THE APPLICATION OF A DEVELOPMENTAL GENOMICS APPROACH TO STUDY THE RESISTANT REACTION OF SOYBEAN TO THE SOYBEAN CYST NEMATODE

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ABSTRACT

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The infection of plants by parasitic nematodes is a ubiquitous problem of agriculture. While all plant tissues can be parasitized by plant parasitic nematodes, the root parasites are the most important, economically. Infections are either compatible, resulting in a susceptible reaction or incompatible, resulting in plant resistance to the nematode. During a compatible reaction, nurse cells that the nematode feeds from are stimulated to form. The nematode can successfully complete its life cycle because ample nourishment is available to the nematode from the nurse cell. In contrast, the resistant reaction that leads to nematode mortality is localized to the nurse cell and sometimes the cells directly surrounding the nurse cell. During the resistant reaction the anatomy of the cells fed on by the nematode initially resemble cells undergoing a compatible reaction. However, a variety of cellular changes occur that results in the collapse of the nurse cell leading to nematode mortality. Gene expression analyses point to jasmonic acid being an important signaling event, locally in the syncytium, during the resistant reaction. This review focuses in on cell isolation procedures and genomics tools that have assisted in the analyzing gene expression of these feeding cells as they undergo a resistant reaction in an agricultural model system soybean-soybean cyst nematode.

Key words: Genomics, *Heterodera glycines,* laser capture microdissection, microarray analysis, *s*oybean cyst nematode, soybean, syncytium.

RESUMEN

Klink, V. P., G. W. Lawrence, P. D. Matsye, and K. C. Showmaker. 2010. La Aplicación de un método de genómica de desarrollo para estudiar la reacción de resistencia de la soya al nematodo quiste de la soya. Nematropica 40:1-11.

La infección de las plantas por nematodos fitoparásitos es un probelma generalizado en la agricultura. A pesar de que los nematodos pueden parasitar todos los tejidos de la planta, los de mayor importancia económica son los parásitos de raíces. Las infecciones pueden ser compatibles, resultando en una reacción susceptible, o incompatibles, resultando en resistancia de la planta al nematodo. Durante la reacción compatible, se estimula la formación de las células de las cuales se alimenta el nematodo. El nematodo completa su ciclo de vida con éxito cuando hay suficiente alimento. En contraste, la reacción de resistencia que ocasiona la muerte del nematodo involucra las células alimenticias y también las células que las rodean. Durante la reacción de resistencia, la anatomía de las células alimenticias inicialmente es igual a la de las células alimenticias de la reacción compatible. Sin embargo, eventualmente ocurren una serie de cambios que resultan en el colapso de las células alimenticias y la muerte del nematodo. Los análisis de expresión génica indican que el ácido jasmónico es una señal importante en la reacción de resistencia, de manera local en el sincicio. Esta revisión se centra en procedimientos de aislamiento de células individuales y en herramientas de genómica que han ayudado a estudiar la expresión de genes en las células alimenticias durante la reacción de resistencia en el sistema modelo soya-nematodo quiste.

Palabras clave: análisis de micromatrices, genómica, *Heterodera glycines,* captura por microdisección láser, nematodo quiste de la soya, sincicio, soya.

Glycine max (soybean), undeniably, is an important agricultural crop on a global scale. Grown worldwide, soybean provides 70% of the world's protein meal. The contribution of soybean to world soybean meal translates to 157.5 million metric tons, annually. No other crop (including rice and corn), provides more than 12% (rapeseed). Soybean provides the second most amount of the world's vegetable oil (30% [37.5 million metric tons]) with palm (32%) providing the most. The third most would be rapeseed (15%). Thus, the worldwide importance of soybean, with the top four producers located on three different continents (U.S. [North America], 84 million metric tons; Brazil [South America], 57 million tons; Argentina [South America], 41 million tons and China [Asia], 18 million tons), with more grown in Europe, Africa and Australia, is undeniable. Soybean is the number 1 value crop export of the U.S. (http://www.soystats.com/2009/page_04.htm). The statistics presented here do not account for the significant contribution of soybean to other industries such as biodiesel production, food additive and industrial uses.

Soybeans are hosts to a number of pests and pathogens. The parasitic nematodes represent the major, dominant and persistent problem for soybean cultivation. Remarkably, soybeans are hosts to over 100 species of nematodes (Sinclair and Backman, 1989). The most devastating pathogen of soybean is the soybean cyst nematode, *Heterodera glycines. Heterodera glycines* was first observed on soybean in Japan in 1881 (Schmitt and Noel, 1984). It has been documented, historically, that *H. glycines* can both invade and spread rapidly into new areas cultivating soybean. *Heterodera glycines* was first identified in the U.S. in 1954 (Winstead *et al.,* 1955). By 1957, *H. glycines* had already been identified as far west as the state of Mississippi (Lawrence and McLean, 1999). Recent reports rank *H. glycines* infection as causing more agronomic loss of *G. max* production than the rest of its pathogens combined (Wrather *et al.,* 2001). *Heterodera glycines* infection, itself, causes more damage to soybeans in the U.S. (~1.5 billion $) than the entire value of some agricultural crops (i.e. strawberries http://www.calstrawberry.com/). *Heterodera glycines* reproduce on at least 97 legume and 63 non-legume hosts. *Heterodera glycines* are nearly ubiquitous (93.5% of total acreage) where *G. max* is cultivated, worldwide (reviewed in Riggs, 2004).

Resistance of *G. max* to *H. glycines* was determined soon after the identification of *H. glycines* in the U.S. (Ross and Brim, 1957). The availability of thousands of *G. max* genotypes, named according to a plant introduction (PI) scheme and a maturity group (MG) hierarchy make the identification of resistance in the germplasm possible. The determination of resistance is complicated by the presence of at least 16 different populations of *H. glycines* (Riggs and Schmitt, 1991; Niblack *et al.,* 2002). However, from numerous studies, over 118 sources of *G. max* resistance to *H. glycines* have been identified (Rao-Arelli *et al.*, 1997; reviewed in Shannon *et al.,* 2004). Only a few of these sources are used for commercial development in the U.S. The reason is because many of these resistant genotypes also carry undesirable traits that can be passed on in classical breeding programs (reviewed in Shannon *et al.,* 2004). This observation demonstrates the impor-

tance of the identification of resistance genes in any *G. max* genotype that exhibits these traits.

The life cycle of H. glycines

Heterodera glycines is an obligate endoparasite of *G. max* whose chronology of its life cycle events (Fig. 1) is well established (Lauritis *et al.*, 1983; Koenning, 2004). Cysts, encasing the eggs, are able to remain dormant in the soil for up to 9 years (Inagaki *et al.*, 1971). After hatching, the second stage pre-infective juveniles (pi-J2s) migrate toward the root and burrow into it. The infective J2s (i-J2s) then migrate toward the root stele. When reaching the targeted cell for nurse cell formation, typically a pericycle cell or neighboring root cell, a stylet emerges from the anterior end of the nematode. The stylet then penetrates the target cell. At this point, the nematodes are parasitic J2s (p-J2). The p-J2 then presumably releases substances into the plant cell that then cause major changes in the physiology of the root cell. The infected root cells subsequently fuse with neighboring cells. The repeated cell fusion events produce a syncytium that contains approximately 200 merged root cells (Jones and Northcote, 1972; Jones, 1981). The syncytium serves as the *H. glycines* nurse cell. After the establishment of the syncytium, p-J2 nematodes that eventually will develop into males feed for several days. The feeding process proceeds until the end of their J3 stage. Meanwhile, the males become sedentary and stop feeding. The process is followed by a molt into vermiform J4 males. The males burrow out of the root in preparation for mating. In contrast to the males, the p-J2s that eventually will develop into females become sedentary after the establishment of their feeding site. The female nematodes then increase in size while undergoing both J3 and J4 molts. During growth and maturation, the posterior of the female will push out of the root boundary, providing access to the male for copulation. After copulation, the adult females will grow into an

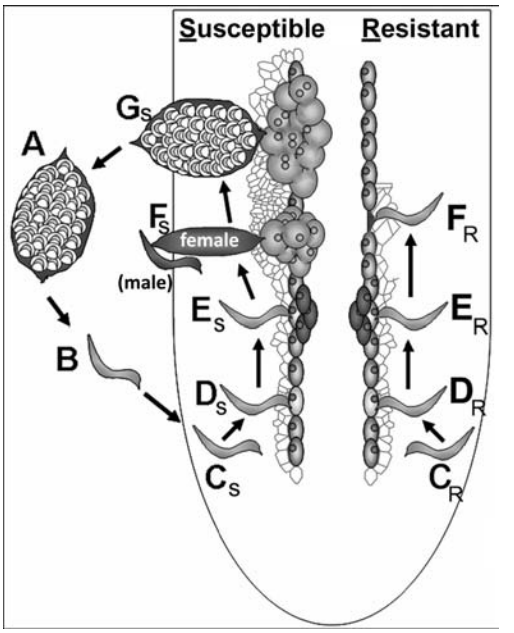

Fig. 1. The life cycle of *H. glycines* during a susceptible and resistant reaction of soybean. Fig. 1A, cysts with eggs (white) hatch. Fig. 1B, second stage pre-infective juveniles (pi-J2) (gray) hatch and migrate toward the root. SUSCEPTIBLE REACTION: Fig. $1C_s$, The infective-J2 (i-J2) nematodes burrow into the root and migrate toward the root stele (dark gray). 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 subsequent 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 because its body emerges from the root tissue. RESISTANT REACTION: Fig. $1C_R$, Like that found during the susceptible reaction, the infective-J2 (i-J2) nematodes burrow into the root and migrate toward the root stele (dark gray). Fig. $1D_R$, feeding site selection by the parasitic J2 (p-J2). Fig. $1E_R$, like the susceptible reaction, the syncytium begins to develop. Fig. $1F_R$, the syncytium has collapsed resulting in nematode mortality. Figure adapted from Klink *et al.* (2009b).

enlarged and hardened structure. Ultimately, the female develops into the cyst that encases the eggs and can remain dormant for years.

Resistance, the cellular processes

The anatomical data of the infection process of *H. glycines* in *G. max* roots clearly demonstrate that the syncytium is undergoing an earlier parasitism phase whose cellular responses to the nematode appear similar during the susceptible and resistant reactions (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). The earlier phase is referred to as phase 1 (Klink *et al.,* 2009a). The cellular processes that occur during the earlier phase of both susceptible and resistant reactions include the dissolution of cell walls, enlargement of nuclei, hypertrophy, the development of dense cytoplasm and increased ER content (Endo, 1965; Riggs *et al.*,1973; Kim *et al.*, 1987; Mahalingam and Skorpska, 1996).

The earlier phase of infection is followed by a later phase that clearly differentiates the resistant from the susceptible reaction. This later phase is known as phase 2 (Klink *et al.*, 2009a). Phase 2 of the susceptible 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).

Three major forms of the resistant reaction are present in the germplasm of *G. max*. All are potent, but vary in how rapidly they result in the collapse of the syncytium and mortality of the nematode. In the *G. max* genotype PI 437654, phase 2 of the resistance reaction becomes evident at both the cytological and ultrastructural levels by 2 days post infection (dpi), the most rapid and potent response, blocking *H. glycines* development at the p-J2 stage (Mahalingam and Skorpska, 1996). Resistance is evident at 4 dpi in *G. max* genotype Peking/PI 548402, blocking *H. glycines* development at the p-J2 stage (Endo, 1965; Riggs *et al.*, 1973). The Peking resistance process involves necrosis of the cells that surround the nematode's head, separating the syncytium from its adjacent cells (Kim *et al.,* 1987). The response is followed by degeneration of the p-J2 nematode within 4-5 dpi (Endo, 1964, 1965; Kim *et al.,* 1987; Kim and Riggs, 1992). However, syncytia are continuing their later stages of the resistant reaction even at 7 dpi (Riggs *et al.,* 1973). Consequently, by 8 dpi the resistant reaction is largely completed.

In contrast to the Peking-type of resistance, the *G. max* genotype PI 88788-type of resistance reaction lacks the development of a necrotic layer (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 or resistant reaction involves nuclear degeneration within the syncytium by 5 dpi (Kim *et al.,* 1987). 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). Thus, the designation of the PI 437654, Peking and PI 88788-types of resistance reactions is justified for the manner that syncytium degenerates and the developmental stage when *H. glycines* experiences its mortality. The important observation from the numerous cytological investigations was that these cellular changes that characterize the resistant reaction lead to a decrease in nematode growth and ultimately their arrest in development at specific stages.

Genetics of the G. max-H. glycines interaction

The resistance reactions of PI 437654, Peking and PI 88788 to *H. glycines* have been defined genetically through decades of genetic mapping research (Wu *et al.* 2009; reviewed in Concibido *et al.*, 2004, Cregan *et al.,* 1999; Diers *et al.,* 1997). Several recessive resistance loci (*rhg1*, *rhg2* and *rhg3* [Caldwell *et al.,* 1960]), and two dominant resistance loci (*Rhg4* [Matson and Williams 1965] and *Rhg5* [Rao-Arelli 1994]) have been identified. Quantitative trait loci [QTL] mapping has identified other minor QTLs that act in genetically-defined ways. 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 and references. The identification of the genes and gene networks that pertain to each type of resistant reaction are the object of intense research efforts.

Nematode feeding site isolation procedures

One method of research that would aid in understanding syncytium biology as it undergoes a resistant reaction would be isolating those cells. After cell isolation, subsequent gene expression studies could be done. As should be imagined, genes pertaining to the resistance reaction would be active specifically in these cells. In contrast, other genes may be turned off by the activities of the nematode. Historically, alternative means to isolate cytoplasm from nematode feeding sites has relied on experiments using hand dissections of infected roots. These experiments have been performed in order to obtain giant cells from galls induced by the root knot nematode (*Meloidogyne incognita*) during a compatible interaction in tomato (*Lycopersicon esculentum*) (Wilson *et al.*, 1994). Those experiments permitted the isolation of cDNA from those cells (Wilson *et al.*, 1994). However, relatively few of them turned out to be gall specific (Bird and Wilson, 1994). Those experiments, nonetheless, demonstrated the efficacy of the approach in isolating RNA from those cell types. Unfortunately, it is not possible to use this method to study syncytium formation during *H. glycines* infection.

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 recalcitrant to their isolation. The original experiments that developed the technology were done in complex animal tissues (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. The first plant-based research followed several years later (Asano *et al.*, 2002). The first use of LCM that studied cyst nematodes was performed by Klink *et al.*, (2005) and demonstrated how the LCM technology would be a major advancement in studying plant pathogen interactions. The advantage of the LCM technology is that it now was possible to study, at the molecular level, only the cells infected by cyst-forming nematodes (Fig. 2). The LCM procedure, thus, allowed for the collection of feeding sites to the exclusion of all other cell types not involved in infection. 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. LCM has proven to be especially valuable to study the development of the syncytium during *G. max* infection by *H. glycines* during both susceptible and resistant reactions (Klink *et al.,* 2005, 2007b, 2009b, 2010a, b). The value of LCM

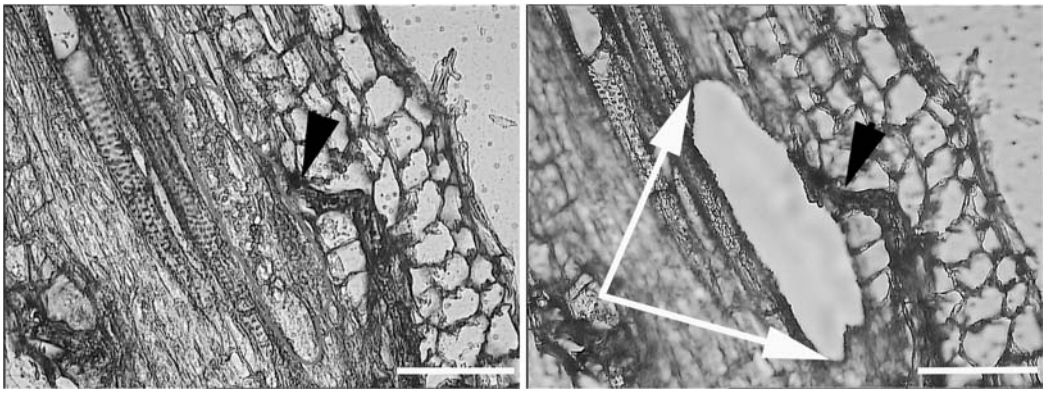

Fig. 2. Laser capture microdissection (LCM) of a syncytium undergoing a resistant reaction. Fig. 2A, Before LCM. Fig. 2B, After LCM. White line, perimeter of the syncytium; black arrow, head of nematode; white arrows, boundary of the microdissected syncytium.

in studying *H. glycines* infection is that *H. glycines* can be used as an *in situ* physical marker that can be used to locate the syncytium in infected tissues prior to LCM.

The genes expressed in the syncytium

One limitation of LCM-based analyses is that the amount of starting material is small, typically in the nanogram scale. The small amount of starting material from syncytia isolated by LCM can be overcome in DNA or RNA-based analyses because the starting material can be amplified in a manner that faithfully retains the relative amounts of genetic material (Klink *et al.,* 2005). These amplification procedures were important in studies that used that starting material in a procedure known as microarray analysis, a procedure where the expression levels of tens of thousands of genes are studied simultaneously (Klink *et al.*, 2007b, 2009a, 2010a, b).

Microarray analysis of the syncytium

A way to study the expression levels of thousands of genes simultaneously is a method known as microarray analysis (Schena *et al.*, 1995). Commercial or home-made microarrays can be analyzed by one of two major methodologies, differential expression (DE) or detection call methodology (DCM). Each method is an important type of microarray analysis. The original microarrays developed for the study of *G. max* infection by *H. glycines* resulted in the identification of genes that pertain to a susceptible reaction (Alkharouf *et al.,* 2006). Those initial analyses were followed up by a series of comparisons that studied both susceptible and resistant reaction in whole infected roots (Klink *et al.*, 2007a). The original microarray analysis that studied syncytium gene expression during both susceptible and resistant reactions in *G. max* was done by Klink *et al.,* (2007b), and followed up in a series of other studies (Klink *et al.,* 2009a, 2010a, b). The microarray studies centered on two major methods, differential expression (DE) (Klink *et al.*, 2007b, 2009a, 2010a) and detection call methodology (DCM) (Klink *et al.,* 2010b). As will be discussed, each method has its advantages, especially when the cells under study are homogeneous populations of cells isolated by methods such as LCM.

Differential Expression (DE) Analyses

The more common method of performing gene expression studies during a microarray-based study 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. Then, a second mRNA sample such as one isolated from a nematode infected root is obtained. For DE analyses to be possible, the gene must be expressed in each sample type in order for it to be possible for statistical analyses to be done (Fig. 3). If the gene is expressed (or present) in each sample type then analyses are performed that determine one of three properties of gene expression of that gene in comparisons between experimental and control samples. If the gene is expressed more actively in the experimental sample than the control, the gene is upregulated or induced in expression. In contrast, if the gene is expressed less actively in the experimental than the control sample, the gene is downregulated or suppressed in expression. The last alternative is that there is no change in expression between the experimental and the control. While sophisticated statistical analyses are available to provide confidence in the results provided by DE analyses, many genes are excluded from the analysis because they are detected in only one of the two sample types. The problem in excluding these genes is that genes expressed only in syncytia (i.e. undergoing a resistant reaction) may actually be the genes that define the resistant reaction. The reason why these genes are important is the syncytium undergoing a resistant reaction may actually be the only cell types where those genes are consistently expressed.

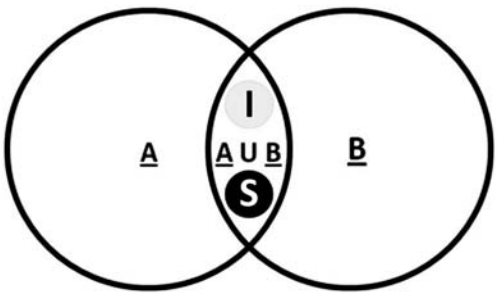

Fig. 3. Comparative analysis of DCM and differential expression analyses. Pool A and B are different sample types with gene pools identified by DCM. Analyses have identified genes that are unique to A, genes that are unique to B and genes common to both pools (A U B). Only genes that are present in both A and B can be used for differential expression analyses. The gray circle represents the genes that are present in both pools that are also differentially expressed, measuring induced (I) or upregulated gene activity. The black circle represents the genes that are present in both pools that are differentially expressed, measuring suppressed (S) or downregulated gene activity. Figure adapted from Klink *et al.* 2010b.

The value of Detection Call Methodology (DCM) in studying homogeneous cell populations

While microarray analyses provide information on differentially expressed genes (Schena *et al.,* 1995), an important facet of microarray analyses is that they provide useful information on the numbers and types of transcripts that are present or absent within samples (Hill *et al.*, 2000). The type of analyses that reveal the transcripts that are present or absent within samples is the DCM (Fig. 3). DCM is a less traditional form of microarray analysis because it results in the identification of all genes that are actively expressed within a sample. However, DCM does not necessarily provide information on how actively expressed the gene actually is in relative terms. 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 (i.e. nematode-infected syncytia undergoing a resistant reaction). Detection calls are typically

used on single microarrays. The DCM is used to answer whether a transcript of a particular gene is present or absent in a sample. The DCM is a statistically sound method based on a four-step procedure. The procedure incorporates [1] removal of saturated probes, [2] calculation of discrimination scores, [3] *p*-value calculation using the Wilcoxon's rank test, and [4] making the detection call. Detection calls are useful when the cost of an experiment is an issue because the method can be performed on a single microarray. Several recent papers have used DCM to understand transcription in various 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. Thus, if a relatively inexpensive assessment is desired to see what genes are actively expressed in a sample, this analysis method may be a cost effective strategy if microarrays are available. In the DCM no cDNA library would have to be made and no DNA sequencing reactions are required. 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 (Fig. 3). 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. As it should be imagined, the different cell types would have a basic gene expression pattern that could be studied between two samples 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 would be expected to be uniquely expressed in one of the two sample types, such as those activated during the resistance reaction in the syncytium (Klink *et al.*, 2010b). The genes would be uniquely expressed since genes involved in the resistant reaction may only be expressed during the resistant reaction. These genes that are detected only in the sample undergoing the resistant reaction would be thrown out of the DE analysis. 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 may actually be a well suited analysis tool for investigations such as those involving homogeneous cell populations undergoing a specific process such as a resistant reaction. Recent DCM analyses of syncytia, amidst susceptible or resistant reactions have been performed, resulting in the identification of genes specific to each reaction (Klink *et al.*, 2010b).

SUMMARY

The resistant reaction of soybean to the soybean cyst nematode is a complex process that with new technologies are being studied at the resolution of the feeding site. Technologies such as LCM, in concert with microarray analysis and other genomic analysis methods, have been identifying genes that are specific not only to the susceptible or resistant reaction, but also to the different resistant reaction types (Klink *et al.*, 2005, 2007b, 2009a, 2010a, b). The application of the technology can be used to study any plant nematode interaction, even those whose agricultural status is

more specialized than soybean. The application of these technologies would allow for the identification of common strategies that the many different plants use to combat plant parasitic nematodes. The impetus is the development of meaningful gene annotation databases that are publicly available and easy to mine so that many labs have the ability to explore the function of genes in functional genomics analyses (Klink *et al.*, 2009c). When that goal is met, real solutions to real agricultural problems such as those presented by plant parasitic nematodes will be achieved.

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