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Characterization of Root-Knot Nematode Resistance in Cowpea and Utilization of Cross-Species Platforms in Legume Genomics

A Dissertation submitted in partial satisfaction
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by

Sayan Das

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Dissertation Committee:
Dr. Timothy J. Close, Co-Chairperson
Dr. Philip A. Roberts, Co-Chairperson
Dr. Mikeal L. Roose

The Dissertation of Sayan Das is approved:

_____

_____

Committee Co-Chairperson

_____

Committee Co-Chairperson

University of California, Riverside

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DEDICATIONS

To Ma and Baba for giving me this wonderful life and guiding me through all adversities.

ABSTRACT OF THE DISSERTATION

Characterization of Root-Knot Nematode Resistance in Cowpea and Utilization of Cross-Species Platforms in Legume Genomics

by

Sayan Das

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Dr. Philip A. Roberts, Co-Chairperson

Cowpea (*Vigna unguiculata* L. Walp) is an important food and fodder legume worldwide. Cowpea is grown extensively in sub-Saharan Africa where it is an important staple. Root-knot nematode (RKN, *Meloidogyne* spp.) is a significant pest in cowpea. Several sources of resistance to RKN have been identified in cowpea, including the widely used *Rk* gene. As part of a study to elucidate the mechanism of *Rk*-mediated resistance, the histological response to avirulent *M. incognita* feeding of a resistant cowpea cultivar CB46 was compared with a susceptible near-isogenic line. It was found that the *Rk*-mediated resistance is a delayed response which lacked an early hypersensitive reaction (HR). Typically in pathogen resistance HR is closely associated with an oxidative burst (OB) in infected tissue. Following a basal OB during early infection in both susceptible and

resistant cowpea roots no significant OB was detected, a profile consistent with the histological observations of a delayed resistance response.

As cowpea does not have a microarray platform, a readily available soybean (*Glycine max*) genome array was used to identify single feature polymorphisms (SFP) in cowpea. An algorithm called Robustified Projection Pursuit was used for data analysis and RNA was used as a surrogate for DNA to detect SFPs between two genetically diverse cowpea genotypes. Cowpea genomic amplicon sequencing was performed for SFP validation and a 71% validation rate was observed. This study demonstrated that the soybean genome array is a satisfactory platform for identification of SFPs in cowpea. Furthermore, this study provided an example of a method to extend genomic resources from well supported species to "orphan" crops like cowpea.

Transcriptome profiles of incompatible and compatible cowpea-RKN interactions for two time points using the Affymetrix soybean GeneChip were generated. The profiles indicated that the RKN resistance pathway was still partially suppressed at 9 days post-inoculation in resistant cowpea roots. Subtle variation of ROS concentration, induction of toxins and other defense related genes were indicated to play a role in this unique resistance mechanism. Further functional analysis of these differentially expressed genes will advance understanding of this intriguing plant-nematode interaction in a more precise manner.

Table of Contents

List of Tables

List of Figures

Chapter 1

General introduction

Cowpea

Cowpea (*Vigna unguiculata* L. Walp) is an important food and fodder legume worldwide. It is grown extensively in West Africa, lower elevation areas of eastern and southern Africa, north-eastern Brazil, parts of the Middle East, India, and the south-eastern and south-western regions of North America (Ehlers and Hall, 1997). Like common bean (*Phaseolus vulgaris* L.), which is combined with maize or other starchy staple crops in other parts of the world, dry grain cowpea is consumed with lower protein cereal and root/tuber staples to provide an adequate protein quantity and quality to hundreds of millions of rural and urban consumers in West Africa (Bressani, 1985; Langyintuo *et al.*, 2003). Cowpea forage is used for livestock and cowpea hay plays a critical role as fodder during the dry season in West Africa (Tarawali *et al.*, 2002). Cowpea is cultivated on an estimated area of 12.5 million hectares with an annual production of approximately three million tonnes of dry grains worldwide (Singh *et al.*, 1997). In the United States cowpea is considered a minor crop grown on an area of about 80,000 hectares (Fery, 1985; 1990).

Cowpea (2N=2X=22) has a genome size of ~ 600 Mb of which about 150 Mb is gene rich (Timko *et al.*, 2008). Until now genomic resources have been very limited in cowpea when compared to the major crop legume soybean (*Glycine max* L.) which is phylogentically close to cowpea (Zhu *et al.*, 2005). For marker-assisted selection in plant breeding, high density genetic linkage maps are an important resource. Since the advent of microarray technology several methods for high throughput genotyping and high

density mapping techniques have been established in different crop species. In cowpea the most comprehensive genetic linkage map is comprised of 423 markers which include AFLP, RAPD, and RFLP markers (Ouédraogo *et al.*, 2002). This map is of limited utility for breeders due to the lack of markers tightly linked to important traits such as root-knot nematode resistance and because AFLP and RAPD markers cannot be applied to additional mapping populations. Currently a major effort is underway at the University of California, Riverside (UCR) as well as by other research groups in different countries to develop comprehensive genomic resources for cowpea, including EST and BAC libraries, microarray platforms, a physical map and high density genetic linkage maps.

Another critical resource for cowpea research is access to a repository of cowpea germplasms. Currently UCR maintains a collection of 5000 cowpea accessions from more than 40 countries. Several recombinant inbred line (RIL) populations have been developed at UCR which are being used for creating genetic linkage maps to help breeders identify the genetic location of economically important traits like drought tolerance, resistance to biotic and abiotic stresses, and improvement of nutritional quality.

Root-knot nematode (RKN), a significant crop pest

Root-knot nematodes (*Meloidogyne* spp.) are sedentary root endoparasites and have a wide host range that encompasses most of the estimated 250,000 flowering plants

(Trudgill and Blok, 2001). Three polyphagus, apomictic RKN species (*M. incognita*, *M. arenaria* and *M. javanica*) along with facultatively parthenogenetic *M. hapla* are serious pests of many crop plants (Sasser and Freckman, 1987). Root-knot nematodes cause significant economic losses by infecting large numbers of important crops every year worldwide. For example, in Ecuador, estimated losses due to *M. incognita* infection in 207 horticultural crops exceed 20% (Trudgill *et al.*, 2001).

The infective stage of root-knot nematode is the second stage juvenile (J2). The J2 penetrates the root and completes three successive molts to become adult male or female. Many of the RKN species, including *M. incognita, M. javanica* and *M. arenaria* reproduce by obligate mitotic parthenogenesis (Jung and Wyss, 1999). The mechanism of feeding site development by root-knot nematodes is not well understood, being a dynamic and complex process involving genes from both the nematode and the host plant. The secretions from the esophageal glands of the nematodes are important in the initiation and maintenance of feeding structures (Davis *et al.*, 2000; Huang *et al*., 2006).

Plant nematode resistance genes

Genes conferring resistance to different plant-parasitic nematodes have been identified and are being used extensively in plant breeding programs. With the rapid progress of

molecular biology, many nematode resistance genes have been genetically mapped and a few genes have been characterized and cloned.

The first nematode resistance gene to be cloned was *Hs1*$^{pro-1}$ which confers resistance to the sugar beet cyst nematode *Heterodera schachtii* (Cai *et al.*, 1997). Other important nematode resistance genes that have been cloned are *Mi-1* in tomato conferring resistance to *M. incognita (*Milligan *et al.*, 1998*)* and *Gpa2* in potato conferring resistance to potato cyst nematode *Globodera pallida* (van der Vossen *et al.*, 2000). Subsequently, the broad-spectrum potato cyst nematode *G. rostochiensis* resistance gene *Hero* was cloned (Ernst *et al.*, 2002). Molecular cloning of the gene *Gro1-4* which confers resistance to pathotype Ro1 of *G. rostochiensis* was also acheived (Paal *et al.*, 2004). *Gro 1-4* is a member of the 15 member *Gro1* gene family in potato. Most of the nematode resistance genes cloned so far are members of the NBS-LRR class of resistance genes except *Hs1* $^{Pro\ 1}$, which lacks a distinct NBS domain (Williamson, 1999) and *Gro1-4,* which is a member of the TIR-NB-LRR family. Evidence of cloning a NB-LRR sequence from wheat conferring resistance to cereal cyst nematode *H. avenae* has also been reported (Lagudah *et al.*, 1998, Ogbonnaya *et al.,* 2001).

Several nematode resistance loci have been mapped using different molecular markers. In soybean two alleles (*rhg1* and *Rhg4)* have been mapped to linkage group G and linkage group A2, respectively (Webb *et al.,* 1995). These alleles confer resistance to the soybean

cyst nematode *Heterodera glycines* and further characterization of the *Rhg4* allele has been made (Lewers *et al.,* 2001). Molecular markers linked to citrus nematode resistance locus *Tyr1* have been identified by Ling *et al.* (2000). High resolution genetic mapping using AFLP markers was carried out in pepper to find markers closely linked to root-knot nematode resistance loci Me_3 and Me_4 (Djian-Caporalino *et al.*, 2001). In cowpea the root-knot nematode resistance locus *Rk* has been mapped to the bottom of linkage group 1 and some AFLP markers are loosely linked to the *Rk* locus (Ouedraogo *et al.,* 2002).

Rk gene in cowpea

The *Rk* locus in cowpea has been used extensively to breed root-knot nematode resistant varieties in the USA and other countries. This gene locus was first designated by Fery and Dukes (1980). Several genetic studies have indicated that this locus may have several alleles, namely *Rk, Rk* and *Rk2.* The *Rk2* allele confers broad-based resistance to different races of *M. incognita* and *M. javanica* (Roberts *et al.*, 1996). Analysis of recombination between *Rk* and *Rk2* failed to generate recombinants among segregating progeny and indicated that the two genes were at most 0.17 cM apart. It remains to be confirmed whether *Rk2* is an allele of *Rk* or a tightly linked separate locus and this provides an interesting target for fine mapping using physical and genetic mapping resources currently under development. A single recessive gene unlinked to *Rk*, which confers broad-based additive resistance when present together with *Rk* was identified and named *rk3* (Ehlers *et al.*, 2000b). Another gene conferring resistance to *M. incognita* root galling

also maps to the *Rk* region and shows low recombination rates with gene *Rk* (Roberts, unpublished data). These resistance loci provide a good resource for future studies and cultivar development.

Plant response to nematode infection

Compatible and incompatible reactions lead to differential plant responses to nematode infection. A complex cascade of plant genes is activated upon nematode invasion and there are some visible plant reactions observed in the plant cells. Based on the limited number of studies available in plant-nematode interactions, a common response to nematode attack in the incompatible reaction is early hypersensitive reaction (HR) mediated cell death around the nematode feeding area, which prevents the nematodes from further feeding and blocks development. Strong early HR responses have been observed in *Mi-1* mediated resistance in tomato (Dropkin, 1969, Williamson, 1999), incompatible plant-root-knot interaction in soybean (Kaplan *et al.*, 1979) and Me_3 mediated resistance in pepper (Pegard *et al.*, 2005). A lack of typical HR was observed in resistant alfalfa infected with root-knot nematodes, where the J2 clumped at the root apex area and could not proceed to the vascular bundle and ended up emigrating from the roots (Potenza *et al.*, 1996). Accumulation of phenolic compounds, especially chlorogenic acid, at the site of infection was also reported in resistant pepper roots by Pegard *et al.* (2005). A recent study by Melillo *et al.* (2006) has shown that an early oxidative burst occurs in the *Mi*-mediated resistance reaction against root-knot nematode in tomato.

Non-hypersensitive reactions have been observed in *Hsp1*$^{pro-1}$-mediated resistance against sugarbeet cyst nematode where the J2 die due to degradation of the feeding structure. A delayed hypersensitive cell death has been observed in the case of the *Gpa2* and *Hero*-mediated gene responses against two potato cyst nematode species, *G. pallida* and *G. rostochiensis,* respectively (Williamson 1999; van der Vossen *et al.*, 2000), in which the nematodes become sedentary and die at a later stage due to HR cell death in the developed syncytium.

Molecular studies and transcriptome analysis

Several molecular studies of nematode feeding sites have been made so far and most have focused on the gene expression in compatible reactions.. The major defense related genes, which are activated upon nematode infection, are peroxidase, chitinase, lipoxygenase, extensin and proteinase inhibitors (Gheysen and Fenoll, 2002; Williamson and Kumar, 2006).

The availability of high throughput microarray technologies has expanded the scope of studying overall gene expression in nematode feeding sites. Microarray technology is being used to study plant gene expression patterns specific to pathogen attacks especially in fungal and bacterial pathogens. There are very few examples of microarray experiments being done to study gene expression in nematode infected roots so far. The

fundamental basis of DNA microarrays is the process of hybridization. When DNA microarrays are used to measure the turnover of mRNA in living cells, a probe of one DNA strand that matches a particular mRNA in the cell is used. The concentration of a particular mRNA is a result of the expression of that corresponding gene and hence this application is often referred to as expression analysis. Through a microarray study all the expressed genes in the genome can be surveyed at once, an approach that is often called global expression profiling. Two commonly used microarray platforms are complementary DNA (cDNA) arrays and oligonucleotide microarrays (Knudsen, 2002). The probes for the cDNA format are generally products of polymerase chain reaction (PCR) generated from cDNA libraries, which are then spotted onto a glass slide. In oligonucleotide arrays, probes are usually 25-40 nucleotides long and are spotted on silicon chips using photolithography. This technique has been pioneered by the company Affymetrix and their 'GeneChips' for several crop and model plants are available commercially. In Affymetrix GeneChips each transcript is represented by 11 pairs of 25-mers which is called a probe set and each probe pair consists of a perfect match and mismatch probe. This probe design strategy provides greater sensitivity to the GeneChips when compared to cDNA arrays and they are much denser also.

A study of the transcriptional profile has been done for the compatible response to *Meloidogyne javanica* in tomato (Bar-Or *et al.* 2005). That study used a microarray chip containing 12,500 clones. Changes were recorded in the steady state level of transcripts

of PR genes, hormone-associated genes and development associated factors. In another study (Khan *et al.,* 2004), expression profiling of susceptible soybean roots infected with soybean cyst nematode *H .glycines* two days post inoculation showed up regulation of different stress induced genes including endoglucanase, SAM22, and peroxidase. Gene expression profiling studies for incompatible plant-RKN interaction were not available until recently when Bhattarai *et al*., (2008) used a tomato cDNA array to explore incompatible tomato-RKN interaction at 24 hours post inoculation.

Outline of this work

In cowpea the the *Rk* locus has been used extensively in breeding programs to develop nematode resistant varieties. Very little was known about the mechanism of this resistance system at the histological and molecular levels. In any plant-pathogen interaction, knowledge about the mechanism of resistance is important in order to use that information in developing resistant cultivars. Chapter 2 of this dissertation focuses on creating a histological profile of RKN-infected roots of two near-isogenic cowpea lines which differ in RKN susceptibility. It was determined that the *Rk*-mediated resistance is a delayed resistance response when compared to some other RKN resistance systems studied and there was no typical HR in resistant roots. These findings were well supported biochemically by assays to detect the production of reactive oxygen species (ROS) in infected roots.

As mentioned earlier, until now cowpea has not been tractable to high throughput genotyping and high density mapping. Chapter 3 of this dissertation reports on the available genomic resources in soybean, which is phylogenetically closely related to cowpea, and these were utilized in developing microarray based markers called single feature polymorphisms (SFP). For that study the commercially available soybean GeneChip from Affymetrix was used to identify some 1000's of SFPs between the parents of a cowpea recombinant inbred line (RIL) population and the statistical predictions were validated with PCR amplicon sequencing. The results of that work showed that the soybean GeneChip can be used effectively in cowpea for SFP discovery.

The success of the SFP discovery project lead to the research reported in Chapter 4, where the Affymetrix soybean GeneChip was used to conduct an expression profiling study of nematode infected feeding sites, in which comparisons of the expression patterns of nematode resistant and susceptible genotypes at 3 and 9 days post-inoculation (dpi) were made. Results from the expression profiling indicated that the root-knot nematode resistance pathway is still partially suppressed at 9 dpi in resistant cowpea roots. There is indication that subtle variation of ROS concentration, induction of toxins and other defense related genes play a role in this unique resistance mechanism. Further functional analysis of these differentially expressed genes could be helpful to understand this intriguing plant-nematode interaction in a more precise manner.

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Chapter 2

Histological characterization of root-knot nematode resistance in cowpea and its relation to reactive oxygen species modulation

Abstract

Root-knot nematodes (*Meloidogyne* spp.) are sedentary endoparasites with a broad host range which includes economically important crop species. Cowpea (*Vigna unguiculata* L. Walp) is an important food and fodder legume grown in many regions where root-knot nematodes are a major problem in production fields. Several sources of resistance to root-knot nematode have been identified in cowpea, including the widely used *Rk* gene. As part of a study to elucidate the mechanism of *Rk*-mediated resistance, the histological response to avirulent *M. incognita* feeding of a resistant cowpea cultivar CB46 was compared with a susceptible near-isogenic line (in CB46 background). Most root-knot nematode resistance mechanisms in host plants that have been examined induced a hypersensitive response (HR). However, there was no typical HR in resistant cowpea roots and nematodes were able to develop normal feeding sites similar to those in susceptible roots up to 9-14 days post inoculation (dpi). From 14 to 21 dpi giant cell deterioration was observed and the female nematodes showed arrested development and deterioration. Nematodes failed to reach maturity and did not initiate egg laying in resistant roots. These results confirmed that the induction of resistance is relatively late in this system. Typically in pathogen resistance HR is closely associated with an oxidative burst (OB) in infected tissue. The level of reactive oxygen species release in both compatible and incompatible reactions during early and late stages of infection was also quantified. Following a basal OB during early infection in both susceptible and resistant roots, which was also observed in mechanically wounded root tissues, no significant OB

was detected up to 14 dpi, a profile consistent with the histological observations of a delayed resistance response. These results will be useful to design gene expression experiments to dissect *Rk*-mediated resistance at the molecular level.

Introduction

Cowpea (*Vigna unguiculata* L. Walp) is a food and fodder legume of significant economic importance worldwide especially in semiarid regions of Africa. It is also grown in North and South America, southern Europe and Asia. Cowpea is cultivated on an estimated area of 12.5 million hectares with an annual production of three million tonnes of dry grains worldwide (Singh *et al.*, 1997). In the United States cowpea is a crop of minor interest grown on an area of about 80,000 hectares (Fery 1985, 1990).

Root-knot nematodes (RKN) are one of the most important nematode pests of crop plants and have a diverse host range. RKN (*Meloidogyne* spp.) are sedentary root endoparasites and are involved in development of specialized feeding structures known as giant cells. The infective stage of the nematode is the second-stage juvenile (J2). The J2 penetrate the roots and go through three successive molts to become adult females or males. Several of the most important root-knot nematode species, including *M. incognita*, reproduce by obligate mitotic parthenogenesis (Jung and Wyss, 1999).

The mechanism of feeding site development by root-knot nematodes is not well understood as it is a very dynamic and complex process involving genes from both the nematode and the host plant. The secretions from the esophageal glands of the nematode are important in initiating the development of feeding structures (Davis *et al.*, 2000,

Williamson and Kumar, 2006). The first sign of giant cell induction by RKN is the formation of a binucleate cell. Rapid divisions of the nuclei continue in the absence of cytokinesis (acytokinetic mitosis) which gives rise to several large multinucleate cells. Surrounding cells divide to form the characteristic galls often known as 'root-knots' (Gheysen and Fenoll, 2002). The xylem parenchyma cells become transfer cells by forming finger-like wall invaginations (Jones and Northcote, 1972). This helps in water transport from xylem to the feeding sites.

Root-knot nematodes are important pests of cowpea worldwide and host plant resistance is a preferred strategy for managing this problem in infested cowpea fields (Roberts *et al.*, 1995; Ehlers *et al.*, 2002). The *Rk* locus in cowpea has been used extensively to breed root-knot nematode resistant varieties in the USA and other countries. This gene locus was first designated as *Rk* by Fery and Dukes (1980) and it confers resistance to many populations of *M. incognita, M. arenaria, M. hapla* and *M. javanica*. Genetic studies have indicated that this locus may have several alleles including *Rk, Rk* and *Rk2*. *Rk2* confers broad-based resistance to different races of *M. incognita* and *M. javanica* (Roberts *et al.*, 1996). It is to be confirmed whether *Rk2* is an allele at the *Rk* locus or is a tightly linked separate locus. A single recessive gene unlinked to *Rk*, which confers broad-based additive resistance when combined with *Rk*, was identified and named *Rk3* (Ehlers *et al.*, 2000). These resistance loci provide a good resource for future studies and cultivar development.

Compatible and incompatible reactions lead to differential plant responses to nematode infection. A complex cascade of plant genes is activated upon nematode invasion and there are some visible reactions observed in the plant cells (Williamson, 1999). Based on the limited number of reported studies, a common response to root-knot nematode attack in host plants carrying a resistance gene is an early hypersensitive reaction (HR) mediated cell death around the nematode feeding site, which prevents the nematode from further feeding resulting in nematode death. For example, strong early HR responses have been observed in *Mi-1*-mediated resistance in tomato (Williamson, 1999), *Mex-1*-mediated resistance in coffee (Anthony *et al.*, 2005), Me_3-mediated resistance in pepper (Pegard *et al.*, 2005), and incompatible interactions in soybean (Kaplan *et al.*, 1979). Accumulation of phenolic compounds, especially chlorogenic acid, at the site of infection was also reported in resistant pepper roots by Pegard *et al.* (2005).

Non-hypersensitive reactions have been observed in $Hsp1^{pro\text{-}1}$-mediated resistance in sugarbeet against the cyst nematode *Heterodera schachtii*, where the J2 died due to degradation of the feeding structure (Holtmann *et al.*, 2000). A delayed hypersensitive cell death was observed in the case of *Hero*-mediated gene responses in tomato against the cyst nematodes *Globodera pallida* and *G. rostochinensis* (Sobczak *et al.*, 2005), in which the nematodes became sedentary and died at a late juvenile stage due to HR mediated cell death in the developed syncytium. Gene *H1* confers resistance to *G. rostochiensis* pathotype Ro1 in potato. Studies of root ultrastructure in resistant potato

plants harboring gene H1 showed an early HR around the J2 (Rice *et al.*, 1985; Williamson, 1999). Here syncytial development was restricted by HR leading to the restriction in the development of the nematode and increased numbers of males and reduced numbers of females.

In cowpea there is no detailed histological documentation of RKN induced changes during compatible and incompatible reactions. This study was done to provide a detailed histological characterization of *Rk*-mediated resistance in cowpea. It is known that an oxidative burst typically is associated with HR in incompatible host pathogen interactions including nematode-plant interactions (De Gara *et al.*, 2003). A significant oxidative burst has been recorded in incompatible tomato (*Mi-1*)-RKN interactions (Melillo *et al.*, 2006). Therefore, the accumulation of reactive oxygen species in the *Rk*-mediated incompatible cowpea-RKN interaction was also investigated.

Materials and methods

Plant material

Two near-isogenic lines (NIL) differing in presence or absence of gene *Rk* were used. The two parents used to develop the NIL were *M. incognita* race 3 resistant cowpea genotype 'CB46' (homozygous resistant, *RkRk*) and a highly susceptible genotype 'Chinese Red' (homozygous susceptible, *rkrk*). The F1 was backcrossed to recurrent

parent CB46 (BC_1) and homozygous *Rk* plants were discarded in BC_1F_2 and non-segregating *rkrk* plants were advanced to the next back-cross (BC_2). Repeated backcrossing and selection was used to recover the *rkrk* line in the CB46 background. BC_4F_4 progenies were used for all the experiments described here. The *rkrk* line is referred to as the null-*Rk* line from here on.

Nematode inoculum

Eggs of *M. incognita* race 3 (isolate Beltran) cultured on susceptible tomato host plants were extracted from roots using 10% bleach solution (Hussey and Barker, 1973). This isolate is avirulent to gene *Rk* in CB46. Eggs were hatched in an incubator at 28° C and J2 were collected in fresh deionized water. The J2 inoculum was prepared according to the experimental requirements.

Histological experiments

Seeds of CB46 and null-*Rk* cowpea genotypes were grown in growth pouches under controlled environmental conditions of 26.7° C ± 0.5° C constant temperature and daily light/da*Rk* cycles of 16/8 hours. This temperature was used because it lies within the optimum temperature range of 26 – 28 °C for development and reproduction of M. incognita on cowpea in growth pouches (Ehlers *et al.*, 2000). Each pouch was inoculated with 3000 J2 in 5 ml of deionized water, 12 days after planting (dap). Three

pouches from each genotype were mock inoculated with 5 ml of deionized water as negative controls. The presence of nematodes in the roots was confirmed by acid fuchsin staining (Byrd *et al.*, 1983) 24 hours post-inoculation (hpi).

Three root tips, each ~50 mm in length, were harvested randomly from two plants of each genotype at 3, 4, 5, 9, 14, 15, 16, 17, 18, 19, 20, and 21 days post-inoculation (dpi) and immersed in half-strength Karnovsky's fixative (2.5% glutaraldehyde and 4% formaldehyde in 50 mM phospahte buffer, pH 7.2). The roots were left overnight in the fixative at 4° C. The roots were dehydrated by passing through a graded ethanol series (10-100%). Infiltration and embedding was done with a JB-4 methacrylate embedding kit (Polysciences Inc., Pennsylvania, USA). Semi-thin sections 4 µm thick were cut using a DuPont-Sorval JB-4 microtome using triangular glass knives. The sections were stained in 0.5% toluidine blue O in borate buffer (pH 4.4). Digital micrographs were taken using a Spot CCD camera (Spot RT color system, model # 2.2.1, Diagnostics Instruments Inc.) attached to a Leica DM LB2 compound bright-field microscope. Giant cell diameters were measured at 5, 9, 14, 19, and 21 dpi. Three well developed giant cells were selected for each time point in both resistant and susceptible cowpea genotypes, being chosen from sections in a sequential series that optimized the giant cell size. The diameter was measured at three positions for each giant cell using a stage micrometer and the mean of the three measurements was used as the diameter for that giant cell. Giant cell measurements from root sections provide a relative measure of cellular changes in

infected resistant and susceptible cowpea roots and do not represent an absolute measurement.

Root penetration studies

Penetration of avirulent nematodes in root tissue was studied on susceptible null-*Rk* and resistant CB46 cowpea genotypes. Plants were grown in growth pouches at 26.7° C constant temperature and daily da*Rk*/light cycles of 16/8 hours. The inoculum level used was the same as for the histological experiments. Each pouch was inoculated with 3000 J2 in 5 ml of deionized water, 12 dap. The inoculated roots were harvested at 24, 48 and 72 hpi and immersed in 1.5% NaOCl solution for 15 minutes followed by rinsing with tap water to remove excess NaOCl. The roots were then stained with 1 ml of 3.5% acid fuchsin stain (Byrd *et al.*, 1983), the solution was heated to boiling, followed by cooling to room temperature, and excess stain was removed by rinsing in running water. The root material was placed in acidified glycerin. The stained roots were pressed between glass slides and observed under the microscope. Three plants were selected for each sampling time point and three root tips from each plant were selected randomly and numbers of J2 inside the root tissue were counted.

An analysis of variance (ANOVA) was used to compare the penetration rate between resistant and susceptible roots. Data from all three root systems for a given genotype × time point were pooled together for analysis.

Egg mass production

Numbers of egg masses per root system were counted using an egg mass specific stain erioglaucine (Omwega *et al.*, 1988; Ehlers *et al.*, 2000) at 30 dpi to confirm the susceptibility of the null-*Rk* line used in the experiments. An inoculum of 3000 J2 per root system was used for the egg mass production assays and 20 plants each from CB46 and null-*Rk* were screened.

Quantitative detection of ROS release

A fluorometric assay was designed to detect ROS accumulation using a membrane permeable probe, dichlorofluoroscein (DCFH). DCFH alone does not have fluorescence but when it reacts with ROS it oxidizes to DCF which is fluorescent. Root pieces were collected from infected root systems of the susceptible and resistant genotypes at 24, 48, and 72 hpi. Also, to detect the presence of a late oxidative burst, root samples were collected at 5, 9, and 14 dpi. The root samples (250 mg) were processed as described by Melillo *et al.* (2006). Phosphate buffered saline (20 mM, pH 7.2) was used in place of potassium phosphate buffer. A 1-ml aliquot of the processed sample was collected and

used to detect the increase in fluorescence (excitation 488 nm, emission 521 nm) caused by oxidation of DCFH using a fluorescence spectrophotometer (SPEX FluoroLog-3, Horiba Jovin Yvon). Mock inoculated roots (5 ml of deionized water per root system) were used as negative controls and mechanically injured roots were used as a positive control. Mechanical wounding was achieved by puncturing plant roots with a hypodermic needle. Five biological replicates were taken and the entire experiment was repeated once. The duplicate experiments did not differ according to ANOVA tests; therefore data from the independent duplicate experiments were combined for analysis. Blank samples without plant material were processed in parallel to eliminate any spontaneous change in fluorescence.

In order to test the specificity of the reaction, an experiment was done using the ROS scavenging reagent diphenilene-iodonium (DPI, an NADPH oxidase inhibitor). Root pieces (250 mg) from non-infected and 24 hour-infected CB46 plants were harvested and pre-incubated for 30 minutes with 100 μM DPI followed by 30 minutes in DCFH reaction medium. The decrease in fluorescence was measured as described earlier and reagent blanks were used as reference.

Results

Root penetration

The presence of gene *Rk* did not affect juvenile penetration into cowpea roots. The avirulent J2 were able to penetrate the roots of both cowpea genotypes and there was no effect of genotype ($p = 0.05$) on the number of J2 in roots up to 72 hpi (Figure 2.1). In both genotypes, up to 24 hpi the number of J2 that had penetrated into roots was low, but the penetration rate was higher at 48 hpi and a gradual increase in penetration was observed up to 72 hpi.

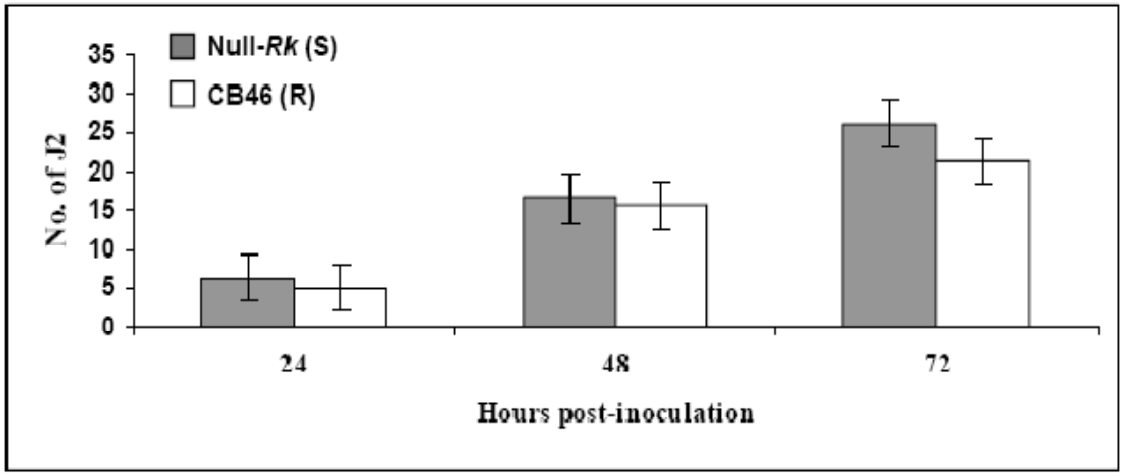


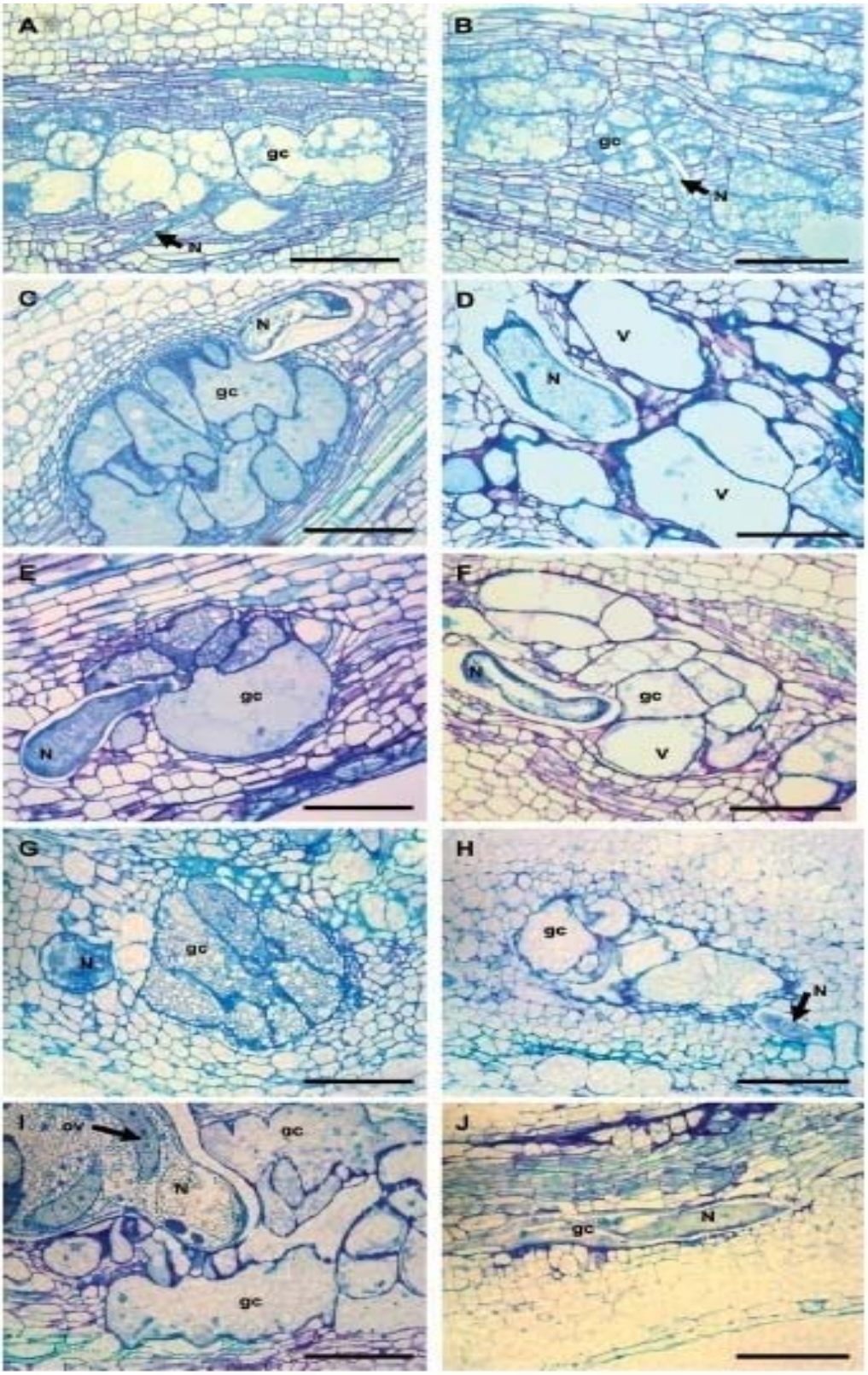
Figure 2.1. Number of J2 penetrating into roots of CB46 (resistant) and null-*Rk* (susceptible) assayed at 24, 48, and 72 hours post-inoculation. Values are means ± SE of two separate experiments.

Histological response to infection

The resistant line CB46 did not show a HR response to nematode infection. HR, in which a programmed cell death around the area of infection occurs and the development of the pathogen is arrested, is a common plant reaction against different pathogens in resistant genotypes. For example, in root-knot nematode-host plant interactions such as the *Mi-1*-mediated resistance in tomato an obvious HR occurs within 24 hours of infection (Dropkin, 1969; Williamson, 1999). In this study when 12 day-old seedlings were inoculated with 3000 J2 and longitudinal sections of roots were examined there was no visible evidence of a HR response in resistant roots up to 21 dpi. The nematodes were able to establish healthy feeding sites in resistant roots in which the giant cells looked similar to those in susceptible roots up to 5 dpi (Figure 2.2A and 2.2B). Lack of HR response was also confirmed by staining the roots with acid fuchsin at various time points (not shown). The first evident differences between the two genotypes were observed at 9 dpi when the giant cells adjacent to the nematode in resistant roots had some larger vacuoles (Figure 2.2C and 2.2D), whereas the giant cells in the susceptible roots had uniformly dense cytoplasm with less vacuolation. The giant cells farthest from the nematode appeared to be metabolically more active than giant cells closer to the nematode in resistant roots (Figure 2.2D). The nematodes at this time point were developing normally in the genotypes based on observations of their size, shape and condition of internal contents. This trend in the giant cell conditions continued up to 14 dpi (Figure 2.2E and 2.2F) when there still was no sign of visible feeding site deterioration in resistant roots. However, the nematodes associated with resistant roots at

14 dpi were arrested in development as they were slightly shriveled and narrower than the nematodes in susceptible roots. This confirmed that although giant cell deterioration was not visible under bright field microscopy at this stage, giant cells were not metabolically active enough to provide optimum nutrients for nematode development. At 19 dpi (Figure 2.2G and 2.2H) the differences in feeding sites between the two genotypes were clearly visible. At this stage most of the giant cells in the resistant roots appeared to be on the verge of collapse as they were devoid of any cytoplasm and the common cell walls between the giant cells were also thin whereas in susceptible roots healthy giant cell complexes with dense cytoplasm and thick cell walls were present. At 21 dpi the giant cell complexes in resistant roots had collapsed completely and nematode development was severely disrupted; they had a shriveled appearance and had not advanced to a mature female stage based on lack of gonad development (Figure 2.2J). In susceptible roots at 21 dpi most of the nematodes had developed to mature females and their well-developed ovaries could be seen in the sections (Figure 2.2I).

Figure 2.2. Longitudinal sections of *Meloidogyne incognita* feeding sites in inoculated cowpea roots. Section are stained with toluidine blue O. A, C, E, G and I are null-*Rk* (susceptible) root sections and B, D, F, H and J are CB46 (resistant) root sections at 5, 9, 14, 19, and 21 dpi, respectively. gc = giant cell, N = nematode, ov = ovary, and V = vacuole. Bar = 200 μm.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Giant cell dimensions

Giant cell diameter did not differ between infection sites in resistant CB46 and susceptible null-*Rk* roots until 19 dpi. Giant cell measurements revealed that by 5 dpi the giant cells were fully developed and overall there was no significant difference in giant cell diameter between the two genotypes up to 19 dpi (Figure 2.3). Although at 14 dpi the

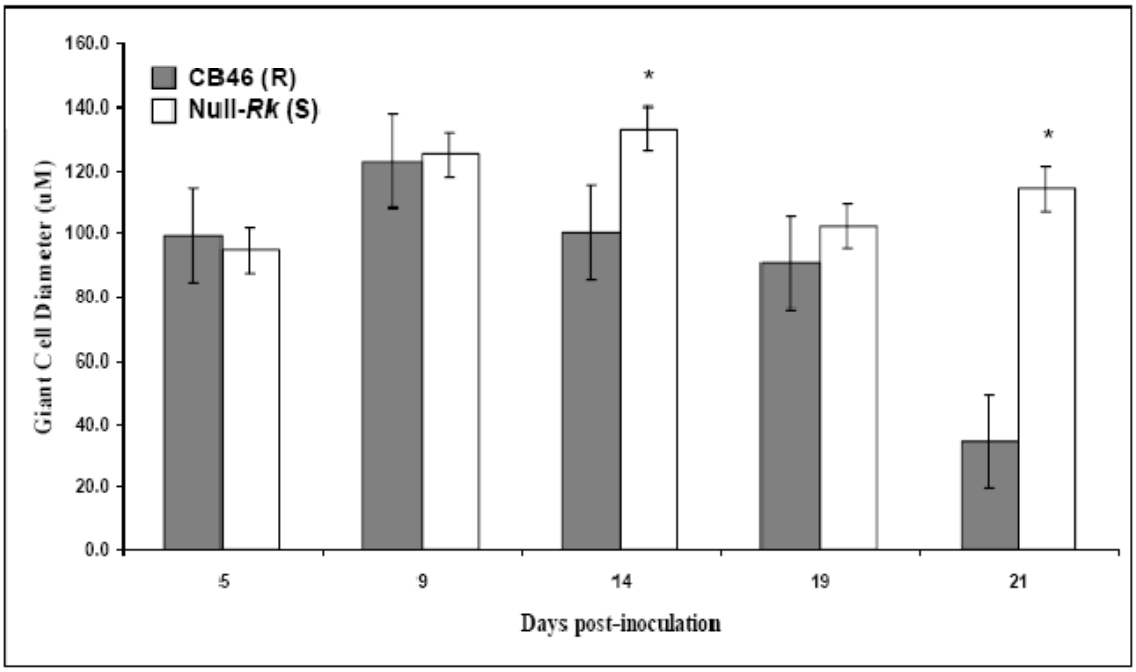

Figure 2.3. Diameters (μM) of nematode-induced giant cells formed in CB46 (resistant) and null-*Rk* (susceptible) inoculated roots over a time period of 5, 9, 14, 19, and 21 days post inoculation. Values are means from measurement of three giant cells. Each giant cell was measured at three different positions. Bars represent ± 1 SE. * Significant at p = 0.05.

giant cells in susceptible roots were found to be larger ($p = 0.05$) than in resistant roots, it was probably because the giant cells selected for the measurement were not fully representative for this time point. However at 21 dpi the mean diameter of the giant cells in resistant roots was much smaller than in susceptible roots due to the collapse of giant cells (Figure 2.3).

Root galling and egg production

Although the *Rk*-mediated resistance reaction was delayed, the development of female nematodes was arrested in resistant roots such that they did not reach reproductive maturity. The cortical cells surrounding the giant cells in resistant roots started to shrink rapidly at 12-14 dpi and at 19-21 dpi the cortical cells were almost normal in size. Therefore at 19 dpi only residual galling was visible on resistant roots even though the giant cells were not collapsed.

External observations of the roots at 21 dpi revealed large well-developed galls in susceptible roots whereas the resistant roots supported only some small residual swelling around the feeding sites (Figuer 2.4). Acid fuchsin staining at 21 dpi revealed that in susceptible roots the females had reached reproductive maturity and started to lay eggs whereas in resistant roots the under-developed female nematodes (approximately 90 % J4 and 10% immature adults) showed no sign of egg production. This confirmed our

observations from the histological root sections. At 30 dpi the number of egg masses per root system ranged from 43 to 99 (mean ± SD = 65.5 ± 15.9) in susceptible roots whereas nematodes failed to produce any egg masses in resistant roots.

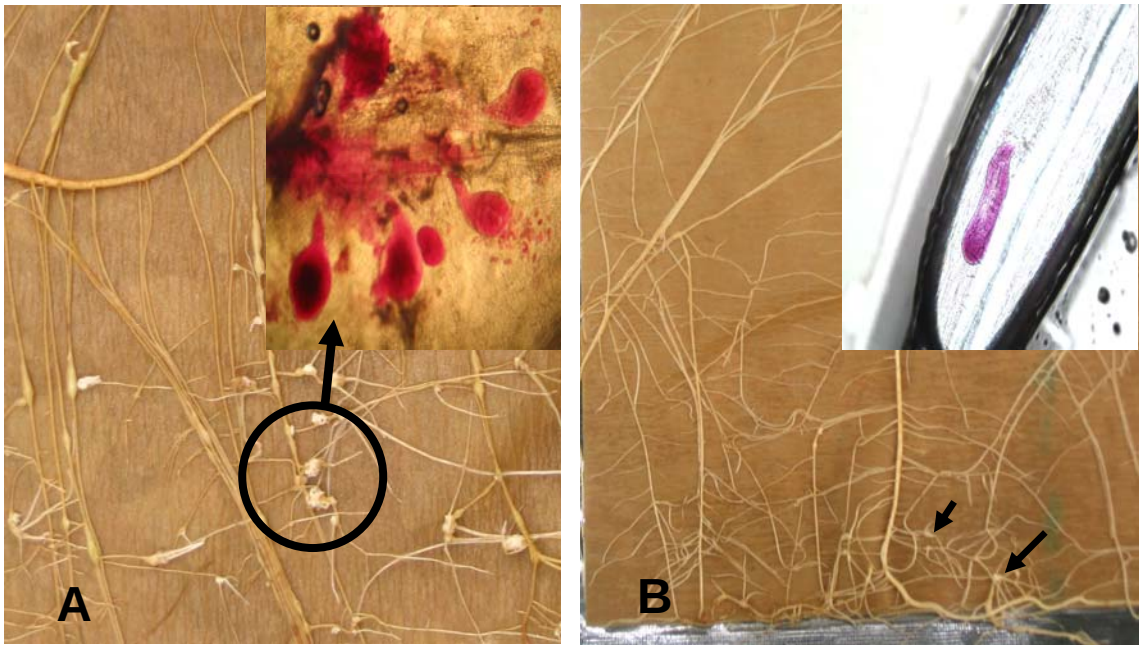

Figure 2.4. Infection symptoms and *M. incognita* females in inoculated roots at 21 days post inoculation. (A) null-*Rk* (susceptible) roots showing nematode induced galling (circled black) on the root surface, inset: a group of egg-laying females stained with acid fuchsin. (B) CB46 (resistant) roots almost free from galling except slight residual swelling indicated by black arrows, inset: a female nematode stained in acid fuchsin that has not developed to maturity and there is absence of egg production.

Quantitative detection of ROS release

In a time-course experiment ROS activity was studied starting at the early stages of infection (24 hpi) until the later stages of infection at 14 dpi. Due to nematode infection an early rise in ROS activity at 24 hpi was observed in root tissue of both resistant CB46 (157% compared with non-infected control) and susceptible null-*Rk* (153% compared with non-infected control) plants as shown in Figure 2.5. This early oxidative burst continued up to 48 hpi in both CB46 (159%) and null-*Rk* (151%). ROS activity decreased after that with readings for ROS in infected roots compared to non-infected control at 72 hpi being 92% in CB46 and 85% in null-*Rk*. There was no differential ROS activity between the resistant and susceptible genotypes during these time points. During later time points (5, 9, and 14 dpi) very low levels of ROS activity were detected in both genotypes in infected and non-infected roots. Compared to non-wounded control plants, mechanically wounded roots (positive control) of both CB46 and null-*Rk* produced a significant early oxidative burst up to 48 hpi, which diminished at 72 hpi (Figure 2.6), similar to the response in nematode-infected resistant and susceptible plants. Oxidation of DCFH in CB46 roots was inhibited by the superoxide ($O2^-$) scavenger diphenilene-iodonium (DPI) (Table 2.1). DPI is an NADPH-oxidase inhibitor and was the most efficient inhibitor of ROS in Mi-1-mediated resistance in tomato (Melillo *et al.*, 2006). Upon DPI treatment ROS activity was reduced to 53% in RKN infected CB46 roots at 24 hpi. This confirmed that the enzymatic origin of superoxide contributed significantly to the early oxidative burst detected in infected cowpea roots. Raw data of two independent

experiments for ROS concentration have been provided in the Appendix (A 2.1, A2.2, A2.3, and A2.4).

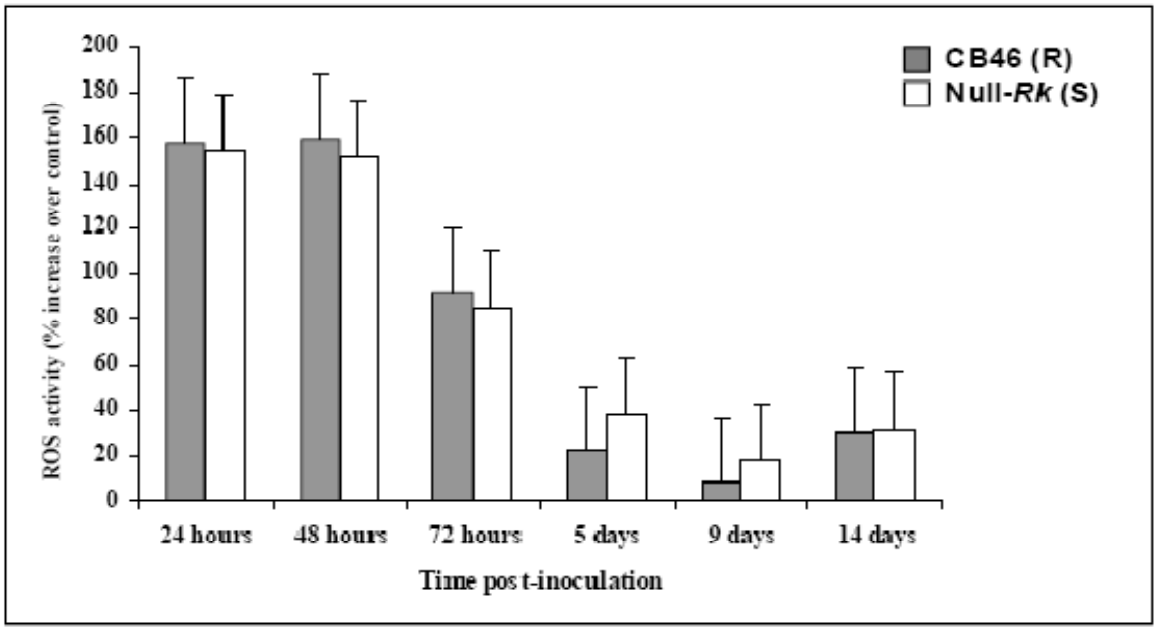

Figure 2.5. Quantification of reactive oxygen species (ROS) in resistant CB46 and susceptible null-*Rk* cowpea root tissue assayed at 1, 2, 3, 5, 9, and 14 days post-inoculation. Increase in dichlorofluoroscein (DCF) is expressed as percent increase over non-infected control. Fluorescence intensity was measured in counts per second (CPS) with excitation wavelength of 488 nm and emission wavelength of 521 nm. Values are means of combined data from two separate five-fold replicated experiments. Bars represent 1 SE.

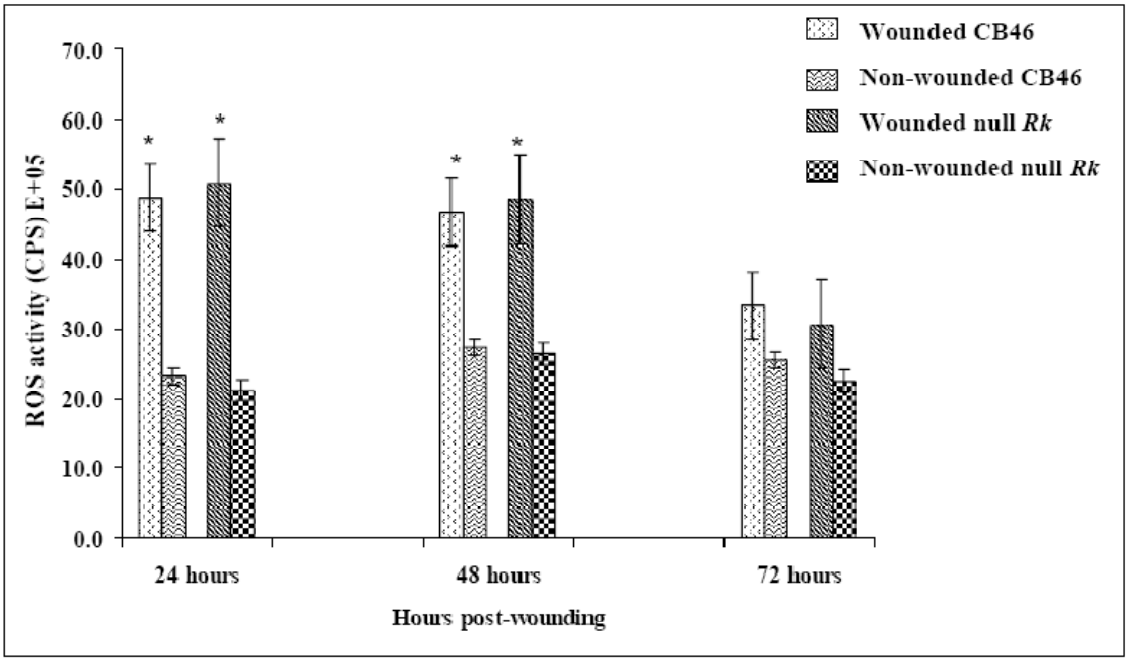

Figure 2.6. Quantification of reactive oxygen species (ROS) in mechanically injured (as a positive control) CB46 (resistant) and null-*Rk* (susceptible) roots over a time period of 24, 48, and 72 hours post-wounding. Fluorescence intensity was measured in counts per second (CPS) at excitation wavelength of 488 nm and emission wavelength of 521 nm. Values are means of combined data from two separate five-fold replicated experiments. Bars represent ± 1 SE. * Significant increase ($p = 0.05$) in ROS activity in wounded roots compared to non-wounded control within a genotype and time point.

Table 2.1. Effect of DPI on ROS release in resistant CB46 roots 24 hours after nematode infection compared to non-infected roots at the same stage.

Inhibitor	ROS release percentage[1]	
	Non infected CB46	Infected CB46
None	100±1	100±2
DPI 100µM	80.7±2	53.2±2

[1] Values are means of combined data from two separate five-fold replicated experiments ± SE

Discussion

The gene *Rk* was identified almost three decades ago (Fery and Dukes, 1980) as a highly effective RKN resistance gene in cowpea. Although the *Rk*-based resistance has been studied genetically and has been used extensively in cowpea breeding, little was known about the mechanism of *Rk*-mediated resistance. Two types of mechanisms for RKN resistance in plants have been reported, including pre-infection resistance, where the nematodes cannot enter the plant roots due to presence of toxic or antagonistic chemicals in root tissue (Haynes and Jones, 1976; Bendezu and Starr, 2003), and post-infection resistance in which nematodes are able to penetrate roots but fail to develop. Post-infection resistance is often associated with an early Hypersensitive Reaction (HR) mediated cell death, in which rapid localized cell death in root tissue around the nematode prevents the formation of a developed feeding site leading to resistance. Tomato (Dropkin, 1969; Williamson, 1999), pepper (Pegard *et al.*, 2005), soybean (Kaplan *et al.*, 1979) and coffee (Anthony *et al.*, 2005) host plants that are resistant show typical HR upon avirulent RKN infection. In tomato HR was observed as early as 24 hpi whereas in pepper, soybean and coffee the HR was visible at 1-3 dpi, 2-3 dpi, and 4-6 dpi, respectively. A lack of typical HR was observed in resistant alfalfa infected with root-knot nematodes, where the J2 clumped at the root apex area and could not proceed to the vascular bundle and ended up leaving the roots (Potenza *et al.*, 1996).

Interestingly in the current study, it was found that the presence of gene *Rk* did not affect the J2 penetration into cowpea roots and there was no evidence of early HR. In fact the nematodes were able to initiate and maintain apparently healthy giant cells in resistant roots for about two weeks before visible signs of deterioration occurred, especially vacuolation and cell wall thinning, leading to giant cell collapse. This mechanism appears to be novel for RKN resistance. The only published report for a delayed resistance response against RKN was in tobacco (Powell, 1962) where a late HR was seen in developed giant cells. In cowpea there was no HR even during the later stages of infection. During this time the nematodes were able to feed and develop into late stage juveniles.

A common feature of pathogen-related HR is that it is preceded by loading of vacuoles with hydrolases and toxins and a calcium flux in the cytoplasm (Jones, 2001). A significant difference in vacuolation between the resistant and susceptible cowpea genotypes starting at 9 dpi was observed, and it is possible that the large vacuoles in resistant cowpea roots were filled with hydrolases and toxins that deprived the nematodes of nutrients and led to giant cell collapse, whereas in susceptible roots nematode feeding did not cause the formation of large vacuoles. In Arabidopsis a mutant called dnd1-1 failed to produce HR response against an avirulent strain of the bacterial pathogen *Pseudomonas syringae* but an effective gene-for-gene resistance was still operative (Clough *et al.*, 2000). *DND1* codes for a cyclic gated ion channel which facilitates

passage of Ca2+, K+ and various other cations. Thus host defense can be effective in the absence of HR and it might be dependent upon subtle changes in ion fluxes.

Reactive oxygen species (ROS) play an important role in plant defense, and during pathogen attack levels of ROS detoxifying enzymes like ascorbate peroxidase (APX) and catalase (CAT) are often suppressed in resistant plants (Klessig *et al.*, 2000). As a result plants produce more ROS and accumulation of these components leads to HR in plant cells. For example, H_2O_2 plays a major role in triggering HR in incompatible interactions (Dangl and Jones, 2001). However, in the cowpea-RKN incompatible interaction mediated by gene *Rk* we did not see a classic HR which is characteristic for most gene-for-gene resistance pathways. Our results of ROS quantification in RKN-infected cowpea roots showed that although there was an early oxidative burst in both the compatible and incompatible interactions in susceptible and resistant roots, respectively, there was no significant difference between the resistant and susceptible genotypes in level of ROS activity. Typically in incompatible interactions ROS is produced in a biphasic manner (Apel and Hirt, 2004), in which the initial rapid accumulation of ROS is followed by a more stable second burst. However, in cowpea we did not see a biphasic pattern of ROS production in the incompatible reaction. These results indicated that the initial burst that was detected in cowpea roots upon RKN infection is a part of basal host defense reaction and is independent of gene *Rk*-mediated resistance. This response, which develops a few days after elicitation, seems to be related to innate immunity in plants (Iriti and Faoro,

2007) and is triggered by changes in membrane potential, ion fluxes, and production of ROS. It is well known now that perception of parasite and/or wounding and modulation of ROS contribute toward the activation of plant defense response (Gechev *et al.*, 2006; Klessig *et al.*, 2000). This pattern of ROS production correlated well with the results of the histological profiles of the feeding site and the giant cell development that we found in the resistant and susceptible cowpea roots. The magnitude of H_2O_2 production apparently was not sufficient enough to trigger HR cell death in cowpea roots. It is also possible that the ROS scavenging mechanism was not suppressed to a level at which enough ROS could be diffused into the cells to trigger HR.

In conclusion we report that *Rk*-mediated resistance in cowpea is a unique resistance mechanism involving the lack of a HR and based on a delayed defense response. The current study provides a strong platform for designing gene expression studies to identify candidate genes which play an active role in this defense pathway. An evaluation of the expression levels of genes coding for enzymes involved in ROS production and ROS scavenging will be useful for understanding the intricacies of redox changes occurring upon RKN infection in resistant cowpea.

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Chapter 3

Detection and validation of single feature polymorphisms in cowpea (*Vigna unguiculata* L. Walp) using a soybean genome array

Abstract

Cowpea (*Vigna unguiculata* L. Walp) is an important food and fodder legume of the semiarid tropics and subtropics worldwide, especially in sub-Saharan Africa. High density genetic linkage maps are required for marker-assisted selection but currently a high density genetic linkage map for cowpea is not available. Single Feature Polymorphism (SFP) is a microarray-based marker which can be used for high density mapping and genotyping. Here the detection and validation of SFPs in cowpea using a readily available soybean (*Glycine max*) genome array is reported. An algorithm called Robustified Projection Pursuit (RPP) was used for data analysis and RNA was used as a surrogate for DNA to detect SFPs between two genetically diverse cowpea genotypes. A total of 1060 polymorphic markers were detected between parents of a recombinant population segregating for important agronomic traits. Cowpea genomic amplicon sequencing was performed for SFP validation and a 71% validation rate was observed. This study demonstrated that the soybean genome array is a satisfactory platform for identification of SFPs in cowpea. Furthermore, this study provides an example of a method to extend genomic resources from well supported species to orphan crops like cowpea.

Introduction

Cowpea (*Vigna unguiculata* L. Walp) is an important food and fodder legume of the semiarid tropics and subtropics worldwide. It is extensively grown in West Africa, lower elevation areas of eastern and southern Africa, northeastern Brazil, parts of the Middle East, India, and the southeastern and southwestern regions of North America (Ehlers and Hall, 1997). Like common bean (*Phaseolus vulgaris* L.) that is combined with maize or other starchy staple crops in other parts of the world, dry grain cowpea is consumed with lower protein cereal and root/tuber staples to provide an adequate protein quantity and quality to hundreds of millions of rural and urban consumers in West Africa (Langyintuo *et al.*, 2003; Bressani, 1985). Cowpea forage is also used to feed livestock and cowpea hay plays a critical role in feeding animals during the dry season in West Africa (Tarawali *et al.*, 2002). 'Longbean' or 'Asparagus bean' of cowpea cultivar group Sesquipedialis is considered one of the top-ten Asian vegetable crops and is grown on at least 400,000 ha worldwide for production of fresh 'green' or 'snap' beans.

Cowpea (2n = 2x = 22) with genome size ~620 Mb belongs to the genus Vigna Savi. (subgenus Vigna sect. Catiang) in the Phaseoleae (Maréchal *et al.*, 1978). Genomic resources such as cDNA libraries, ESTs and BAC libraries have been meager in cowpea (Timko *et al.*, 2007). High-resolution genetic maps provide breeders the ability to analyze the inheritance of genes of interest, monitor the transmission of specific genes or genomic regions from parents to progeny, and accelerate map-based cloning (Kumar, 1999;

Simpson, 1999). However, relatively few genetic resources are available to cowpea breeders, and modern molecular marker-based selection is only possible for a few traits (Timko *et al.*, 2007; Ouédraogo *et al.*, 2001; Ouédraogo *et al.*, 2002a; Ouédraogo *et al.*, 2002b, Boukar *et al.*, 2004). Efforts made previously for linkage mapping in cowpea include 92 RFLP markers (Fatokun *et al.*, 1997), 181 markers that are mostly RAPDs (Menéndez *et al.*, 1997), and 242 markers that are mostly amplified fragment length polymorphisms (AFLP) together with 17 biological resistance traits and resistance gene analogs (Ouédraogo *et al.*, 2002a). The previous genetic maps generated for cowpea are of limited utility for breeders due to the lack of markers tightly linked to important traits such as root-knot nematode resistance (Ouédraogo *et al.*, 2002a).

Any marker system with increased throughput, decreased cost per data-point, and greater map resolution is highly desirable (Kumar, 1999; Gupta and Rustgi, 2004). Oligonucleotide-based microarrays have been used recently to identify genetic polymorphisms (Hazen and Kay, 2003). Winzeler *et al.* (1998) reported the hybridization of labeled genomic DNA to oligonucleotide microarrays to identify sequence polymorphisms in haploid yeast. Borevitz *et al.* (2003) demonstrated that this approach can be applied to organisms with somewhat larger genomes, such as Arabidopsis thaliana, to analyze sequence polymorphisms termed single feature polymorphisms (SFPs). Similarly, DNA-based SFP detection and validation has been accomplished in rice (Kumar *et al.*, 2007) which has a genome size of 440 Mb. To reduce target

complexity in a large genome organism, Cui *et al.* (2005) and Rostoks *et al.* (2005) hybridized barley expression microarrays with cRNA, enabling detection of thousands of SFPs. Array based genotyping by hybridizing with cRNA instead of DNA was accomplished initially in yeast (Ronald *et al.*, 2005) and subsequently in Arabidopsis (West *et al.*, 2006). SFPs were also used for genome-wide association mapping or linkage disequilibrium studies (Kim *et al.*, 2006), and to estimate mutation and recombination parameters in population genetics studies (Jiang *et al.*, 2006).

As an important crop for millions of resource-poor farmers and consumers in developing countries, and with scant budgets for agricultural research, cowpea has been identified as an "orphan crop" recommended for increased support for biotechnology research (Naylor *et al.*, 2004). There is a major opportunity to apply knowledge being gained from genome research on "model species" such as *Arabidopsis*, rice (*Oryza sativa*), and *Medicago truncatula* to cowpea. Relatively large genetic gains can be made with only modest investments in both applied plant breeding and molecular genetics in cowpea (Timko *et al.*, 2007). In this context, we explored an SFP approach to distinguish two genetically diverse parents of a recombinant inbred line (RIL) mapping population.

Materials and Methods

Plant materials

The genotypes used in this study were California Blackeye No. 46 (CB46) and IT93K-503-1. CB46 was developed at the University of California, Davis, released in 1989 (Helms *et al.*, 1991) and is currently the most widely grown blackeye-type cowpea cultivar in the United States. IT93K-503-1 is a breeding line developed by the International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria to produce high grain yield in the savanna agro-ecological zone of West Africa. These parental genotypes are inbred pure lines and differ in a number of important agronomic traits including grain size, photoperiod sensitivity, seedling drought tolerance, and resistance to *Fusarium* wilt race 4 and brown blotch fungal diseases. In order to determine the inheritance of these traits and to develop nearby markers, a RIL population with 135 lines was created.

RNA extraction from cowpea

Seeds were germinated in two sets of pots and grown in controlled glasshouse conditions during spring 2005 under natural photoperiod. The temperature was maintained at day/night cycle of 35/18°C. The growing axillary buds were harvested at 14 days after planting (DAP) from one set of pots to provide tissues for control samples (flash frozen and stored at -80°C). Plants in the second set of pots were exposed to drought stress induced by withholding water after germination. The growing axillary buds were

harvested from drought-stressed seedlings at 14 DAP (flash frozen and stored at -80°C). With two genotypes, two treatments and two replicates, there were a total of 8 samples. Total RNA was isolated using TRIzol (Gibco BRL Life Technologies, Rockville, MD) reagent. The RNA was purified using an RNeasy spin column (Qiagen, Chatsworth, CA) and an on-column DNase treatment. RNA integrity was assessed prior to target preparation using RNA Lab-On-A-Chip (Caliper Technologies Corp., Mountain View, CA) evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

Soybean genome array

Phylogenetic relationships based on the conserved sequences within Papilionoideae legumes imply that *Vigna* (cowpea) is closely related to soybean (Zhu *et al.*, 2005). Since a cowpea genome array was not available, a soybean genome array was used to identify SFPs in cowpea. The soybean genome array contains 37,500 probe sets derived from soybean *(Glycine max* L.) unigenes. This represents 61% of the total probe sets on the chip, with the remainder targeting two pathogens important for soybean research of which 15,800 (26%) probe sets target *Phytophthora sojae* (a water mold) and 7,500 (12%) probe sets target *Heterodera glycines* (soybean cyst nematode). This array uses probe sets composed of 11 probe pairs to measure the expression of each gene. Each probe pair consists of a perfect match (PM) probe and a mismatch probe.

Labelling and hybridization of cowpea RNA on soybean genome array

Cowpea RNA samples were used to make biotin tagged cRNAs. These were hybridized to an Affymetrix soybean genome array as recommended by Affymetrix (Affymetrix GeneChip Expression Analysis Technical Manual; Affymetrix Inc., Santa Clara, CA) at the Institute for Integrative Genome Biology Microarray Facility at the University of California, Riverside (http://www.genomics.ucr.edu). The hybridization data were scanned for visible defects and then extracted using default settings and tabulated as CEL files using Affymetrix GeneChip Operating Software (GCOS 1.2). A global scaling factor of 500, a normalization value of 1, and default parameter settings were used for the soybean genome array. The detection calls (present, absent, or marginal) for the probe sets were made by GCOS as follows (abridged from the Affymetrix GeneChip Expression Analysis Technical Manual; Affymetrix Inc., Santa Clara, CA). The detection algorithm uses probe pair intensities to generate a detection p-value and assign a "present", "marginal", or "absent" call. Each probe pair in a probe set has a potential vote in determining whether the measured transcript is or is not "present". The vote is described by the discrimination score (R). R is calculated for each probe pair and compared to a predefined threshold, Tau. Probe pairs with R higher than Tau vote "present" and the voting result is summarized as a p-value. The greater the number of discrimination scores (R) that are above Tau, the smaller the p-value and the more likely the given transcript is truly present in the sample.

Method for identifying SFPs

Expression data were generated by hybridizing cowpea cRNA to the soybean genome array. A statistical method called Robustified Projection Pursuit (RPP) was used for SFP analysis (Cui *et al.*, 2005). Only the values from the PM probes were utilized. The use of RNA as a surrogate for genomic DNA eliminated interference from highly repetitive DNA as a technical impediment to SFP detection. An important aspect of the RPP method is that it first utilizes a probe set level analysis to identify SFP-containing probe sets and then chooses individual probes from within each SFP-containing probe set. The net result is the identification of probes that directly overlay polymorphic sequences.

Separate comparisons were made for drought stress and control treatments, resulting in two SFP lists. In the context of SFPs, there is no relevance to the production of separate stress and control lists. Two separate lists were available simply as a consequence of another study that is not described here which compared gene expression patterns in stressed and control plants. A top 15% outlying score cut-off was considered optimum for this experiment (Cui *et al.*, 2005). At this cut-off, we detected 489 SFPs in stress and 663 SFPs in control treatments. There were 92 SFPs in common between these lists.

Genomic DNA isolation

Young leaves from one–week-old cowpea seedlings were ground with liquid nitrogen in mortar and pestle. Approximately ~0.1 g of ground tissue was used for genomic DNA isolation using a DNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's protocol. Eluted genomic DNA was tested by spectrophotometer and on 1.0% agarose for quality and quantity evaluation.

Primer design and PCR

The soybean genome array unigenes were used to query (using blastx) the *Arabidopsis* translated gene models version 7.0 from The *Arabidopsis* Information Resource (TAIR). Annotations for the Affymetrix soybean probe sets were compiled into a browser http://www.harvest-web.org called HarvEST:Soybean, which can be downloaded for windows installation from http://harvest.ucr.edu/ or accessed online at http://www.harvest-web.org.

Cowpea ESTs available from GenBank were assembled using the CAP3 program (Huang and Madan, 1999) and compiled into a browser called HarvEST:Cowpea. This software is also available for download from the HarvEST website (http://harvest.ucr.edu) or online at http://www.harvest-web.org. Cowpea methyl-filtered sequences were obtained from Dr. Michael Timko, Department of Biology, University of Virginia

(http://cowpeagenomics.med.virginia.edu/). The soybean unigenes corresponding to each SFP probe set were used to query cowpea unigenes in HarvEST:Cowpea and cowpea methyl-filtered sequences. The cowpea sequences corresponding to SFPs were then used for PCR primer design in the flanking regions of SFP position. Primers were designed using Primer3 web version software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The strategy used for primer design is illustrated in Appendix A3.1. All primer pairs were designed with Tm ~55 $^{\circ}$C.

PCR was performed in 20 μl reactions containing 20-25 ng of genomic DNA, 0.1 μM of primers, 0.2 mM dNTPs, and 1 unit of Taq DNA polymerase (New England Biolabs, USA). The reaction included an initial 5 min denaturation at 95°C, followed by 35 cycles of PCR (94°C, 30 sec; 55°C, 45 sec; 72°C, 1 min), and a final 5 min extension at 72°C. Aliquots (4 μl) of the PCR products were loaded and separated on 1.2% agarose gel by electrophoresis. A higher Tm was used when non-specific bands were amplified. PCR products were purified using QIAquick PCR purification Kit (Qiagen, USA) after confirming their uniformity on agarose gels.

DNA sequencing and analysis

DNA sequencing was performed using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Both strands of the amplified PCR products were sequenced with

an ABI-PRISM 3730xl Autosequencer (Applied Biosystems, USA) at the Core Instrumentation Facility of the UC Riverside Institute for Integrative Genome Biology (http://www.genomics.ucr.edu). The forward and reverse sequence reads for each genotype for a particular primer pair were assembled using Contig Express (Invitrogen, USA) and the consensus sequence was used for alignment. These sequences were then compared with each soybean Sequence Information File (SIF, the target sequence which extends from the 5' end of the 5'-most probe to the 3' end of the 3'-most probe) and cowpea sequence using AlignX (Invitrogen, USA). Comparisons of nucleotide sequence similarity were displayed using GeneDoc (Nicholas *et al.*, 1997). Cowpea genomic amplicon sequences have been deposited into the dbGSS Data Library under GenBank accession numbers ET041523 to ET041556.

Functional categorization of SFPs

The soybean unigenes corresponding to each SFP probe set were used to query HarvEST:Soybean (http://harvest.ucr.edu) and the corresponding *Arabidopsis* gene models were recorded. *Arabidopsis* gene models were then used to query Munich Information Centre for Protein Sequences (MIPS) (http://mips.gsf.de/) for functional categorization of the genes.

Results

Cross species platform for array hybridization

When cowpea transcripts were hybridized to the soybean genome array, the frequency of present calls ranged from 11 to 14.7% of all probe sets on the chip (consult Affymetrix manual, 2001 for the details of "present" calls). This is a frequency of 18 to 24.7% of all probe sets specific to soybean transcripts (Table 3.1) since only 61% of the probe sets target soybean genes (see Materials and Methods). Therefore, given that 37,500 soybean genes are represented by soybean probe sets, the soybean genome array provides a satisfactory tool to detect SFPs in approximately 7,500 cowpea genes for a typical RNA sample.

SFP detection and validation

As described earlier, the purpose of RPP method was to detect polymorphisms between two parental genotypes of a RIL population segregating for important agronomic traits. This method first measures the overall extent of outlying of each probe set. Probe sets with significantly high outlying scores are then analyzed at the probe level and the probes that make a sufficiently large contribution to overall outlying extent of the probe set are identified as SFP probes (Cui *et al.*, 2005). At a top 15% outlying score a list of 1060 putative SFPs between genotypes CB46 and IT93K-503-1 was generated. A full list of these SFPs and their outlying scores is provided in Supplementary Table S3.1.

A schematic diagram of SFP detection and validation is given in Figure 3.1. Plots of the log intensities, affinity differences and individual outlying score for a representative probe set (Gma.1863.1.S1_at) under both drought-stress and non-stress conditions are shown in Figure 3.2. The intensity differentiation is highest at probe 2 between CB46 and IT93K-503-1, indicating polymorphism at this probe position. Probe 2 was selected as the best SFP probe by the RPP method based on having the highest outlying score.

Table 3.1. Percent present calls from cowpea transcript hybridization to soybean genome array**.**

Genotype	Treatment*	Replicate	% Present call*	% Present call* *Glycine max*
CB46	unstressed	1	14.7	24
CB46	unstressed	2	13.9	22.8
CB46	stressed	1	11.5	18.7
CB46	stressed	2	12.5	20.4
IT93K-503-1	unstressed	1	14.3	23.4
IT93K-503-1	unstressed	2	15.2	24.7
IT93K-503-1	stressed	1	13.9	22.8
IT93K-503-1	stressed	2	11	18
		Range	11 - 14.7	18 - 24
		Mean	13.4	21.9

*Stressed = plants exposed to drought; Unstressed = plants watered regularly

*See Methods for definition of "present call"

PCR and gel electrophoresis

A list of primers used and the expected and observed PCR amplicon sizes for the validated SFP probe sets are provided in Table 3.2. PCR amplicon sizes ranged from 400 - 450 bp. Larger PCR fragments were due to non-specific amplification. A representative gel image of PCR amplification is shown in Figure 3.3. For nine primer pairs (GS 1 to 9), the amplicon sizes were as predicted by Primer3. For one primer pair (GS 10), non-specific bands were detected in both genotypes. For this primer pair the 'Tm' was increased and the PCR was tested again but still no specific band of the predicted size was amplified. Those products were not used for sequencing. Four of the primer pairs failed to produce amplicons (data not shown).

Alignment of amplicon sequences from two genotypes for SFP validation

A representative alignment of genomic amplicon sequences with the target sequence of Gma.1863.1.S1_at probe set is shown in Figure 3.4. Probe position 2 spans a SNP as predicted by the RPP method. Alignments of the 20 validated SFP probes are shown in Figure 3.5. In three cases (Gma.8053.1.S1_at, GmaAffx.93250.1.S1_s_at,Gma.1449.1.S1_s_at), the SFP probe spanned an insertion or deletion (INDEL). Among the 28 putative SFPs examined by amplicon sequencing, 20 were found to cover polymorphisms (~71% validation success). Three out of 25 probe sets contained two SFP probes each. Among the 20 confirmed SFPs from 17 probe sets, 15 (75%) probes were positioned over a single SNP, 3 (15%) were positioned over an

INDEL, 2 (10%) spanned one SNP and one INDEL and 1 (5%) spanned more than one SNP (Figure 3.5). From these data we conclude that at the threshold of top 15 percentile outlying scores, the detection method is correct about 71% of the time in identification of SFPs from the Affymetrix soybean genome array data using cowpea RNA-based datasets.

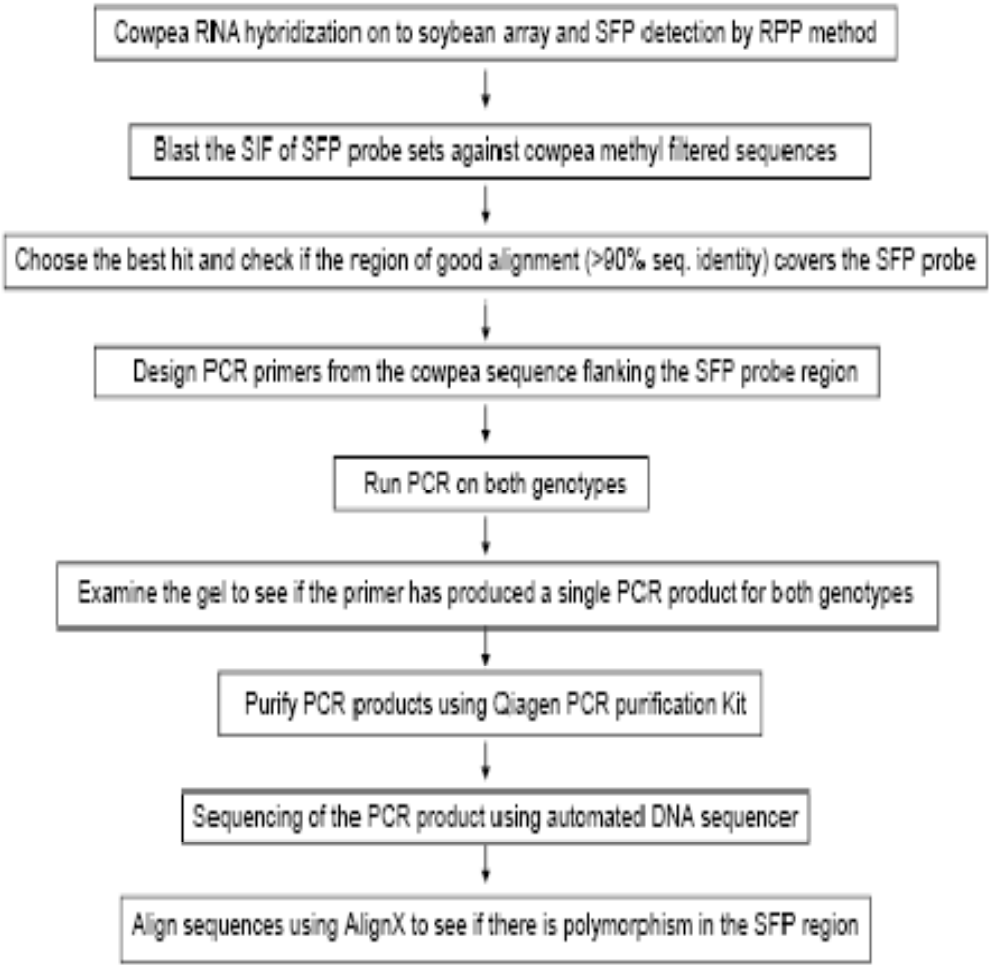

Figure 3.1. Schematic diagram of SFP validation protocol

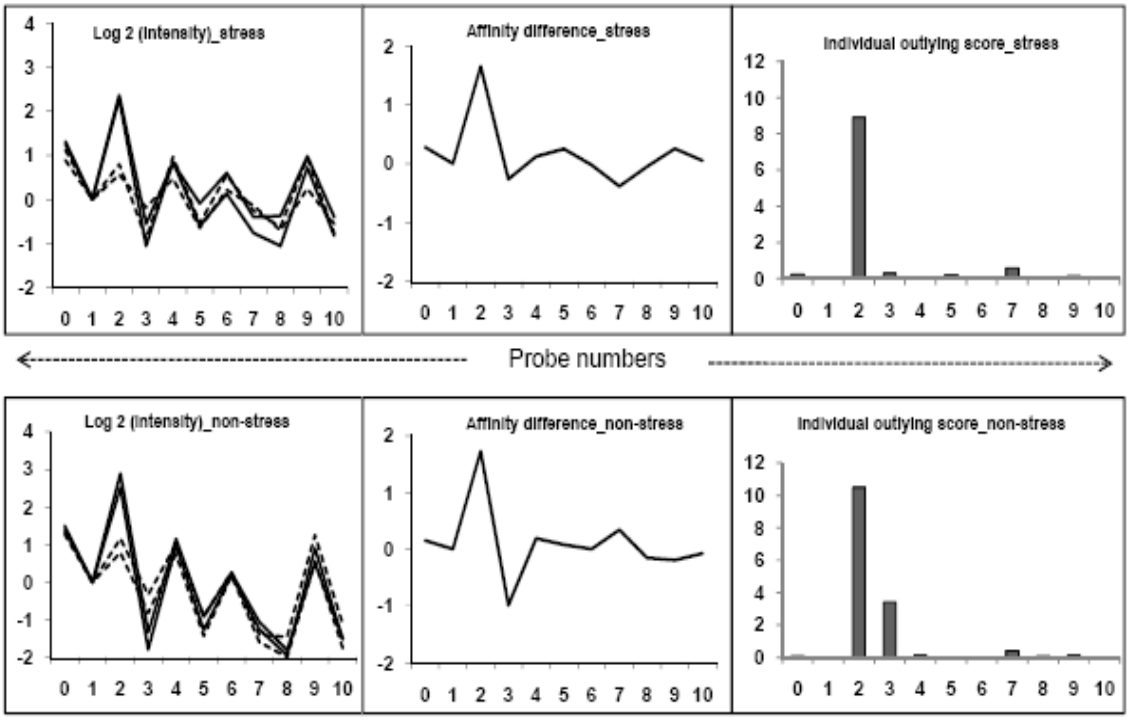

Figure 3.2. Plots of signal intensities, affinity differences and individual outlying scores. Left panels: log intensities (PM, perfect match) for a representative probe set (Gma.1863.1.S1_at) from two genotypes. Middle panels: the differences of average log intensities between two genotypes. Right panel: individual outlying scores for each probe. Dotted lines indicate IT93K-503-1 and solid lines indicate CB46. This SFP was identified both in stress (upper layer) and non-stress (lower layer) datasets

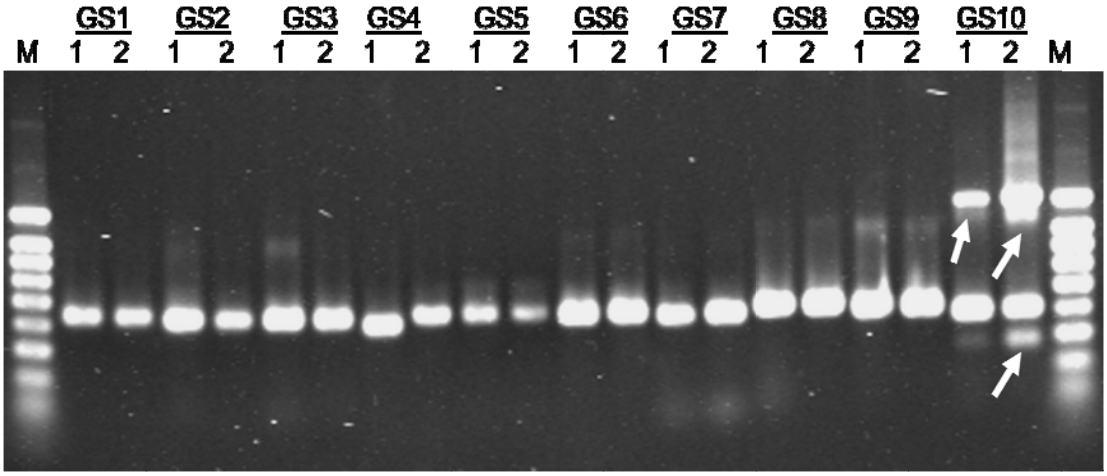

Figure 3.3. A representative gel image of PCR amplification**.** Aliquots of the PCR products of ten (GS1 to GS10) representative primer pairs were loaded and separated on a 1.2 % agarose gel. 1, CB46; 2, IT93K-503-1; M, size marker. The arrows indicate non-specific amplification.

Table 3.2. Primer list and amplicon lengths for 17 validated SFP-containing amplicons.

Affymetix probe set	Primer name	Forward primer (5' - 3')	Reverse primer (5 - 3')	Predicted* amplicon size (bp)	Observed# amplicon size (bp)
Gma.7528.1.S1_s_at	GS01	GTACCTTCCGGTGGATTCAA	TGGAATGATCACTTGGCAGA	435	439
GmaAffx.93250.1.S1_s_at	GS02	AATCCGTGTGCCAGGAATAG	ACCCAATCAGACCAATGGAG	416	420
Gma.1164.2.S1_at	GS03	GTCTTTTTGGCCTTGCATGT	TCTCAGCTTCTCCGTCCATT	422	424
Gma.1555.1.S1_a_at	GS04	AACAACAGAGTGAGCCAGCA	ACCAGGGTACCTCCCTTGAC	400	402
Gma.1863.1.S1_at	GS07	GCAGGAGTGCGTTTACCCTA	TGCTTTCACACCGCAAATTA	417	424
GmaAffx.86591.1.S1_at	GS08	TGGGAGCTGTGTCAACAGAA	GGCCTCTCAAACTGAACGTC	431	433
Gma.4431.1.S1_at	GS12	TAGCAGCCAGCCTGTGTATG	CGAAGGGTCCTAAACAACCA	447	449
Gma.8053.1.S1_at	GS16	ATCTGAGGCAGCAGCAAAAG	TGCCATGGCCACTTTAGATT	402	407
GmaAffx.62051.1.S1_at	GS21	GAAGCGTTGCATGCTTATCC	CATTCCAGTCACACCACCAG	429	432
GmaAffx.58155.1.S1_at	GS22	GTTAAACGCACCGATGGACT	ACACACTCGCCAAACAATGA	404	406
Gma.5674.1.S1_a_at	GS23	GCGGTGTTTCTTTCATGGTT	TCCCTCGTATATTCGGCATT	429	433
Gma.1449.1.S1_s_at	GS24	GCCTTTCTTCAGTGGATTGG	TGATTCACAACCCCATTTGA	442	444
Gma.13293.1.S1_at	GS27	TCTGCATTAAGCCACTGCAC	AATAGCAGCACCACGATTCC	442	441
GmaAffx.14067.1.S1_at	GS29	GGCATCCCTCTCAAGAATGT	GCAACAAAAATGGGGTGAGA	424	427
GmaAffx.69322.1.S1_at	GS31	AGTCTTATGTTGGCACAAAAACA	GCCAACTCTACCCACCAAGA	433	433
GmaAffx.28120.1.s1_at	GS36	CATCAGACACAGACGGCACT	TCACACCAATCTCCCAAACA	414	413
GmaAffx.70836.1.S1_at	GS37	CGTTCCAGTGGACATTATGC	AGATTCTTTTTGCCCAAGCA	441	431

*Predicted size indicates the PCR product size anticipated by the primer3 program. #Observed size indicates size of amplicon sequences after assembling the bidirectional sequence reads.

Functional categorization of SFPs

Since microarray based SFP markers are derived from genic regions, they can be assigned into functional categories. Arabidopsis gene models corresponding to soybean SFP probe sets were categorized into 23 different groups (see Appendix A3.2). Out of 968 unique SFP probe sets, 886 (91.5%) had a significant hit in the MIPS database (http://mips.gsf.de/). A maximum of 479 (54%) genes were grouped within the functional

Figure 3.4. Alignment of cowpea amplicon sequences related to a probe set (Gma.1863.1.S1_at) and its target sequence from the soybean SIF**.** Polymorphic residues between CB46 and IT93K-503-1 are highlighted in grey, polymorphic residues between cowpea methyl filtered sequence and soybean SIF are in black. The position of SFP probe number 2 detected by the RPP method is underlined. Arrows indicate SNPs. I, IT93K-503-1; C, CB46; G, cowpea methyl filtered sequence; S, target sequence from soybean SIF.

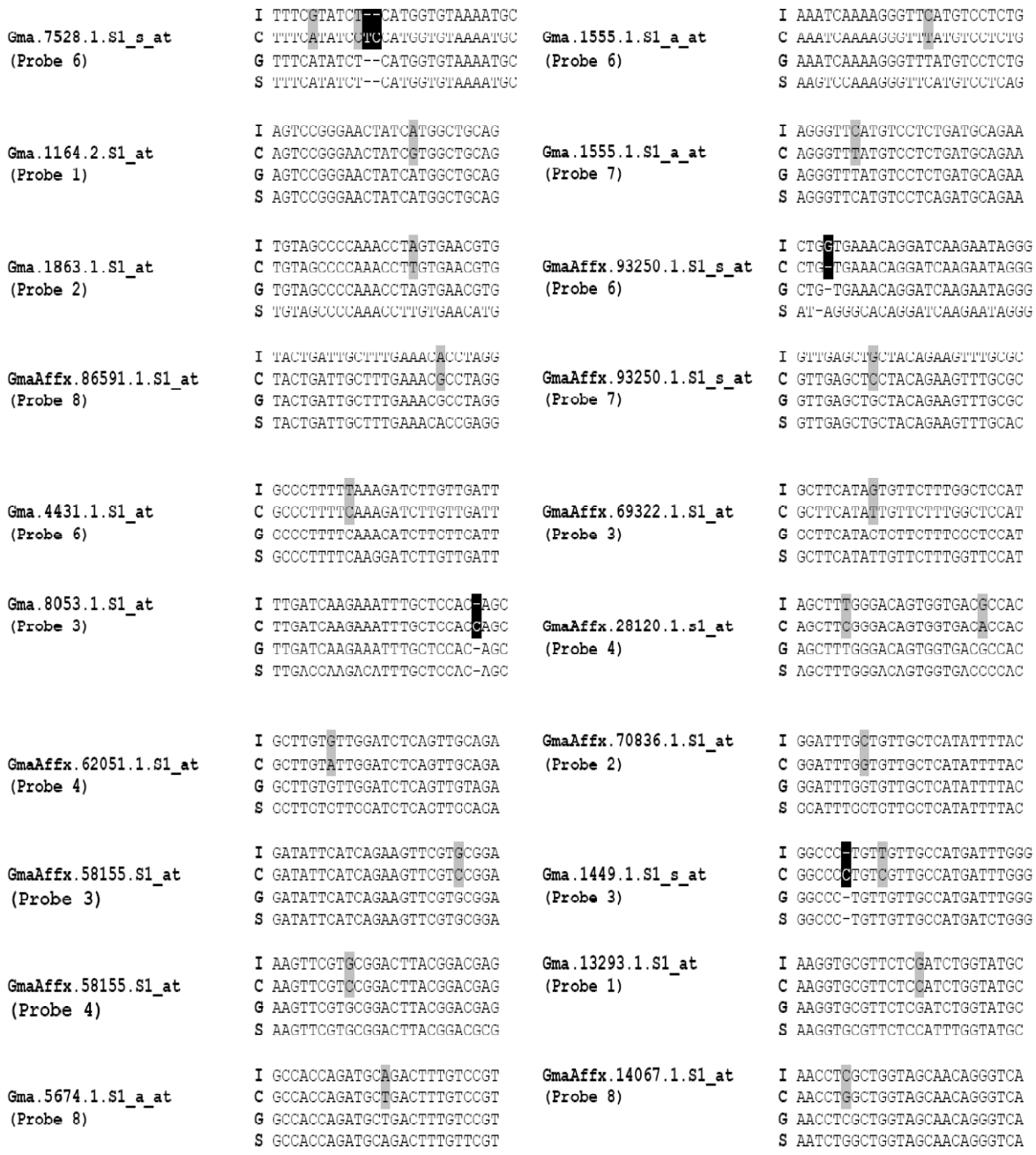

Figure 3.5. Alignments of validated SFPs**.** Grey background indicates SNP and black background indicates INDEL. I, IT-93K-503-1; C, CB46; G, methyl filtered cowpea sequence; S, target sequence from soybean SIF. The target SFP probe number is given in parentheses.

category “sub-cellular localization” followed by 296 genes (33.4%) in structural or catalytic proteins with binding function and 230 genes (25.9%) in metabolism. Several genes were classified under more than one category. A total of 77 (8.7%) genes were grouped under the “cell rescue, defense and virulence” category. Seventeen validated probe sets contained 20 SFPs and have important putative biological roles (see Table 3.3) such as defense and metabolism.

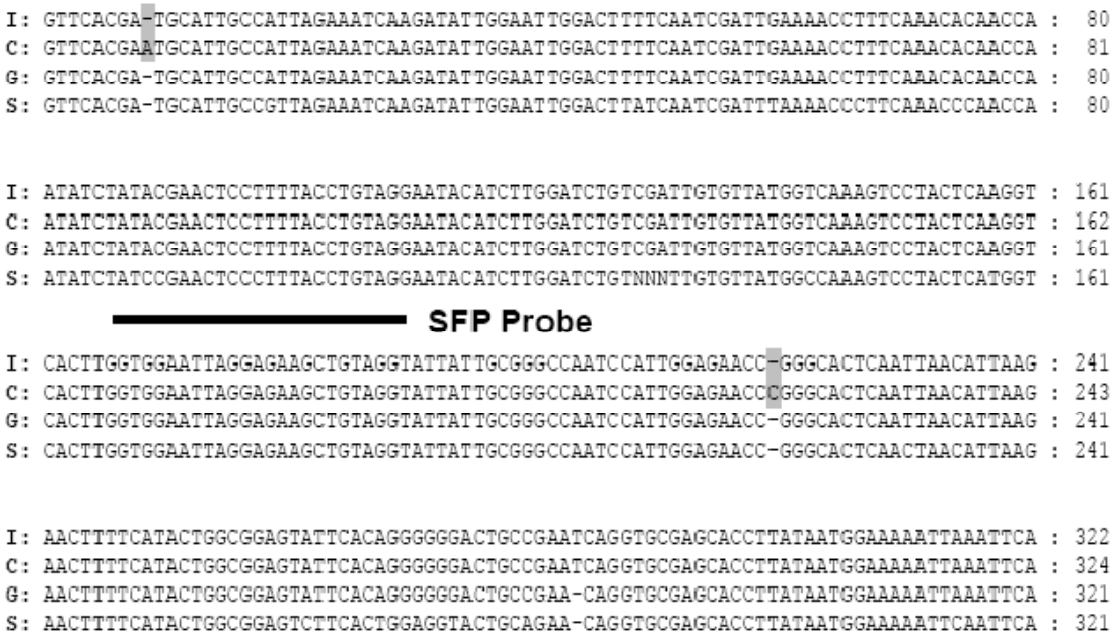

Figure 3.6. Useful by-products of genomic amplicon sequencing for probe set GmaAffx.24893.1.S1_at. Residues highlighted in gray indicate polymorphism between two cowpea genotypes though no polymorphism was detected in the SFP probe region detected by RPP (underlined). I, IT93K-503-1; C, CB46; G, cowpea methyl-filtered sequence; S, target sequence from soybean SIF.

Table 3.3. Annotation for probe sets containing validated SFPs

Primer name	Probe set name	Annotation from HarvEST:Soybean	E-Score
GS01	Gma.7528.1.S1_s	Putative importin alpha related cluster	1.00E-52
GS02	GmaAffx.93250.1.S1_s	ATP citrate lyase, putative; 3734-7120 related cluster	0
GS03	Gma.1164.2.S1_at	FRO1-like protein related cluster	5..00E-89
GS04	Gma.1555.1.S1_a_at	Early light-induced protein related cluster	5.00E-58
GS07	Gma.1863.1.S1_at	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor related cluster	1..00E-113
GS08	GmaAffx.86591.1.S1_at	Hypothetical protein F22K18.40 related cluster	5.00E-63
GS12	Gma.4431.1.S1_at	VHS domain-containing protein / GAT domain-containing protein,	1.00E-103
GS16	Gma.8053.1.S1_at	cryptochrome 2 apoprotein (CRY2) / blue light photoreceptor (PHH1)	1.00E-105
GS21	GmaAffx.62051.1.S1_at	N'-5'-phosphoribosyl-formimino-5-aminoimidazole-4-carboxamide ribonucleotide isomerase	2.00E-75
GS22	GmaAffx.58155.1.S1_at	synaptobrevin family protein, similar to Synaptobrevin-like protein 1	8.00E-66
GS23	Gma.5674.1.S1_a_at	ketol-acid reductoisomerase, identical to ketol-acid reductoisomerase	1.00E-164
GS24	Gma.1449.S1_s_at	No annotation found	-
GS27	Gma.13293.1.S1_at	Hypothetical Protein related cluster	3.00E-59
GS29	GmaAffx.14067.1.S1_at	cytosine methyltransferase (DRM2), identical to cytosine methyltransferase GI:7658293 from (*Arabidopsis thaliana*)	0
GS31	GmaAffx.69322.1.S1_at	potassium transporter, putative, similar to potassium transporter HAK2p (Mesembryanthemum crystallinum) gi:14091471:gb:AAK53759;	3.00E-93
GS36	GmaAffx.28120.1.s1_at	UDP-glucoronosyl/UDP-glucosyl transferase family protein, similar to UDP glucose:flavonoid	1.00E-37
GS37	GmaAffx.70836.1.S1_at	Chloroplast DNA-dependent RNA polymerase B subunit.	2.00E-68

Discussion

Array-based genotyping

The availability of genome arrays for a wide range of species has spawned considerable interest in identifying single feature polymorphisms (SFPs) from transcriptome data (Lou *et al.*, 2007). SFPs are genetic markers which potentially offer a physical link to the structural genes themselves. Array based genotyping methodologies are particularly valuable for species with large or un-sequenced genomes, for example in large genome hexaploid wheat a high variance probe set method has been applied to the mapping of translocation breakpoints (Bhat *et al.*, 2007). Here cross-species application of the Affymetrix soybean genome array for polymorphism survey in parents of a cowpea recombinant inbred line mapping population was investigated.

Several publications have described detection of SFPs using oligonucleotide microarrays. The error rate in SFP detection is an important criterion for its direct application in genetic mapping. An error rate of about 5% in SFP detection was reported in yeast (Winzeler *et al.*, 1998). Similarly a 3% error rate was reported in *Arabidopsis* SFP detection (Borevitz *et al.*, 2003) using genomic DNA. Approximately a 25% error rate was reported in SFPs detected in rice using genomic DNA (Kumar *et al*., 2007) and the error rate in SFP validation in barley using RNA based datasets was about 20% (Cui *et al.*, 2005).

Here the RPP method was applied to data obtained by hybridization of cRNA synthesized from cowpea RNA to the Affymetrix soybean genome array. The high validation rate (~71%) that was obtained indicates that this method works efficiently in cowpea. This validation rate is comparable to the studies cited above in yeast, *Arabidopsis* and rice for which total genomic DNA was hybridized to arrays. In rice a 97% validation rate was attained using RNA as a surrogate for DNA (Kim, S.H., personal communication). The apparent SFP validation rate in cowpea is lower than rice, but the availability of sequences overlapping the SFP probe is a limitation in cowpea. Unlike rice, where the whole genome sequence is available, only ~70% of cowpea genomic sequences are available in cowpea methyl-filtered sequences. Therefore, the validation of cowpea SFPs is more likely to be confounded by multi-gene families, as was the case with barley (Cui *et al*., 2005). In the current study, at least 50% of the non-validated cowpea SFPs are in genes that are multi-gene family members. When the full sequence of an organism is not available, it is sometimes impossible to design primers that will amplify the specific member of the gene family in which an SFP is detected. An extreme case comes from transcriptome analysis of *Medicago truncatula* nodules, which revealed a gene family named NCR (nodule-specific cysteine rich) with more than 300 members (Mergaert *et al.*, 2003).

Cross-species platform for orphan crops/organisms

Recently a number of interspecies comparisons of gene expression have been carried out in various phylogenetic branches, including human versus monkey (Gilad *et al.*, 2005; 2006), among rodents (Voolstra *et al.*, 2007), human versus mouse (Liao and Zhang, 2006), within *Xenopus* (Sartor *et al.*, 2006), and within *Drosophila* (Moehring *et al.*, 2007). Cross-species analysis of gene expression in non-model mammals was reported by Nieto-Díaz *et al.*, 2007. The reproducibility of probe data obtained from hybridizing deer, Old-World primates, and human RNA samples to the Affymetrix human GeneChip® U133 Plus 2.0 was compared. The results showed that cross-species hybridization affected neither the distribution of the hybridization reproducibility among different categories nor the reproducibility values of the individual probes. Cross-species comparisons of gene expression levels also have been informative in plants. For example, conservation and divergence of light-regulated gene expression patterns during seedling development were revealed in a comparison between rice and *Arabidopsis* (Jiao *et al.*, 2005). These successful cross-species studies provided the rationale for extending this approach to genotyping and marker development for cowpea using a readily available soybean genome array.

The SFP validation process identified many other polymorphic sites including SNPs or INDELs in sequences neighboring the SFP probe positions. Such polymorphisms, which generally represent haplotypes, also can be used for mapping and therefore are useful by-

products of genomic amplicon sequencing for SFP validation. As shown in Figure 3.6, there are even instances of neighboring polymorphisms when no polymorphism is detected in an SFP position. In general cowpea seems to have abundant polymorphism, making it compatible with a SFP marker approach.

Cost of array-based genotyping

The total cost involved in marker development is an important aspect of any marker system. At a current price of $600 per GeneChip for purchase and labeling, the cost of genotyping 100 RILs from two cowpea genotypes using one chip per RIL would be $60,000 USD. If this approach would yield 1000 good SFP markers, then the cost per marker would be $60 USD per marker. Depending on the circumstances, this cost may or may not be attractive relative to other marker options.

Another possible application of cross-species SFP analysis would be simply to compare any two genotypes within a species in order to focus amplicon sequence-based marker development on a set of target sequences that have a high chance of revealing a polymorphism. A modest expenditure to produce two datasets from each genotype of interest could provide a sizeable cost savings in materials and labour by increasing the success rate of amplicon sequencing in search of polymorphisms

It is concluded that the soybean genome array is a satisfactory platform for identification and validation of SFPs in cowpea. Furthermore, the readily available soybean genome array also could be used for SFP marker development in other legumes for which a genome array is not yet available. This study demonstrated an efficient way of generating genetic markers for orphan crops. The two genotypes used here are parents of a RIL population segregating for important agronomic traits. Therefore, the SFPs detected between these two genotypes could be used for high density mapping and association genetics studies. If some of these SFPs co-segregated with useful phenotypes like drought, disease or insect tolerance, then they could be used for marker-assisted selection in cowpea breeding programs.

Data Availability

All expression data are available through the Gene Expression Omnibus (GEO) under platform GPL 4592, Series GSE 10284 (http://www.ncbi.nlm.nih.gov/geo/).

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Chapter 4

Transcriptional profiling of root-knot nematode induced feeding sites in cowpea (*Vigna unguiculata* L. Walp) using a soybean genome array

Abstract

Cowpea (*Vigna unguiculata* L. Walp) is grown extensively as a food and fodder crop in West Africa, lower elevation areas of eastern and southern Africa, north-eastern Brazil, parts of the Middle East, India, and the south-eastern and south-western regions of North America. Root-knot nematodes (RKN, *Meloidogyne* spp.) are sedentary endoparasites with a wide host range and are one of the world's most damaging crop pests. The *Rk* locus in cowpea has been used extensively to breed root-knot nematode resistant varieties in the USA and other countries since its identification. The *Rk*-mediated resistance in cowpea has a delayed resistance response during the incompatible interaction and there was an absence of typical Hypersensitive Reaction (HR) mediated cell death in the resistant roots upon nematode infection. This is in contrast to most other plant-RKN systems studied so far. In this study the transcriptome profile of both incompatible and compatible cowpea-RKN interaction for two different time points was studied using the Affymetrix soybean GeneChip. It provides a broad insight into the *Rk*-mediated resistance in cowpea and creates an excellent dataset of potential candidate genes involved in both nematode resistance and parasitism. The results of this study have shown that the root-knot nematode resistance pathway is still partially suppressed at 9 dpi in resistant cowpea roots. There is an indication that subtle variation of ROS concentration, induction of toxins and other defense related genes play a role in this unique resistance mechanism. Further functional analysis of these differentially expressed

genes will help us to understand this intriguing plant-nematode interaction in a more precise manner.

Introduction

Cowpea (*Vigna unguiculata* L. Walp) is grown extensively as a food and fodder crop in West Africa, lower elevation areas of eastern and southern Africa, north-eastern Brazil, parts of the Middle East, India, and the south-eastern and south-western regions of North America (Ehlers and Hall, 1997). In West Africa cowpea is mainly cultivated as a rainfed crop from April to November depending on the location. Cowpea (2N=2X=22) has a genome size of ~600 Mbp (Timko *et al.*, 2007).

Root-knot nematodes (RKN, *Meloidogyne* spp.) are sedentary endoparasites with a wide host range and are one of the world's most damaging crop pests (Trudgill and Blok, 2001). RKN feeding in plant roots leads to development of specialized feeding structures in the vascular parenchyma called "giant cells". The infective stage of this nematode is second stage juvenile (J2). J2 penetrate the roots and go through three successive molts to become adult females. In most cases root-knot nematodes reproduce by obligate mitotic parthenogenesis (Jung and Wyss, 1999).

In cowpea RKN is an important pest worldwide and host plant resistance has been a preferred strategy along with cultural practices to control the nematode population in infested cowpea fields (Roberts *et al.*, 1995; Ehlers *et al.*, 2002). The *Rk* locus in cowpea has been used extensively to breed root-knot nematode resistant varieties in the USA and

other countries. This gene locus was first designated as *Rk* by Fery and Dukes (1980) and it confers resistance to many populations of *M. incognita, M. arenaria, M. hapla* and *M. javanica.*

The *Rk*-mediated resistance in cowpea has been characterized histologically by Das *et al.* (2008b; see also Chapter 2). An important finding from that study was that the resistance response was much delayed during the incompatible interaction and there was an absence of typical Hypersensitive Reaction (HR) mediated cell death in the resistant roots upon nematode infection. This is in contrast to most other plant-RKN systems studied so far (e.g. *Mi-1* mediated resistance in tomato triggers a rapid HR as early as 24 hours post-infection; Williamson, 1999).

Whole genome microarrays provide the means for scanning for genes involved in particular biological processes on a global scale. Unfortunately cowpea does not have a commercially available microarray platform. It was shown previously that the commercially available soybean GeneChip from Affymetrix can be used effectively in cowpea to identify single feature polymorphisms (SFPs) (Das *et al.*, 2008a; see also Chapter 3). Cowpea RNA was used as a surrogate for DNA to identify SFPs in the cowpea study, which established the utility of the soybean genome array as a satisfactory platform for use in examining cowpea transcripts. In the current study the soybean

platform was used to study the global plant responses to nematode infection at the molecular level.

There have been several microarray studies of the nematode infection process in plants in last few years. The RKN-plant compatible interactions have been studied using microarrays by several groups in *Arabidopsis* (Jammes *et al.*, 2005; Fuller *et al.*, 2007) and tomato (Bar-Or *et al.*, 2005; Bhattarai *et al*., 2008). Global gene expression levels have also been studied during the infection process of another important plant parasitic nematode, soyben cyst nematode (SCN, *Heterodera glycines*) (Puthoff *et al.*, 2003, 2007; Alkharouf *et al.*, 2006; Ithal *et al.*, 2007a and 2007b; Tucker *et al.*, 2007). There have been relatively fewer studies which looked into incompatible plant-nematode interactions (Bhattarai *et al.*, 2008; Klink *et al.*, 2007a, 2007b). In this study the transcriptome profile of both incompatible and compatible cowpea-RKN interaction for two different time points was studied using the Affymetrix soybean GeneChip. This is the first study of this kind in cowpea-RKN interaction. It provides a broad insight into the *Rk*-mediated resistance in cowpea and creates an excellent dataset of potential candidate genes involved in both nematode resistance and parasitism, which can be tested further for their role in this biological process using functional genomics approaches.

Materials and methods

Plant material

Two near-isogenic lines (NIL) differing in presence or absence of gene *Rk* were used. The two parents used to develop the NIL were *M. incognita* race 3 resistant cowpea genotype 'CB46' (homozygous resistant, *RkRk*) and a highly susceptible genotype 'Chinese Red' (homozygous susceptible, *rkrk*). The F_1 was backcrossed to recurrent parent CB46 (BC_1) and homozygous *Rk* plants were discarded in BC_1F_2 and non-segregating *rkrk* plants were advanced to the next back-cross (BC_2). Repeated backcrossing and selection was used to recover the *rkrk* line in the CB46 background. BC_6F_4 progenies were used for all the experiments described here. The *rkrk* line is referred to as the null-*Rk* line from here on.

Nematode inoculum

Eggs of *M. incognita* race 3 (isolate Beltran) cultured on susceptible tomato host plants were extracted from roots using 10% bleach solution (Hussey and Barker, 1973). This isolate is avirulent to gene *Rk* in CB46. Eggs were hatched in an incubator at 28° C and J2 were collected in fresh deionized water. The J2 inoculum was prepared according to the experimental requirements.

Root infections for microarray analysis

Seeds of CB46 and null-*Rk* were surface sterilized using 10% (v/v) bleach solution and planted singly in growth pouches. Plants were grown under controlled environmental conditions of 26.7°C ± 0.5°C constant temperature and daily light/dark cycles of 16/8 hours. This temperature was used because it lies within the optimum temperature range of 26 – 28 °C for development and reproduction of *M. incognita* on cowpea in growth pouches (Ehlers *et al.*, 2000). Each pouch was inoculated with 3000 J2 in 5 ml of deionized water 12 days after planting (dap). An equal number of pouches were mock inoculated with 5 ml of deionized water to use as non-infected controls. Nematode infected root tissue was excised using a sterile scalpel at 3 days post-inoculation (dpi) and 9 dpi respectively under a magnifying glass and flash frozen immediately in liquid nitrogen. Approximately an equal amount of root tissue was also excised from the similar root regions of the control plants and flash frozen. Galled tissue was excised by cutting immediately adjacent to the root-gall in order to minimize the amount of non-infected tissue included in the assays. The harvested tissue was stored in -80°C until RNA isolation. A few infected root pieces were stained in acid fuchsin (Byrd *et al.*, 1983) to confirm the nematode infection.

RNA isolation

RNA from nematode infected and non-infected root tissue was isolated using RNeasy plant mini kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's

protocol. One volume of Plant RNA Isolation Aid (Ambion, Austin, TX, USA) per unit mass of frozen tissue (ml/gm) was added before the tissue homogenization step for removal of common contaminants such as polysaccharides and polyphenolics. RNA was treated with RNase-Free DNase set (QIAGEN Inc., Valencia, CA, USA) to digest any genomic DNA which might be present. RNA was quantified using a UV-spectrophotometer. RNA quality and integrity was examined using RNA Lab-On-A-Chip (Caliper Technologies Corp., Mountain View, CA, USA) evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA).

Soybean genome array

Phylogenetic relationships based on the conserved sequences within Papilionoideae legumes imply that *Vigna* (cowpea) is closely related to soybean (Zhu *et al.*, 2005). Since a commercial cowpea genome array was not available, a soybean genome array (Affymetrix Inc., Santa Clara, CA, USA) was used for transcriptome profiling in cowpea. The soybean genome array contains 37,500 probe sets derived from soybean (*Glycine max* L.) unigenes. This represents 61% of the total probe sets on the chip, with the remainder targeting two pathogens important for soybean research, of which 15,800 (26%) probe sets target *Phytophthora sojae* (a water mold) and 7,500 (12%) probe sets target *Heterodera glycines* (soybean cyst nematode). This array uses probe sets composed of 11 probe pairs to measure the expression of each gene. Each probe pair consists of a

perfect match (PM) probe and a mismatch (MM) probe (see also http://www.affymetrix.com/products_services/arrays/specific/soybean.affx).

Array hybridization

Double-stranded complementary deoxyribonucleic acid (cDNA) was synthesized using SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) and T7-oligo (dT) promoter primers. The IVT Labeling Kit (Affymetrix) was then used to synthesize biotin-labeled complementary RNA (cRNA) from template cDNA by in vitro transcription. Twelve to 16 μg labeled cRNA was fragmented by metal-induced hydrolysis to 35–200 base fragments following Affymetrix protocols. Ten micrograms labeled, fragmented cRNA was then hybridized at 45°C with rotation for 16 h in an Affymetrix microarray Hybridization Oven 320 on Affymetrix soybean genome arrays. The arrays were washed and stained using streptavidin phycoerythrin on an Affymetrix Fluidics Station 450. The arrays were scanned on a Hewlett-Packard GeneArray scanner. cRNA synthesis and array hybridizations were performed in the Genomics Core Facility (http://www.genomics.ucr.edu) at the University of California, Riverside.

Data analysis

For 9 dpi samples three biological replicates were used for each of the four treatments (*Rk* infected & non-infected and *Rk*-null infected and non-infected), requiring 12 soybean

GeneChips. For the 3 dpi samples two biological replicates were used for each treatment requiring 8 GeneChips. The data from all 20 chips (CEL and CHP files) are publicly available in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, platform GPL 4592, series GS13631). Expression signals were first analyzed in GeneChip operating software 1.3 (GCOS, Affymetrix Inc.) to determine the "present" probe set list. The detection algorithm uses probe pair intensities to generate a detection p-value and assign a "present", "marginal", or "absent" call. Each probe pair in a probe set has a potential vote in determining whether the measured transcript is or is not "present". The vote is described by the discrimination score (R), which is calculated for each probe pair and compared to a predefined threshold, Tau. Probe pairs with R higher than Tau vote "present" and the voting result is summarized as a p-value. The greater the number of discrimination scores (R) that are above Tau, the smaller the p-value and the more likely the given transcript is truly present in the sample. Only probe sets with a "present" call in all three replicates of at least one treatment were considered to be "expressed".

Data normalization and further analysis was carried out in GeneSpring GX 7.3 (Agilent Technologies, Palo Alto, CA, USA). Robust Multiarray Average (RMA, Irrizary *et al.*, 2003; Bolstad *et al.*, 2003) normalization was performed. The data transformation was set from less than 0.01 to 0.01. Each chip was normalized to the 50th percentile and each gene was normalized to the median. As we were only interested in plant response to

nematode infection, all the probe set data from *P. sojae* and *H. glycines* were excluded from any further analysis.

Principal component analysis (PCA) is often used to reduce multidimensional data sets to lower dimensions for summarizing the most important part of the data while simultaneously filtering out the background errors. PCA involves the calculation of the eigenvalue decomposition of a data covariance matrix or singular value decomposition of a data matrix, usually after mean centering the data for each attribute. The results of a PCA are usually discussed in terms of component scores and loadings (Knudsen, 2002). PCA on conditions (treatments) based on all genes which were present in at least one chip in the 9 dpi samples were carried out to visualize the overall genome response to nematode infection in the resistant and susceptible cowpea genotypes.

For the 3 dpi samples, with only two biological replicates available, a Pearson correlation coefficient was calculated for normalized values of all probe sets between the two replicates of each treatment to determine the robustness of the data. This analysis was carried out in dChip software (Li and Wong, 2001).

Differentially expressed genes were identified using a one-way analysis of variance (ANOVA) with a p-value cut off 0.05. A multiple testing correction was performed using

the Bonferroni error correction model (Bender and Lange, 2001). False discovery rate (FDR) was set at 5.0%. Subsequently differentially expressed genes were filtered for 1.5 fold change in expression level between the control and nematode infected treatment for both genotypes and also between the nematode infected treatments of the resistant and susceptible genotypes.

Annotations and functional classification of genes

The soybean genome array unigene sequences were used to query (using blastx) *Arabidopsis* translated gene models (version 7.0) from The *Arabidopsis* Information Resource (TAIR, www.arabidopsis.org) and *Medicago truncatula* 2.0 assembly release (www.medicago.org). Annotations for the Affymetrix soybean probe sets were compiled into a browser called HarvEST:SoyChip which can be accessed online (www.harvest-web.org) or downloaded for Windows installation (http//harvest.ucr.edu/).

Gene ontology based classification was obtained by transferring the corresponding *Arabidopsis* gene models to Munich Information Center for Protein Sequences *Arabidopsis thaliana* FunCat database (MIPS, http://mips.gsf.de/proj/funcatDB/search_main_frame.html). *Arabidopsis* gene models were taken from HarvEST:SoyChip.

Results

Heterologous microarray platform

In order to elucidate the plant response to root-knot nematodes, infected CB46 (incompatible interaction) was compared with non-infected CB46 and infected null-*Rk* (compatible interaction) was compared with non-infected null-*Rk.* Two time points were chosen for this analysis i.e., 3 and 9 dpi. Nine dpi was selected as a critical time point because sequentially assayed histological sections during 21 days of infection revealed that at 9 dpi the first subtle differences appeared between incompatible and compatible interactions (Das *et al.*, 2008b; see also chapter 2). The 3 dpi samples provided a time point prior to visible differences histologically, between incompatible and compatible interactions.

The average number of soybean probe sets which had "present" call in 9 dpi samples was 10,521, which comprised 28% of the total number of soybean probe sets present in the soybean GeneChip. Similarly, the average "present" call in 3 dpi samples was 10,685 (~28.5% of all soybean probe sets). When soybean RNA was used to hybridize with the soybean GeneChip, the "present" call percentage ranged from 70-75% (Alvord *et al*., 2007). Considering that we used a heterologous genome array, these "present" call-rates are satisfactory and confirm the assumption that cowpea has a significant sequence similarity with soybean.

Data quality

PCA was done on triplicate data for each treatment at 9 dpi in order to visualize the overall genome response to nematode infection in resistant and susceptible cowpea genotypes. PCA on conditions (treatments) for the 9 dpi sample are shown in Figure 4.1. PCA component 1 (40.32% of total variance) and PCA component 2 (33.62% of total variance) comprised the majority of the described variance (73.94%). The PCA showed a clear separation between the two genotypes when infected with nematodes whereas the two genotypes clustered together when there was no external stimulus (non-infected control). Because we used near-isogenic lines for this analysis, clustering of the non-infected control samples was expected. This confirmed the robustness of the experimental design.

Because there were only two biological replicates for the 3 dpi samples, a correlation analysis was performed between the two replicates of each treatment. A representative MA-scatter plot is shown in Figure 4.2. Correlation coefficients between replicates ranged from 0.932 to 0.973 showing the robustness of the data (correlation coefficients more than 0.9 are considered to be high, Alvord *et al*., 2007).

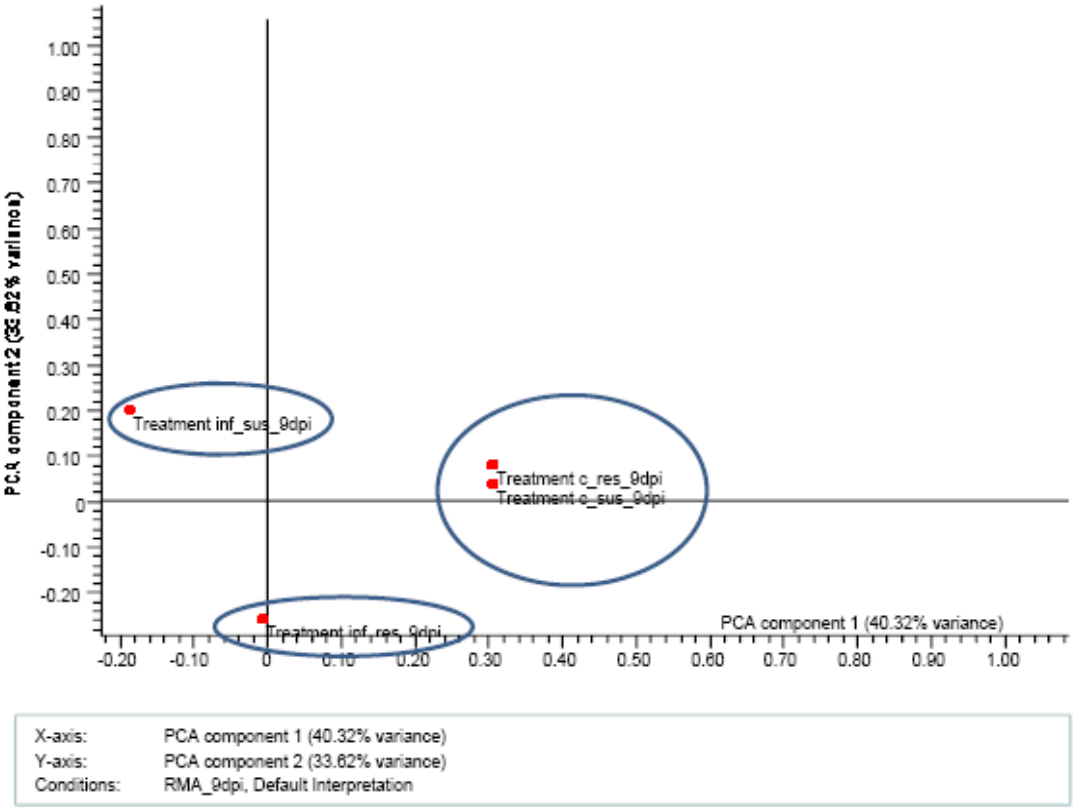

Figure 4.1. PCA plot of genome response to nematode infection. Each dot represents mean of a particular condition (treatment).

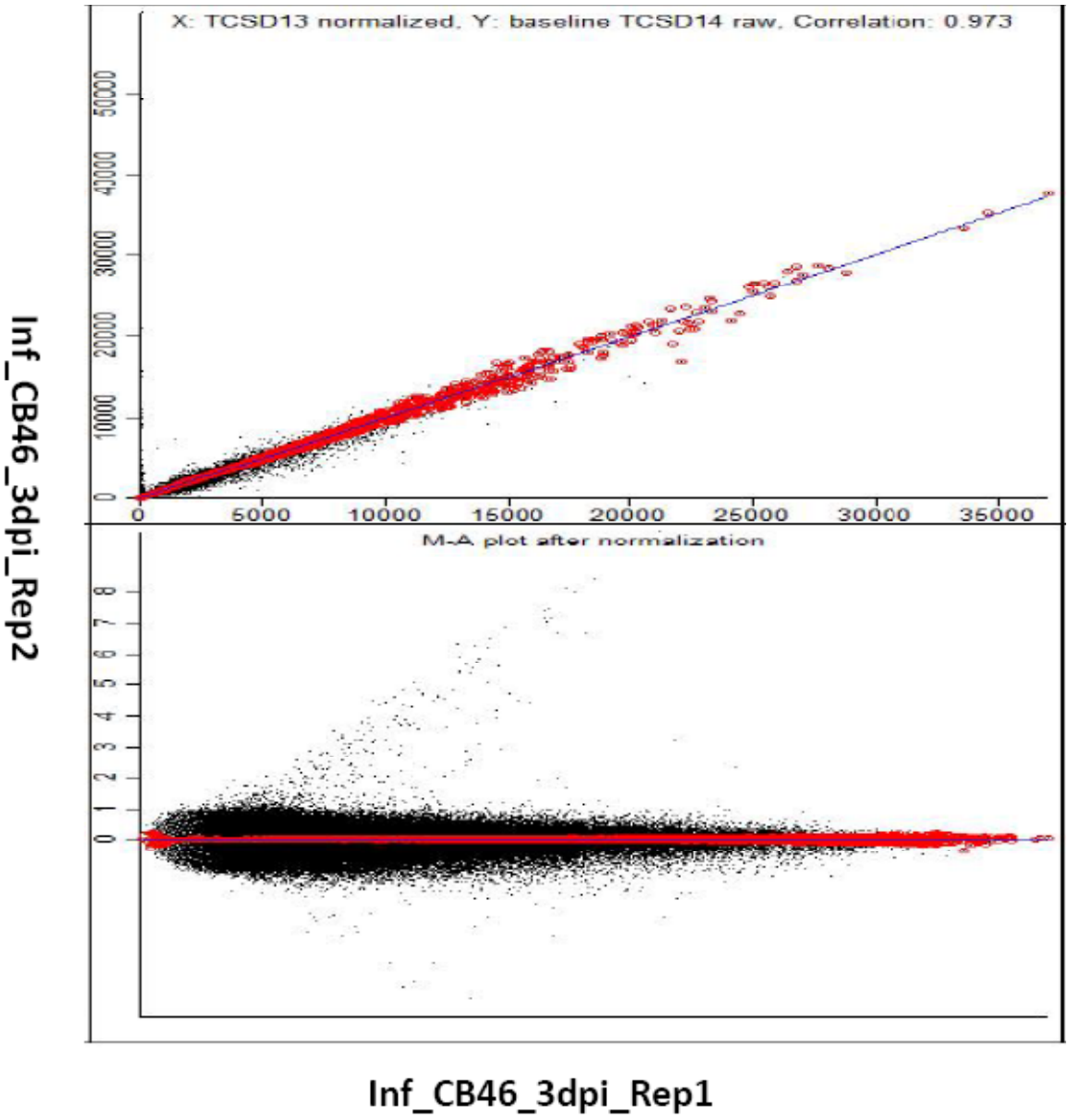

Figure 4.2. MA-scatter plot of all probe sets of two replicates of nematode infected CB46 at 3 dpi. The correlation was calculated on normalized expression values. Top panel represents the invariant probe sets to calculate the median. In the bottom panel all the probe sets were plotted around the median line.

Gene expression in incompatible and compatible interactions in 9 dpi root samples

At 9 dpi three different comparisons were made. In the first comparison gene expression in the resistant CB46 infected with root-knot nematodes (incompatible interaction) was compared with non-infected CB46. Secondly, a comparison in gene expression was made between the infected null-*Rk* (compatible interaction) and non-infected null-*Rk*. Finally, a comparison of gene expression was made between the infected *Rk* and null-*Rk* near-isogenic lines. The final comparison was important because the near-isogenic lines were predicted to show differential gene expression for the genes which are critical for nematode resistance or nematode parasitism.

In the incompatible interaction 552 (~5.3% of total expressed probe sets) genes were significantly differentially expressed between the *Rk*-infected and non-infected treatments based on the statistical test. These genes were then passed through a fold-change filter. The geometric mean of the normalized expression intensities of all samples under one condition were used to calculate the fold-change ratios. If the ratio of geometric means of infected sample and non-infected control for a probe set is ≥ 1.5, then that particular probe set is categorized as 1.5-fold or more up-regulated and if the ratio is ≤ 0.67 then the probe set is categorized as down-regulated by 1.5 fold or more. 141 genes showed 1.5-fold or more up-regulation and 59 genes were down-regulated by 1.5-fold or more in the *Rk*-infected compared with the *Rk*-non-infected treatment (Figure 4.3a and 4.3b). In the compatible interaction 1,060 genes passed the statistical filter (~10% of total expressed

probe sets). Among these 1,060 genes 218 were 1.5-fold or more up-regulated and 41 genes were 1.5-fold or more down-regulated in the infected null-*Rk* compared to the non-infected null-*Rk* treatment (Figure 4.3a and 4.3b). In this context it can be noted that in the current study the number of differentially expressed genes is lower than some of the other microarray studies previously published in the field of plant-microbe interactions. One of the reasons may be that due to the use of a heterologous GeneChip we might have lost some information. Nevertheless, the information generated will be very valuable as this is the first report on the cowpea root-knot nematode interaction. In the final comparison between the two near-isogenic lines infected with *Rk*-avirulent root-knot nematodes, 638 genes (~6% of total expressed probe sets) passed the statistical filter. Among the differentially expressed genes only 20 genes were 1.5-fold or more up-regulated in the infected *Rk* compared to the infected null-*Rk* treatment and 100 genes were 1.5-fold or more down-regulated in the infected *Rk* compared to the infected null-*Rk* treatment.

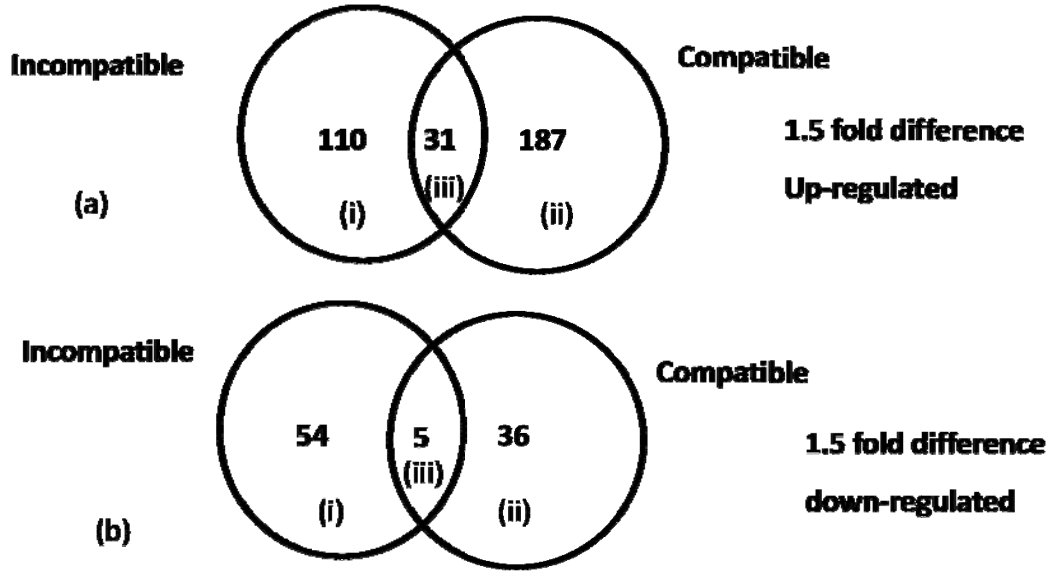

Figure 4.3. Venn diagram of differentially expressed genes in incompatible and compatible interactions at 9 dpi. (a) Genes 1.5-fold or more up-regulated in incompatible (i) and compatible (ii) interactions and genes overlapping between both interactions (iii). (b) Genes 1.5-fold or more down-regulated in incompatible (i) and compatible (ii) interactions and genes overlapping between both interactions (iii).

Selected genes from all three above comparisons are presented in Tables 4.1, 4.2 and 4.3 with their fold-change ratios and *Medicago* annotations. The genes were selected based on their fold-change level assuming that the genes with highest fold-change ratio will be likely to have a biological role in the plant- nematode interaction. Lists of all genes passing the 1.5-fold filter are provided in supplementary Tables S4.1, S4.2 and S4.3.

A gene ontology based analysis was carried out to categorize the differentially expressed genes into different functional classes. In the incompatible interaction the most abundant functional class observed for the up-regulated probe sets (Figure 4.4a) was genes involved in metabolism (32.8%), followed by proteins with binding function (28.8%) and genes involved in cell rescue and defense (13.7%). In the compatible interaction the most abundant functional classes in up-regulated probe sets (Figure 4.4a) were metabolism (30.6%), proteins with binding function (26.9%), and genes involved in protein fate (13.2%). For down-regulated probe sets the most abundant functional classes in the incompatible interaction (Figure 4.4b) were proteins with binding function (39.2%), metabolism (21.5%), and interaction with the environment (15.6%), whereas in the compatible interaction the most abundant classes (Figure 4.4b) were proteins with binding function (23.6%), cellular transport (18.4%), and systemic interaction with the environment (13.1%). A significant number of probe sets were also categorized under unclassified or unknown proteins in all the above comparisons.

Genes differentially expressed between the two infected NILs were also functionally classified (Figure 4.5). In the probe sets up-regulated in infected resistant CB46 over infected susceptible null-*Rk*, the most abundant classes were cellular transport (36.8%), proteins with binding function (21%), and proteins involved in transcription (15.7%). Among the probe sets which were down-regulated in the resistant genotype over the susceptible genotype, the most abundant functional classes were metabolism (35.1%),

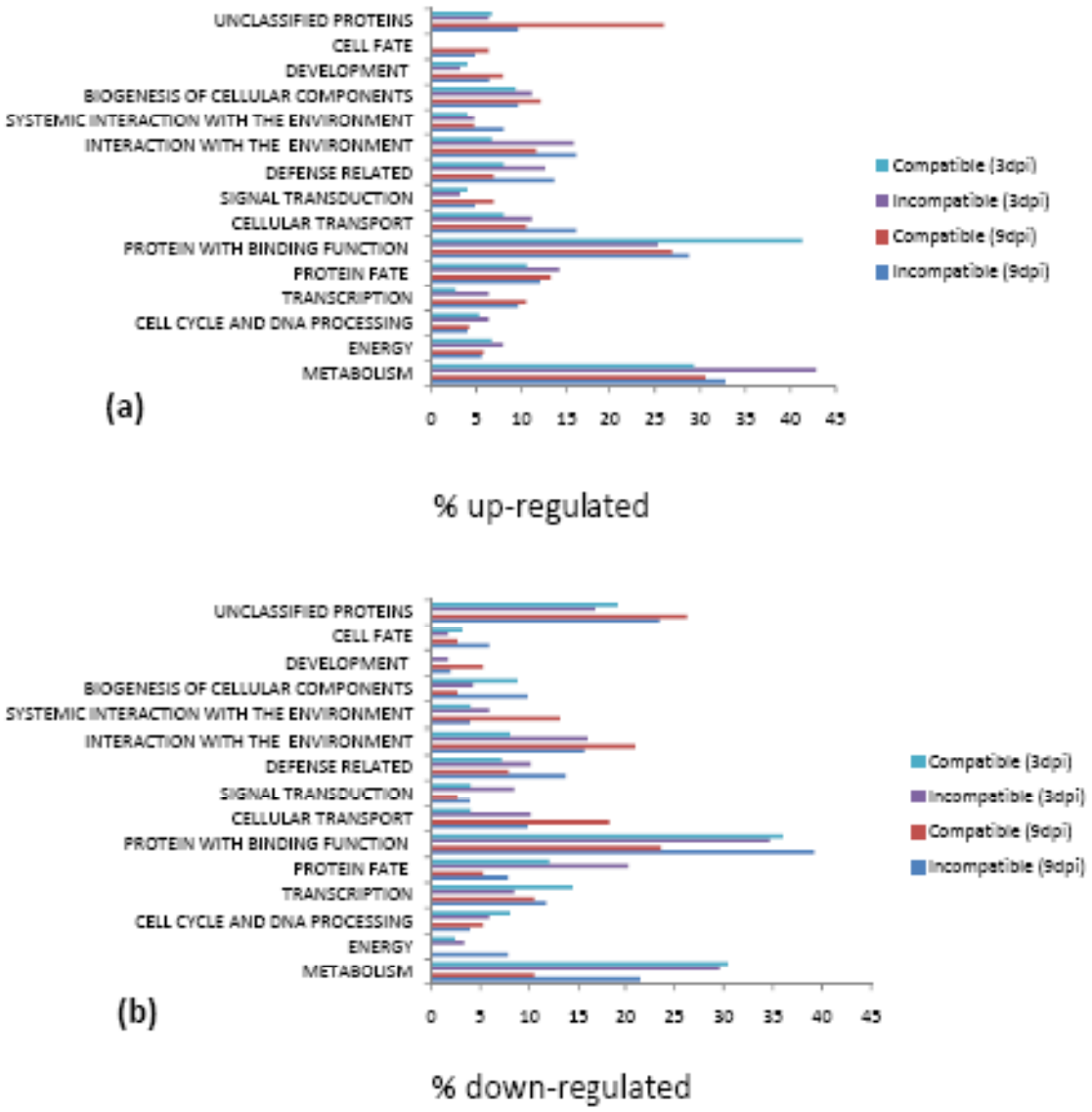

Figure 4.4. Functional classification based on MIPS using homologous sequence of *Arabidopsis.* Only the main functional categories are listed. (a) 1.5-fold or more up-regulated genes in both 9 and 3 dpi samples, (b) 1.5-fold or more down-regulated genes in both 9 and 3 dpi samples.

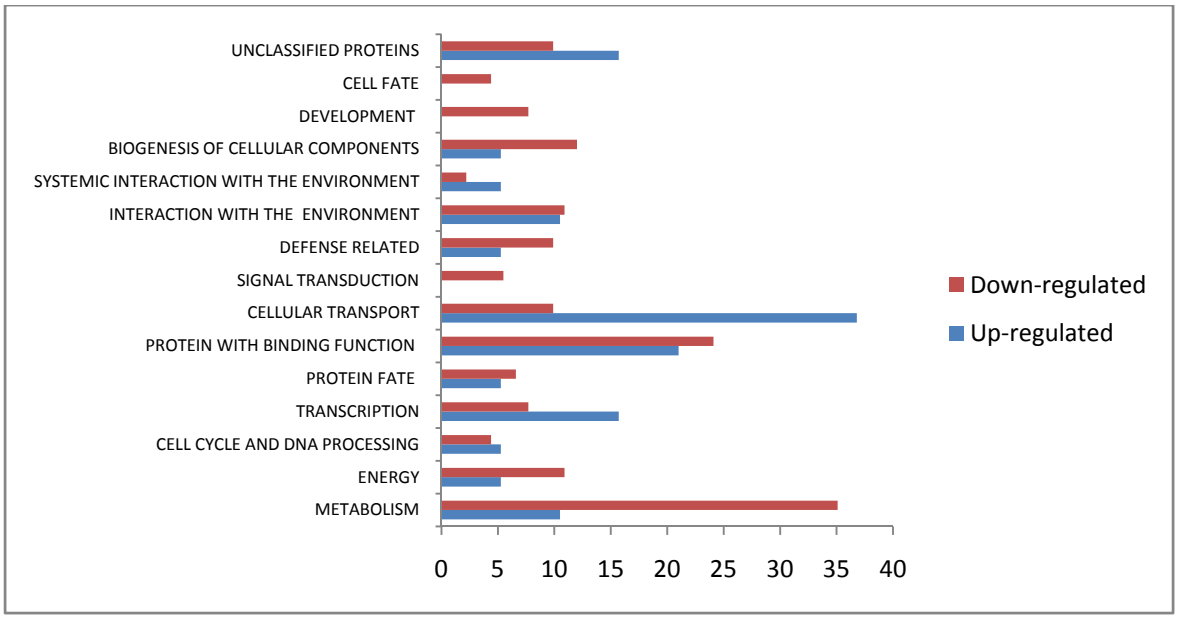

Figure 4.5. Functional classification of genes differentially expressed between infected CB46 (resistant) and infected null-*Rk* (susceptible) at 9 dpi, based on MIPS using homologous sequence of *Arabidopsis.* Only the main functional categories are listed.

proteins with binding function (24.1%), and systemic interaction with the environment (10.9%).

Gene expression in incompatible and compatible interactions in 3 dpi root samples

Similar to the 9 dpi samples, 3 different comparisons were made for 3 dpi root samples. In the first comparison gene expression in the resistant *Rk* plants (incompatible interaction) infected with root-knot nematodes was compared with non-infected *Rk* plants. A comparison in gene expression also was made between the infected null-*Rk*

(compatible interaction) and non-infected null-*Rk*. Finally, a comparison of gene expression was made between the infected *Rk* and null-*Rk* near-isogenic lines.

In the incompatible interaction 746 (~6.9% of total expressed probe sets) genes were significantly differentially expressed based on the statistical test. These genes were then passed through a fold-change filter based on $\log_2$ ratios. 65 genes showed 1.5 fold or more up-regulation and 129 genes were down-regulated by 1.5-fold or more in the incompatible interaction (Figure 4.6a and 4.6b). In the compatible interaction 623 genes passed the statistical filter (~5.8% of total expressed probe sets). Among these 623 genes 81 were 1.5-fold or more up-regulated and 148 genes were 1.5-fold or more down-regulated in the compatible interaction (Figure 4.6a and 4.6b). In the final comparison between the two near-isogenic lines infected with *Rk*-avirulent root-knot nematodes, 197 genes (~1.8% of total expressed probe sets) passed the statistical filter. Among the differentially expressed genes only 4 genes were 1.5-fold or more up-regulated in the resistant CB46 compared to null-*Rk*, and 10 genes were 1.5-fold or more down-regulated in the resistant CB46 compared to susceptible null-*Rk* plants.

Selected genes from incompatible and compatible interactions are presented in Tables 4.4 and 4.5 with their fold-change ratios and annotations based on the *Medicago* annotation database. Lists of all genes passing the 1.5-fold filter are provided in supplementary Tables S4.4, S4.5, and S4.6.

A gene ontology based analysis was carried out to categorize the differentially expressed genes into different functional classes. In the incompatible interaction the most abundant functional class observed for the up-regulated probe sets (Figure 4.4a) was genes involved in metabolism (42.8%), followed by proteins with binding function (25.3%), and genes involved in interaction with the environment (15.8%). In the compatible interaction the most abundant functional classes in up-regulated probe sets (Figure 4.4a) were proteins with binding function (41.3%), metabolism (29.3%), and genes involved in protein fate (10.6%). For down-regulated probe sets the most abundant functional classes in the incompatible interaction (Figure 4.4b) were proteins with binding function (34.7%), metabolism (29.6%), and genes involved in protein fate (20.3%), whereas in the compatible interaction the most abundant classes (Figure 4.4b) were proteins with binding function (36%), metabolism (30.4%), and genes involved in transcription (14.4%).

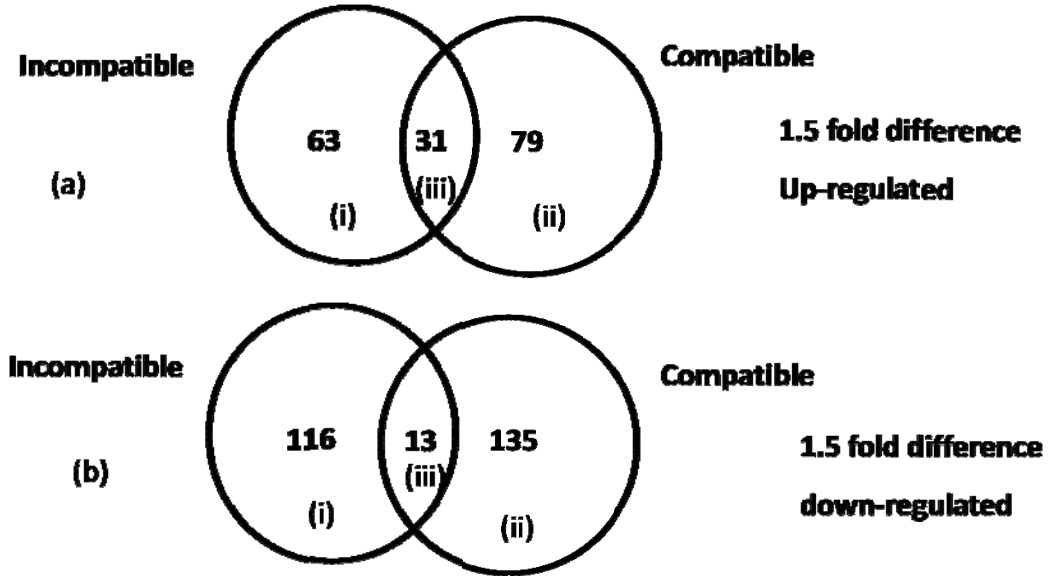

Figure 4.6. Venn diagram of differentially expressed genes in incompatible and compatible interactions at 3 dpi. (a) Genes 1.5-fold or more up-regulated in incompatible (i) and compatible (ii) interactions and genes overlapping between both interactions (iii). (b) Genes 1.5-fold or more down-regulated in incompatible (i) and compatible (ii) interactions and genes overlapping between both interactions (iii).

A significant number of probe sets were also categorized under unclassified or unknown proteins in all the above comparisons.

Comparison of gene expression between 9 dpi and 3 dpi root samples

A comparison of differentially expressed genes was done between the 9 dpi and 3 dpi samples. It was found that in the incompatible interaction 188 genes (137 up-regulated by 1.5-fold or more and 51 down-regulated 1.5-fold or more) were uniquely expressed at 9 dpi and 182 (62 up-regulated by 1.5-fold or more and 120 down-regulated 1.5-fold or

more) genes were unique for 3 dpi. 12 genes were differentially expressed at both time points. In the compatible interaction 238 genes (198 up-regulated by 1.5-fold or more and 40 down-regulated 1.5-fold or more) were uniquely expressed at 9 dpi and 208 genes (61 up-regulated by 1.5-fold or more and 147 down-regulated 1.5-fold or more) were unique for 3 dpi. 21 genes were differentially expressed at both time points.

Discussion

Recently a number of interspecies comparisons of gene expression have been carried out including human versus monkeys (Gilad *et al.*, 2005; Gilad *et al.*, 2006), between rodents (Voolstra *et al.*, 2007), human versus mouse (Liao and Zhang, 2006), within *Xenopus* (Sartor *et al.*, 2006), and within *Drosophila* (Moehring *et al.*, 2007). Cross-species analysis of gene expression in non-model mammals was reported by Nieto-Díaz *et al.*, 2007. The reproducibility of probe data obtained from hybridizing deer, Old-World primates, and human RNA samples to the Affymetrix human GeneChip® U133 Plus 2.0 was also compared. Cross-species hybridization affected neither the distribution of the hybridization reproducibility among different categories nor the reproducibility values of the individual probes. In plants, the use of heterologous platforms for transcriptome profiling also is becoming more popular. Recently the Affymetrix *Arabidopsis* GeneChip was used to analyze gene expression during seed germination in *Brassica* (Hudson *et al.*, 2007). The Affymetrix tomato GeneChip was used to survey the early events associated with the potato tuber cold sweetening (Bagnaresi *et al.*, 2008). The usefulness of the

soybean genome array to study cowpea has already been shown when the Affymetrix soybean GeneChip was used successfully to identify and validate single feature polymorphisms in cowpea (Das *et al.*, 2008a; also see Chapter 3).

There have been several microarray studies done so far to elucidate the molecular mechanism of the root-knot nematode infection process. Bar-Or *et al.*, (2005) reported a transcriptome profile of the compatible interaction in susceptible tomato roots infected with root-knot nematodes. Jammes *et al.*, (2005) and Fuller *et al.*, (2007) have done similar studies in *Arabidopsis* infected with root-knot nematodes. Several GeneChip microarray studies have also been made recently to investigate the infection process by soybean cyst nematode, *Heterodera glycines* (Puthoff *et al.*, 2007; Tucker *et al.*, 2007; Ithal *et al.*, 2008a; Ithal *et al.*, 2008b).

Microarray studies available so far in the area of plant-nematode interactions mostly examined the compatible or susceptible interaction. There is a dearth of information available for the incompatible plant-root-knot nematode interaction. Very recently Bhattarai *et al.* (2008) reported the expression profile of *Mi-1*-mediated incompatible interaction in tomato infected with root-knot nematodes. They reported the gene expression profile for only one time point, as early as 24 hours-post inoculation. Incompatible interactions have been monitored for soybean cyst nematode by Klink *et al.* (2007a and 2007b) for two different time points. In our study both compatible and

incompatible interactions were studied using the soybean genome array. This is the first insight into the cowpea-root-knot interaction at the transcriptome level. It has already been shown in related work that *Rk*-mediated resistance in cowpea is a very delayed but a strong and effective resistance response (Das *et al.*, 2008b; see also Chapter 2).

For this study the 9 dpi time point was critical as histologically there are some subtle differences between compatible and incompatible interaction at 9 dpi. Though the nematodes were able to maintain normal giant cells in resistant roots at 9 dpi, more vacuolation was evident in those giant cells when compared to the giant cells in the susceptible roots at the same stage (Das *et al*., 2008b; see also Chapter 2). Among the highly up-regulated genes in the incompatible interaction at 9 dpi there was an Alcohol dehydrogenase (Adh). *Adh* genes are widely known to respond to different biotic stimuli like fungal elicitors (Mitchell *et al.*, 1994) and cyst nematodes (Ithal *et al.*, 2007a). This leads to heavy lignification of cell walls and creates a mechanical barrier for the pathogen. In rice the sequencing of the 340 kb region surrounding the two *Adh* gene loci *Adh1* and *Adh2* revealed the presence of 33 putative genes, several of them being resistance gene analogues (Tarchini *et al.*, 2000). Also among highly up-regulated genes there was a serine threonine protein phosphatase which is known to play a role in negative regulation of defense response in *Arabidopsis* (Schweighofer *et al.*, 2007). Also several plant lectins were highly up-regulated in the incompatible interaction at 9 dpi. Plant lectins are carbohydrate binding proteins which are supposed to be toxic to several

plant pathogens (Van Damme *et al.*, 1998). These findings are consistent with the indications made by Das *et al.* (2008b) that the vacuoles of giant cells in the resistant genotype might be loaded with certain toxins which leads to developmental and reproductive arrest of the female nematode. Among the down-regulated genes in the incompatible interaction was a superoxide dismutase which generates super oxides (Apel and Hirt, 2004). Down-regulation of this gene by the plant prevents reactive oxygen species-mediated cell death which is combined with up-regulation of peroxidases (involved in breakdown of H_2O_2) in both incompatible and compatible interactions. These results are consistent with the absence of hypersensitive response (HR)-mediated cell death in the incompatible interaction (Das *et al.*, 2008b). In the incompatible interaction an expansin was highly down-regulated. Expansins are found to be important for maintaining the specialized feeding structures in plant parasitic nematodes (Klink *et al.*, 2007a, 2007b). It seems that though there is a visible effect of resistance at 9 dpi, the plant is able generate some defense response against the nematode feeding but the nematode is able to partially suppress the plant defense at this time and continue development.

When the response of the two near-isogenic lines infected with nematodes was compared, it was interesting to see that a greater number of genes were suppressed in the resistant genotype than were induced or up-regulated genes compared to the susceptible genotype. This observation is novel as usually more genes were induced in the resistant genotype

when near-isogenic lines were compared in sugarcane mosaic virus infected maize plants (Shi *et al.*, 2006) and wheat plants infected with leaf rust fungus (Hulbert *et al.*, 2007). A possible explanation for this observation is that the defense machinery in the plant is still suppressed to a large extent and as a result the nematodes are able to maintain functional giant cells even at the 9 dpi stage.

At 3 dpi in both compatible and incompatible interactions, more genes were down-regulated than up-regulated. Jammes *et al.*, (2005) also reported that there is a significant number of genes down-regulated during giant cell formation in *Arabidopsis* roots, indicating that these suppressed genes might be important negative regulators of nematode parasitism. Though in both incompatible and compatible interaction at 3 dpi there are many induced genes involved in basal defense, only a few genes were common in both interactions. This indicates that in spite of the similar nature of the infection process in both compatible and incompatible interactions, these two responses have their own molecular signature, unlike *Mi-1* mediated resistance in which many genes are shared between incompatible and compatible interactions (Bhattarai *et al.*, 2008).

In conclusion the results of this study have shown that the root-knot nematode resistance pathway is still partially suppressed at 9 dpi in resistant cowpea roots. There is an indication that subtle variation of ROS concentration, induction of toxins and other defense related genes play a role in this unique resistance mechanism. Further functional

analysis of these differentially expressed genes will help us to understand this intriguing plant-nematode interaction in a more precise manner.

Table 4.1. Selected up and down-regulated genes in infected compared with non-infected resistant plants (incompatible interaction) at 9 dpi.

Probe set name	***Medicago* annotation**	**E-value**	**Fold ratio**
Genes up-regulated by 4 fold or more			
Gma.4097.1.S1_at	Alcohol dehydrogenase superfamily, zinc-containing	1E-127	11.912
GmaAffx.7738.1.S1_s_at	Homeodomain-related	5E-87	11.64
Gma.17019.1.S1_at	Unknown		10.513
GmaAffx.1441.1.S1_at	Peptidase aspartic	5E-53	10.32
GmaAffx.5726.1.S1_at	Actin/actin-like	1E-68	9.831
Gma.3579.1.S1_at	Serine/threonine protein phosphatase	2E-68	9.656
Gma.11273.1.S1_s_at	Pyruvate decarboxylase	0	9.231
GmaAffx.50860.1.S1_at	Ribosomal protein	2E-42	9.001
Gma.2715.1.S1_at	Concanavalin A-like lectin	0	7.907
GmaAffx.91087.1.S1_s_at	Glycoside transferase	1E-145	7.782
GmaAffx.31196.1.S1_s_at	Proteinase inhibitor I9	1E-164	7.52

Gma.876.1.S1_at	Haem peroxidase	1E-100	5.921
Gma.1326.1.S1_a_at	Pectolytic enzyme, Pectin lyase	1E-48	5.814
Gma.8765.1.S1_at	Auxin responsive SAUR protein	1E-72	4.763
GmaAffx.83378.1.S1_at	FAD linked oxidase	1E-104	4.361
GmaAffx.89508.1.A1_s_at	Phenylalanine/histidine ammonia-lyase	1E-107	4.112
Gma.5689.3.S1_s_at	Peptidase, metallopeptidases	3E-63	4.031

Genes down-regulated by 3 fold or more

Gma.3233.1.S1_s_at	iron superoxide dismutase	1E-95	0.228
Gma.7006.1.S1_at	Expansin 45, endoglucanase-like	1E-119	0.274
Gma.1619.1.S1_at	WD-40 repeat family protein	1E-113	0.303
GmaAffx.80492.1.S1_at	Response regulator receiver	3E-91	0.309
Gma.13643.1.A1_at	unknown		0.314
Gma.17650.2.S1_at	hypothetical protein	1E-16	0.319
GmaAffx.89596.1.S1_at	hypothetical protein	8E-13	0.323

Table 4.2. Selected up and down-regulated genes in infected compared with non-infected susceptible plants (compatible interaction) at 9 dpi.

Probe set name	***Medicago* annotation**	**E-value**	**Fold ratio**
Genes up-regulated by 4 fold or more			
GmaAffx.8712.1.S1_s_at	Haem peroxidase	1E-130	10.98
Gma.17805.1.A1_s_at	Haem peroxidase	7E-47	9.492
Gma.289.1.S1_s_at	Alpha/beta hydrolase	1E-107	8.21
GmaAffx.20156.1.S1_s_at	Glycoside hydrolase	4E-96	6.855
GmaAffx.7738.1.S1_s_at	Homeodomain-related	5E-87	6.594
Gma.4674.1.A1_at	Esterase/lipase/thioesterase	3E-12	4.573
Gma.8525.1.S1_s_at	Haem peroxidase	1E-156	4.351
Gma.2446.1.S1_a_at	Rhodanese-like	5E-08	4.26
GmaAffx.84607.2.S1_at	Phosphate-induced protein 1	2E-19	4.09

Genes down-regulated by 2 fold or more

GmaAffx.89665.1.A1_s_at	hypothetical protein	4E-64	0.273
GmaAffx.47611.1.S1_s_at	Pollen Ole e 1 allergen	3E-70	0.42
Gma.4750.1.S1_at	Protein of unknown function	4E-56	0.429
GmaAffx.6711.1.S1_at	Auxin Efflux Carrier	8E-61	0.439
Gma.3429.1.S1_at	Dehydrogenase, E1 component	1E-158	0.464
GmaAffx.20418.1.A1_s_at	similar to unknown protein [*Arabidopsis thaliana*]	1E-19	0.5

Table 4.3. Selected up and down-regulated genes in infected CB46 (resistant) when compared to infected null-*Rk* (susceptible) at 9 dpi.

Probe set name	***Medicago* annotation**	**E-value**	**Fold ratio**
Select up-regulated genes			
GmaAffx.89665.1.A1_s_at	hypothetical protein	4E-64	2.605
GmaAffx.89786.1.A1_s_at	hypothetical protein	4E-43	2.343
GmaAffx.76516.1.S1_at	Major facilitator superfamily	2E-21	2.165
GmaAffx.6711.1.S1_at	Auxin Efflux Carrier	8E-61	2.07
GmaAffx.46592.1.S1_s_at	Rhamnogalacturonate lyase	6E-67	1.905
Gma.10150.1.A1_at	2OG-Fe(II) oxygenase	3E-51	1.806
Gma.17019.1.S1_at	Unknown		1.804
Gma.5057.1.S1_a_at	Ubiquinol cytochrome reductase transmembrane region	1E-124	1.798
GmaAffx.42856.1.S1_at	Peptidase S10, serine carboxypeptidase	1E-40	1.693
GmaAffx.31196.1.S1_s_at	Proteinase inhibitor I9	1E-164	1.687

Select down-regulated genes

Gma.289.1.S1_s_at	Alpha/beta hydrolase	3E-75	0.124
Gma.3233.1.S1_s_at	iron superoxide dismutase	1E-95	0.152
GmaAffx.8712.1.S1_s_at	peroxidase, putative	1E-130	0.221
Gma.17805.1.A1_s_at	Haem peroxidase	7E-47	0.232
GmaAffx.91763.1.S1_s_at	Xyloglucan endo-transglycosylase	1E-155	0.243
Gma.8525.1.S1_s_at	Haem peroxidase	1E-156	0.279
Gma.2801.1.S1_at	Glycoside hydrolase	1E-124	0.29
Gma.9086.2.S1_at	Cellulose synthase	5E-74	0.3
Gma.1955.4.S1_a_at	Photosystem II oxygen evolving complex protein	3E-13	0.312
Gma.2446.1.S1_a_at	Rhodanese-like	5E-8	0.318

Table 4.4. Selected up and down-regulated genes in infected compared with non-infected resistant plants (incompatible interaction) at 3 dpi.

Probe set name	***Medicago* annotation**	**E-value**	**Fold ratio**
Genes up-regulated by 3 fold or more			
Gma.1555.1.S1_a_at	Early light-inducable protein	5E-58	5.126
Gma.18079.1.S1_s_at	Protein kinase	7E-82	4.692
Gma.7289.1.S1_at	Glycosyl transferase	2E-36	4.091
Gma.289.1.S1_s_at	hydrolase, alpha/beta	3E-75	4.039
GmaAffx.47649.1.S1_at	S-adenosyl-L-methionine:carboxyl methyltransferase	1E-78	3.64
Gma.7224.1.S1_at	C-terminal; Protein kinase	3E-82	3.499
Gma.12211.2.S1_at	Phytochelatin synthetase-like	2E-48	3.463
Gma.8441.1.S1_at	Copper-resistance protein	0	3.263

Genes down-regulated by 2.5 fold or more

GmaAffx.92973.1.S1_s_at	hypothetical protein	3E-15	0.209
GmaAffx.80492.1.S1_at	Response regulator receiver	3E-91	0.22
Gma.2313.2.S1_s_at	aluminum-induced protein	9E-91	0.273
Gma.10406.1.S1_a_at	hypothetical protein	2E-81	0.325
GmaAffx.81362.1.S1_at	endoglucanase-like	7E-60	0.326
GmaAffx.55568.1.S1_at	Histidine kinase related protein	5E-70	0.332
Gma.5992.2.S1_at	Aldehyde dehydrogenase	2E-67	0.334
GmaAffx.57046.1.S1_at	Zinc finger, RING-type	1E-63	0.378
Gma.3712.1.S1_s_at	AKIN gamma - Medicago truncatula	0	0.38

Table 4.5. Selected up and down-regulated genes in infected compared with non-infected susceptible plants (compatible interaction) at 3 dpi.

Probe set name	***Medicago* annotation**	**E-value**	**Fold ratio**
Genes up-regulated by 3 fold or more			
Gma.5785.2.S1_at	Glycoside transferase	3E-32	7.464
GmaAffx.84607.2.S1_at	Phosphate-induced protein	2E-19	4.498
Gma.6152.1.S1_at	Multicopper oxidase	2E-98	3.569
GmaAffx.60283.1.S1_at	Zinc finger, RING-type	2E-36	3.416
Gma.15048.2.S1_at	Zinc finger, RanBP2-type	8E-35	3.409
Gma.16367.2.S1_a_at	Ras GTPase	2E-94	3.406
GmaAffx.33748.1.S1_at	Translation factor	1E-76	3.335
GmaAffx.84607.1.S1_at	Phosphate-induced protein 1	3E-24	3.146
Genes down-regulated by 3 fold or more			
Gma.10580.2.S1_a_at	PDS1 (phytoene desaturation 1)	1E-106	0.195
Gma.1746.1.S1_s_at	Isocitrate lyase and phosphorylmutase	7E-9	0.265

Gma.2079.3.S1_at	Adenosine/AMP deaminase	1E-158	0.268
Gma.4182.1.S1_s_at	CMP/dCMP deaminase, zinc-binding	9E-89	0.294
Gma.10456.2.S1_a_at	Zinc finger, CCHC-type	1E-161	0.302
GmaAffx.89665.1.A1_s_at	hypothetical protein	4E-64	0.303
GmaAffx.80492.1.S1_at	Response regulator receiver	3E-91	0.306
GmaAffx.89425.1.A1_s_at	hypothetical protein	4E-24	0.312

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Chapter 5

General conclusions

In crop plants host-plant resistance has been a preferred strategy to control important pests. In cowpea root-knot nematode (RKN) is a damaging pest. The identification of *Rk*-mediated resistance in cowpea has enhanced the breeding efforts to develop RKN resistant cultivars. However, very little was known about the mechanism of this resistance at the cellular and molecular levels. Until recently genomic resources in cowpea were poorly developed and cowpea does not have a commercially available microarray platform. This limits the scope of doing array-based high density genetic linkage mapping and global gene expression profiling in cowpea, though these studies are routinely done in model species and important crops. The goals of this thesis research were to investigate the mechanism of RKN resistance in cowpea and to find a suitable heterologous genomic platform which can be used until the genomic resources are well developed in cowpea.

When the incompatible cowpea-RKN interaction was compared histologically to the compatible interaction, it was established that the *Rk*-mediated resistance is a delayed resistance response, unlike most other well characterized plant-RKN interactions. There was no hypersensitive reaction (HR) mediated cell death in resistant roots upon nematode infection and nematodes were able to initiate and maintain functional giant cells and develop to late juvenile or premature adult stages. However, even though the resistance reaction was delayed, it was effective because it completely arrested the reproduction of the nematode. Further studies confirmed that *Rk*-mediated resistance is independent of

any early oxidative burst in the infected cells unlike some other plant-pathogen interactions. It could be concluded that *Rk*-mediated resistance in cowpea is a unique resistance mechanism involving the lack of a HR and based on a delayed defense response. The histological and oxidative burst profiles in infected resistant plants provided a strong platform for designing gene expression studies to identify candidate genes which play an active role in this defense pathway.

A study was designed to determine the utility of the commercially available soybean microarray platform to identify single feature polymorphisms (SFP) in cowpea. Soybean is phylogenetically closer to cowpea compared to the model legumes *Medicago truncatula* and *Lotus japonicus*. More than 1000 putative SFPs were identified between two reasonably genetically diverse cowpea genotypes. A validation success rate of ~70% was achieved by PCR amplicon sequencing. It was concluded that the Affymetrix soybean genome array is a satisfactory platform for identification of several hundreds of SFPs for cowpea. This study provided an example of extension of genomic resources from a well supported species to an orphan crop. Presumably, other legume systems are similarly tractable to SFP marker development using existing legume array resources. This study also supported the assumption that the soybean array also could be used effectively for transcriptome profiling in cowpea.

In order to elucidate the plant response to nematode infection, an expression profiling study was conducted. RNA from nematode infected feeding sites in both susceptible and resistant cowpea genotypes was purified and hybridized to the soybean genome array, using root samples collected at 9 days post inoculation (dpi) and 3 dpi. Results from this study have showed that the root-knot nematode resistance pathway is still partially suppressed at 9 dpi in resistant cowpea roots. The gene expression profiles indicated that subtle variation of ROS concentration, induction of toxins and other defense related genes play a role in this unique resistance mechanism. Further functional analysis of these differentially expressed genes will help to understand this intriguing plant-nematode interaction in a more precise manner.

Functional characterization of the potential candidate genes is required to confirm their role in the resistance or parasitism process. For the functional studies, virus induced gene silencing (VIGS) or RNAi mediated gene silencing could be used to transiently silence the candidate genes one by one to determine their effects on the nematode resistance. Unfortunately at present, there is no standard gene silencing system available in cowpea but efforts are underway to develop a suitable system in this important crop plant.

In the current research whole cowpea roots were used for RNA isolation which includes non-infected cortical tissue as well. As a result a large number of systemic genes were expected to be differentially regulated in the microarray experiment. In order to identify

differentially regulated transcripts which are locally expressed in the giant cells, laser capture micro-dissection (LCM) can be used to specifically excise the giant cells for isolation of RNA. This approach would also facilitate identification of rare transcripts and more genes involved in regulation i.e., transcription factors.

Limited information is available about the genetic location of locus *Rk* in the cowpea genome and there is a lack of markers closely associated with this locus which will be required for fine mapping of gene *Rk*. Co-segregating markers will provide the opportunity for marker-assisted selection in cowpea breeding programs and also the locus will become amenable for positional cloning of the gene. The marker resources being developed currently through SNP genotyping and physical mapping efforts in a related project shall greatly enhance the genomic resources required for these applications to cowpea.

APPENDIX

A 2.1. ROS concentration in counts per second (CPS) in root-knot nematode infected and non-infected CB46 (resistant) roots at different time points post-inoculation (experiment 1).

CB46							
Treatment	REP	24 hours	48 hours	72 hours	5 days	9 days	14 days
Infected	R1	42.0	45.3	44.7	34.6	38.8	33.2
Infected	R2	35.0	54.0	43.7	20.6	27.8	33.2
Infected	R3	33.3	45.0	41.0	27.3	27.4	31.9
Infected	R4	55.3	66.0	46.4	16.3	35.4	32.5
Infected	R5	61.3	44.3	33.7	29.9	28.4	27.5
Non-infected	R1	25.7	37.8	27.7	20.8	34.9	19.2
Non-infected	R2	20.7	28.1	35.7	23.8	28.9	16.8
Non-infected	R3	25.7	19.8	18.3	35.8	29.6	32.2
Non-infected	R4	16.4	24.1	18.0	28.4	26.6	27.8
Non-infected	R5	27.7	26.8	27.3	30.8	24.6	15.5

A 2.2. ROS concentration in counts per second (CPS) in root-knot nematode infected and non-infected null-*Rk* (susceptible) roots at different time points post-inoculation (experiment 1).

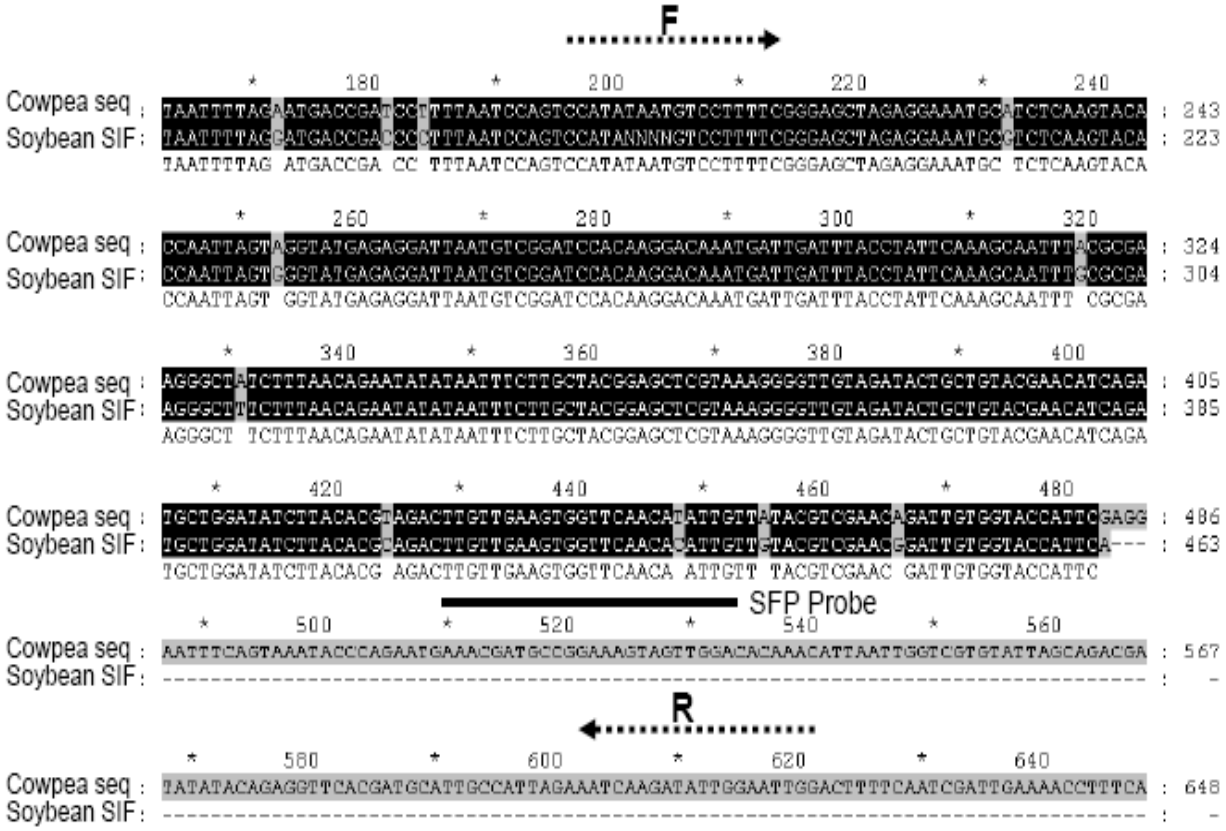
Null-*Rk*							
Treatment	REP	24 Hrs	48 hours	72 hours	5 days	9 days	14 days
Infected	R1	59.0	59.3	25.4	25.9	16.1	38.5
Infected	R2	58.3	55.7	37.0	13.9	20.4	37.5
Infected	R3	45.3	51.7	58.4	25.3	39.8	31.9
Infected	R4	50.0	48.7	28.4	34.9	18.1	34.9
Infected	R5	50.3	53.0	27.4	22.3	25.4	29.2
Non-infected	R1	18.4	31.4	16.3	36.4	19.6	34.8
Non-infected	R2	16.0	16.4	27.3	19.1	27.6	28.8
Non-infected	R3	28.4	20.8	20.7	30.8	19.2	18.8
Non-infected	R4	26.7	37.1	26.7	17.4	15.9	16.2
Non-infected	R5	19.4	26.4	21.3	16.4	28.6	24.2

A 2.3. ROS concentration in counts per second (CPS) in root-knot nematode infected and non-infected CB46 (resistant) roots at different time points post-inoculation (experiment 2).

CB46							
Treatment	REP	24 hours	48 hours	72 hours	5 days	9 days	14 days
Infected	R1	65.8	72.9	44.0	29.7	28.8	42.9
Infected	R2	72.8	72.2	53.0	29.0	32.1	31.3
Infected	R3	71.2	72.5	45.3	38.7	28.1	20.6
Infected	R4	75.5	72.2	49.0	27.3	33.8	29.3
Infected	R5	72.8	70.2	27.3	33.3	29.1	31.3
Non-infected	R1	24.7	19.0	11.9	16.7	28.9	21.8
Non-infected	R2	22.4	24.7	20.9	21.7	32.5	20.8
Non-infected	R3	12.4	15.0	15.3	26.4	27.5	29.1
Non-infected	R4	25.4	30.0	29.6	23.7	22.5	31.4
Non-infected	R5	27.4	19.7	21.6	28.1	29.2	25.4

A 2.4. ROS concentration in counts per second (CPS) in root-knot nematode infected and non-infected null-*Rk* (susceptible) roots at different time points post-inoculation (experiment 2).

Null-*Rk*							
Treatment	REP	24 hours	48 hours	72 hours	5 days	9 days	14 days
Infected	R1	73.8	65.9	54.7	25.7	37.8	32.9
Infected	R2	66.8	67.5	56.0	29.7	25.1	29.3
Infected	R3	62.5	75.2	46.3	40.7	32.1	31.9
Infected	R4	76.8	74.2	48.3	42.3	29.8	31.6
Infected	R5	70.8	56.5	47.0	33.7	29.1	23.3
Non-infected	R1	30.7	19.4	27.9	27.7	22.2	25.4
Non-infected	R2	14.0	24.7	28.6	13.7	19.9	33.4
Non-infected	R3	33.0	30.0	14.3	15.7	30.9	31.8
Non-infected	R4	17.7	15.7	17.9	29.4	28.5	24.8
Non-infected	R5	17.4	19.0	20.6	28.4	19.2	21.1



A 3.1. Strategy for primer design. F is forward primer, R is reverse primer and solid line indicates SFP probe position.

A 3.2. Functional categories of putative SFP containing probe sets.

Functional Category	**Number of genes**	**% genes**
METABOLISM	230	25.9
ENERGY	64	7.22
CELL CYCLE AND DNA PROCESSING	32	3.61
TRANSCRIPTION	83	9.36
PROTEIN SYNTHESIS	67	7.56
PROTEIN FATE	140	15.8
PROTEIN WITH BINDING FUNCTION	296	33.4
REGULATION OF METABOLISM AND PROTEIN FUNCTION	34	3.83
CELLULAR TRANSPORT	98	11
CELLULAR COMMUNICATION	33	3.72
CELL RESCUE, DEFENSE AND VIRULENCE	77	8.69
INTERACTION WITH THE ENVIRONMENT	88	9.93
SYSTEMIC INTERACTION WITH THE ENVIRONMENT	25	2.82
CELL FATE	16	1.8
DEVELOPMENT (Systemic)	33	3.72
BIOGENESIS OF CELLULAR COMPONENTS	41	4.62
CELL TYPE DIFFERENTIATION	6	0.67
TISSUE DIFFERENTIATION	5	0.56
ORGAN DIFFERENTIATION	7	0.79
SUBCELLULAR LOCALIZATION	479	54
TISSUE LOCALIZATION	3	0.33
ORGAN LOCALIZATION	4	0.45
UNCLASSIFIED PROTEINS	117	13.2