



Differentially expressed genes in cotton plant genotypes infected with *Meloidogyne incognita*

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ABSTRACT

Meloidogyne incognita is a nematode responsible for huge losses of economically important crops. The control of this pathogen is heavily centered on chemical nematicides, which are toxic to humans and environment, besides being very expensive. Alternatively, resistant varieties of cotton generated from conventional breeding programs represent an attractive strategy for the control of *M. incognita*. In this context, the goal of the work reported here was to analyze the gene expression profile of one resistant and one susceptible cotton genotype infected with *M. incognita* aiming to understand the mechanisms involved in resistance. EST libraries of cotton in both resistant and susceptible to infection by *M. incognita* were constructed and sequenced, generating 2261 sequences that were assembled into 233 contigs and 1593 singlets. Genes differentially expressed were observed in both resistant and susceptible cotton. Twenty genes were found to be expressed exclusively in the resistant cotton genotype, with functions related to pathogen recognition, signal transduction, defense mechanisms and protein synthesis transport and activation. The coordinated action of these genes suggests the existence of a complex defense pathway towards nematode attack in cotton. Our data indicate some candidate genes for validation and use through transformation in other agronomically important plants.

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1. Introduction

The worldwide crop losses caused by plant-parasitic nematodes reach numbers over US\$ 157 billion annually [1]. The most economically important group comprises the root-knot nematodes (RKN), of the genus *Meloidogyne*. Around 95% of all root-knot nematode infestations are caused by a small number of species, including the most harmful nematode, *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*, in which, together, attack more than 1700

plants species. Among this group, *M. incognita* is considered the most severe phytopathogen [2], and recently, the protein profile of its infective form was reported, highlighting the mode of action of this pathogen [3].

Traditionally nematode control has been driven by using tolerant varieties, crop rotation, cultural practices and chemical nematicides. However, these chemicals are toxic or volatile, non-specific, and are known to pose risks to human health and the environment. Furthermore, the majority of plant-parasitic nematodes live in soil or within plant roots, hindering the action of chemical nematicides. In addition, the cuticle and other surface structures of the nematodes are impermeable to many organic molecules [4].

During millions of years of evolution, plants have developed, through natural selection, a complex response system to protect themselves from nematodes, including physical and chemical

Abbreviations: RKN, root-knot nematode; ROS, reactive oxygen species; HR, hypersensitive response.

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barriers, as well as particular resistance genes that allow recognition of specific pathogens. Part of this defense reaction is released by specialized groups of gene products known as resistance proteins (R) [5,6]. The greater part of R proteins are characterized by several domains, which include nucleotide-binding site (NBS), leucine-rich repeat (LRR) regions, coiled coil (CC) domain and a domain that has homology to the Toll/Interleukin-1 Receptor (TIR). The NBS domain may be related to downstream signaling, while LRR domain is involved in pathogen recognition [7]. The ability of R genes to confer resistance against the major classes of plant pathogens, including bacteria, virus, fungi and nematodes, is already reported for a huge number of plant species [8], and the characterization of new genes involved in resistance to phytonematodes is of great interest in crop improvement.

Root-knot nematode (RKN) resistance markers have already been characterized for many plants including, *Prunus cerasifera* [9], *Arachis* [10], rice [11] and cotton [12]. They are important tools for understanding the host–pathogen relationship and for incorporating RKN resistance into elite cotton cultures [12]. Secondary metabolites can also be responsible for plant resistance to root-knot nematodes [13].

Cotton is one of the most economically important crops worldwide. However, its production is highly affected by diverse pests, including nematodes, which lead to a decrease in quality and fiber length [14–17]. In this context, EST libraries from resistant and susceptible cotton genotypes infected with *M. incognita* were sequenced and analyzed. The putative genes related to nematode resistance analyzed here are available for validation and introgression into other economically important crops.

2. Materials and methods

2.1. Plant materials

Two cotton varieties (*Gossypium hirsutum* L.), IAC 98/708 (a susceptible line) and IAC 96/414 (a resistant line), were generated by the Campinas Agronomic Institute, São Paulo (Brazil), using classical breeding and were grown under greenhouse conditions. 30 days after sowing plant roots were inoculated with 1200 second stage juveniles of *M. incognita* per plant, and maintained in a greenhouse. Roots were collected 3 days after inoculation and immediately frozen in liquid nitrogen. Tissues were stored at -80°C until use.

2.2. RNA preparation and construction of cDNA library

Total RNA was extracted from frozen root tissues using CONCERT Large-Scale RNA Isolation (Invitrogen) according to the manufacturer's instructions. Poly-A+ mRNA was isolated using an Oligotex mRNA Bath Protocol kit according to the manufacturer's manual (QIAGEN). A cDNA library was constructed from 4 μg of poly-A+ mRNA, measured by absorbance at 260 nm, in the pSPORT 1 vector exploiting the *NotI* and *Sall* restrictions sites at the 5' and 3' ends, respectively. The Superscript Plasmid System with Gateway Technology was used for cDNA Synthesis and Cloning (Invitrogen) according to the manufacturer's instructions.

2.3. Plasmid isolation and sequencing

The library was amplified in *Escherichia coli* DH-5 α (Invitrogen), placed on LB agar and grown overnight at 37°C . Plasmid preparations of the individual transformants were performed in 96-well plates according to the protocol described at the web site <http://www.cenargen.embrapa.br/laboratorios/psd/psd.html>. cDNA inserts were sequenced using SP6 and T7 primers by DNA

Automatic Sequencers (Applied Biosystems model 3700, at Embrapa Genetics Resources and Biotechnology, Brazil).

2.4. EST processing and sequence analysis

Trace files were submitted to the GENOMA system at Embrapa Genetic Resources and Biotechnology Bioinformatics Laboratory (www.genoma.embrapa.br). Base calling and quality assignment of each base of the EST sequences were performed using the program PHRED [18]. All contaminating sequences originating from nematodes, fungi or bacteria were eliminated from the pool. The resulting sets of cleaned sequences were assembled into clusters using the TGICL program of the TIGR (Institute for Genomic Research). The criteria applied were a minimum of 30 bases of similarity between sequences and 90% identity. The semi-automatic annotation was performed by BLASTX 2.2.3 [19] using the following databases in GenBank: complete and non-redundant database (with 2,452,561 sequences), all protein sequences from *Arabidopsis thaliana* (MIPS with 25,458 sequences), KOG.v.1.0 (with 4825 sequences), Swissprot (with 170,940 sequences) [20] and Pfam.v.11.0 (with 7255 sequences) [21]. These results were displayed on a web page: <http://www.genoma.embrapa.br/algo-dao/anotacao/>. For all sequences the results were analyzed by annotators to decide the final annotation.

2.5. Electronic subtraction

To identify specific transcripts from susceptible and resistant cotton varieties, each sequence was sorted by reads composition with a PERL script. Sequences exclusively from susceptible or resistant plants were placed into two databases, and the clusters containing both resistant and susceptible ESTs were sorted into a third database by the same script. Each contig could then be described by its percentage of R (resistant) and S (susceptible) sequences, which defined specific, preferential and common transcripts. These percentages were used to identify putative differentially expressed genes in cotton resistant plants and thereby to pinpoint genes related to pathogen resistance.

3. Results

Analysis of sequenced cDNA libraries is one of the most efficient methodologies to identify the expression profile of genes in specific biological situations [22]. Here this approach was used to detect genes in cotton which respond to infection by *M. incognita*. Nematode infected roots were collected 3 days after inoculation with the aim to detect early expressed genes at the beginning of the infection process.

After sequencing and assembly, 1826 cDNA sequences were obtained, comprising 233 contigs and 1593 singlets. From these, 789 singlets came from resistant plants and 804 singlets from susceptible plants. The majority of the sequences formed contigs containing reads from resistant and susceptible cotton plants (122 contigs). Surprisingly, a larger number of contigs were formed with ESTs from susceptible plants (75 contigs), rather than from resistant plants (36 contigs) (Fig. 1). These data suggest that susceptible cotton plants expressed more genes than the resistant ones. A similar proportion was also observed in the composition of singlets, suggesting that it is probably not the quantitative levels of expression, but instead, the types of genes expressed that might interfere with nematode survival. Comparable data were observed in tomato, in which the susceptible plant expressed more genes than the resistant plant after nematode infection [23].

KOG classification allowed the identification of 768 unigenes, as well as 180 genes with an imprecise classification. 588 of the

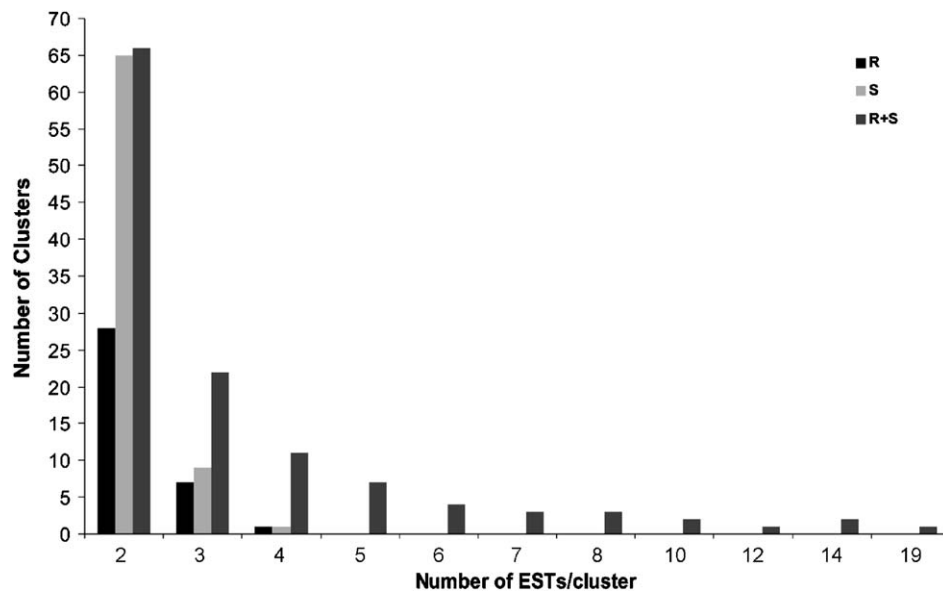


Fig. 1. Number of generated clusters, classified by composition and number of reads in each cluster. R, clusters formed only with reads that came from resistant plants; S, clusters formed only with reads that came from susceptible plants; R + S, clusters formed with reads that came from both plants.

classified clusters were divided into three categories, designated cellular processes and signaling (257 sequences), metabolism (215 sequences) and information storage and processing (116 sequences) (Fig. 2). In relation to the category of cellular processes and signaling, the KOG annotations (Fig. 2) identified 2.33% genes related to defense mechanisms and 30.74% related to signal transduction mechanisms. Biosynthesis, transport and catabolism of secondary metabolites are another gene category that could be involved in pathogen defense (Fig. 2), reaching 14.88% of the genes in KOG's annotation.

4. Discussion

Functional characterization using bioinformatic tools of the sequenced contigs and singlets revealed several genes related to

biotic and abiotic stresses that were differentially expressed in resistant and susceptible cotton plants. In this work the analyses were concentrated on genes related to biotic stress, which may be involved in pathogen defense and signaling.

Several genes were expressed exclusively in resistant plants, and some among them were identified as being related to pathogen resistance and its roles were described below (Table 1). They were functionally grouped to (i) defense signal transduction, (ii) protein activation and transport, (iii) pathogen recognition, as well as, (iv) action against the pathogen, and all of them are described below. However, six contigs, with reads found only in resistant plants, did not have hits in any data bank used in this work.

Related to defense signal transduction function (i), the most expressed gene in resistant cotton plants was pyruvate decarbox-

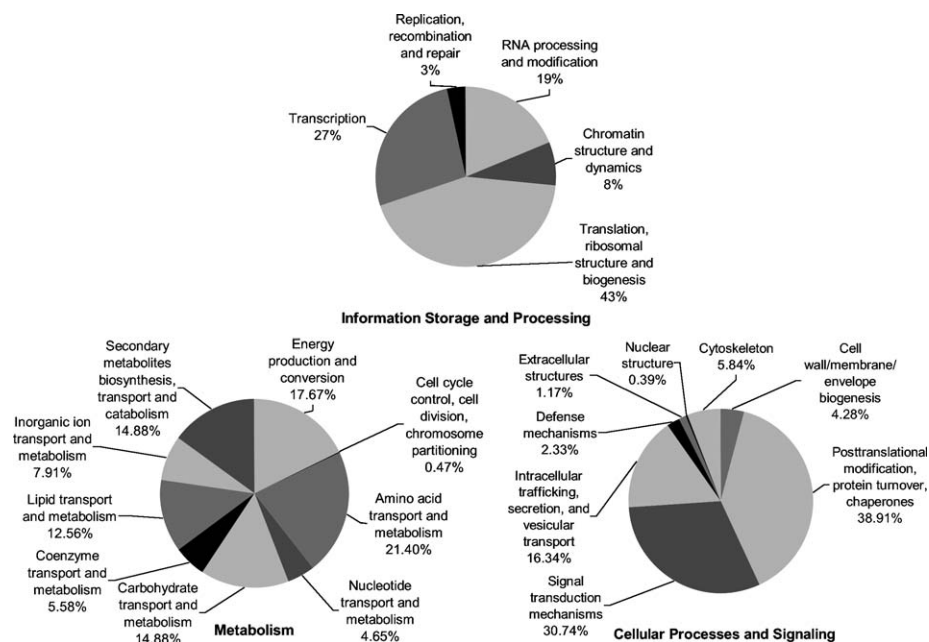


Fig. 2. Graphic representation of the functional annotation using the KOG database. Cotton ESTs were classified into three KOG categories, as follows: (1) information storage and processing, (2) metabolism and (3) cellular processes and signaling.

Table 1
BLASTX results of the clusters and singlets expressed only in resistant cotton roots infected with *M. incognita*.

GenBank accession	No. of reads	Putative gene	Related taxon	E-value
Defense signal transduction				
FL684413	4	Pyruvate decarboxylase	<i>Lotus corniculatus</i>	0.0
FL684423	2	ZF-HD homeobox protein	<i>Flaveria bidentis</i>	9e–37
FL684434	1	Calcium-dependent protein kinase	<i>Arabidopsis thaliana</i>	9e–97
FL684435	1	Cinnamoyl-CoA reductase	<i>Arabidopsis thaliana</i>	1e–58
FL684426	1	Brassinazole resistant 1 protein	<i>Arabidopsis thaliana</i>	9e–78
Protein activation and transport				
FL684416	2	Molecular chaperone Hsp90	<i>Nicotiana benthamiana</i>	e–108
FL684414	3	Translation initiation factor 5A	<i>Hevea brasiliensis</i>	4e–84
FL684429	1	Vesicle transport V-SNARE 13	<i>Arabidopsis thaliana</i>	7e–78
Pathogen recognition				
FL684432	1	Hypersensitive-induced response protein	<i>Cucumis sativus</i>	2e–78
FL684418	2	Leucine-rich repeat protein	<i>Citrofortunella mitis</i>	6e–81
Action against pathogens				
FL684415	2	Superoxide dismutase	<i>Fagus sylvatica</i>	8e–80
FL684425	1	Peroxiredoxin	<i>Populus tremula</i> × <i>P. tremuloides</i>	9e–52
FL684417	2	Reversibly glycosylated polypeptide	<i>Gossypium hirsutum</i>	e–110
FL684412	1	Isoflavone reductase	<i>Lupinus albus</i>	3e–93
FL684424	1	Endo-1,3-β-D-glucosidase	<i>Arabidopsis thaliana</i>	5e–82
FL684427	1	Skp1	<i>Medicago sativa</i>	1e–67
FL684430	1	Class IV chitinase	<i>Arabidopsis thaliana</i>	3e–28
FL684433	1	Dirigent protein	<i>Gossypium barbadense</i>	8e–81
FL684428	1	Cyclophilin	<i>Oryza sativa</i>	7e–81
FL684431	1	Actin depolymerizing factor 1 (ADF1)	<i>Petunia</i> × <i>hybrida</i>	2e–66
Genes no associated with defense response				
FL684421	2	MATE efflux family protein	<i>Arabidopsis thaliana</i>	1e–64
FL684420	2	TOC33	<i>Brassica napus</i>	2e–101
FL684419	2	Cold acclimation specific protein 15	<i>Medicago truncatula</i>	8e–32
FL684422	2	Glycerophosphodiester phosphodiesterase	<i>Arabidopsis thaliana</i>	3e–70

ylase (FL684413). This enzyme is mainly related to alcohol metabolism in response to oxygen absence [24], and its expression has already been observed in stress signal transduction pathway and in disease-resistance response [25]. Additionally, changes in sugar metabolism during disease response have also been documented in several plants [25]. In plants as soybean [26], proteins with Zinc-finger domain are transcriptional factors responsible for activating genes in pathogen response pathways. Remarkably, a gene with this domain (FL684423) was observed in cotton resistant plants. Furthermore, calcium-dependent protein kinase (FL684434) acts in plant defense response [27], as well as, cinnamoyl-CoA reductase (FL684435), which participates in defense signaling in rice [28]. In addition, brassinazole resistant protein (FL684426) is involved in the synthesis of brassinolide, and this hormone is responsible for inducing disease resistance in plants [29].

Another three genes, with functions associated with the activation, synthesis and transport of proteins (ii) were found. First is the molecular chaperone Hsp90 (FL684416) that participates in folding of R proteins, which act to protect the plant against pathogen attack [30]. Another essential role of Hsp90 is the activation of *Mi-1* protein, a *M. incognita* resistance gene found in tomato plants [31]. A translation initiation factor 5A (FL684414), the second most expressed gene in resistant plants, had been reported to be expressed in response to bacterial attack in a resistant variety of rice [32]. The last gene is the vesicle transport V-SNARE (FL684429) gene, associated with plant immune responses [33].

Concerning to the pathogen recognition (iii), the gene (FL684432) coding for a hypersensitive-induced response protein was found. This protein can act recognizing the pathogen, inducing a rapid cell death and activating other pathogen related genes [34]. In the same way, the expressed gene FL684418, containing the LRR domain within its sequence, related too with pathogen recognition [35].

Other genes could be grouped by their action against pathogens (iv). The enzyme superoxide dismutase (FL684415)

usually participates in defense against reactive O₂ species (ROS) [36], and can also be expressed in plants as tomato [37], barley [38] and in yellow lupine [39] infected with pathogens. Recently, research involving *Vigna unguiculata* showed the importance of production and degradation of ROS in the resistance against RKN [40]. Equally, the peroxiredoxin (FL684425) acts in pathogen defense response in poplar and also against oxidant stress [41]. On the other hand, the function of the reversibly glycosylated polypeptide (FL684417) remains unclear, but the interaction of this polypeptide with tomato leaf curl virus has already been observed, suggesting a possible defense function [42]. Concerning to the enzyme isoflavone reductase (FL684412), high expression levels in an alfalfa cultivar resistant to *M. incognita* have been observed [13]. Additionally, the enzyme endo-1,3-β-D-glucosidase (FL684424) may be involved in resistance to fungi [43] and to the phynematode, *Heterodera rostochiensis* [44]. In parallel, the protein Skp1 (FL684427) was expressed in rice resistant to bacterial leaf blight and gall midge [24]. Chitinases are generally expressed in several plant–pathogen interactions, as has been observed for a spruce plant resistant to *Ceratobasidium bicorne*, a root pathogen [45]. In our work, the chitinase (FL684430) was found only in resistant cotton plants challenged with *M. incognita*. The expression of a dirigent protein (FL684433) is noted in wounding [46]. A cyclophilin with antifungal activity was found in *Brassica campestris* [47], and in our analysis a cyclophilin (FL684428) was expressed only in resistant cotton plants. Another gene that was characterized with antifungal defense is the actin depolymerizing factor (FL684431) [48].

The contigs showing similarity to cold acclimation specific protein (FL684419), TOC33 (FL684420), MATE efflux protein (FL684421) and glycerophosphodiester phosphodiesterase (FL684422) have not apparently been associated with any pathogen resistance mechanism. Interestingly, these genes were expressed only in cotton resistant plants (Table 1).

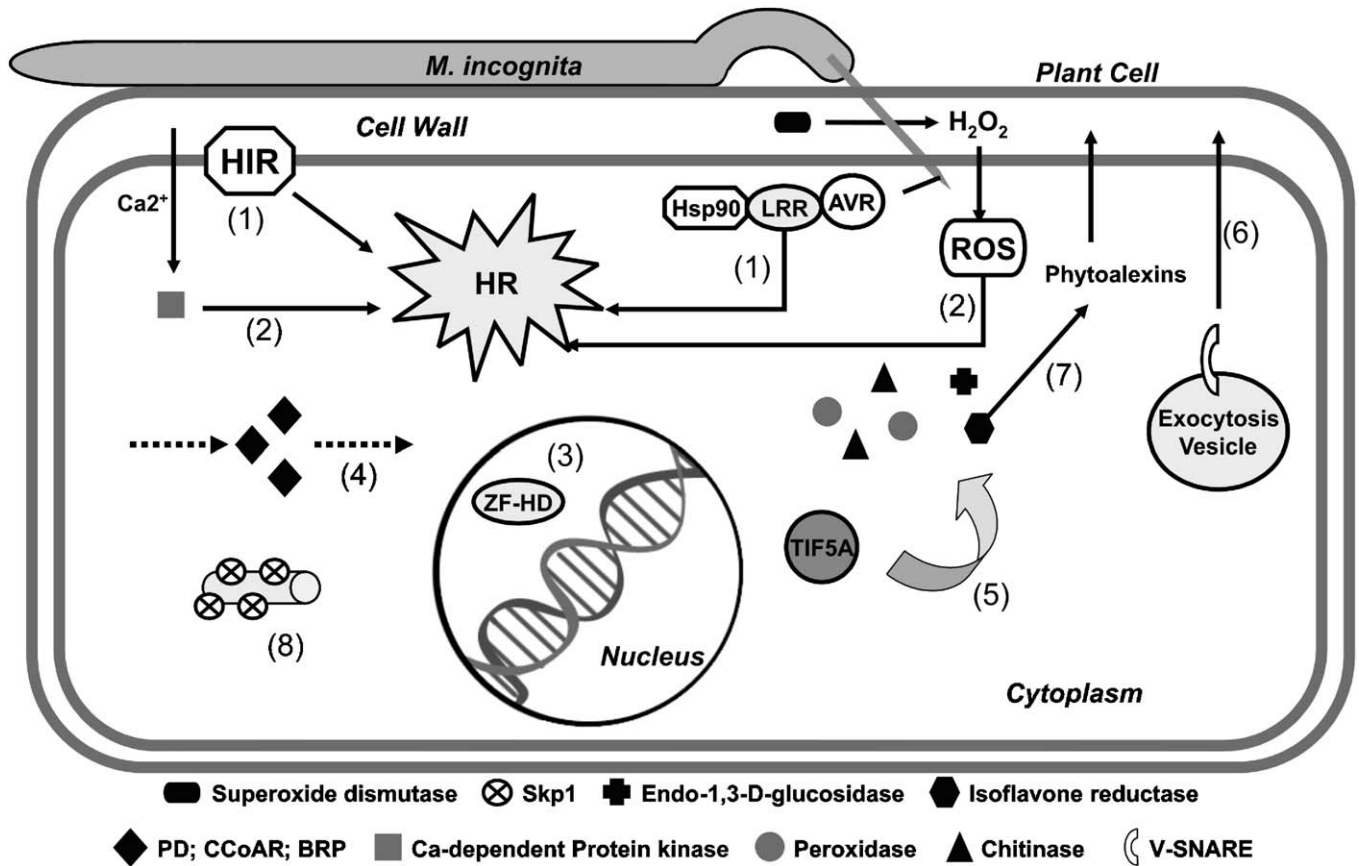


Fig. 3. Putative signaling and defense response pathway against *M. incognita* in cotton. In this model, the presence of nematode can be detected by LRR or HIR, with possible LRR activation by Hsp90 (1). This detection triggers the HR response that can be also activated by Ca-dependent protein kinase and ROS (2). After HR induction, ZF-HD initiates the transcription of PR and defense genes (3), with possible action of TIF5A in translation of them (4). Furthermore, PD, CCoAR and BRP could also make the signal transduction activating the transcription of resistance genes (5). Some defense proteins (PR) can be transported to extra cellular medium by V-SNARE (6) and others, like isoflavone reductase, can produce toxic compounds to nematode (7). Finally, Skp1 might be responsible to select target proteins for degradation (8).

The resistance mechanisms against nematode attack in plants could be explained due to complex responses, which are well described in recent papers [23,31,49]. This work identified several putative genes expressed only in the resistant cotton genotype that are related to pathogen recognition, signaling and defense (Table 1). According to their functions it was possible to infer a putative response pathway against nematodes (Fig. 3). In this scenario, LRR protein might be interacting with Avr from nematode, detecting its presence and also triggering the plant hypersensitive response (HR) [35]. In parallel, Hsp90 possibly binds to LRR protein enhancing its activity [31]. Furthermore, HR could be induced by hypersensitive-induced response (HIR) protein [34] as well as by Ca-dependent protein kinase [27] (Fig. 3). Additionally, the enzyme superoxide dismutase promotes the synthesis of the H₂O₂ contributing to the HR-mediated cell death with the accumulation of reactive oxygen species (ROS) in the cells [50].

On the other hand, the ZF-HD homeobox protein could be activating the defense PR genes transcription [26], for instance, the class IV chitinase [51]. Subsequently, the PR proteins might be transported to the extra cellular medium with the participation of V-SNAREs, which promotes the anchor of the vesicles in the cellular membrane to release the PRs in the extracellular medium [33]. Besides PRs synthesis, the cotton resistant plants also expressed isoflavone reductase, which is related to the production of phytoalexins toxic to *M. incognita* [13], and endo-1,3-D-glucosidase that showed activity against *H. rostochiensis* [44].

During the plant–pathogen interaction, Skp1 protein could be responsible to select target proteins for degradation. These target proteins could be produced by nematode during the infection

process, or even by the plant cell that might be inhibiting the defense response [24].

Additionally, protein pyruvate decarboxylase (PD), cinnamoyl-CoA reductase (CCoAR) and brassinazole resistant protein (BRP) could activate signal transduction (ST) and trigger transcription of resistance genes [24,28,29]. Subsequently, the translation initiation factor 5A could act in synthesis of resistance proteins [32].

This work provides data on the identification of several putative genes involved in nematode resistance in cotton. Despite the fact that the cultivars used in this work are not isogenic, a great number of genes identified in the resistant cotton line have been frequently related with pathogen responses in plants, mainly in response to phytonematodes. Moreover, these genes have been previously reported as being involved in response to a broader pathogen attack. Therefore, we believe that our data in cotton suggest the implication of a network of genes acting as a response to pathogen attack. Future experiments will be necessary to validate the genes responsible for nematode resistance in cotton plants. Better understanding of the molecular mechanisms of resistance will allow the deployment of nematode resistance in commercially important crops worldwide.

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