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RESISTANCE OF TOMATO AGAINST THE ROOT KNOT NEMATODE, *MELOIDOGYNE INCOGNITA*, IS ENHANCED BY SOIL DRENCH APPLICATION WITH SALICYLIC ACID

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Abstract

Root knot nematode (RKN) is one of the most economically important pests worldwide. It is important to determine the key regulatory genetic elements of plant resistance against nematodes. We evaluated the effect of the plant hormone salicylic acid (SA) application as soil drench on resistant (cv. Beef master) and susceptible (cv. Rutgers) tomato cultivars. The soluble proteins of the shoots and the expression of three pathogenesis-related (*PR*) genes (*PR2*, *PR3*, and *PR9*) in roots and shoots were measured by SDS-PAGE and by Real Time-Polymerase Chain Reaction (RT-PCR), respectively, to track the systemic acquired resistant (SAR) in both cultivars. Three unique protein bands were present in the resistant cultivar, which may represent new candidate nematode-resistance proteins. In addition, another band at ~50 kDa was present in the control sample of the resistant plants, disappeared in the infected resistant plants and appeared in the infected resistant plants treated with SA prior to nematode infection. This ~ 50-kDa band may be one of the plant pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) proteins suppressed by nematode effectors. The expression of *PR* genes increased in the roots and shoots in response to SA treatment. These bands could represent new proteins as candidate defense proteins that contribute to nematode resistance in the resistant tomato cultivar. Moreover, SA application increased the expression of *PR* genes in both roots and shoots, with higher expression in the roots compared to the shoots, and in resistant cultivar compared with susceptible cultivar. The results reported here indicated that resistance to nematode may be due to the function of at least the three genes examined, where PR2 and PR3 break down or weaken the cell wall of the invading nematode, and PR9 enhances the hypersensitivity of the infected plant cells. Therefore, PR2, PR3 and PR9 collectively enhanced plant resistance to the root knot nematode. Therefore, SA application may be used as an environmentally safe approach towards effective management strategy against RKN in tomato.

Keywords : *Meloidogyne incognita*, Pathogenesis-Related (PR) Proteins, Real Time-Polymerase Chain Reaction (RT-PCR), Salicylic Acid (SA), SDS-PAGE, Tomato (*Solanum lycopersicum*).

Introduction

The world's food supply poses a major challenge due to the high and rapid growth rate of the world population in recent decades, and due to pathogens and plant pests (Savary *et al.*, 2019). Plant nematodes are of the economically most important pathogens worldwide. The crop losses resulting from nematode infections worldwide were about 157 billion dollars annually (Williamson and Kumar, 2006; Abad *et al*., 2008), with the root knot nematodes (RKN), *Meloidogyne* spp., being among the species causing high crop losses (Jones *et al*., 2013). Nematodes existed for about one billion years. They are members of the phylum Nematoda (Lambert and Bekal, 2002). Among the *Meloidogyne* spp., *Meloidogyne incognita* is known to exhibit a broad host range and high reproduction rate (Abad and Williamson, 2010; Jones *et al*., 2011). Five *Meloidogyne sp*., most importantly *M. incognita*, can infest tomato, *Solanum lycopersicum,* (Khanzada *et al*., 2012; Onkendi *et al.*, 2014) which is cultivated worldwide (Arie *et al*., 2007), with 80 billion dollars annual losses caused by nematodes (Nicol *et al*., 2011). Due to the world's climate change, the nematode life cycle may be accelerated leading to increase in nematode population. In addition, the host plant physiology may change in response to heat or drought stress, allowing increased infestation (Somasekhar and Prasad, 2012). Thus, there is an increasing interest worldwide in studying the relationship between RKN and their host plants (Cabrera *et al*., 2015).

Upon nematode infection, the recognition of nematodes at the extracellular plant cell membrane activates the plant innate immune system. Nematode infection activates programmed cell death to prevent the spread of nematodes (Williams and Dickman, 2008). Also, a variety of receptors activate additional signal transduction pathways leading to several chemical defense responses, such as reactive oxygen species (ROS), plant-elicitor peptides (Peps), and plant growth regulators biosynthesis, e.g. of salicylic acid (SA) (Goverse and Smant, 2014). SA plays a role in accumulating H_2O_2 and other ROS that lead to hypersensitivity of the infected host cells (Shirasu *et al.*, 1997; Mur *et al*., 2006). SA fine-tunes ROS scavenging enzymes such as peroxidases to regulate ROS levels, and in turn regulates plant defenses against different pathogen attacks (Torres *et al.*, 2006). Rice peroxidase is responsible for the resistance of rice to *Xanthomonas oryzae* pv. *oryzae* (Chittoor *et al.*, 1997). *Phaseolus vulgaris* L. plants treated with SA showed increased levels of peroxidases after infection with the white clover mosaic potex virus (WClMV) (Clarke *et al*., 2002).

Also, infection with nematodes activates the expression of defense-related genes encoding proteins that include enzymes and/or pathogenesis-related (PR) proteins that activate other defense pathways (Dixon *et al*., 1994). Thus, total soluble protein profiles may provide indications of the changes that take place in plants, such as tomato, in response to *M. incognita* infection, with prior application of SA in resistant and susceptible tomato cultivars. In particular, resistant tomato cultivars may show a different protein profile appropriate for an effective response to the nematode threat, while a susceptible cultivar would be incapable of providing such response. There are 17 identified PR protein

families, most of which are controlled by plant phytohormones such as SA, jasmonic acid and ethylene (van Loon *et al.*, 2006). Five *PR* families (*PR1-PR5*) were first identified in tobacco (Bol *et al.*, 1990). *PR6* and *PR7*, encoding proteinase inhibitors and endo-proteinases, respectively, were first identified in tomato (Green and Ryan, 1972; Vera and Conejero, 1988). Some PR proteins show a defense response against abiotic in addition to biotic stresses (Cruz *et al*., 1992; Wu *et al*., 2016). Examples of *PR* genes with a significant effect on tomato resistance against nematodes are; 1) the *PR2* gene family encoding the enzyme β-1,3-endoglucanase; 2) the *PR3* gene family encoding a chitinase enzyme that may have a significant role in the defense response of plants to pathogens (chitin is an essential component of nematode eggshell and pharynx); and 3) the *PR9* gene family encoding a peroxidase enzyme (Van Loon *et al.*, 2006; and Andolfo *et al*., 2014). According to NCBI, tomato *PR2* is a 1250 base pairs gene consisting of two exons (NM_001247229). It encodes a 263 amino acid-long protein (NP_001266258). The *PR3* gene of tomato is a 989 base pairs gene consisting of three exons (NM_001279329) and encodes a 344 amino acids protein (NP_001234158.2). *PR9* is 1111 base pairs in the coding region (XM_004233394) and encodes 326 amino acids (XP_004233442). β-1,3-endoglucanases (PR2 proteins) are among the most important PR proteins that confer resistance to nematodes at the early stages of nematode infection. *Arabidopsis thaliana* mutant lines that were not able to express *PR2* were more susceptible to the cyst nematode *Heterodera schachtii*, while overexpressing lines of *PR2* were less susceptible to the same cyst nematode (Hamamouch *et al*., 2012). The chitinase *PR3* is another important defense enzyme. The level of the *PR3* enzyme is increased in potato plants after infection with *Globodera pallida* (Rahimi *et al*., 1996). In addition, expression of class III chitinase genes increased in sugar cane in response to the fungus *Sporisorium scitamineum* (Su *et al*., 2014). PR9 peroxidases are usually stimulated in the plant to constrain the pathogen at the infection site by causing necrosis of the infected cells because of the hyper-sensitive response (HR) (Dietrich *et al*., 2004).

Different PR families may be co-expressed in response to pathogen infection in order to maximize the effectiveness of the defense response. For example, the peroxidase PR9 and the chitinase PR3 are co-expressed in sugar cane in response to the fungal pathogen *Colletotrichum falcatum* (Ramesh Sundar *et al*., 2008), Chinese chili in response to pepper mild mottle virus infection (Elvira *et al.*, 2008), wheat in response to the leaf rust pathogen *Puccinia triticina* pv. UVPt9 (Cawood *et al*., 2010), and tomato in response to the early blight *Alternaria solani* (Salim *et al*., 2011).

Systemic acquired resistance (SAR) is the phenomenon of priming systemic plant tissues after a local infection towards a more effective response against secondary infections (Conrath, 2006). SAR is associated with increased expression and faster induction of *PR* genes, increased levels of SA and the active compound Pipecholic acid, priming of defense-related genes, and enhanced ROS burst, callose deposition and calcium influx in systemic leaves, leading to increased resistance against infections by a multitude of pathogens. Irrigation of plants with SA or Pipecholic acid induces SAR as well, leading to increased resistance in *Arabidopsis* (Bernsdorff *et al.*, 2016; Návarová *et al.*, 2012). Application of SA via soil drench primed tomato against *M. incognita* resulting in increased resistance against subsequent infections (Molinari and Baser, 2010).

The expression of the β-1,3-endoglucanase-encoding gene *PR2*, the chitinase-encoding gene *PR3* and the peroxidase-encoding gene *PR9* are all induced by salicylic acid in the context of SAR, thus improving plant defense against pathogen infection (Coqueiro *et al.*, 2015; Falcioni *et al.*, 2014). Thus, these three genes are directly, and co-involved in the resistance to plant nematodes such as *M. incognita.* The aim of this study was to examine the effect of SA application on enhancing tomato resistance against nematode infection and the response of resistant and susceptible tomato cultivars (Beef master and Rutgers, respectively) to nematode infection. The total soluble proteins and the differential expression of the three PR genes, PR2, PR3, and PR9 were utilized to quantify the response of bot vultivars to nematodeinfection and SA application prior to infection.

Materials and Methods

Plant materials

Seeds of the nematode-resistant tomato cultivar Beef master (Jaiteh *et al.*, 2012) and the susceptible tomato cultivar Rutgers (Melakeberhan, 1998) were purchased from Burpee Seed Co. (Pennsylvania, USA) and Baker Creek Heirloom Seeds Rareseeds Co. (Mansfield, USA), respectively. The seedlings were germinated in peat moss in the greenhouse (23-25 °C, 14 h:10 h light/dark cycle) at Faculty of Agriculture, Cairo University, Giza, Egypt, then transferred into sand/soil (1:1) filled pots (1.5 liter-15 cm) and cultivated in the greenhouse under the same conditions.

Nematode cultivation

M. incognita was reared on susceptible eggplants for three months at the Nematology branch, Zoology Department, Faculty of Agriculture, Cairo University, Egypt. At the infection time *M. incognita* larvae were harvested from infected eggplant roots, counted under the light microscope (4x lens, Zeiss, Jena, Germany). Approxmately, 3,000 larvae were used to infect each plant(Yang *et al.*, 2016).

Preparation of SA soil drench solution

A fresh aqueous solution of potassium salicylate (Alpha Chemika, India) was prepared one day prior to drenching the soil with a concentration of 150 ppm/g of soil, i.e. about 22 mg of SA/plant (Molinari and Baser, 2010). Control plants (uninfected, untreated), and nematode infected plants (without SA treatment) were drenched with the same amount of water. One day after drenching, SA soil-drenched plants were infected with 3,000 larvae each. Three days after nematode infection, root and shoot samples were collected for further analysis.

Experimental design and sampling

Four weeks-old plants were assembled into three groups. The first group was the untreated controls, the second group was nematode-infected (no SA drenching), and the third group was SA-treated one day prior to nematode infection. The seedlings were divided into three treatments and two genotypes (six samples): 1) control susceptible plants, 2) control resistant plants, 3) susceptible plants infected with nematodes, 4) resistant plants infected with nematodes, 5) susceptible plants pre-treated with SA and

infected with nematodes, 6) resistant plants infected pre-treated with SA and with nematodes. Each treatment was represented in three replicates. Three days after nematode infection, plant leaves and roots were collected separately submerged in liquid nitrogen, and stored at -80 ºC until further analysis.

RNA extraction and cDNA synthesis

Total RNA was isolated from leaves of tomato plants using the RNeasy Plant Mini Kit (QIAGEN, Germany). Leaf samples (50 mg) were ground to fine powder in liquid nitrogen. Ground plant tissues were homogenized in 600 µl lysis buffer containing β-Mercaptoethanol. RNA isolation was performed according to the manufacturers' instructions. The purity of total RNA was examined by the 260/280 nm ratio (at least 2.0 for pure RNA) using Nanodrop (Thermo Fisher Scientific Waltham, USA). RNA integrity was examined using ethidium bromide-stained agarose gel electrophoresis to visualize the 28S RNA and 18S RNA bands. Aliquots of total RNA were used immediately for reverse transcription (RT) or otherwise stored at -80 °C.

The complete Poly (A)+ RNA isolated from plant tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) using 5 µg of total RNA. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was terminated by heating for 5 min at 99 °C. cDNA was used for real time-polymerase chain reaction (RT-PCR) or stored at -20 °C.

Real Time-Polymerase Chain Reaction (RT-PCR)

The StepOne™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine relative transcript abundance. PCR reactions were set up in 25 µl reaction mixtures containing 12.5 µl of 1× SYBR® Premix Ex Taq^{TM} (TaKaRa Biotech, Japan), 0.5 µl of 0.2 µM sense primer, 0.5 µl of 0.2 µM antisense primer, 6.5 µl distilled water and 5 µl of cDNA template. Each experiment included a distilled water control. The reaction program consisted of the following profile: Initial denaturation at 95°C for 3 min; 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. Then, 71 cycles of 10 s with incremental increase of temperature by 0.5 °C from 60.0 °C to 95 °C were run. At the end of each qRT-PCR, a melting curve analysis was performed at 95 °C to analyze the primer efficiency (Table 1). *PR3* and *PR9* primers were designed using the primer3 primer design tool (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) while the *PR2* and *Elongation factor-1* (*EF-1*) primer sequences were obtained from (Molinari *et al.*, 2014) and (Rotenberg *et al.*, 2006), respectively. The relative quantification of the target to the reference was determined by using the $2^{-\Delta\Delta CT}$ method to measure the relative expression of the *PR* genes in relation to the housekeeping gene *EF-1* (Livak and Schmittgen, 2001). *EF-1* was used as a reference as it was found to be more stable to experimental conditions than other reference genes (Rotenberg *et al.*, 2006). The ΔΔCT values of the two qRT-PCR experiments are represented in figure 2.

Table 1: Sequences of gene primers of the PR genes and the reference gene used for qRT-PCR.

Gene	Primer sequence	Accession number	Gene Length (bp)*
PR2	F 5'-AAGTATATAGCTGTTGGTAATGAA-3' R 5'-ATTCTCATCAAACATGGCGAA-3'	NM_001247229	1,250
PR9	F 5'-CCTCGGTCAGGGAGGACTAA-3' R 5-'CAGAACCATCACAACCCCGA-3'	XM_004233394.4	1,111
PR3	F 5'-AATTATGGGGCAGCAGGGAG-3' R 5'-TCATCCAGAACCACAACGCT-3'	NM_001279329.2	989
EF-1	F 5'-GATTGGTGGTATTGGAACTGTC-3' R 5'-AGCTTCGTGGTGCATCTC-3'	X14449	1,692

*Gene sequences used to design the primers were obtained from www.ncbi.nlm.nih.gov

Total soluble protein extraction and SDS-PAGE

Tomato leaves were ground in liquid nitrogen and used to extract total soluble proteins. The plant tissues were transferred into a 2 ml centrifuge tube and mixed thoroughly in 100 mM phosphate buffer (pH 7) at 1:1 (w/v) ratio. The extract was centrifuged in the reaction tube at 11,000-x g for 20 minutes at 4 °C. The supernatants were transferred to a new tube.

SDS-PAGE was performed according to (Sambrook and Russell, 2006). Protein samples were boiled with an equal volume of 2x sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 4% β-mercaptoethanol, 0.01% bromophenol blue) for 3 min before loading on 12% gradient sodium dodecyl sulfate (SDS)/polyacrylamide gels (Laemmli, 1970). The total soluble proteins of the fresh leaves in the three treatments (control, nematode infected, SA treated prior to nematode infection) of resistant and susceptible tomato were estimated according to (Bradford, 1976) using fixed concentrations for gel loading. Electrophoresis was performed at 50 V, then at 100 V to resolve the gel. The protein bands were stained overnight with Coomassie brilliant blue R250 (Sigma Aldrich, St. Louis, Missouri, United States).

Statistical analysis

The effect of treatments on the levels of gene expressions, using data obtained by qRT-PCR of *PR2*, *PR3* and *PR9* were analyzed using the two-way ANOVA method (Kim, 2014), followed by a Tukey post-hoc ($p<0.05$). Statistics was performed in R v3.5.1 (Team, 2018) using the functions aov and TukeyHSD. The LSD test was used to confirm the relationship between all studied parameters. P-values of <0.05 were considered as statistically different. Treatments having different letters means that they are significantly different. The experiments were performed twice to ensure the reproducibility of the results.

Results and Discussion

SDS-PAGE profile of nematode-infected leaves of tomato

We compared the total soluble proteins of nematode resistant (Beef master) and susceptible (Rutgers) tomato cultivars upon nematode infection and SA treatment by SDS-PAGE. Tomato leaves were collected to monitor the proteome of the systemic tissue in the context of SAR. The high molecular weight proteins (ranging from 50 to 200 kDa) exhibited detectable differences between the susceptible and resistant tomato cultivars, while the low molecular weight proteins (ranging from 10 to 50 kDa) did only show differences (Fig. 1). The protein samples of the resistant plant leaves showed three bands not found in the samples from the susceptible plant leaves (Fig. 1). Two of these were unique bands at ~160 kDa and ~80 kDa. The ~ 160-kDa band was apparent only in resistant plants treated with SA prior to infection with nematodes (arrow 1, Fig.1). This protein band present only in tomato plants treated with SA prior to nematode infection could be one of the downstream gene products activated in response to SA application, such as PR2, PR3 and PR9, possibly due to SAR induced by SA (Coqueiro *et al.*, 2015; Falcioni *et al.*, 2014). As a result, resistance against *M. incognita* is enhanced in tomato cv. Beef master. The ~80 kDa band appeared only in resistant plants infected with nematodes and was absent in the other samples (arrow 2, Fig. 1). We estimated the molecular weight of PR2, PR3 and PR9 proteins (www.bioinformatics.org /sms2/protein_mw.html) as 37.81 kDa for PR2, 28.45 kDa for PR3 and 35.45 kDa for PR9. None of the separated low molecular weight proteins corresponds to this estimated molecular weight. However, (Rahimi *et al.*, 1998) identified several chitinases ranging between 18 and 80 kDa in potato in response to infection with potato cyst nematode. Therefore, the ~80 kDa protein band may be a result of the upregulation of *PR3* gene expression in the nematode infected resistant tomato, and may be one of the chitinases reported by (Rahimi *et al.*, 1998). This band does not appear in the resistant plants drenched with SA, possibly because the resistant plants require more time to mount an effective resistance response against the nematode if they are not primed, thus the nematode matures further than in the SA-treated plants. The higher number of both nematode effectors and elicitors possibly triggers a stronger or different response in systemic tissues (G. Huang *et al.*, 2006; Guozhong Huang *et al.*, 2006). Another band at ~50 kDa was present in the control sample of the resistant plants, disappeared in the infected resistant plants and appeared in the infected resistant plants treated with SA prior to nematode infection (arrow 3, Fig. 1). The absence of this ~ 50-kDa band in plants infected with nematodes may be because it is one of the plant pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) proteins suppressed by nematode effectors such as MiMsp40 in *Arabidopsis*. MiMsp40 suppresses the PTI response in *Arabidopsis* and overexpression of *MiMsp40* also suppressed the expression of defense-related genes including FRK1, PAD4, WRKY29, WRKY33, and CYP81F2 (Niu *et al.*, 2016). Nematode effectors also target other plant proteins, e.g. transcription factors such as SCARECROW-like transcription factors in *Arabidopsis* to facilitate nematode infection (Guozhong Huang *et al.*, 2006; G. Huang *et al.*, 2006). Taken together, we show that two proteins of ~80 kDa and ~160 kDa are present only in the resistant cv. Beef master, suggesting that these two proteins contribute to the resistance against nematodes in this cultivar.

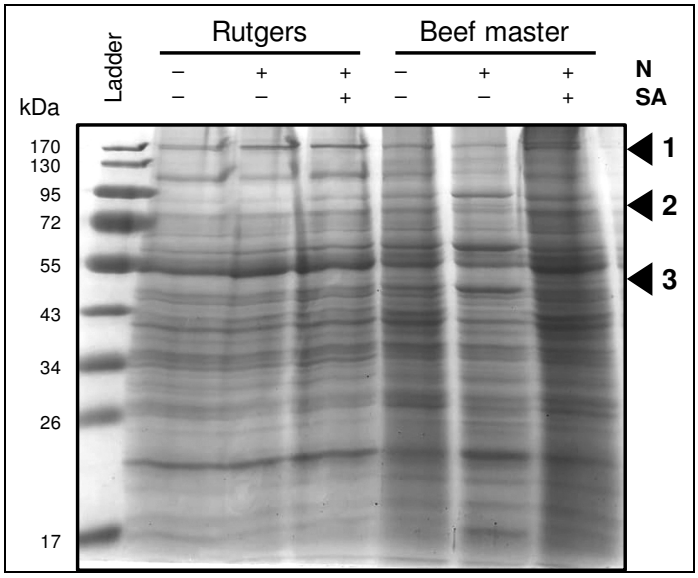

Fig. 1: Protein profile of leaves from resistant (Beef master) and susceptible (Rutgers) tomato plants. Ladder = protein marker (EZ-Run™ Pre-Stained Rec Protein Ladder, Fisher BioReagents™). N, samples infected with the nematode *M. incognita*; SA, plants pre-treated with SA.

PR2, *PR3* and *PR9* expression profiles in roots and shoots of susceptible and resistant tomato varieties

To investigate the response of tomato to nematode infection, the expression levels of the genes *PR2*, *PR3* and *PR9* in two different tomato cultivars (the resistant cv. Beef master and the susceptible cv. Rutgers) were measured using quantitative real time PCR (qRT-PCR) analysis. We examined the transcript abundance of these *PR* genes in control plants, after nematode infection, and upon salicylic acid application prior to nematode infection. The expression levels of *PR2*, *PR3*, and *PR9* were higher in the root (the infected tissue) than in the shoot (the systemic tissue), particularly upon nematode infection (Fig. 2). This is in line with SAR, which primes systemic plant tissues against future attack, but does not result in a complete defense response that would overwhelm the plant (Zhang *et al.*, 2010). As a result, the leaves showed a quantitatively lower expression of the *PR* genes than the roots in our experiments. A reason for the observed difference in basal levels of expression of these *PR* genes lies in the observation that many *PR* genes are of higher expression in roots than in shoots (Nahar *et al.*, 2011).

All three *PR* genes, *PR2*, *PR3*, and *PR9*, were consistently induced in expression upon nematode infection, about two-times in fold change in both the root and the shoot (Fig. 2). This is consistent with earlier reports (Ham, 1991). In addition, pre-treatment with SA further induced expression of the three *PR* genes because of priming by SAR. Additionally, the expression levels of those *PR* genes were higher in the resistant tomato cv. Beef master compared to the susceptible cv. Rutgers in response to nematode infection, in both the root and the shoot (Fig. 2). On the other hand, soil drench application could also have a negative effect on the nematode itself. For instance, SA soil drench application affected also the number of root knot nematode eggs developing into the effective juveniles, as well as gall and egg mass formation (Bakr & Hewedy, 2018).

β-1,3-endoglucanase and chitinase are the enzymes encoded by *PR2* and *PR3*, respectively (van Loon *et al.*, 2006). Both β-1, 3-endoglucanases and chitinases are able to degrade complex carbohydrates that constitute the cell wall, i.e. callose and chitin, respectively. Chitin is the major component of fungal cell walls, while callose is formed by plant cells in response to pathogen recognition in the context of pattern-triggered immunity (PTI) (Underwood, 2012). Chitin also makes up the shell of insects and is an essential component of the nematode eggshell and pharynx. Plant chitinases not only disturb the growth of fungi and nematodes, but also release degradation products that are recognized by receptor-like kinases such as CERK1 (Brotman *et al.*, 2012), thus reinforcing the defense response. Consequently, a high number of chitinase-encoding genes are induced upon pathogen infection and especially upon nematode infection (Rahimi *et al.*, 1998). Overexpression of the β-1, 3-endoglucanase *PR2* in *Arabidopsis* resulted in decreased infection rates by the nematode *H. schachtii*. On the other hand, knockdown of the *PR2* gene exhibited increased susceptibility to *H. schachtii*. The *H. schachtii* effector 30C02 was found to directly suppress *PR2* expression as *Arabidopsis* plants over expressing *30C02* exhibited increased susceptibility to *H. schachtii* (Hamamouch *et al.*, 2012). Chitinase expression increased in the tall fescue herb in response to *Meloidogyne marylandi* infection, and it was found to be expressed systemically, not only confined at the infection sites (Roberts *et al.*, 1992).

The role of PR9 as peroxidase is related to ROS production in response to pathogen infection. ROS have several functions in plant defense, ranging from oxidative stress for the pathogen, activating downstream defense signaling, inducing host cell death, and short-range danger signaling by hydrogen peroxide (Bindschedler *et al.*, 2006). We diagnostically demonstrated that *PR* genes involved in cell wall degradation and in ROS production are concordantly upregulated upon nematode infection in tomato plants (Fig. 2). This response was quantitatively stronger in the resistant tomato cv. Beef master. Beef master is reported to be of the highest resistance against *Meloidogyne* spp. compared to other 33 genotypes, and exhibited with the lowest nematode reproduction rate (Jaiteh *et al.*, 2012). In addition, specific protein bands only appearing in cv. Beef master suggest the specific presence or expression of at least two proteins. These proteins may be identified, in future studies, which will reveal the source of resistance in cv. Beef master. Given the sparse number of sources of resistance against root-knot nematodes such as *M. incognita* (Ibrahim *et al.*, 2019), novel resistance genes or mechanisms are needed towards engineering environmentally-friendly nematode-resistance crops in the future.

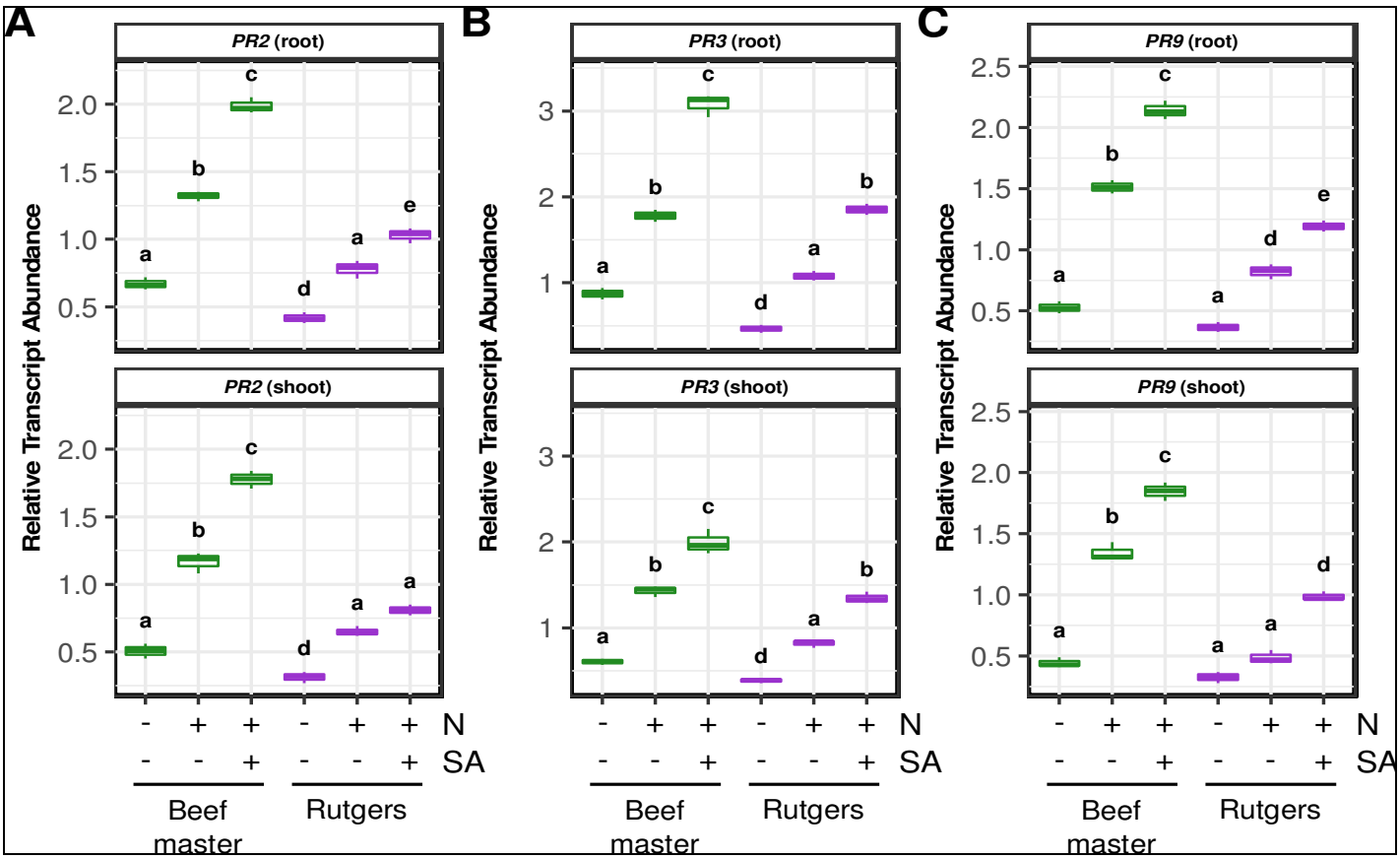

Fig. 2: Relative fold change expression of *PR2*, *PR3*, and *PR9* in roots and shoots of resistant (Beef master) and susceptible (Rutgers) tomato cv.s, respectively. Three treatments representing control, nematode-infected plants (N) using 3,000 J2 *M. incognita* larvae, and plants pre-treated with salicylic acid (SA) one day prior to nematode infection. Root and shoot samples were collected 3 days post nematode infection (dpi). The reference gene was *EF-1* and expression levels of *PR2*, *PR3*, and *PR9* were determined using the $2^{-\Delta\Delta CT}$ method. Data is represented as boxplots. Statistical testing was performed by the two-way ANOVA method, followed by a Tukey post-hoc ($p<0.05$).

Nguyễn, *et al.* (2014) reported a model for the interaction between *Meloidogyne incognita* - rice (*Oryza sativa*), a monocotyledonous plant using RT-PCR. They showed that *M. incognita* expressed the calreticulin *Mi-CRT* gene all along its infection cycle in Nippon bare roots, which plays a role as immune modulator in the suppression of plant basal defenses. Calreticulins are highly conserved calcium-binding proteins in plants and animals that act as Ca^{2+}-binding chaperones, regulating Ca^{2+} storage and signaling in the cell. However, they reported that it was not known how

Mi-CRT contributes to the infection process of the nematode, and suggested it should be further investigated in particular during rice infection. Kyndt *et al.* (2014) reviewed the molecular and cellular aspects of plant- nematode interactions in rice. They discussed the interactions between the different plant hormones in relation to plant defense against nematode infection, and concluded that effectors secreted from rice-infecting nematodes could suppress plant defense. Our results with tomato, a dicotyledonous plant, are in agreement with these reports. The results reported here indicate that resistance to nematode may be due to the function of at least the three genes examined, where PR2 and PR3 break down or weaken the cell wall of the invading nematode, and PR9 enhances the hypersensitivity of the infected plant cells. Therefore, PR2, PR3 and PR9 collectively enhance plant resistance to the root knot nematode.

Conclusion

In this study we attempted to evaluate the effect of soil drench application with salicylic acid on resistant (beef master) and susceptible (Rutgers) tomato cultivars by analyzing the soluble proteins of tomato shoots after challenging the plant roots with nematode, as well as by measuring the expression of three pathogenesis-related (*PR*) genes, *PR2*, *PR3*, and *PR9* in roots and shoots to track the systemic acquired resistant (SAR) response in the resistant and susceptible tomato cultivars upon treatment. Here, we identified three unique protein bands in resistant tomato in response to SA application or nematode infection. These bands could represent new proteins as candidate defense response proteins that contribute to nematode resistance in the resistant tomato cultivar. Moreover, SA application increased the expression of *PR* genes in both roots and shoots, with higher expression in the roots compared to the shoots, and in resistant cultivar compared with susceptible cultivar. The results reported here indicate that resistance to nematode may be due to the function of at least the three genes examined, where PR2 and PR3 break down or weaken the cell wall of the invading nematode, and PR9 enhances the hypersensitivity of the infected plant cells. Therefore, PR2, PR3 and PR9 collectively enhance plant resistance to the root knot nematode. Based on these findings, it may be suggested that future studies should aim to identify the proteins reported here, and analyze their potential as sources of nematode resistance in agriculture. In addition, we suggest that SA application may be used to control nematode infections, as it is environmentally safe and may provide an effective management strategy against root-knot nematodes in tomato by enhancing the systemic acquired resistance (SAR) priming of the plant.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

E.M. Ahmad, planned and performed the experiment, collected the literature, contributed to writing the first draft of the manuscript. H.M.M. Ibrahim planned and designed the experiments, performed the statistical analysis, supervised the project, collected the literature, and wrote and critically revised the manuscript. H.M. Elshabrawi helped in designing the experiment, provided the lab equipment and chemicals for the protein analysis, and participated in the manuscript writing, A. H. Hussein, provided the nematode, participated the student supervision, and revised the manuscript. M.A.M. Aly, contributed to planning the research, writing and reviewing of the manuscript, added references, supervised the project, and provided critical feedback. All authors have read and approved the manuscript.

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