

RESEARCH ARTICLE

Cowpea–*Meloidogyne incognita* interaction: Root proteomic analysis during early stages of nematode infection

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Cowpea (*Vigna unguiculata* L. Walp) is an important legume species well adapted to low fertility soils and prolonged drought periods. One of the main problems that cause severe yield losses in cowpea is the root-knot nematode *Meloidogyne incognita*. The aim of this work was to analyze the differential expression of proteins in the contrasting cultivars of cowpea CE 31 (highly resistant) and CE 109 (slightly resistant) during early stages of *M. incognita* infection. Cowpea roots were collected at 3, 6, and 9 days after inoculation and used for protein extraction and 2-DE analysis. From a total of 59 differential spots, 37 proteins were identified, mostly involved in plant defense, such as spermidine synthase, patatin, proteasome component, and nitrile-specifier protein. A follow-up study was performed by quantitative RT-PCR analysis of nine selected proteins and the results revealed a very similar upregulation trend between the protein expression profiles and the corresponding transcripts. This study also identified ACT and GAPDH as a good combination of reference genes for quantitative RT-PCR analysis of the pathosystem cowpea/nematode. Additionally, an interactome analysis showed three major pathways affected by nematode infection: proteasome endopeptidase complex, oxidative phosphorylation, and flavonoid biosynthesis. Taken together, the results obtained by proteome, transcriptome, and interactome approaches suggest that oxidative stress, ubiquitination, and glucosinolate degradation may be part of cowpea CE 31 resistance mechanisms in response to nematode infection.

Keywords:

2-DE / Differential expression / MS / Nematode–plant interaction / Plant proteomics



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1 Introduction

Cowpea (*Vigna unguiculata* L. Walp) is an important legume crop, adapted throughout its evolution to a vast range of climate conditions such as drought, high humidity, and extreme temperatures. This crop grows in warm and hot regions worldwide, mainly in tropical areas of Africa, Asia, Southern Europe, and Central/South America where it is broadly consumed. Cowpea beans are considered a highly nutritious

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Abbreviations: DAI, days after inoculation; HR, hypersensitive response; LSU, large subunit; qRT-PCR, quantitative RT-PCR; RKN, root-knot nematodes; UPS, Ubiquitin/26S proteasome degradation system

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legume with antioxidant properties since it is rich in minerals, vitamins, and folic acid (vitamin B9) [1]. Moreover, cowpea dry beans can be stored, rapidly cooked, and eaten in many forms, which are considered important aspects for populations living in poverty.

In Brazil, the production of cowpea beans is mainly concentrated in the North and Northeast regions which are low fertility soil and semiarid zones, respectively. However, expansion to tropical savannah zones of the country, characterized by a marked dry season during winter, has been recently observed. In contrast to its high ability to adapt to adverse environmental conditions such as drought, heat, and salt stresses [2, 3], cowpea harbors low resistance to biotic stresses and can be severely attacked by different pathogens such as bacteria, virus, and nematodes, especially the root-knot nematodes (RKN) *Meloidogyne* spp. [4]. RKN are highly polyphagous plant pathogens and are responsible for severe yield losses in many economically important crops worldwide, such as cotton, soybean, and coffee, and the gall nematode *M. incognita* is considered the most destructive species [5, 6]. *Meloidogyne incognita* colonizes plant roots leading to a loss in the efficiency of water and nutrients uptake by the root system that causes symptoms similar to nutritional deficiency, such as reduced vigor with smaller leaves. The control of RKN parasites occurs mainly by the application of soil nematicides, which are usually inefficient, expensive, and extremely harmful to human and animal health as well as to the environment. The use of resistant plants, associated with integrated pest management programs, has been considered one of the main alternatives for nematode control, minimizing the harmful effects of toxic agrochemicals.

Resistance (R) genes that confer resistance to nematodes have been mapped and identified in different plant species [7, 8]. These R-genes mediate highly specific pathogen recognition in compatible or incompatible plant–RKN interaction. The first RKN R-gene described was *Mi-1.2* from tomato (*Solanum lycopersicum*) that belongs to the nucleotide binding-leucine-rich repeats (LRR) family of plant R-gene-associated products [9]. In cowpea, a dominant single R-gene or locus (*Rk*) for resistance to *M. incognita* and other RKNs was also described [10], but it seems that the resistance conferred by *Rk* is not effective to some RKN populations [11].

Studies on global gene expression and protein accumulation are interesting approaches to identify genes involved in resistance and may be a valuable resource for the characterization of new alleles leading to the development of cultivars with a broader resistance/tolerance against nematodes. Molecular studies have shown that intense gene expression changes occur in response to nematode infection [12–14], particularly in the early stages after pathogen perception. One strategy used for discovering potential candidates for nematode resistance is by analyzing the proteome of infected plants by 2-DE, which is a classical approach routinely used in proteomic studies. Although 2-DE has been widely applied in the field of plant–pathogen interactions, only few proteins from host–nematode interactions were identified to date by pro-

teomic surveys [15]. Considering the need to better understand the molecular processes triggered after nematode infection, particularly in resistant plant genotypes, we have investigated the proteomic changes in two contrasting cowpea cultivars [16] infected with the RKN *M. incognita* in an attempt to identify genes and proteins involved in resistance/defense. Additionally, gene expression profiling by quantitative RT-PCR (qRT-PCR) of some identified proteins and interactome analysis were also performed to complement the proteomic study and help understand the cowpea resistance mechanisms triggered in response to RKN infection.

2 Materials and methods

2.1 Plant material and nematode challenge

Meloidogyne incognita population was maintained in tomato roots under greenhouse conditions for approximately 3 months. After this period, roots were washed and triturated in a blender with 0.5% v/v sodium hypochlorite to extract nematodes, as previously described [17]. Freshly hatched second-stage juveniles (J2) were collected using modified Baermann funnels and nematode counting was done under a light microscope using Peter's counting slides. Cowpea plants from CE 31 and CE 109 cultivars, previously classified [16] as highly and slightly nematode resistant, respectively, were challenged with 5,000 *M. incognita* J2 in three independent experiments (biological replicates). Infected cowpea roots were collected at 3, 6, and 9 days after inoculation (DAI), all early stages of infection, as indicated by previous studies [16, 18], ground in liquid nitrogen and stored at -80°C .

2.2 Protein extraction and 2-DE analysis

Infected cowpea roots collected at 3, 6, and 9 DAI were pooled for each cultivar and used for protein extraction with phenol, as previously described [19] and suspended in solubilization buffer (7 M urea; 1 M thiourea; 4% w/v CHAPS; 2% v/v IPG buffer pH 4–7; 40 mM DTT). Protein quantification was performed using the Protein Assay Dye Reagent Concentrate (Bio-Rad), according to the manufacturer's instructions. Approximately 400 μg of extracted proteins were used to rehydrate 11 cm *Immobiline DryStrips*, pH 4–7 for 16 h. IEF was performed using a Multiphor II Electrophoresis system (GE Healthcare), according to the manufacturer's instructions (phase 1: 300 V, 2 mA, 5 W, 0:01 h; phase 2: 3500 V, 2 mA, 5 W, 1:30 h; phase 3: 3,500 V, 2 mA, 5 W, 3:00 h). IPG strips were maintained in equilibration buffer (1.5 M Tris-HCl pH 8.8; 6 M urea; 30% v/v glycerol; 2% w/v SDS; 1% v/v bromophenol blue) with 1 M DTT for 15 min followed by additional 15 min in the same buffer containing 2.5% w/v iodoacetamide. The second dimension was performed using 10% w/v polyacrylamide gels and the electrophoresis was run in a vertical system (Biometra V2) with glycine buffer (20 mM Tris-HCl pH 8.3, 192 μM glycine,

and 0.1% w/v SDS) and the molecular mass marker “Benchmark Protein Ladder” (Invitrogen). Gels were stained with colloidal Coomassie Blue (0.1% w/v Coomassie G250; 2% v/v phosphoric acid; 10% w/v ammonium sulphate, and 20% v/v methanol) and at least four gels from each of the three biological replicates were digitalized with the ImageScanner III (GE Healthcare). One gel from each biological replicate was analyzed using the software Image Master 2D Platinum version 7.05 (GE Healthcare). Spots were automatically detected and a manual adjustment was performed to minimize technical artifacts. Automated matching was performed and matches were manually checked in order to minimize possible errors. Only proteins present in at least two out of three replicates were considered for analysis. Proteins were considered as differentially expressed only when differences were significant by Student’s *t*-test at a significance level of 95%.

2.3 Protein identification

Differentially expressed proteins were excised from the gels and hydrolyzed with trypsin using the Trypsin Profile IGD kit (Sigma), according to the manufacturer’s instructions. Gel spots were submitted to hydrolysis at 37°C for 19–22 h and 1 μ L of the hydrolyzed solution was mixed with 1 μ L of alpha-cyano-4-hydroxycinnamic acid (10 mg/mL dissolved in 50% v/v ACN and 0.1% v/v TFA) and applied manually onto an Anchorchip 800/384 MALDI target plate. Peptides were analyzed using UltraFlex III or AutoFlex Speed MALDI TOF-TOF mass spectrometers (Bruker Daltonics) operating in positive reflector (MS) and LIFT (MS/MS) modes.

MS and MS/MS peak lists were generated using the FlexAnalysis 3.3 software (Bruker Daltonics) and individually searched using the MASCOT search engine (Matrix Science) with the NCBI nr protein database against all entries, Viridiplantae (or plant ESTs), *Caenorhabditis elegans* and other Metazoa. When a positive identification to Viridiplantae was obtained, an additional search of the unmatched proteins was performed against *C. elegans* and other Metazoa in order to verify the presence of nematode proteins in protein mixtures. The protein identification parameters for PMF searches were as follows: 150 ppm mass tolerance, one missed cleavage, and carbamidomethylation of cysteine residues as fixed modification and oxidation of methionine residues as variable modification. For MS/MS, the parameters used were the same described for PMF with an ion mass tolerance of 0.6 Da and charge state +1. When *pI* and molecular weight were not available, these values were calculated using ExpASY Compute *pI*/molecular weight tool (http://ca.expasy.org/tools/pi_tool.html). The cutoff value for the Probability Based Mowse score calculated by MASCOT (at $p < 0.05$) was used to accept the identification. When only single peptides were identified, the sequence generated by the MASCOT program was checked by manual *De novo* sequencing with the help of FlexAnalysis 3.3 software (Bruker

Daltonics). Some protein spots not identified by MALDI TOF-TOF were submitted to LC-MS. The peptide mixture eluted from the gel was desalted in a Poros R2 (Applied Biosystems) microcolumn, packed in a gel loader pipette tip. Peptides were separated using a Dionex Ultimate 3000 (Thermo Scientific) chromatographic system with a linear gradient of 5–35% solvent B (0.1% w/v formic acid in ACN) in 15 min at a flow rate of 250 nL/min on a C_{18} reversed phase column (75 μ m \times 15 cm). Peptides were eluted directly to an Orbitrap Elite mass spectrometer (Thermo Scientific). The mass spectrometer performed a full spectrum scan from *m/z* 350 to 1650 at a resolution of 120,000, and then the 15 most intense ions were fragmented by high energy collision dissociation approach. The fragment ions were detected at a resolution of 15,000; dynamic exclusion was set to 10 s; and charge states assigned as single or unassigned were rejected.

The resulting raw files containing MS and MS/MS data were analyzed using Peaks v.7 [20] and MaxQuant v.1.4.1.2 [21] against the NCBI EST database, filtered by the taxonomy ID 3917 applying standard settings. The precursor mass tolerance was 15 ppm and the fragment mass tolerance was 0.1 Da. Two missing cleavages were allowed and carbamidomethylation (C), oxidation (M), and acetylation (N-term) were also allowed as modifications. The list of identified proteins was filtered by FDR < 0.01 at the peptide level and presence of at least two unique peptides. Identified proteins were sorted based on intensity and when the most abundant protein was ten times over the second abundant protein, it was selected as a positively identified protein.

2.4 RNA extraction and cDNA synthesis

Total RNA was extracted from infected CE 31 roots at 3, 6, and 9 DAI and from noninoculated control using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer’s instructions. RNA quality/integrity was checked on ethidium bromide stained 1.5% w/v agarose gel and quantified using NanoDrop[®] ND-1000 spectrophotometer (Thermo Scientific). RNA samples were subsequently treated with DNase (TURBO DNA-free, Ambion), according to manufacturer’s instructions. For cDNA synthesis, 2 μ g of treated total RNA was mixed with 500 ng oligo d(T) primer and 0.5 mM dNTP and the reaction was incubated for 5 min at 65°C. A total of 4 μ L 5 \times First strand buffer, 1 μ L 10 mM DTT, and 1 μ L Superscript Reverse Transcriptase III (200 U/ μ L; Invitrogen) were added and the reaction incubated for 60 min at 50°C, followed by 15 min at 70°C.

2.5 Primer design and qRT-PCR analysis

Proteins were selected from the proteome analysis here obtained (six proteins) and from previous unpublished studies performed by our group (three proteins) for a follow-up study of gene expression by qRT-PCR analysis. The nucleotide sequences of transcripts corresponding to the selected proteins

was obtained by performing a TBLASTN search using the protein sequence identified by the MASCOT program against the NCBI EST database of *V. unguiculata*. For each candidate gene, primers were designed using Primer 3 Plus software [22] and its specificity confirmed using BLASTN searches on *Vigna* spp. transcript database resources (EST and TSA) from NCBI. Primer sequences, length of amplicons, and source of sequences are listed in Supporting Information Table 1.

qRT-PCR reactions were conducted using the Platinum[®] SYBR[®] Green qPCR Super Mix-UDGw/ROX kit (Invitrogen), in two biological and three technical replicates, as previously described [23], on a StepOnePlus[™] Real-Time PCR System (Applied Biosystems). Primer efficiency and optimal cycle threshold (C_q) values were estimated using the online real-time PCR Miner tool [24]. Average C_q values were normalized using two reference genes: an actin (ACT), previously identified as reference gene in *V. radiata* [25], and a glyceraldehyde 3-phosphate dehydrogenase (GAPDH), identified as stably expressed in previous qRT-PCR analysis conducted in *V. unguiculata* roots in our laboratory (unpublished data). Expression ratios of mRNA transcripts at 3, 6, and 9 DAI, relative to control condition (day 0), were calculated and statistically tested using REST 2009 v. 2.0.13 software [26].

2.6 In silico prediction of protein–protein interactions

The protein–protein interaction network of differentially expressed proteins was predicted from *Arabidopsis thaliana* proteins homologous to *V. unguiculata* using String v. 9.1 (<http://string-db.org/>) with default parameters (medium confidence) and GeneMania [27] with co-expression evidences. No more than 20 interactions were allowed to predict the interactions between the *V. unguiculata* differentially expressed proteins and other *A. thaliana* proteins. GeneMania was used to complement the network predicted with String v. 9.1 since this software retrieves information from a different database (NCBI's GEO). The Arabidopsis Information Resource (TAIR) was used to define the locus and extract gene ontology annotations.

3 Results and discussion

The complex interaction between the host plants and the parasitic nematodes has been widely studied in the search for more efficient strategies to control this important agricultural pest. Although the use of transcriptomic approaches to analyze global gene expression in nematode infected plants has been reported for many hosts, proteomic methods have been rarely employed [15, 18, 28]. In this study, we used 2-DE analysis to identify proteins differentially accumulated in two contrasting cowpea cultivars (highly *M. incognita*-resistant CE 31 and slightly *M. incognita*-resistant CE 109, as determined

by Oliveira et al. [16, 18]) in response to nematode inoculation. In a previous field study, Oliveira et al. [16] classified ten cowpea cultivars according to nematode resistance and showed that the most numerous *M. incognita* egg masses were present in the root system of CE 109 cultivar (41.3 ± 16.5) whereas in CE 31 such values were much lower (0.7 ± 0.4). These data allowed distinguishing these two contrasting cultivars, used in the present work, as highly and slightly *M. incognita* resistant, respectively, according to Sasser et al. [29].

Oliveira et al. [16, 18] also analyzed the kinetics of antioxidant enzymes and protein accumulation in these contrasting cultivars and showed that the antioxidant enzyme superoxide dismutase increased and catalase decreased in CE 31, leading to accumulation of hydrogen peroxide in inoculated roots in comparison to the noninoculated control, from 2 DAI. Moreover, the pathogenesis-related proteins chitinase, β -1,3-glucanase, peroxidase, and cysteine proteinase inhibitor increased in CE 31 cultivar in comparison to CE 109 at 2 DAI with *M. incognita*. These changes persisted up to the end of the experimental period (10 DAI) and were associated to the smaller final population of *M. incognita* in CE 31. Based on these previous results, we have sampled CE 31 challenged roots at 3, 6, and 9 DAI for proteomic and transcriptomic analysis.

In the present study, the analysis of the obtained 2-DE maps from both cultivars showed approximately 500 protein spots per gel, varying in mass from 10 to 160 kDa and in pI from 4 to 7 (Fig. 1). The comparative analysis between CE 31 and CE 109 showed a total of 59 differential proteins, including 13 unique to CE 31 and 15 to CE 109, as well as 18 increased and 13 decreased in CE 31 (Fig. 1; Supporting Information Table 2). Some of the differential spots were very small and could hardly be individualized and therefore were not excised for identification by MS. Other spots did not return reliable MS identification probably due to reduced ionization capacity or absence of information in the database. Therefore, from the 59 differential proteins, 37 (63%) could be identified by MS (Table 1; Supporting Information Tables 2 and 3). Interestingly, four of the 37 differential spots (spots 313, 352, 436, and 520) were identified as a mixture of plant and nematode proteins (Table 1 and Supporting Information Table 3). It is expected that the amount of plant material present in inoculated cowpea root samples is much higher than that of the nematode. However, the detection of nematode proteins in root samples indicates that it is also important to perform searches against the infecting organism even if the study aims at plant proteins. In the case of spot 352 (increased in CE 31) and spot 520 (unique to CE 31), it is probable that the difference in protein abundance may indeed correspond to the plant proteins, since the transcriptomic analysis, performed with specific primers for plant genes, confirmed the upregulation, as discussed below. For the two other spots, which were increased (spot 313) and unique (spot 436) to CE 109, additional studies need to be performed, since we cannot determine which protein, if any, is being differentially expressed.

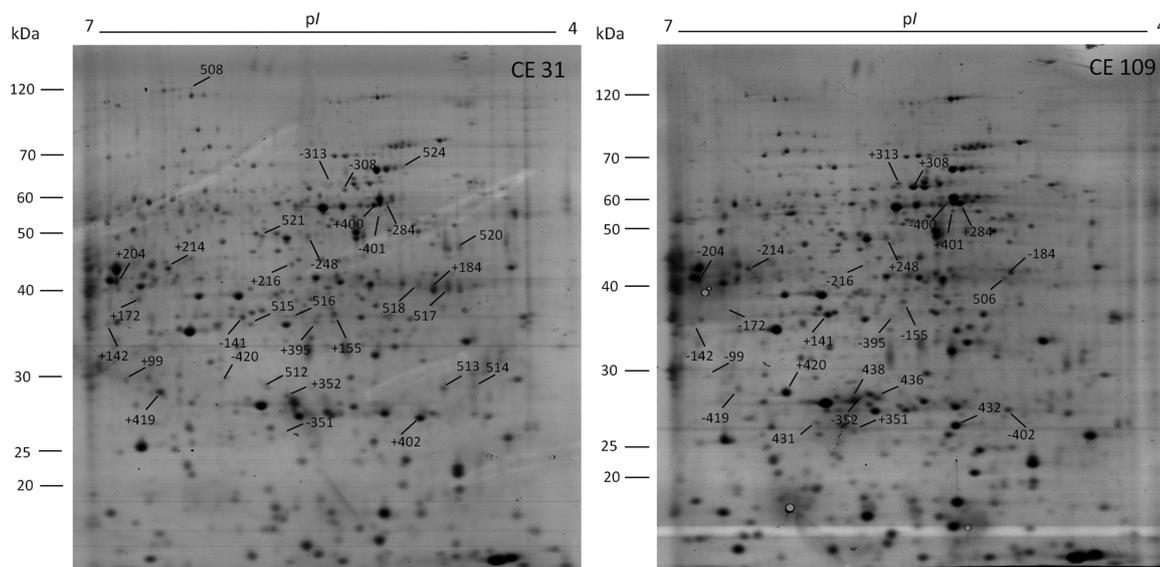


Figure 1. 2-DE maps of CE 31 and CE 109 cultivars. The symbols + and – indicate increased and decreased proteins, respectively, in response to *M. incognita*. Numbers with no symbols indicate unique proteins.

One of the major challenges in plant–nematode interaction studies is to know how host proteins interact with pathogen effectors and which are the first signaling events triggered upon the recognition of the nematode by the plant that lead to resistance. Therefore, we have focused our analysis in the early stages of nematode infection (3–9 DAI). As a follow-up study of the proteomic analysis, we have further investigated the transcription profile of selected proteins showing increased expression in the resistant cultivar. Studies involving gene expression analysis of cowpea by qRT-PCR are limited and hence there is a lack of reliable reference genes to normalize the expression quantification in the genus *Vigna*. The two genes used herein as reference (ACT and GAPDH) showed stable expression levels in cowpea roots along the time-course assay in both challenged and control plants and, therefore, were validated as a good combination of reference genes for the pathosystem cowpea/nematode.

Below, we present a comprehensive analysis of the proteome data and the expression profiles of the corresponding genes, correlating the primary functions of the identified proteins to nematode infection response in an attempt to understand their role during the plant–nematode interaction.

3.1 Proteins increased in the highly resistant cultivar CE 31

Several studies on expression changes in the host plant upon RKN infection have shown the involvement of hormones in the regulation of cell growth and stress tolerance by modulation of ROS levels [30–32]. Although the occurrence of oxidative stress upon nematode infection is a known phenomenon, the identification of genes and proteins being modulated during this process is still an important field of investigation.

Moreover, incompatible interactions are rarely studied and to date only eight nematode resistance genes have been isolated and shown to confer resistance to cyst or RK nematodes [33]. Therefore, the comparison of contrasting cultivars in response to *M. incognita* can provide unique contributions for a better understanding of nematode resistance processes involving oxidative stress response.

At the first stages of cowpea infection by *M. incognita*, the levels of several proteins associated to oxidative stress response were increased in CE 31, including a multicatalytic endopeptidase complex (proteasome), hydroxyacid oxidase, gamma-type carbonic anhydrase family protein, ferredoxin-NADP reductase isozyme 2, and glutathione S-transferase (Fig. 2; Table 1). All these proteins, although associated to ROS scavenging, are also involved in other important processes for plant defense. The multicatalytic endopeptidase complex (proteasome) protein identified in this study, for example, is part of the plant 26S proteasome, which is an essential component of the Ubiquitin/26S proteasome degradation system (UPS). UPS plays a crucial role in the degradation of short-lived regulatory proteins and in host immune responses triggered by pathogen attack [34]. It has also been shown that the proteasome is responsible for the degradation of oxidant-damaged proteins [35, 36] and the control of plant development and responses to stress [34]. The activation of UPS is an important defense mechanism and has been reported in other plant–pathogen interactions [37, 38]. Additionally, the expression behavior of cowpea proteasome-encoding genes in response to *M. incognita* inoculation obtained by qRT-PCR (Fig. 3) was in complete accordance with the proteome analysis, showing a very similar profile with enhanced expression approximately sevenfold from 3 to 6 DAI and keeping up these levels until 9 DAI.

Table 1. Differentially expressed proteins from CE 31 and CE 109 infected with *M. incognita*, analyzed by MS

Spot number	Protein identification ^{a)}	Acession (NCBI ^{nr}) ^{b)}	Organism	Fold change in CE 31 related to CE 109 ^{c)}
99	Gama carbonic anhydrase-like (F18O14.34)	gi 8778427	<i>Arabidopsis thaliana</i>	+2.31
141	Cinnamyl-alcohol dehydrogenase family protein (CPRD 14 protein)	gi 1854445	<i>Vigna unguiculata</i>	−1.55
142	UCRVU07_CCNP15642_b1 (Annexin)	gi 190493106 ^{d)}	<i>Vigna unguiculata</i>	+4.84
155	UCRVU07_CCNP3199_b1 (Fructokinase)	gi 190468481 ^{d)}	<i>Vigna unguiculata</i>	+9.62
172	UCRVU05_CCNN18825_b1 (Ferredoxin)	gi 190455501 ^{d)}	<i>Phaseolus vulgaris</i>	+9.40
184	Patatin-17-like	gi 356520766 gi 565342133	<i>Glycine max</i> <i>Solanum tuberosum</i>	+4.28
204	Glyceraldehyde-3-phosphate dehydrogenase	gi 543176466 gi 62816190	<i>Phaseolus vulgaris</i> <i>Lupinus albus</i>	+2.66
214	Hypothetical protein EUTSA_v10028651mg (Elongation factor Tu)	gi 567161651	<i>Eutrema salsugineum</i>	+1.29
216	Isovaleryl-CoA-dehydrogenase precursor	gi 5596622	<i>Arabidopsis thaliana</i>	+1.96
248	S-Adenosylmethionine synthase	gi 1709006 gi 558695842 gi 543176428 gi 558695842	<i>Actinidia chinensis</i> <i>Phaseolus vulgaris</i> <i>Phaseolus vulgaris</i> <i>Phaseolus vulgaris</i>	−1.42
284	MOPEW79TF (ribulose 1.5-bisphosphate carboxylase/oxygenase large subunit)	gi 182658412 ^{d)}	<i>Vigna unguiculata</i>	−2.70
308	MOPF213TF (pyruvate dehydrogenase E1 component beta subunit)	gi 182660042 ^{d)}	<i>Vigna unguiculata</i>	−3.32
313	Hypothetical protein PHAVU_010G163900g (vacuolar H ⁺ -ATPase) Protein SET-5, isoform d	gi 593267358 gi 453232574	<i>Phaseolus vulgaris</i> <i>Caenorhabditis elegans</i>	−2.19
351	Hypothetical protein PHAVU_011G084400g (Glyoxalase_I)	gi 593194743	<i>Phaseolus vulgaris</i>	−4.31
352	Multicatalytic endopeptidase complex. proteasome component (alpha subunit) Protein PAS-1	gi 2511588 gi 17562788	<i>Arabidopsis thaliana</i> <i>Caenorhabditis elegans</i>	+7.63
395	UCRVU05_CCNN15860_b1 (F0F1 ATP synthase subunit beta)	gi 190451397 ^{d)}	<i>Vigna unguiculata</i>	+3.20
400	Hypothetical protein PHAVU_007G034800g (ATP synthase subunit beta)	gi 593627323	<i>Phaseolus vulgaris</i>	+7.03
401	UCRVU09_CCNT13871_b1 (ribulose 1.5-bisphosphate carboxylase/oxygenase large subunit)	gi 190556563 ^{d)}	<i>Vigna unguiculata</i>	−5.28
402	Glutathione transport system permease protein GsiC-like (partial)	gi 449468157	<i>Cucumis sativus</i>	+5.59
419	Hypothetical protein PHAVU_010G011300g (NAD (P) binding site)	gi 593263658	<i>Phaseolus vulgaris</i>	+2.81
420	UCRVU08_CCNS9838_b1 (Chaperonin 10)	gi 190521803 ^{d)}	<i>Phaseolus vulgaris</i>	−6.38
431	Glutathione S-transferase F9-like Hypothetical protein PHAVU_006G020800g (glutathione S-transferase)	gi 356526968 gi 593691257	<i>Glycine max</i> <i>Phaseolus vulgaris</i>	Unique to CE 109
432	Chalcone isomerase (fragment)	gi 81864	<i>Phaseolus vulgaris</i>	Unique to CE 109
436	Unknown (proteasome-subunit alpha) Hypothetical protein PHAVU_004G073800g (proteasome subunit alpha) Protein LNP-1	gi 255648341 gi 593702733 gi 17550174	<i>Glycine max</i> <i>Phaseolus vulgaris</i> <i>Caenorhabditis elegans</i>	Unique to CE 109
438	UCRVU08_CCNS4917_b1 (cytosolic ascorbate peroxidase)	gi 190516707 ^{d)}	<i>Vigna unguiculata</i>	Unique to CE 109
506	Hypothetical protein PHAVU_011G053800g (Fructokinase)	gi 593154227 gi 585973	<i>Phaseolus vulgaris</i> <i>Solanum tuberosum</i>	Unique to CE 109
508	UCRVU05_CCNN15860_b1 (F0F1 ATP synthase subunit beta)	gi 190451397 ^{d)}	<i>Vigna unguiculata</i>	Unique to CE 31
512	Unknown (chorismate mutase)	gi 255640032	<i>Glycine max</i>	Unique to CE 31

Table 1. Continued

Spot number	Protein identification ^{a)}	Accession (NCBIInr) ^{b)}	Organism	Fold change in CE 31 related to CE 109 ^{c)}
513	UCRVU04_CCNI1299_b1 (Glutathione S-transferase)	gi 190463341 ^{d)}	<i>Phaseolus vulgaris</i>	Unique to CE 31
514	UCRVU09_CCNT13871_b1(ribulose 1.5-bisphosphate carboxylase/oxygenase large subunit)	gi 190556563 ^{d)}	<i>Vigna unguiculata</i>	Unique to CE 31
515	UCRVU04_CCNI1065_b1 (protein disulfide isomerase)	gi 190415885 ^{d)}	<i>Vigna unguiculata</i>	Unique to CE 31
516	Nitrile-specifier protein 5	gi 356525790	<i>Glycine max</i>	Unique to CE 31
517	Spermidine synthase	gi 6094335	<i>Coffea arabica</i>	Unique to CE 31
518	Os11g0652100/hypothetical protein (putative NB-ARC domain-containing protein)	gi 115486473	<i>Oryza sativa Japonica</i>	Unique to CE 31
520	Hydroxyacid oxidase 1 Protein SWSN-1, isoform a	gi 226500726 gi 392923158	<i>Zea mays</i> <i>Caenorhabditis elegans</i>	Unique to CE 31
521	6-Phosphogluconate dehydrogenase. decarboxylating-like	gi 449447452	<i>Cucumis sativus</i>	Unique to CE 31
524	UCRVU08_CCNS1576_b1 (F0F1 ATP synthase subunit beta)	gi 190511123 ^{d)}	<i>Vigna unguiculata</i>	Unique to CE 31

a) Protein identification by MASCOT. Using database of plant ESTs.

b) More than one accession cited indicates different hits for peptides identified from the same protein.

c) The “+” and “-” signals indicate increased and decreased proteins, respectively.

d) Protein Identification by LC-MS, using ESTs database (*Vigna unguiculata*).

Proteins potentially involved in hypersensitive response (HR) were also identified in this study with increased expression in inoculated CE 31 (Fig. 2; Table 1), such as hydroxyacid oxidase (or glycolate oxidase), which is a peroxisomal enzyme that participates in the generation of ROS and is involved in the oxidative stress signal transduction cascade in response to pathogen attack [39]. It has been demonstrated that glycolate oxidase is an alternative source for the production of H₂O₂ and plays a main role in both gene-for-gene-mediated and nonhost resistance responses [40]. Similarly, aldo-keto reductase is also involved in ROS signaling network by detoxifying stress-associated reactive carbonyl products that mitigate the effects of ROS, preventing oxidative damage [41]. The qRT-PCR expression profiles of hydroxyacid oxidase and aldo-keto reductase showed a gradual increase in transcript levels in the days subsequent to nematode infection (Fig. 3). Therefore, the induction of hydroxyacid oxidase and aldo-keto reductase in response to oxidative stress triggered by the RKN inoculation could be associated with the transition between defense responses and basic metabolism to restore homeostasis and return to normal metabolic conditions as a mechanism of resistance in CE 31 cultivar.

Another identified protein, possibly involved in HR, was a protein that belongs to the gamma-type carbonic anhydrase family (Table 1), formed by five enzymes displayed in the plant mitochondrial Complex I [42]. Carbonic anhydrase has been associated to resistance in the interaction between

Brassica carinata and the fungal pathogen *Leptosphaeria maculans* [43]. Moreover, it has been demonstrated that the silencing of this gene in tomato resulted in suppression of pto:avrpto-mediated HR [44]. In cowpea CE 31, carbonic anhydrase is positively regulated in response to nematode infection (Fig. 2) and could also be related to its resistance response.

Plants, when infected by nematodes, present loss of vigor, leaf chlorosis, general compromised development, among other symptoms. Consequently, the expression of the proteins involved in these processes is also affected (reviewed in [45]). In this study, we have identified proteins in CE 31 associated to plant growth and development that may have an important role in resistance, including spermidine synthase and nitrile-specifier protein 5, unique to CE 31 and patatin 17-like, increased in CE 31 (Table 1).

Hewezi et al. [46] showed that spermidine synthase 2 is a target of a cyst nematode effector that inhibits plant defense responses. It has also been reported that the overexpression of spermidine synthase induces the expression of stress-related genes, such as the transcriptional factor DREB, associated with increased tolerance to environmental stresses [47]. Cowpea spermidine synthase revealed to be unique in CE 31 and its transcript levels determined by qRT-PCR displayed a gradual increase in the days subsequent to nematode infection (Fig. 3), with a maximum level of gene expression (4.85-fold) at 9 DAI.

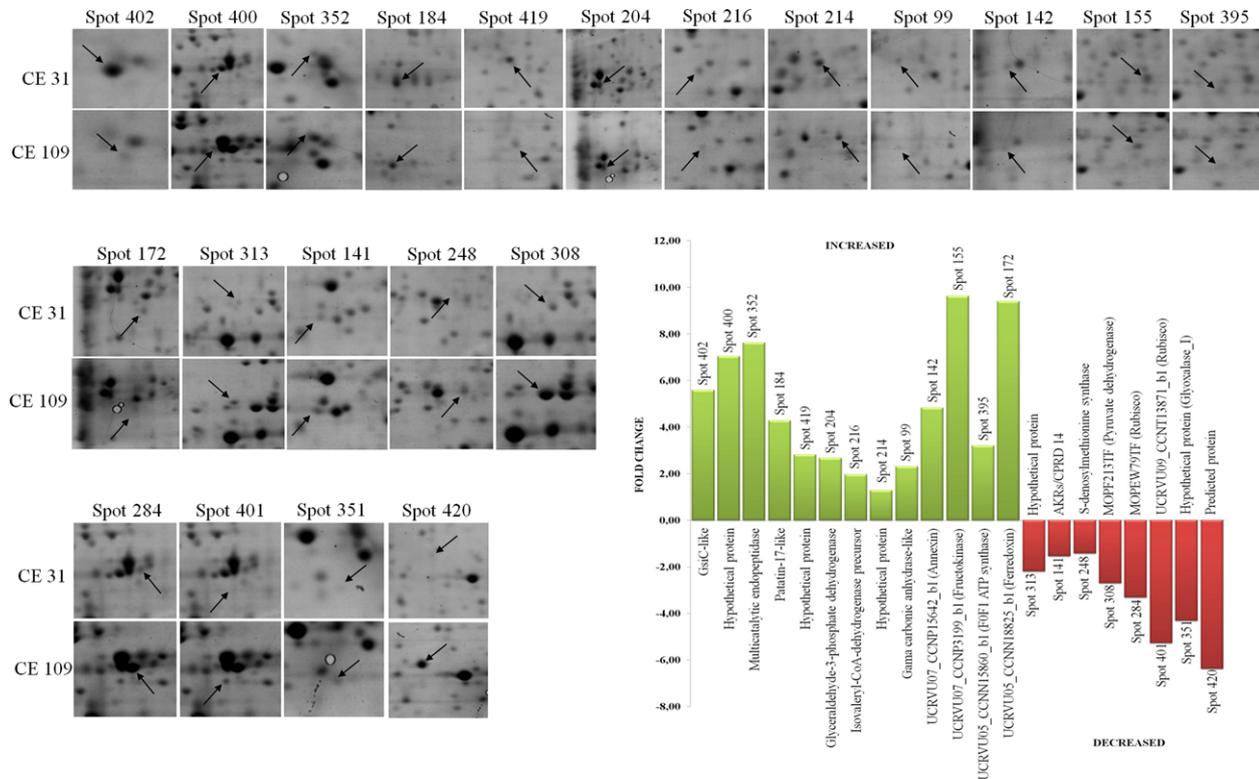


Figure 2. Overview of increased and decreased proteins in the highly resistant cowpea cultivar CE 31. Arrows indicate the protein spots identified. The graph represents the fold change of the differential expression of these proteins in CE 31 when compared to CE 109.

Nitrile specifier was another protein identified in CE 31, involved in resistance, which had not been yet reported in cowpea–nematode interaction studies. This protein is involved in the hydrolysis of glucosinolates, secondary metabolites present in Brassicaceae plants. It has been suggested that intact glucosinolates are biologically inactive, while the products of its degradation after hydrolysis may have multiple functions in the regulation of growth and plant defense [48]. Therefore, the identification of a nitrile-specifier protein in CE 31 may indicate activation of the plant secondary metabolite pathways as a defense mechanism against *M. incognita*. Notably, nitrile-specifier protein showed the highest transcriptional induction in response to the nematode infection in the qRT-PCR analysis, in particular at 9 DAI (26.52-fold; Fig. 3). Nitrile-specifier protein was here identified as unique in the highly resistant CE 31 cultivar (Table 1), which is in accordance with its strong gene expression induction at 9 DAI.

As nitrile-specifier protein, isopropylmalate dehydrogenase is also involved in the glucosinolate pathway and these proteins are probably under the control of the same regulatory system. Although not detected in our proteome analysis, isopropylmalate dehydrogenase transcripts showed a high induction in its expression (19.53-fold; Fig. 3) in response to the nematode infection. These results could indicate that the expression of nitrile-specifier protein and isopropylmalate dehydrogenase genes are simultaneously triggered by the

nematode infection and lead to the activation of glucosinolate metabolism to circumvent pathogen attack.

Another protein increased in CE 31 was patatin (Fig. 2; Table 1), which is a storage glycoprotein that has lipid acyl hydrolase activity and is also associated with defense against plant pests [49, 50]. Camera et al. [51] showed that a patatin-like protein 2 is an important component of the plant defense machinery to combat the cucumber mosaic virus. Regarding the patatin transcripts, a modest increase was observed along the bioassay time course, beginning by a downregulation response to nematode attack at 3 DAI and reaching a maximum level of expression at 9 DAI (Fig. 3). The results showed that both transcription and protein profiles of patatin were similar, however, the magnitude of fold change was smaller in the qRT-PCR analysis (from -1.67 to $+2.22$; Fig. 3) when compared to the proteome results ($+4.28$; Table 1).

Interestingly, we also identified the resistance protein RPP13, which is a member of the NBS-LRR family of plant R-genes and confers resistance to the causal agent of downy mildew in *A. thaliana* [52]. This protein was unique to CE 31 and may play a crucial role in resistance. The transcription profile of RPP13 obtained by qRT-PCR revealed an increasing upregulation from 3 to 9 DAI. These results are compatible with its expected role in the initiation of a complex defense response to pathogen infection often leading to host immune response.

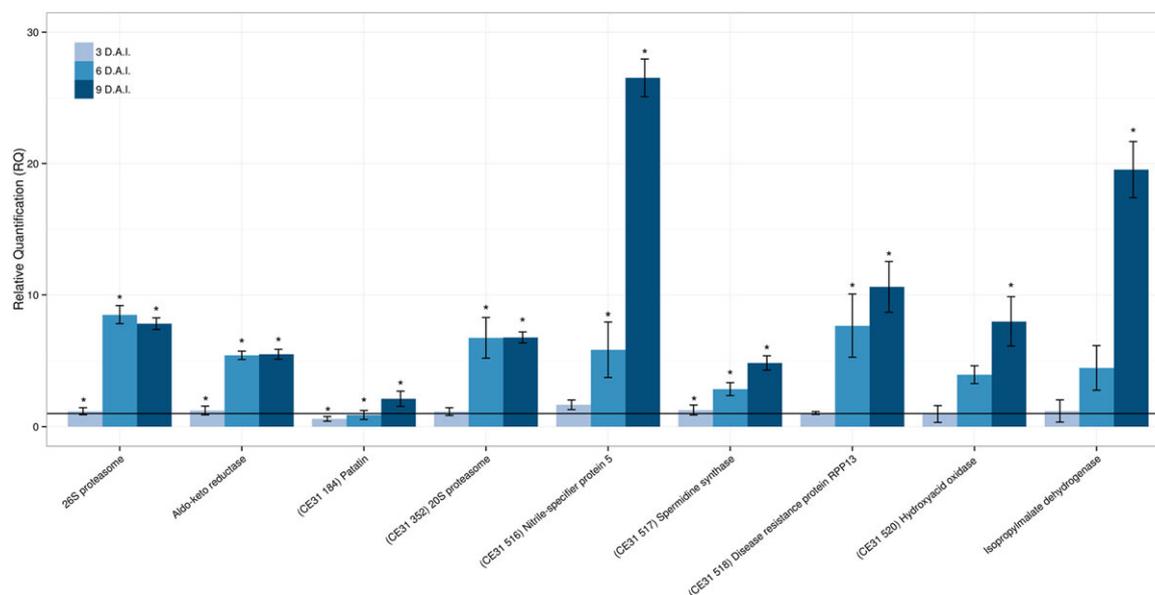


Figure 3. Relative quantification (RQ) of mRNA levels of nine candidate genes differentially expressed in cowpea roots challenged with *M. incognita* at 3, 6, and 9 days after inoculation (DAI), relative to day 0. Bars represent the standard error of the mean of two biological replicates for each sample and the horizontal line indicates RQ = 1.0. Significantly ($p \leq 0.05$) up- or downregulated genes are indicated by asterisk (*).

Overall, the results obtained by proteome and transcriptome approaches give strong evidence that oxidative stress is part of the resistance mechanisms of CE 31 cultivar. Similar results were recently obtained by Oliveira et al. [18] using the same cultivar CE 31 in the comparison between *M. incognita* inoculated and noninoculated plants. These authors observed an increase in several oxidative stress proteins in inoculated plants such as superoxide dismutase and peroxidase. In the current study, we have identified additional processes implicated in plant resistance and programmed cell death including ubiquitination and hydrolysis of glucosinolates, which may be part of the complex resistance mechanism of CE 31 cultivar to combat nematode infection.

3.2 Proteins decreased in the highly resistant cultivar CE 31

Some proteins showed a negative regulation after *M. incognita* inoculation in the highly resistant cultivar CE 31, such as the stress-related proteins glyoxalase and chaperonin 10 (Table 1). We also observed two differential protein spots with decreased expression in CE 31 (spot 284 and spot 401; Fig. 2; Table 1) corresponding to Rubisco large subunit (LSU). Despite the fact that the partial hydrolysis of Rubisco LSU has been associated to oxidative stress in barley chloroplasts [53], the expression of Rubisco subunit in roots is still intriguing. Reports have already described the presence of photosynthesis-related genes/proteins in nonphotosynthetic tissues [54] however, additional studies need to be performed in order to further investigate the expression of Rubisco in cowpea roots. It

is possible that the oxidative stress induced in the resistant response lead to the decreased intensity of LSU in CE 31. Another spot (514) identified as Rubisco LSU was unique to CE 31 (Fig. 1; Table 1); however, the molecular mass was lower than the theoretical mass (27 kDa instead of 52 kDa; Supporting Information Table 3), indicating some degradation or processing of this protein.

Some unique proteins were also identified in the slightly resistant cultivar CE 109 such as cytosolic ascorbate peroxidase (Fig. 1; Table 1). These results contrast those obtained by Oliveira et al. [18] that observed an increase in ascorbate peroxidase in CE 31 inoculated with *M. incognita* when compared to noninoculated plants. Contrasting results were also obtained for chalcone synthase, a key enzyme of the flavonoid/isoflavonoid biosynthesis pathway, which was also identified in this study as unique in CE 109. Oliveira et al. [18] again observed an increase in this protein in CE 31 inoculated with *M. incognita*. Different sampling points were analyzed in both studies (4 and 6 DAI vs. 3–9 DAI) and may have influenced the levels of these proteins, which seem to be highly variable and dependent on the infection process. It is possible that at 9 DAI these proteins are no longer needed to cope with *M. incognita* infection in CE 31, since other resistance processes are activated, such as ubiquitination and glucosinolate hydrolysis.

Two different protein spots, one unique to CE 31 and the other unique to CE 109, were identified as glutathione *S*-transferase, which is a major cellular detoxification enzyme and plays important roles in plant protection against biotic and abiotic stresses [55]. It is possible that these different spots represent isoforms or PTMs and their distinct

expression may result in different responses to *M. incognita* infection.

Unlike the highly resistant cultivar CE 31, it seems that CE 109 showed a basal defense response by triggering the increase in general stress-related proteins such as peroxidase, glyoxalase, and chaperonin. In order to determine resistance, signaling processes of defense and the chain of events that occur in the host plants upon the pathogen perception must be immediate to allow the induction of early or long-lasting resistance mechanisms, which may not have occurred in CE 109. Flavonoids represent a class of secondary metabolites with diverse functions in plant development and response to stresses, including wounding and pathogen attack [56]. It seems that oxidative stress, as well as stress and wounding response are prevalent in CE 109 and that more sophisticated resistance mechanisms, such as those presented by CE 31, need to be activated in order to cope with *M. incognita* infection.

3.3 Prediction of *M. incognita*–cowpea interactome

The investigation of the interactions among proteins potentially involved in the response to nematode infection can provide new insights for pathogen control. As cowpea genetic and genomic resources are still limited, the gold-standard gene product annotation of the model plant *A. thaliana* was used to predict the interactome of the differentially expressed proteins identified between the two cowpea cultivars (Fig. 4; Supporting Information Table 4). Out of the 37 proteins identified in this study, 35 showed homology to *A. thaliana* proteins. Two proteins were identified in multiple spots (spots 284, 401, 514 and 395, 400, 508; Table 1 and Supporting Information Table 4) and hence were considered only once. Two proteins did not show orthologs in *A. thaliana* and were therefore not included in the interactome analysis. The interactome was therefore performed with 31 different proteins: 20 for CE 31 and 12 for CE 109.

The results obtained using the String program showed that for the highly resistant CE 31 cultivar, ten out of 20 proteins interact with at least one protein (Fig. 4) based on evidence from co-expression, experiments, and text-mining information. On the other hand, for the slightly resistant cultivar CE 109, only a few direct interaction between differentially or uniquely expressed proteins was predicted (Fig. 4). However, when up to 20 protein interactions were added, it was possible to visualize two protein interaction clusters for CE 31 and three for CE 109 (Fig. 4). In order to identify possible metabolic pathways, a Kegg enrichment analysis was performed and revealed that proteasome (Supporting Information Fig. 1) and oxidative phosphorylation pathways could be activated in both cultivars while flavonoid biosynthesis pathway (Supporting Information Fig. 2) could be activated only in CE 109. As evidenced here in protein and gene expression analyses, these pathways seem to be important processes in response to *M. incognita* attack in cowpea cultivars.

An additional network based on mRNA co-expression evidences was built using GeneMania (Fig. 4), which revealed a higher number of protein co-expression when compared to the results obtained using the String program. The GeneMania results suggest that most of the differentially expressed proteins (14 out of 20 proteins in CE 31 and nine out of 12 in CE 109) co-express with at least one product during biotic stresses. Although the results are based on a predictive interaction, the data obtained show that most proteins identified in the present study are part of a network activated during plant–pest interaction. At present, experimental evidence on protein–protein interaction is highly limited, even in the well-studied *A. thaliana* [57], which hinders a more profound comprehension on the interactome. Further studies need to be conducted in order to confirm the interactions predicted for cowpea upon *M. incognita* infection.

Based on gene ontology classification, noteworthy is the fact that ten of the 31 differentially expressed protein spots (Supporting Information Table 4) were associated with response to cadmium ion (GO:0046686), most of which (seven proteins) were increased or unique in CE 31 (Fig. 4, red-colored nodes). This GO term was by far the most common term among the homologous proteins. Plants can be classified as hyperaccumulators and nonhyperaccumulators in accordance with the unusual metal element concentration in their aboveground tissues. There are evidences suggesting that hyperaccumulated ions could confer tolerance to abiotic stress, as drought, and resistance to biotic stress, as nematodes. The “defense hypothesis,” defined by Boyd and Martens [58] proposes, on a molecular basis, that accumulated ions could induce common or complementary pathways to enhance the plant defense response against insect pests and pathogens. The crosstalk involving metal accumulation/homeostasis and biotic stresses signaling pathways was reviewed by Poschenrieder et al. [59]. Few studies have associated the presence of metals and plant resistance to fungus or bacteria, but not yet to nematodes [60–62]. There is no evidence that *V. unguiculata* is a cadmium hyperaccumulator, but recent studies indicated that this legume can be considered as a phytoremediator plant in zinc contaminant soils [63].

4 Concluding remarks

In this study, we have investigated the proteins expressed during early stages of *M. incognita* infection in highly and slightly resistant cowpea cultivars and have found several proteins differentially expressed. Regarding the global pattern of protein expression upon *M. incognita* infection, we observed that the majority of the 37 identified proteins are unique (11) or increased (13) in CE 31 (Fig. 2 and Table 1). These results seem to be in contrast with previous studies that reported a trend toward a downregulation of genes and partial suppression of typical defense response in incompatible cowpea–RKN interaction when compared to the compatible

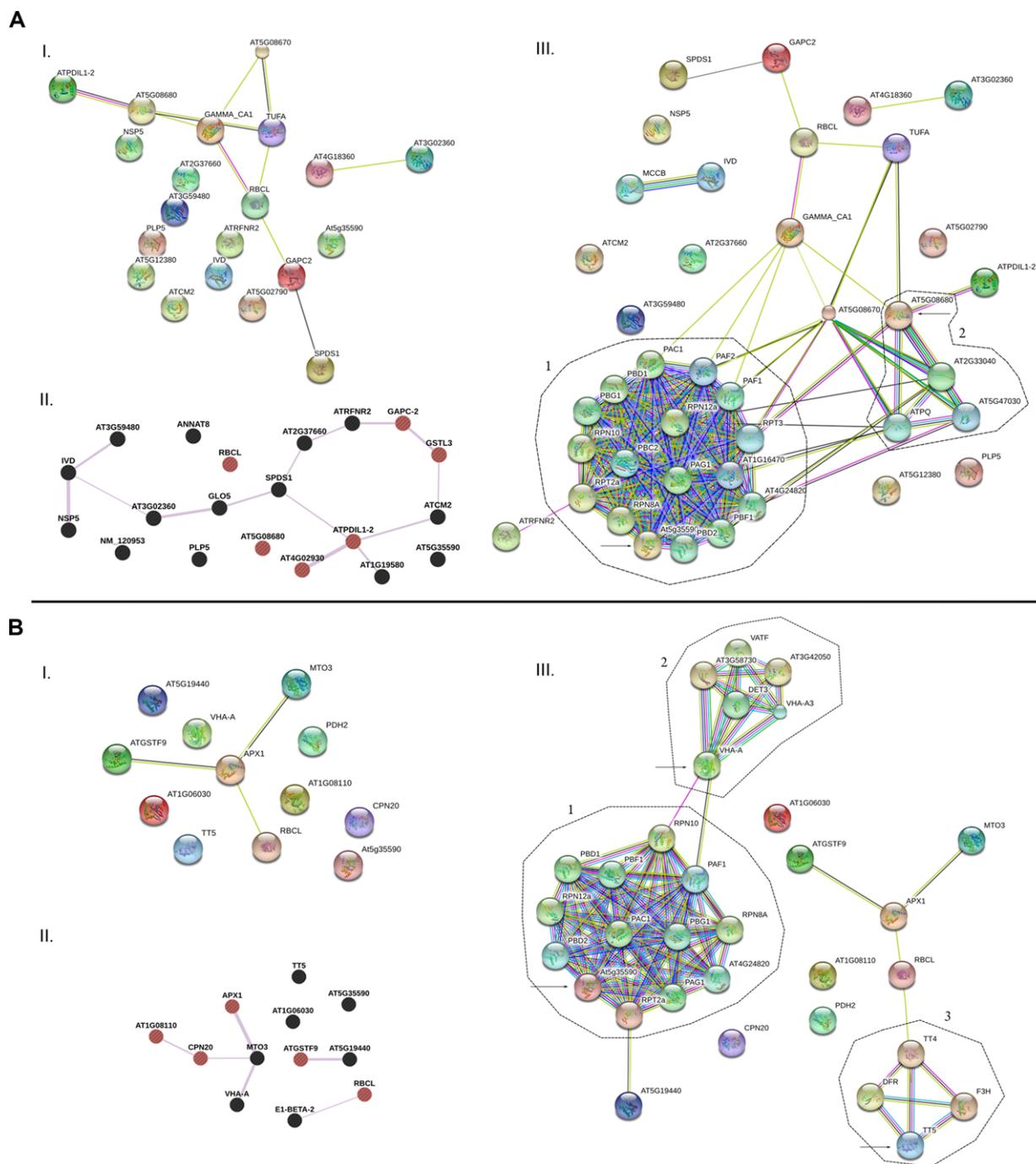


Figure 4. Interactome prediction using *A. thaliana* homologous genes. (A) Highly resistant CE 31 cultivar. (B) Slightly resistant CE 109 cultivar. Networks showing interactions among proteins belonging to our dataset were predicted using String v. 9.1 (I and III) and GeneMania (II). The type of evidence is as follows: dark green line—neighborhood; red line—gene fusion; dark blue line—co-occurrence; black line—co-expression; pink line—experiments; light blue—databases; light green—text mining. Dotted lines indicate predicted Kegg pathways: 1—proteasome; 2—oxidative phosphorylation; 3—flavonoid biosynthesis. Arrows indicate proteins belonging to our dataset that participate in the predicted Kegg pathways. In figures AII and BII, proteins associated with response to cadmium ion (GO:0046686) are indicated in red.

interaction [64]. However, the majority of these studies analyzed differential expression profiles only at the transcript level. Therefore, one of the main advantages of proteomic global profiling is that the final product of gene expression is detected, showing that the protein is indeed produced to play its biological role. In this study, we identified several proteins increased or unique to highly resistant CE 31 that could be involved in nematode defense and resistance mechanisms. Interestingly, qRT-PCR analyses showed that the transcripts and proteins had a very similar upregulation trend in CE 31, including the LRR resistance gene. These data are in accordance with previous reports showing that upregulated transcripts usually result in increased abundance of proteins [65]. For example, the majority of the analyzed transcripts corresponding to upregulated proteins showed basal gene expression levels at the beginning of nematode infection (3 DAI) followed by enhanced transcription levels as nematode infection progressed (6–9 DAI). As observed in the proteome and transcriptome analysis, the interactome prediction also revealed that oxidative stress is crucial for resistance, since oxidative phosphorylation was affected by nematode infection. Additionally, the interactome prediction revealed a potential role of several proteins in metal resistance, which might also be associated to resistance to biotic stresses. Further studies need to be performed in order to confirm this hypothesis.

Overall, this study showed that each approach used (proteomics, relative quantification of transcripts, and interactome predictions) provided additional contributions to the understanding of the cowpea–nematode interaction. Taken together, these results indicate that some proteins with a putative role in RKN resistance (such as nitrile-specifier protein, RPP13, and spermidine synthase) showing similar transcription and protein expression behavior could be good candidates for engineering nematode resistance in agriculturally important crops. It seems that oxidative stress, ubiquitination, and glucosinolate degradation are part of cowpea CE 31 resistance mechanisms in response to nematode infection.

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