of

time in the absence of its host or could live on alternate hosts. Both cases are true. *Heterodera glycines* can survive in the soil as hardened, desiccated cysts for up to nine years (Inagaki and Tsutsumi, 1971). Other research has demonstrated that *H. glycines* can reproduce on at least 97 legume and 63 non-legume hosts (Epps and Chambers, 1958; Riggs and Hamblen, 1962, 1966a, b; reviewed in Sinclair and Backmon 1989; reviewed in Riggs 1992). New hosts are identified on a regular basis (Creech and Johnson, 2006). Thus, even though its name implies a tight relationship with soybean as a specialist nematode, its mode of existence more closely relates to that of a polyphagous or generalist pathogen because it can successfully reproduce on multiple species and plant families. This additional facet of the *H. glycines* life cycle poses a significant problem for control because *H. glycines* can survive and reproduce on weeds in and around fields throughout the year. This characteristic of their biology is not a minor issue because as high as 93% of fields infected with *H. glycines* in some locales have such alternate winter annual weed hosts with *H. glycines* growing at high densities (Creech and Johnson, 2006). Also, *H. glycines* will infect and detrimentally affect yield in other crops without even completing their life cycle. However, Riggs (1992) noted that many resistant plant species actually prevent penetration altogether. This observation has important ramifications for the development of resistance because it demonstrates that infection is not a default mechanism of the nematode in the presence of any root.

3. The life cycle of *H. glycines*

Heterodera glycines are an obligate endoparasite of soybean with a life cycle of approximately 30 days, depending on temperature and other factors. The timing of the different juvenile stages and associated events (**Figure 1**) is well established (Lauritis et al. 1983; Koenning, 2004). Prior to hatching, eggs are contained within a cyst. The cyst is a hardened structure composed of the carcass of the female that encases the egg mass. After hatching, the second stage pre-infective juveniles (pi-J2s) emerge from the cyst and migrate toward and burrow into the root. The infective J2s (i-J2s) (Noel 2004) then burrows through the cortex intracellularly toward the root stele by using a tubular mouthpiece called a stylet to slice through the cells. When *H. glycines* reaches a targeted cell for nurse cell formation (typically a pericycle cell or neighboring root cell), the stylet is used to penetrate the targeted cell. At this point, the nematodes are parasitic J2s (p-J2). The p-J2 then presumably injects substances into the plant cell. These substances are synthesized in esophageal and/or subventral gland cells with each gland cell providing certain substances at specific times during parasitism. The substances initiate the major physiological changes that are observed in the root cell. Shortly after, the infected root cell fuses with neighboring cells. The process begins by breakdown of cell wall material at or near plasmodesmata. The wall openings increase in size, permitting the free flow of cytoplasm, organelles and even nuclei in and out of former cellular boundaries. The repeated cell fusion events produce a syncytium, defined as a multinucleate cell. The mature syncytium, acting as a nurse cell, contains approximately 200 cells sharing a common cytoplasm (Jones and Northcote 1972; Jones, 1981). The p-J2 nematodes that are destined to develop into males then feed for several days. During feeding, the males become sedentary. The feeding process proceeds until the end of their J3 stage. The males then stop feeding and subsequently molt within the former J2 and J3 cuticles into vermiform J4 males. Of note, the J4 males remain encased within the J2 and J3 cuticles until they burrow out of the cuticles and root in preparation for mating. In contrast to the males, the p-J2s that eventually will develop into females become and remain

sedentary during and after the establishment of their nurse cell. During feeding, the female nematodes increase in size. The process is followed by J3 and J4 molts. During growth, the posterior of the female will erupt out of the root boundary, beyond the root epidermis. This feature of female development provides access to the male that is present outside the root boundary for copulation. After copulation, the adult females will continue to grow while the eggs develop internally. As the life cycle ends for the female its color changes from a creamy white to yellow-tan. This is a visual sign of mortality of the female. However, the eggs within her carcass remain viable. The dead female carcass that encases the eggs, including the eggs, is known as the cyst. The cyst can remain dormant in the field, acting as a repository for viable eggs, for up to 9 years (Inagaki and Tsutsumi, 1971).

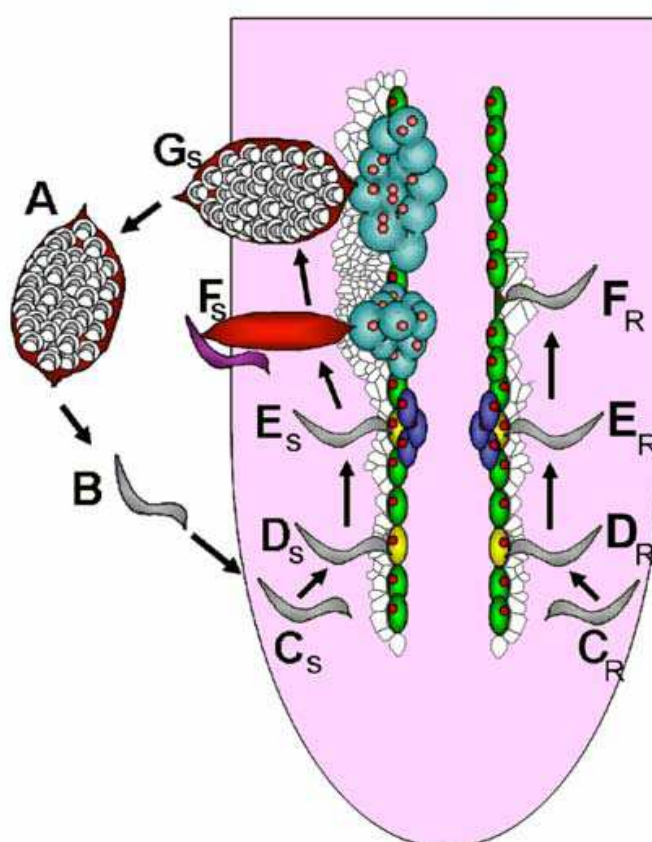


Fig. 1. The life cycle of *H. glycines* during a susceptible and resistant reaction of soybean. **Fig. 1A**, cysts with eggs (white) hatch. **Fig. 1B**, second stage pre-infective juveniles (pi-J2) (gray) hatch and migrate toward the root. **SUSCEPTIBLE REACTION:** **Fig. 1C_S**, The infective-J2 (i-J2) nematodes (dark gray) burrow into the root and migrate toward the stele. **Fig. 1D_S**, feeding site selection by the parasitic J2 (p-J2). **Fig. 1E_S**, p-J2 nematodes molt into J3, subsequently, they undergo a molt into J4. During this time, the original feeding site is incorporating adjacent cells via cell wall degradation and fusion 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**, After ~30 days, the female is clearly visible externally. **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. Figure adapted from Klink et al. (2009c).

4. Soybean that resist *H. glycines* infection

The United States Department of Agriculture (USDA) collects and maintains thousands of accessions of soybean within the National Plant Germplasm System (<http://www.ars-grin.gov/npgs/>). The accessions are named according to a plant introduction (PI) scheme. The collections have proven to be a valuable resource because the vast majority of soybean accessions are susceptible to *H. glycines* (Winstead, 1955). The availability of thousands of soybean accessions (genotypes) makes the identification of resistance in the germplasm possible. For example, resistance of soybean to *H. glycines* was determined soon after the identification of the nematode in the U.S. (Ross and Brim 1957). The identification of these sources of resistance was initially accomplished by analyzing over 5,800 soybean accessions in two successive large screens (reviewed in Shannon et al. 2004). Most of these sources of resistance play little or no role in commercial breeding programs. The reason is because many of these genotypes carrying resistance genes also carry undesirable traits that can be passed on in classical breeding programs (reviewed in Shannon et al. 2004). Altogether, various screens over the years have identified approximately 118 *H. glycines*-resistant accessions (Rao-Arelli et al. 1997; reviewed in Shannon et al. 2004). However, the two large screens provided nearly all of the resistance germplasm that is currently bred into commercial varieties.

The first screen was performed on 2,800 PIs from this collection. The screen resulted in the identification of the resistant PI 548402 (Peking), PI 84751 PI 209332, PI 90763 and PI 548349 (Ilsoy), each having the undesirable black or brown seed coat (Ross and Brim 1957). From these resistant genotypes, Peking was selected for introgression of resistance germplasm. Thus, Peking is an archetypal source for resistance. Of note, the resistance process of Peking was documented shortly thereafter through histological techniques, revealing the cytology of the resistance reaction to *H. glycines* (Ross, 1958). This work is described in detail in a subsequent section. The introgression of resistance germplasm was facilitated by genetic crosses with susceptible genotypes having the desirable yellow colored seed coat. The genetic crosses resulted in the development and registration of PI 548988 (Pickett) (Brim and Ross 1966) and PI 548546 (Custer) (Luedders et al. 1968). Unfortunately, field variations in *H. glycines* were documented by their ability to fully develop on Peking and its progeny lines even before the release of Pickett. The observations prompted a subsequent screen. The second screen of over 3,000 PIs resulted in the identification of additional resistant accessions including PI 88788 (among others) (Reviewed in Shannon et al. 2004). PI 88788 is another archetypal genotype having a different form of resistance that is evident at the cellular level (Kim et al. 1987). The PI 88788 genotype was used to develop and register PI 548974 (Bedford) (Hartwig and Epps, 1978) and PI 518674 (Fayette) (Bernard et al. 1988).

5. Resistance, the cellular processes

Plants, composed of numerous cell types, react to and interact with other organisms in a tightly regulated manner whether the outcome is a compatible reaction as in susceptible plants or an incompatible reaction like what happens during a resistant reaction. Thus, the cellular response to the interaction will likely be accompanied by cytological features that characterize the process at each step during its development. The cytology of the resistant reactions in these different accessions has revealed that while commonalities exist in how

the different soybean genotypes react to *H. glycines*, features unique to the different accessions are present (Endo, 1965, 1991; Riggs et al. 1973; Acido et al. 1984; Kim et al. 1987; Kim and Riggs 1992; Mahalingam and Skorpska, 1996). Those observations have led to a generic classification scheme of resistance that is based off of those cytological observations (Niblack et al. 2008). The cytological observations along with experimental research demonstrating the effects on *H. glycines* have proven essential for a molecular dissection of the process at the cellular level (Klink et al. 2007a, 2009a, 2010a, b, 2011).

The cytological data of the infection process of *H. glycines* in soybean roots clearly demonstrate that the syncytium is undergoing two separate phases that lead to a susceptible or resistant reaction (**Figure 2**), depending on the activity of resistance genes and outcome of any resistance reaction. The two distinct phases during parasitism are an earlier phase referred to as phase 1 and a later phase referred to as phase 2 (Klink et al. 2009b, 2010a, 2011).

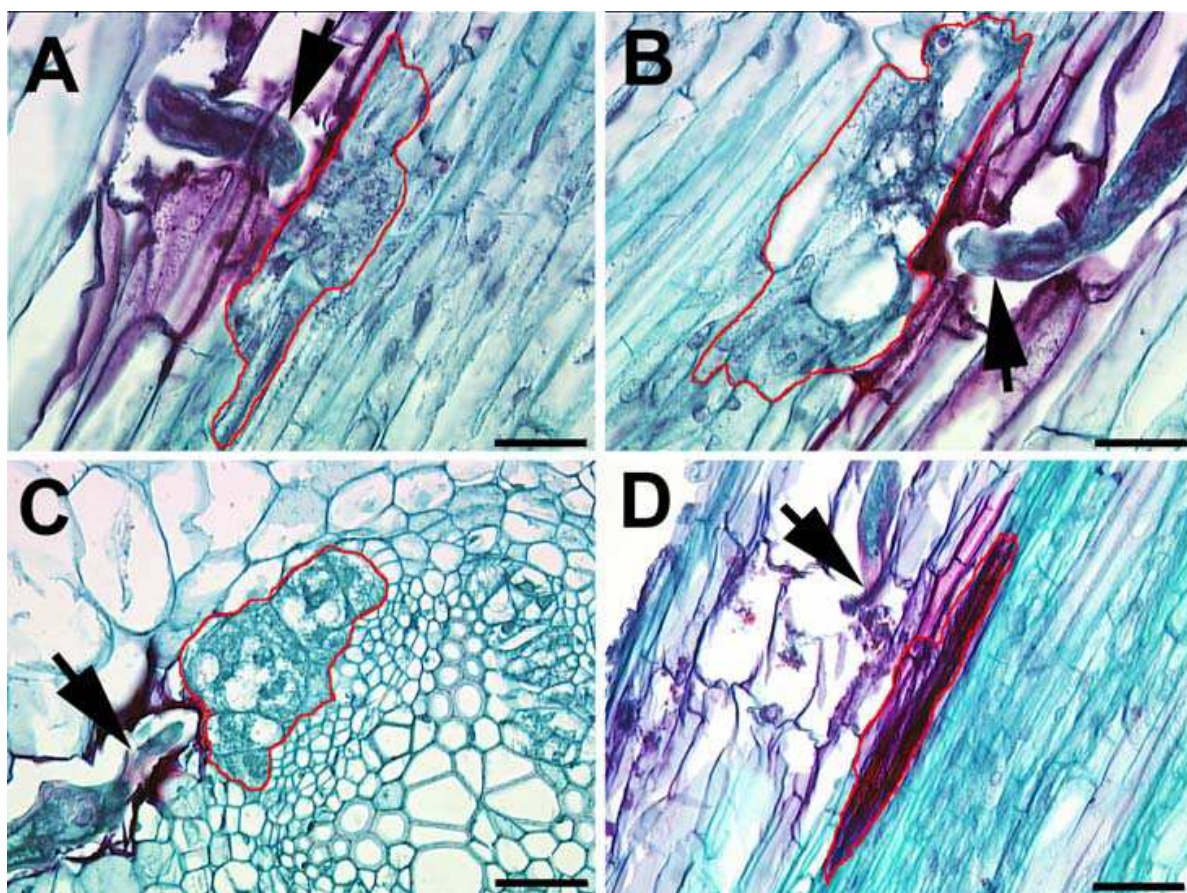


Fig. 2. Histology of *H. glycines* infection at stages during a compatible and incompatible reaction. Peking seedlings were inoculated with incompatible or compatible *H. glycines* J2 nematodes. Roots were harvested and prepared for histological observation to confirm the establishment of feeding sites at 3 and 6 dpi. **Fig. 2A**, 3 dpi Peking infected with a compatible nematode, black arrow; area encircled in red, syncytial cell. **Fig. 2B**, 6 dpi Peking infected with a compatible nematode, black arrowhead; area encircled in red, syncytial cell. **Fig. 2C**, 3 dpi *G. max* Peking] infected with an incompatible nematode, black arrowhead; area encircled in red, syncytial cell. **Fig. 2D**, 6 dpi Peking infected with an incompatible nematode, black arrowhead; area encircled in red, syncytial cell. Bar = 50 μ m. Images obtained from Klink et al. 2009a, 2010b.

During phase 1 the cytological features in the developing nurse cell, that eventually respond by undergoing compatible or incompatible reactions, appear the same (Endo, 1965, 1991; Riggs et al. 1973; Acido et al. 1984; Kim et al. 1987; Kim and Riggs 1992; Klink et al. 2007a, b, 2009a, 2010a, b, 2011). The cellular processes that occur during phase 1 of both compatible and incompatible reactions include the dissolution of cell walls, enlargement of nuclei, hypertrophy, the development of dense cytoplasm and increased ER and ribosome content (Endo, 1965; Riggs et al. 1973; Kim et al. 1987; Mahalingam and Skorpska, 1996). Thus, phase 1 appears the same or nearly so during compatible or incompatible reactions at the cytological level (**Figure 2**).

During phase 2, the cellular response to infection of the compatible and incompatible reactions differs dramatically (Ross, 1958; Endo, 1965, 1991; Riggs et al. 1973; Acido et al. 1984; Kim et al. 1987; Kim and Riggs 1992; Mahalingam and Skorpska, 1996; Klink et al. 2007a, b, 2009a, 2010a, b, 2011) (**Figure 2**). Phase 2 of the compatible reaction is characterized by hypertrophy of nuclei and nucleoli, proliferation of cytoplasmic organelles, reduction/dissolution of the vacuole and cell expansion as it incorporates adjacent cells (Endo and Veech 1970; Gipson et al 1971; Riggs et al 1973). Eventually these recruited cells merge to form a syncytium that incorporates approximately 200 cells (Jones and Northcote, 1972; Jones, 1981). At this point, it is unclear if different forms of the susceptible reaction exist.

In contrast, two major forms of the incompatible reaction have been revealed through screening of the PI collection, followed by cytological examination. All of the different forms of the incompatible reaction can be potent, meaning that almost all nematodes are affected (depending on the population of nematode used in the study). However, the two forms of the incompatible reaction vary in how rapidly they result in the collapse of the syncytium and the timing (juvenile stage) of nematode mortality. Two of the major cytological routes for the incompatible reaction are found in cohorts of soybean accessions that are defined by the archetypal PIs, Peking and PI 88788. A third type of incompatible reaction found in PI 437654 bridges the cytological features of Peking and PI 88788. These forms of the resistant reaction are discussed below.

One form of the resistant reaction is found in the archetype Peking (Ross, 1958). The cytology of the Peking-type of reaction is common to Peking, PI 90763, PI 89772 and partially PI 437654. Cytologically, the Peking-type of resistance reaction is evident at the cellular level at 4 dpi. The Peking resistance process involves necrosis of the cells that surround the head of the nematode. This process separates the syncytium from the cells that surround it (Kim et al. 1987). However, syncytia are continuing their later stages of the resistant reaction even at 7 dpi (Riggs et al. 1973). Peking has cell wall appositions. Cell wall appositions are structures defined as physical and chemical barriers to cell penetration (Aist et al. 1976, Schmelzer, 2002; Hardham et al. 2007). Consequently, by 8 dpi the resistant reaction is largely completed. The stage of nematode development that is affected by the resistant reaction has also been determined, occurring at the p-J2 nematode within 4-5 dpi (Endo 1964, 1965; Riggs, 1973; Kim et al, 1987; Kim and Riggs 1992; Colgrove and Niblack, 2008). Molecular analyses of this reaction have been presented (Klink et al. 2007a, 2009a, 2010b, 2011) and will be discussed in a later section.

A second form of resistant reaction is found in the archetype PI 88788. In contrast to the Peking-type of resistance, the PI 88788-type of resistance reaction lacks the development of a necrotic layer that surrounds the head of the nematode (Kim et al. 1987). This is an important distinction between the Peking- and PI 88788-types of resistant reactions. The

initial stages of the PI 88788-type of resistant reaction involves extensive accumulation of cisternae and rough ER and nuclear degeneration within the syncytium by 5 dpi (Kim et al. 1987). There are no thickened cell walls or appositions. Degradation of the cytoplasm is observed by 10 dpi (Kim et al. 1987). The PI 88788-type of resistance reaction results in nematode death at the J3 and J4 stages (Acido et al. 1984; Kim et al. 1987; Colgrove and Niblack, 2008) which is later than that observed for the Peking-type of reaction. Molecular analyses of this reaction have been done (Klink et al. 2010b, 2011) and will be presented in a later section.

A third type of resistant reaction occurs in PI 437654. The PI 437654 accession was collected from China much later than those used in the initial screens that determined resistance for Peking and PI 88788. The PI 437654 genotype is a highly underutilized source of resistance. However, new breeding efforts are being done using PI 437654 (Diers et al. 2010). The cytological features of the PI 437654 resistance reaction bridge those of Peking and PI 88788 (Mahalingam and Skorpska, 1996). The PI 437654 resistance reaction becomes evident at both the cytological and ultrastructural levels by 2 dpi, thus, the PI 437654 resistant reaction is the most rapid response. The PI 437654 resistant reaction is characterized by the presence of cell wall appositions, irregular cell wall thickenings, nuclei that degenerate and necrosis of cells (Mahalingam and Skorpska, 1996). *Heterodera glycines* development is blocked at the p-J2 stage (Mahalingam and Skorpska, 1996; Colgrove and Niblack, 2008). Based on HG-type tests, it was reported that PI 437654 was highly resistant to most populations of *H. glycines*. Thus, PI 437654 is also the most potent resistant reaction because its activity spans all traditionally accepted *H. glycines* races. However, *H. glycines* populations have been identified that will reproduce (albeit at lower levels than a fully susceptible reaction) on PI 437654 (Klink and Lawrence personal observations).

6. Genetics that underlie different forms of the resistant reaction

The resistance reactions of Peking, PI 88788 and PI 437654 to *H. glycines* have been defined through decades of genetic mapping research (Diers et al. 1997; Cregan et al. 1999; reviewed in Concibido et al. 2004; Wu et al. 2009). This information can be highly informative because of the availability of the sequenced soybean genome (Schmutz et al. 2010). Several recessive resistance loci (*rhg1* [linkage group G], *rhg2* [linkage group M] and *rhg3* (Caldwell et al. 1960), and two dominant resistance loci (*Rhg4* [linkage group A2]) (Matson and Williams 1965) and (*Rhg5* [linkage group J]) (Rao-Arelli 1994) have been identified. Importantly, The Peking, PI 88788 and PI 437654 harbor both common and unique genes (reviewed in Concibido et al. 2004). Peking resistance is explained by four genes. Three of the genes, *rhg1*, *rhg2*, and *rhg3*, are recessive. The three recessive genes are accompanied by the dominant gene *Rhg4*. Other quantitative trait loci (QTLs) are present. The PI 88788 resistance is explained by four genes. Two of the resistance genes, *rhg1* and *rhg2* are recessive in nature. In addition to these two recessive genes, PI 88788 resistance involves the dominant genes *Rhg4* and *Rhg5*. Other QTLs are present. The underlying nature of PI 437654 is the recessive genes *rhg1*, *rhg2* and the dominant *Rhg4* and *Rhg5* genes. A less well understood locus, linkage group I, apparently is the only QTL other than the *rhg1*, *rhg2*, *Rhg4* and *Rhg5* genes found in PI 437654 to be associated with resistance to *H. glycines* race 14 (Wu et al. 2009). It must be noted that the race 14 designation of *H. glycines* in Wu et al. (2009) is part of the original and very commonly used classification schemes of Riggs and Schmitt (1988, 1991). However, those races were subsequently redefined as populations because they can only be

maintained through a sexual cycle (Niblack et al. 2002). As can be imagined, numerous populations can be isolated from a single locale and selected in the greenhouse during a genetic bottlenecking procedure. The procedure that includes numerous generations of single cyst selection and descent results in the generation of numerous populations that behave in unique manners when grown on a panel of soybean genotypes with resistance genes. Thus, the quantity of the *H. glycines* populations can go well beyond the original 16 races that were identified by Riggs and Schmitt (1988, 1991). Most notably, the HG-type populations do not always easily cross reference back to the original 16 *H. glycines* races without extensive testing.

The observations show that PI 437654, Peking and PI 88788 share *rhg1*, *rhg2*, and the *Rhg4* genes. In contrast, Peking has the recessive *rhg3* while PI 437654 and PI 88788 have the dominant *Rhg5*. Quantitative trait loci [QTL] mapping have identified other minor QTLs that act in various ways on the different populations of *H. glycines*. Different combinations of these genes are required for resistance, depending on the soybean genotype and the population of *H. glycines* involved in the interaction. For details, please refer to Concibido et al. (2004).

7. Genetic variability of *H. glycines* permits the transcriptomic analysis of both the susceptible and resistant reaction in the identical soybean genetic background

Even closely related soybean genotypes can have varying abilities to resist infection by *H. glycines* (Figure 3). The use of genetic maps and the soybean genome, however, would allow for association mapping of gene expression data directly on to the sequence of the genome (Stolc et al. 2005). This procedure can be done because the map locations of the loci that regulate the resistant reaction of soybean to *H. glycines* are available. However, additional layers of complexity are added to the analysis by the 16 different known populations of *H. glycines* and numerous QTLs found in the different soybean genotypes harboring unique resistance genes. For details please refer to (Concibido et al. 2004).

There are three major strategies that can be used to perform meaningful cell-based transcriptomic studies of host-pathogen interactions. Two of these very useful methods employ genetic variants of the host to understand compatible and incompatible reactions. The first analyzes near isogenic lines (NILs) and the second analyzes recombinant inbred lines (RILs). Near Isogenic Lines (NILs) are identical at the genetic level except at one or a few genetic loci. Recombinant Inbred Lines (RILs) are formed by crossing two inbred strains followed by repeated selfing or sib mating to create a new inbred line whose genome is a mosaic of the parental genomes. The problem with NIL or RIL-based strategies is that even minor differences in the genome that are present can greatly alter gene expression. For example, even in the absence of any pathogen, different NILs have been shown to possess alterations in gene expression in specific cell types (Hinchliffe et al. 2010). To circumvent this issue, whole genome sequencing can be performed to understand the genetic and transcriptomic nature of the host. Whole genome sequencing is getting to be less expensive and has been done to understand different ecotypes of *A. thaliana* (Santuari et al. 2010). The analyses are revealing the genetic basis underlying their varying phenotypes (Santuari et al. 2010). Unfortunately, it is typically prohibitive to sequence NILs or RILs, especially for agricultural plants like soybean that have highly duplicated genomes (Schmutz et al. 2010). It is also unclear what differences in genotype are actually responsible for the observed

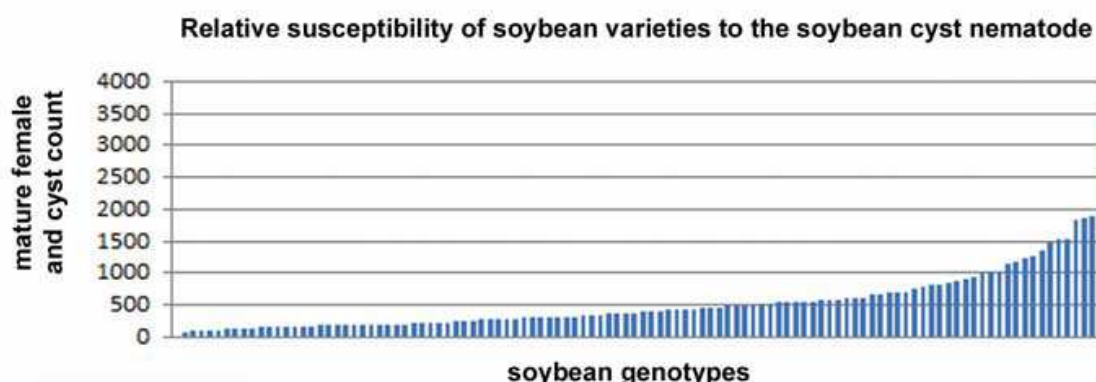


Fig. 3. Relative susceptibility of soybean varieties to *H. glycines* race 15. One hundred twenty five varieties were evaluated for their host response to *H. glycines*. Only one variety out of 125 examined was considered resistant to this population of the *H. glycines*. Seeds of each variety were germinated in germination paper for three days. Seedlings were then transplanted singly to pots containing 500cc of sand: soil mix (50:50 v/v). Each soybean variety was inoculated with 2,500 *H. glycines* eggs recovered from stock plants maintained in the greenhouse. Each variety was replicated 6 times and placed in a randomized complete block design. Tests ran for 50 days prior to harvest. At harvest, the white females and mature cysts are extracted from each pot by hand and then enumerated using a stereomicroscope. Egg and cyst counts are numbers per 500cc soil. The susceptible variety Hutcheson was included in the test to compare the relative susceptibility of each entry to a widely known soybean variety.

phenotype. Such a problem was revealed in soybean for the *rhg1* locus. The *rhg1* locus containing a leucine rich repeat gene believed to be the actual resistance gene for over a decade has been shown to be duplicated with both copies of the duplicated gene having no role in resistance (Melito et al. 2010). However, for example, if the genetic loci that map to the resistance phenotype are known, that region of the genome can be sequenced specifically (Innes et al. 2008). In the case of host-pathogen interactions, the hope is that the alteration in resistance can be attributed to those regions of the genome that are sequenced. The gene(s) are identified in mapped regions experiencing duplicated or lost genes that may or may not be in the sequenced reference genome (Innes et al. 2008). If not, the phenotype may be caused by genes at unmapped loci that lack genetic lesions but experience transcriptomic alterations that account for resistance. In these cases it is possible that the phenotype is caused by alterations in gene expression that are not directly associated with the alterations in genotypes of the NILs or RILs. In this case, alternative approaches need to be used.

An alternative approach that provides the purest analyses would be to use the identical plant genotype to accomplish both compatible and incompatible reactions in the presence of resistance genes (i.e. Peking, PI 88788 and PI 437654). In this manner, the activity of the gene itself is used as the marker of the compatible or incompatible reaction. A long history of such analyses exists for studies in soybean and other pathogens (Staskawicz et al. 1984; Bonhoff et al. 1986; Dhawale et al. 1989; Whalen et al. 1991). During the course of those studies it was determined that plant pathogens have specific variations in their genetic

makeup that underlie their patterns of virulence, influencing how they infect soybean (Staskawicz et al. 1984; Bonhoff et al. 1986; Dhawale et al. 1989; Whalen et al. 1991).

Unlike many systems where plant-nematode interactions are studied, related experiments to those already presented are actually possible in soybean because of the availability of different *H. glycines* field races Golden et al. (1970). The original race designation was based on their ability to reproduce on a panel of four resistant varieties (i.e. Peking, Pickett, PI 88788 and PI 90763). The scheme was later expanded to include sixteen races (Riggs and Schmitt 1988, 1991). Subsequently the races were renamed as populations (Niblack et al. 2002) of *H. glycines*, based on their sexual life cycle. Currently, there are nine resistant genotypes commonly used in *H. glycines* type (HG-type) tests that include PI 88788, PI 548402 (Peking), PI 437654, PI 90763, PI 548988 (Pickett), PI 89772, PI 538316 (Cloud), PI 209332 and PI 438489B. Other *H. glycines* populations have been reported with some of them specifically selected for their virulence patterns on highly resistant soybean genotypes (Niblack et al. 2002). The early studies of Staskawicz et al. (1984) demonstrating virulence factors in other pathogens, established the framework to perform molecular analyses of nematode feeding sites. This framework was strengthened by the demonstration of genetic (Bekal et al. 2008) and transcriptional (Klink et al. 2009b, c) differences in the various *H. glycines* populations that may underlie their patterns of virulence and ability to infect the different soybean genotypes. Therefore, the available populations, differing in their virulence, can be used to accomplish compatible and incompatible reactions in the same soybean genotype (Mahalingam and Skorpska, 1996; Klink et al. 2007a, 2009c, 2010b), providing the most genetically pure experiments possible for examining the different forms of the resistant reaction.

8. Cell isolation procedures

Endo (1971) determined that nucleic acids are synthesized within syncytia. The experiments meant that both DNA and RNA were synthesized and present in these feeding sites. Since compatible and incompatible reactions appear different cytologically for the different forms of the resistant reaction, the variations in the virulence of *H. glycines* populations can be studied. These observations permit a remarkable series of experiments to be performed that determine how the nematode specifically alters localized gene regulatory mechanisms both spatially and temporally, allowing it to complete its life cycle in an otherwise resistant plant (Klink et al. 2007a, 2009a, 2010b, 2011). The analyses, because they are done in the identical host genotype, can identify the transcriptomic programs that underlie this switch from a genotype's resistance and susceptibility that is accomplished through the modulation of plant stress responses (Klink et al. 2007a, 2009a, 2010b, 2011). Genomics-based studies of whole roots infected by *H. glycines* identified different suites of genes that accompanied the susceptible and resistant reactions during the course of infection (Klink et al. 2007b). Those analyses became a model for the cell-type specific studies presented in a later section (Klink et al. 2007a, 2009a, 2010b, 2011).

From cytological analyses it is clear that the cellular changes that characterize the incompatible reaction lead to a decrease in nematode growth and ultimately their arrest in development at specific stages. The specificity and consistency of the cytological reactions in the different PIs at the site of infection suggest that while basic aspects of the reaction are likely to be conserved, specific differences are present. This is in agreement with the genetic mapping data (reviewed in Concibido et al. 2004). The obvious problem with the genomics-

based molecular study of cells embedded within complex tissues is that each cell type, composing the tissue or organ, contributes its own gene expression profile (Birnbaum et al. 2002). Thus, isolating the cells of interest (i.e. the syncytium undergoing compatible or incompatible reactions) that represent a far minority of the cells within the tissue, organ or organism to some level of purity could be done to unravel the intricacies of gene expression that define syncytium biology in each reaction type (**Figure 1**). After cell isolation, subsequent gene expression studies can be done. As should be imagined, genes pertaining to the resistance reaction may be active or even de-activated specifically in these cells. In contrast, other genes may be influenced by the activities of the nematode, promoting the formation and maintenance of the nurse cell. Whether studying the compatible or incompatible reaction, the genes involved in those processes will be concentrated at the site of infection, the syncytium.

Where possible, methods to isolate the cytoplasm from nurse cells have relied on experiments using hand dissections. The first experiments obtained giant cells from galls induced by the root knot nematode (*Meloidogyne incognita*) during a compatible interaction in tomato (*Lycopersicon esculentum*) (Wilson et al. 1994). The advantage of experiments using the root knot nematode is that infected root regions are obvious because of the formation of enlarged galls on the roots that can be dissected. The experiments permitted the isolation of RNA from those cells and the construction of cDNA (Wilson et al. 1994). Relatively few of the cDNAs turned out to be gall specific. This outcome would be expected since they were plant genes involved in basic metabolic processes (Bird and Wilson 1994). However, the significance of the experiments was that they demonstrated the efficacy of the approach in isolating RNA from those cell types. Other more recent experiments performed in the model organism *A. thaliana* have demonstrated that it is possible to dissect out root regions where giant cells were developing during infection by *M. incognita* (Jammes et al. 2005). The experiments provided a genomics-based blueprint for giant cell development over a wide range of time points (Jammes et al. 2005). The work also confirmed the expression of a panel of genes through promoter-reporter assays (Jammes et al. 2005).

In contrast to plants experiencing root knot nematode infection, hand dissections cannot be used to isolate syncytia induced to form by the activities of *H. glycines*. The reason is because syncytia formation is not accompanied by the development of obvious swollen root regions as happens during infection by root knot nematodes. Thus, the identification of *H. glycines*-infected root regions is not an obvious or a straightforward procedure. Also, the root region is not dominated by cytoplasm from the nurse cell as in the case with the root knot nematodes. Therefore, alternative methods would have to be adapted to accomplish the same task for genomics-based analyses in soybean. Such a method had its roots in experiments performed in the 1970s.

Laser capture microdissection (LCM) is an alternative means that affords a high degree of precision and accuracy to isolate homogeneous cell populations that are otherwise technically difficult to isolate. The original work that developed the technology was done in research that studied complex animal tissues. The technological achievement involved engineering a microscope with a laser beam to isolate cells from freeze-dried samples (Isenberg et al. 1976; Meier-Ruge et al. 1976). The procedure was largely forgotten about until Emmert-Buck et al. (1996) redesigned the microscope so that it could be used to isolate cells from histological sections for molecular work. Later work used single cell gene profiling (in the absence of LCM) in animal pancreatic samples (Chiang and Melton, 20002)

showed the importance of single cell profiling techniques in investigating developmental processes. The first plant-based research followed (Asano et al. 2002) showing the efficacy of the LCM approach for molecular analyses.

The successes gained by use of LCM in studying *H. glycines* infection is based on the ability to use *H. glycines* itself as an *in situ* physical marker to locate the syncytium in infected tissues prior to LCM (Klink et al. 2005) (**Figure 2**). The use of LCM in studying cyst-forming nematodes demonstrated how the LCM technology would be a major advancement in investigating plant pathogen interactions at the genomics level (Klink et al. 2005). The LCM procedure allowed for the collection of feeding sites to the exclusion of other cell types not involved in infection (**Figure 4**). LCM has proven to be especially valuable to study the development of the syncytium during soybean infection by *H. glycines* in comparative analyses of compatible and incompatible reactions (Klink et al. 2005, 2007b, 2009b, 2010a, b). It has also been extremely useful in studying the various forms of the resistant reaction (Klink et al. 2011). Thus, like in animal studies, LCM would be of value in studying plant developmental and pathological processes (Klink et al. 2005, 2007b, 2009b, 2010a, b; 2011). This capability of LCM would allow for the identification of genes specifically involved in either the resistant or susceptible reaction without other cell types interfering in the analysis procedures.

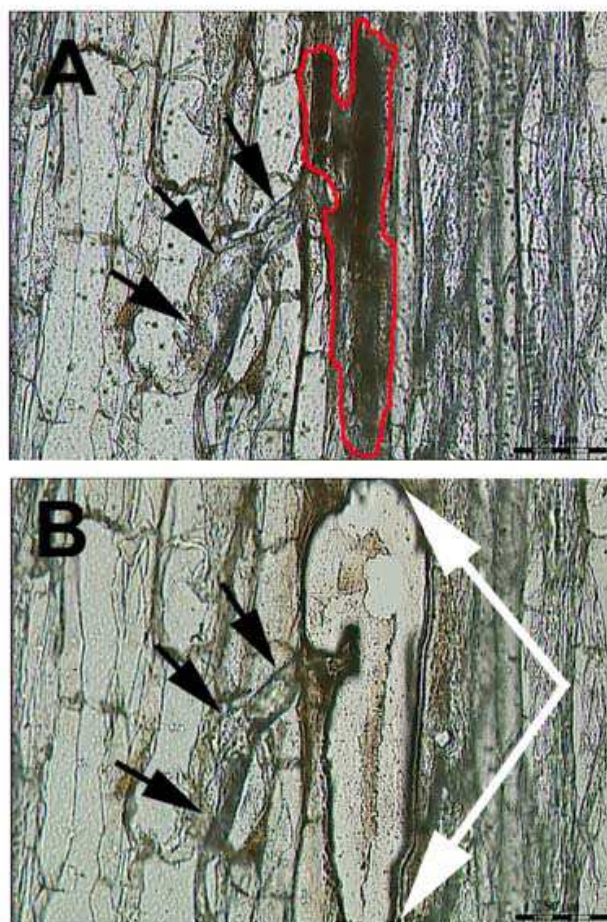


Fig. 4. Microdissection of a syncytial cell. **Fig. 4A**, a 3 dpi time point syncytial cell (area encircled in red) prior to microdissection was identified by their proximity to *H. glycines* (three black arrows). **Fig. 4B**, the same syncytial cell section from **Fig. 4A** after microdissection; microdissected syncytial cell (area between white arrows).

9. Genomics- based analyses of the developing syncytium undergoing a resistant reaction

Prior to genomics-based methods, gene expression studies of nurse cells relied heavily on reporter-based assays (reviewed in Gheysen and Fenoll, 2002; de Almeida Engler et al, 2004; Jammes et al. 2005; Klink et al. 2005). Such experiments are important for a variety of reasons including the demonstration of enhanced expression in the nurse cell as compared to the surrounding cells. Unfortunately, the expression of only a few genes could be demonstrated at any one time. In contrast, genomics-based analyses procedures involving microarrays (Schena et al. 1995) are a way to study the expression levels of thousands of genes simultaneously. Since the LCM procedure purifies the cell populations to the exclusion of the unassociated cells, the procedure will demonstrate gene expression that is specific to the nurse cell. However, even though the RNA is isolated from the nurse cells, it does not mean that these genes cannot be expressed at higher or lower levels or not at all in other cell types within the plant. Methodologies to analyze gene expression occurring during syncytium development have employed two major procedures; (1) differential expression (DE) and (2) detection call methodology (DCM). Each method is an important type of microarray analysis. The original microarray analysis that studied syncytium gene expression during both compatible and incompatible reactions in soybean employed both methodologies. The differential expression (DE) (Klink et al. 2007a, 2009a, 2010a, 2011) and detection call methodology (DCM) (Klink et al. 2010b) have various advantages because the cells under study are homogeneous populations.

10. Differential Expression (DE) Analyses in studying homogeneous cell populations

The more common gene expression analysis procedure is the DE analysis. The premise of DE analyses is that one mRNA sample, such as an uninfected root sample is used as a base line (control) for gene expression experiments. The comparison is made to a second experimental mRNA sample such as one isolated from a nematode infected root. For DE analyses to be possible, the gene must be expressed in each sample type in order for statistical analyses to be possible (**Figure 5**). If the gene is expressed (or present) in each sample type then differential expression analyses can be performed. Otherwise, if the gene is present on one sample and absent in the other, the probe set is discarded from the analysis. This is because statistics cannot be done on that probe set even though, biologically speaking, it may be differentially expressed to an extent even more so than many genes that make it into DE analyses.

If the gene is present in both sample types, one of three properties of gene expression can be determined in comparisons between experimental and control samples. Through statistical analyses, if the gene transcript is present in higher quantities in the experimental sample than the control, the gene is considered up regulated or induced in expression. In contrast, if the gene transcript is lower in quantity than in the experimental than the control sample, the gene is considered down regulated or suppressed in expression. The last alternative is that there is no change in quantity of transcript between the experimental and the control. Thus, its expression is unaltered.

While sophisticated statistical analyses are available to provide confidence in the results provided by DE analyses, many genes are excluded from the analysis because, as mentioned

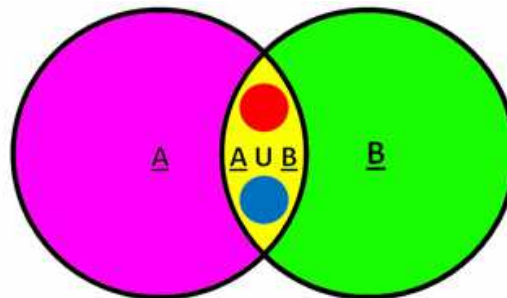


Fig. 5. Sorting microarray data through differential expression (DE) and detection call methodology (DCM). Pools A and B are different sample types. Analyses have identified genes that are unique to A (purple), genes that are unique to B (green) and genes common to both pools ($A \cup B$). Only genes that are present in both A and B can be used for differential expression analyses. The red circle represents the genes that are present in both pools and are also differentially expressed, measuring induced gene activity. The blue circle represents the genes that are present in both pools that are differentially expressed, measuring suppressed gene activity. The remaining genes (yellow) are present in both samples (common) but are not differentially expressed. Image adapted from Klink et al. 2010b.

earlier, they are detected in only one of the two sample types. In these cases, especially when different cell types are under study, it is important to investigate why a particular transcript is present in one sample type and absent in another. This is especially important when the genes are expressed only in syncytia undergoing an incompatible reaction. The reason why it is important to go back and investigate these pools of discarded genes is that they may actually be the genes that define the resistant reaction. Those analyses (see below) have been performed, revealing genes expressed specifically within the syncytium undergoing a resistant reaction (Klink et al. 2010b).

11. Detection Call Methodology (DCM) in studying homogeneous cell populations

An important facet of microarray methods is that they provide useful information on both the numbers and types of transcripts that are present or absent within samples (Hill et al. 2000) (**Figure 5**). DCM is an underutilized form of microarray analysis because it identifies genes that are actively expressed within a sample (Seo et al., 2004; McClintick and Edenberg, 2006; Rème et al., 2008; Klink et al. 2010b; Archer and Reese, 2010). The DCM may not necessarily provide information on how actively expressed the gene actually is like that provided by the DE analysis. However, DCM has been used in a variety of studies to simply answer the question of what genes are actively expressed within a particular sample under study (Hill et al. 2000; Birnbaum et al. 2003; Poroyko et al. 2005). DCM-based studies include comparative analysis of syncytium development during a compatible and incompatible reaction (Klink et al. 2010b). DCM may be a good way to identify rare transcripts that are lost in DE analyses (Klink et al. 2010b).

The DCM is a statistically sound method. Several recent papers have used DCM to understand transcription in various model experimental systems (Seo et al., 2004; McClintick and Edenberg, 2006; Rème et al., 2008). The utility of the method is that genes that are actively expressed can be detected within a particular sample type. This procedure provides a relatively inexpensive assessment of what genes are actively expressed in a cell

type. An extension of the DCM is to use them to compare transcripts between different cell types or of the same cell type and at different points during a time course (Hill et al. 2000; Birnbaum et al. 2003; Poroyko et al. 2005). The DCM takes into consideration only the presence of the transcript as measured by the probe set on the microarray. Thus, DCM can be used as a measurement of the diversity of transcripts within those samples during a developmental process (**Figure 5**). This is a very important aspect of the DCM because complex tissues and organs are composed of numerous cell types, each with its own gene expression pattern (Birnbaum et al. 2003). As it should be imagined, each of the different cell types would have a basic gene expression pattern that could be studied between two samples. This is because many genes would be expected to be expressed within a sample pair (i.e. control and experimental). However, in the case of studying gene expression of individual homogeneous cell populations, many genes (including rare transcripts) would be expected to be uniquely expressed in one of the two sample types, such as those activated during the incompatible reaction in the syncytium (Klink et al. 2010b). Unfortunately, genes experiencing this expression profile, detected only in the sample undergoing the resistant reaction and not the control sample, would be thrown out of the DE analysis. Unless cognizant of DE methodologies, the genes are not considered in downstream analyses. As can be imagined, these discarded genes may actually define the resistance reaction since they are expressed only in cells undergoing that reaction as compared to the control that lack their expression. While DE analyses dominate biological investigations, DCM-based analyses are a well suited analysis tool for investigations such as those involving homogeneous cell populations undergoing a specific process such as a resistant reaction (Klink et al. 2010b).

12. Cellular analyses of the resistant reaction of Peking and PI 88788

Genomics-based analyses of the resistant reaction of the Peking-type of reaction have identified highly induced activities of genes including jasmonic acid signaling that include lipoxygenase (LOX) and JASMONATE RESISTANT 1 (JAR1) (Klink et al. 2007a, 2009a). LOX expression is also highly active in PI 88788 (Klink et al. 2010a). LOX is not only associated with plant development and responses to environmental change, but are known well for their involvement in plant defense to pathogen challenge (Song et al. 1993). LOXs are non-heme dioxygenases, catalyzing the oxygenation of polyunsaturated fatty acids. This activity leads to hydroperoxide generation. Hydroperoxides can be converted into (1) stable aldehydes, (2) hydroxy- and epoxy-fatty acids that exhibit antimicrobial activity and (3) jasmonic acid. Jasmonic acid is derived from the LOX product 13-hydroperoxy-octadecatrienoic acid and has been shown genetically to be involved in plant defense responses (Vijayan et al. 1998; Rancé et al. 1998). Importantly, ZmLOX3 is shown to control resistance to *M. incognita* (Gao et al. 2008). In agreement with this observation, a homolog of the *A. thaliana* gene JASMONATE RESISTANT 1 (JAR1) is induced during the incompatible reaction (Klink et al. 2009b). JAR1 is an ATP-dependent JA-amido synthetase (Staswick et al. 2002; Staswick and Tiriyaki 2004) that is essential for defense against the soil fungus, *Pythium irregulare* (Staswick et al. 1998). JAR homologs are present in various plant families. Therefore the genes and their activities are conserved in response to pathogens, having various roles in the defense response (Wang et al. 2007, 2008). Other highly induced genes include S-adenosylmethionine synthetase (SAM) (Klink et al. 2009a). SAM catalyzes the formation of S-adenosyl-L-methionine. This product is an

important methyl group donor in most transmethylation reactions that participate in plant lipid, nucleic acid, polysaccharide, secondary products and polysaccharide synthesis. S-adenosyl-L-methionine is a precursor of ethylene and is also necessary for constituents of the phenylpropanoid pathway, a pathway involved in lignin biosynthesis. The formation of cell wall appositions in Peking are consistent with the induced activity of genes involved in lignin biosynthesis such as SAM. Cell wall appositions have been known for over a century (Aist, 1976). The structures have been measured and are well known to be involved in plant defense to fungal pathogens (Israel et al. 1980). This is an important observation because of the presence of cell wall appositions during the resistant reaction in Peking and PI 437654 but not in PI 88788. The cell wall appositions contain several substances such as phenolics, callose, lignin, chitin, lipids, suberin, silicon and other cell wall proteins such as hydroxyproline-rich glycoproteins and peroxidases (McLusky et al. 1999). All of these substances or the enzymes involved in their synthesis have been found in genomics analyses of the syncytium undergoing an incompatible reaction in Peking and PI 88788 (Klink et al. 2007a, 2009a, 2010b, 2011). An important facet of this activity is that the metabolic process is accompanied by a local release of reactive oxygen species (ROS) (Levine et al. 1994; Tenhaken et al. 1995; Hueckelhoven et al. 1999). This activity includes the release of hydrogen peroxide (Levine et al. 1994; Tenhaken et al. 1995), consistent with experiments of the resistant reaction in Peking that compared the incompatible syncytia to the compatible syncytia (Klink et al. 2007a). It was noted that the soybean actin homolog, ACT-7, was also induced during the incompatible reaction. Both hormone treatment and wounding induces ACT-7 expression, being required for hormone-induced callus formation in *A. thaliana* (Hardham et al. 2008), in agreement with cell wall apposition development in Peking (Klink et al. 2007b, 2009b).

In agreement with these observations, components of the phenylpropanoid pathway are induced in their activity in syncytia undergoing an incompatible reaction (Klink et al. 2009b). Some of these genes included phenylalanine ammonia lyase (PAL), Cinnamoyl-CoA reductase (CCR) and caffeic acid O-methyltransferase (COMT). PAL is a key enzyme in the phenylpropanoid pathway, catalyzing the first reaction of phenylpropanoid biosynthesis. PAL converts phenylalanine into trans-cinnamic acid, a substance that can be both hydroxylated and methylated. These derivatives can produce compounds such as coumaric, caffeic, and ferulic acids, substances that are toxic to both herbivores and pathogens (Cole 1984; Leszczynski et al. 1989; Verpoorte and Alfermann 2000; Morello et al. 2005; Wang et al. 2006). CCR activity occurs several steps downstream of PAL of the lignin biosynthesis pathway. CCR catalyzes the conversion of cinnamoyl-CoA esters to their respective cinnamaldehydes. Of note, CCR is the first enzyme of the monolignol-specific part of the lignin biosynthetic pathway (Raes et al. 2003; Mir Derikvand et al. 2008). COMT is involved in the later steps of lignin biosynthesis (Goujon et al. 2003; Raes et al. 2003; Ferrer et al. 2008). In *A. thaliana*, COMT mutants are deficient in lignin biosynthesis and production of soluble phenolics (Goujon et al. 2003). The COMT mutant, *atomt1*, also affects the expression of genes pertaining to monolignol biosynthesis (Goujon et al. 2003). The induced states of these enzymes (Klink et al. 2007a, 2009a) are consistent with the presence of cell wall appositions in Peking and observations of thickened cell walls in cells undergoing a resistant reaction. The induced states of key enzymes of the lignin biosynthesis are just one pathway of many that exhibit altered states of expression as a consequence of the resistant reaction (Klink et al. 2007a, 2009a, 2011). In addition to the jasmonic acid and

phenylpropanoid biosynthetic pathways, numerous genes are also specifically suppressed during the interaction between *H. glycines* and soybean (Klink et al. 2009b).

The potent but prolonged resistant reaction of PI 88788 was also studied, demonstrating similarities in gene expression exist between Peking and PI 88788 (Klink et al. 2010a, 2011). The gene expression analyses revealed highly induced activities of components of the jasmonic acid signaling pathway. The analyses also identified induced activities of the phenylpropanoid pathway. However, direct comparisons of the two forms of the resistant reaction were needed to determine what differences existed between Peking and PI 88788.

13. Comparative genomics analyses of the different forms of the resistant reaction

As mentioned in an earlier section, two major forms of the resistant reaction exist in soybean, Peking and PI 88788. Between the Peking and PI 88788 forms, the rate at which the potent reaction occurs is more rapid in Peking. Altered *H. glycines* development is observed in the p-J2 stage in Peking (Endo 1964, 1965; Riggs, 1973; Kim et al, 1987; Kim and Riggs 1992), followed by altered development at the J3 and J4 stages in PI 88788 (Kim et al. 1987). In determining the different forms of the incompatible reaction, it is possible through bioinformatics procedures to make comparative analyses between different genotypes at the cellular level (Klink et al. 2011). Research in other plant-pathogen systems has also demonstrated that this is possible. In interactions between the pathogen *Fusarium graminearum* (Fusarium head blight) and the wheat genotypes *Triticum aestivum* L. cv. Ning (resistant) 7840 and *T. aestivum* L. cv. Clark (susceptible), comparative studies have been performed using whole plant organs. However, as discussed earlier, the studies began by identifying genotype-specific differences in gene expression that are present even prior to pathogen invasion (Bernardo et al. 2007). Variations in gene expression are present between Peking and PI 88788 (Klink et al. 2011). In some cases, these differences in gene expression occur in the absence of a pathogen prior to infection (Klink et al. 2011). The differences in gene expression may be an important basic property of the different genotypes to consider since it is possible that the relative differences in gene activity presages their ability to resist infection by *H. glycines*. For example, substantially higher levels of the differentially expressed in response to arachidonic acid 1 gene (*DEA1*) and a protease inhibitor are present in Peking as compared to PI 88788 (Klink et al. 2011). In tomato, *DEA1* exhibits organ-specific expression, being highly expressed in roots, stems, and leaves (Weyman et al. 2006a). The *DEA1* gene is induced by arachidonic acid (AA). This observation could be an important clue to the rapid and potent resistant reaction of Peking. AA is a polyunsaturated fatty acid molecule that is produced by various pathogens (i.e. *Phytophthora infestans*). The molecule is known to trigger programmed cell death (PCD), a hallmark of the resistant reaction in Peking (Kim and Riggs 1992). In other plant-pathogen interactions, AA is shown to be released from germinating *P. infestans* spores (Ricker and Bostock, 1992) and can mimic the PCD response (Bostock et al. 1981; Bostock et al. 1986). While there are many gaps remaining in the role of *DEA1*, it has been shown to have a conserved, shared domain found in the eight-cysteine motif superfamily of protease inhibitors. The domain is also found in proteins such as alpha-amylase inhibitors, lipid transfer proteins and seed storage proteins (Weyman et al. 2006a). Other experiments including reporter analyses involving normal protoplasts and protoplasts undergoing plasmolysis show that *DEA1* is associated with the cell membrane (Weyman et al. 2006a). In a heterologous yeast system, *DEA1* protects the

yeast from freezing death (Weyman et al. 2006b). AA, since it functions upstream of jasmonate signaling (Blee, 2002), may provide a way to amplify the signal leading to the rapid and potent resistant reaction of Peking. The possibility that AA is associated with resistance is consistent with knowledge of the involvement of jasmonate signaling in the resistance of plants to parasitic nematodes (Gao et al. 2008). The analyses done on Peking and PI 88788 demonstrated that the plant-pathogen interactions are, requiring better ways to visualize the data. This gap was filled by the development of customized pathway visualization aids that could take the gene expression data and overlay them on visual gene pathway charts.

14. Pathway analyses

The genomics-based analyses of the cell-specific interactions occurring between soybean and *H. glycines* during their respective compatible and incompatible reactions resulted in a substantial amount of data. The data requires useful processing in order to put it into context. Even when annotated, the genes are typically presented in list form. Without extensive knowledge of gene function or computational power it can be difficult to determine the context of the genes or the significance of their activity. Earlier analyses that have studied plant-pathogen interactions in *A. thaliana* during infection by *Pseudomonas syringae* pv. *Tomato* determined the pathways whose expression was altered during infection (Scheideler et al. 2001). An important outcome of those analyses was that the pathway analyses identified a major metabolic shift from gene expression centering on housekeeping to pathogen defense (Scheideler et al. 2001). The far more reaching impact was that the analyses showed how complex genomics-based information can be massaged into a useful form in less genetically tractable agricultural plants such as soybean.

For some organisms (i.e. soybean), it is difficult to take large amounts of genomics-based information and cluster the information into useful pathway information. Part of the problem is that, to date, most molecular-based plant research has been generated in the model organism *A. thaliana*. Data for *A. thaliana* is housed in the data warehouse found at <http://www.arabidopsis.org/>. While *A. thaliana* is the model for molecular and genetic information in plants, the vast majority of molecular research and data warehouse development that has identified gene function has been done in the animal genetic models; *Caenorhabditis elegans* (<http://www.wormbase.org/>) and *Drosophila melanogaster* (<http://flybase.org/>) with other work done in the budding yeast model *Saccharomyces cerevisiae* (<http://www.yeastgenome.org/>). Thus, to make use of the massive amount of functional data as it pertains to soybean, it would be important to have a useful annotation of the soybean expressed sequence tag (EST) annotation. This information has recently been provided for the EST collection (Le et al. 2007) and an annotated reference genome (<http://www.phytozome.net/soybean>) (Schmutz et al. 2010). Through cross comparative analyses, genes that are conserved in primary DNA or protein structure can be identified. However, most of the work for soybean resides in customized data warehouses that are not really easily accessible to most labs.

One of the original ways to visualize gene pathways was the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.ad.jp/kegg/>) (Goto et al., 1996). KEGG was designed to computerize the current knowledge of metabolic and regulatory pathways and is an exceptional platform to obtain a deeper understanding of the genetic

circuitry within the cells under study. KEGG has been expanded to include a plant database (Masoudi-Nejad et al., 2008) that is useful for a variety of purposes, including useful secondary metabolism maps <http://www.genome.ad.jp/kegg/atlas/>. To further explore these genes, the Database for Annotation, Visualization and Integrated Discovery (DAVID) can be used as a platform to obtain additional information. DAVID (<http://david.abcc.ncifcrf.gov/home.jsp>) was erected by the National Institute of Allergy and Infectious Diseases (NIAID) with the mission of providing laboratory and bioinformatics support. To take full advantage of the well-known KEGG pathways, the DAVID pathway viewer can display genes from a user's list on pathway maps to facilitate biological interpretation in a network context. Once the KEGG annotations are complete, those data can be organized and stored into a relational database. The expression levels of genes can be correlated to a particular biochemical, physiological or metabolic role as it pertains to what developmental process or pathological interaction the cell is undergoing (Figure 6).

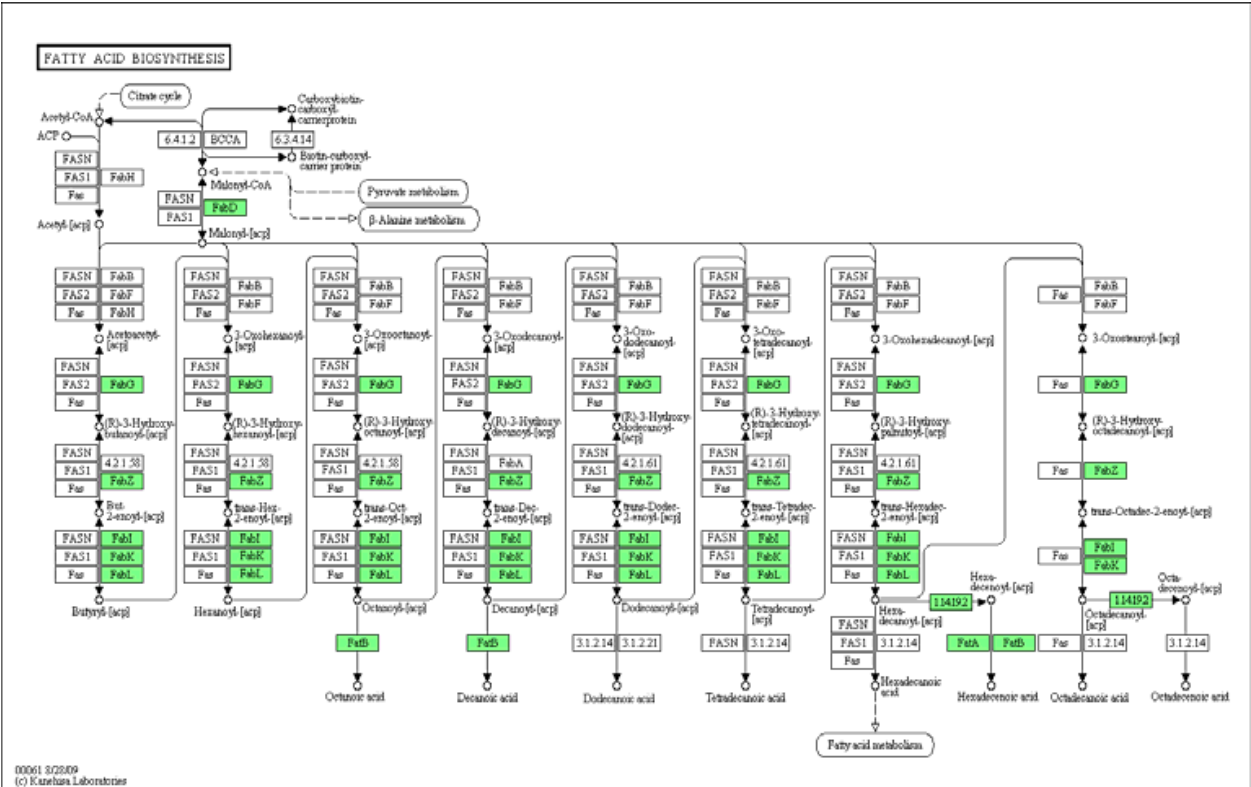


Fig. 6. Pathway analyses of genes involved in fatty acid biosynthesis of Peking. Pathway analyses were performed by determining the soybean homologs of genes found in the different pathways available in KEGG. Expression data from both Peking and PI 88788 genotypes were combined. Differential expression data obtained from laser captured syncytia was then used to color code genes found in the KEGG pathways. Legend: Green = induced gene; Red = suppressed gene; yellow = induced but not at a statistically significant level; white = no expression data. The expression map represents the combined Peking + PI 88788 expression data from the 6 dpi time point as compared to the combined Peking + PI 88788 expression data from uninfected pericycle cells (control). Image adapted from Klink et al. 2011.

15. Caution should be exhibited to pathway-based analyses

Bioinformatics-based comparisons of different forms of the resistant reaction to *H. glycines* at single cell type resolution are revealing commonalities and unique features of the different forms of the resistant reaction in soybean. Customized pathway analyses have presented the activity of specific genes composing various pathways in Peking as compared to PI 88788 (Klink et al. 2011). An example of one of the custom analyses is provided (Figure 6). However, the major problem with pathway analyses is that many genes lack homology to known genes. These genes may be conserved in primary nucleotide sequence or even protein sequence but lack any known function. The genes may lack known protein domains. The genes may have organism-specific functions (i.e. soybean). In these cases, the genes will escape meaningful analyses or placement in a pathway because they have no annotation. Genes of unknown function or annotation pose an important problem in obtaining knowledge on the cellular features of the incompatible reaction. The observations reveal a significant gap in knowledge between the ability to identify gene expression (and hence genes) and the capability to test the function of those genes in important agricultural crops like soybean.

16. Functional genomics research: the development of a rapid cycling, small soybean plant

In contrast to their field grown counterparts, dwarf varieties of agricultural plants are an avenue that allows large numbers of plants to be grown in small areas. Sometimes, the dwarf varieties have the added benefit of a rapid life cycle. For example, the dwarf tomato, *Lycopersicon esculentum* cv. Micro-Tom (Scott and Harbaugh 1989; Meissner et al. 1997) has proven extremely useful in rapidly identifying genes that control tomato development (Meissner et al. 2000; Marti et al. 2006). Some of the studies involve a procedure for rapid and efficient transformation (Sun et al. 2006; Orzaez et al. 2006). The development of the soybean genotype MiniMax (PI 643148), a dwarf, rapid cycling, MG 000 plant that grows as quickly and is as tall as the genetic model *A. thaliana* (Matthews et al. 2007; Klink et al. 2008) may fill that void (Figure 7). MiniMax is well suited for forward genetic analyses that include the development of NILs and RILs because they can be grown year-round in the greenhouse with little effort (Klink et al. 2008). MiniMax can also be used for mutational analyses. An additional advantage of MiniMax is that the rapid growth habit carries over into the tissue culture process (Klink et al. 2008). This property of MiniMax makes the isolation of transformed somatic embryos much more rapid than standard soybean genotypes, allowing for rapid reverse genetic analyses to be performed.

17. An alternative strategy for developmental genomics-based screens to study root pathogens

Soybean presents many technical challenges to studies testing gene function because of how problematic plant transformation is to most labs. These technical problems have been addressed by massive efforts over the years in various labs focusing in on various aspects of soybean transformation (Ranch et al. 1986; Hinchey et al. 1988; Parrott et al. 1989; Santarem et al. 1997; Santarem and Finer 1999; Tomlin et al. 2002; Ko et al. 2004; Collier et al. 2005; Klink et al. 2008, 2009b). A problem encountered during the early stages of the development



Fig. 7. MiniMax (PI 643148) three weeks after planting with young pods and flowers.

of a plant transformation pipeline for the study of *H. glycines* was the features to design into the vectors. At a minimum, it would be important to have vectors in hand that could be used for the overexpression of genes, the knock down gene activity through RNA interference (RNAi) (Fire et al. 1998; Klink and Wolniak 2000, 2001) and promoter analyses. However, the vectors would have to actually function in soybean under a variety of well-defined conditions.

An advantage for *H. glycines* research was that transformed soybean roots could be kept in tissue culture (Savka et al. 1990; Cho et al. 2001). In addition, modifications of those procedures have led to the development of chimeras that had transformed roots and untransformed aerial portions of the plant (Collier et al. 2005; Klink et al. 2008, 2009b). The ability to obtain transformed roots through their interaction with microorganisms had their beginnings in the work of Tepfer (1984). The experiments used the known influence that *A. rhizogenes* had on root development. The influence is known as hairy roots because of the dense clustering of root development that was caused by the transmission of genes from the root inducing (Ri) plasmid of *A. rhizogenes* into the genome of the plant cell. The most efficient hairy root transformation procedure in soybean for *H. glycines* research relies on the bacterium, *A. rhizogenes* strain K599 (Haas et al. 1995). Initial tissue culture-based analyses using the hairy root system have been employed in studying *H. glycines* development (Savka et al. 1990; Cho et al. 2001; Li et al. 2010a). The hairy root technology could be used for overexpression, promoter analyses and RNAi studies Limpkins et al. (2004). However, it was possible that overexpression and RNAi-based studies could become negatively influenced if the presence of the nematode in the infected root shut down or suppressed the activity of the promoter driving transgene expression. The figwort mosaic virus sub genomic transcript (FMV-sgt) promoter (Bhattacharyya et al. 2002) allows for high levels of expression of transgenes throughout the course of nematode infection of soybean roots in numerous genotypes (Klink et al. 2008). New Gateway® compatible versions of the original plasmid DNA vectors used in those studies facilitate high throughput functional studies of the genes identified through the gene expression studies (Klink et al. 2009b).

A different strategy that obtains whole plants uses the hairy root procedure to obtain transformed roots that develop on the untransformed seeding. The method produces plants known as composite plants due to their chimeric nature (Collier et al. 2005; Klink et al. 2008, 2009b). This procedure was a major advancement for studying root biology (Collier et al. 2005). An important feature of the composite plant technology is that the transformation procedure can be done in the absence of tissue culture or axenic conditions (Collier et al. 2005; Klink et al. 2008, 2009b). Tissue culture is not needed because the plants are selected by the presence of the enhanced green fluorescent protein (GFP) (Haseloff et al. 2007) as a visual marker. Gateway®-compatible vectors (Klink et al. 2009b) designed for their use in soybean make highthroughput analysis a reality. During this transformation procedure in soybean, dozens of roots are produced on any individual plantlet (Klink et al. 2009b). However, only those roots that are transformed as observed by their GFP expression are allowed to grow (Klink et al. 2009b). Roots lacking fluorescence are trimmed off of the stock and replanted (Klink et al. 2009b). Transformation efficiencies are usually around 100% of the stocks exhibiting positive GFP fluorescence. The composite plants are ready within 40-45 days for experiments in soybean (Klink et al. 2009b). Quantitative real time PCR (qRT-PCR) is used to demonstrate the expression of the transgene. Of note, whole transformed plants have been generated from transformed hairy roots (Tepfer 1984; Zdravkovic-Korac et al. 2004; Crane et al. 2006). This observation provides an additional route that can be taken to

obtain whole transformed plants. The method has been used to obtain soybean roots that are resistant to *H. glycines* infection by engineering *H. glycines* genes in as inverted hairpin repeats, resulting in an RNAi phenocopy of gene activity (Klink et al. 2009b; Li et al. 2010b).

18. Summary

With new technologies, the resistant reaction of soybean to the *H. glycines* is being studied at the resolution of its nurse cell known as the syncytium. LCM, in concert with genomics-based analysis methods, is identifying genes that are specific not only to the susceptible or resistant reaction, but also to the different forms of the resistant reaction found in different soybean genotypes (Klink et al. 2005, 2007b, 2009a, 2010a, b, 2011). The application of the technologies and procedures outlined in this review are allowing for the identification of common strategies that the many different soybean genotypes use to combat plant parasitic nematode infection. The method has application even to study other pathogens or symbionts. However, the methods underscore the importance of the development of meaningful gene annotation databases that can maximize the usefulness of the information. Equally important are public availability of the data and that it is easy to mine. The development of the tools will allow many labs access to explore the function of genes in meaningful genomics analyses that are actually functional in nature (Klink et al. 2009a). Databases housing and storing data in model genetic organisms have already demonstrated the value of such databases to the research community. When these resources are developed, real solutions to real agricultural problems, whether local and specialized or a global and generalist scale, such as those presented by plant parasitic nematodes will be achieved.

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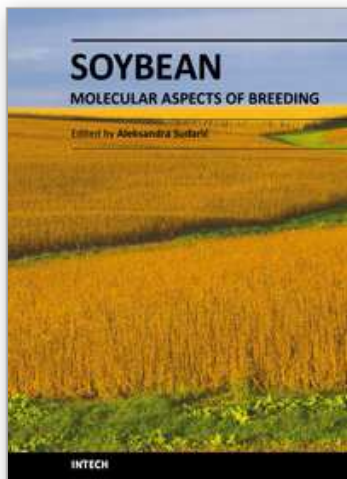
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