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Research Note

Defense gene expression in root galls induced by *Nacobbus aberrans* in CM334 chilli plants

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Summary

Capsicum annuum L. CM334 is susceptible to *Nacobbus aberrans* but highly resistant to *Phytophthora capsici.* Resistance to *P. capsici* is associated with the over-expression of various defense genes such as those encoding pathogenesis-related proteins. The transcriptional alterations of defense-related genes were determined in galls induced by *N. aberrans* (*Na*) in CM334 chili roots. Transcripts accumulation of *WRKY-a*, *WRKY1*, *POX* (peroxidase), *PR-1* (pathogenesis-related protein 1), and *EAS* (5-epiaristolochene synthase) was estimated by qRT-PCR, and they were compared with those recorded in the incompatible CM334- *P. capsici* (*Pc*) interaction. The levels of all studied genes were significantly ($P \leq 0.05$) lower (*WRKY1*, *POX* and *PR-1*) or down-regulated (*WRKY-a* and *EAS*) in the presence of *N. aberrans*; in contrast, in the incompatible interaction, all genes were significantly up-regulated. The alterations induced by *N. aberrans* could be necessary to ensure the successful completion of its life cycle in CM334 chili roots.
Keywords: *Capsicum annuum*; qRT-PCR; transcription factors; gene reprogramming

Introduction

Plants possess defense mechanisms to respond to pathogen attack, which usually involve the transcriptional activation of several defense-related genes that subsequently leads to the *de novo* synthesis of various proteins and antimicrobial compounds (Rushton & Somssich, 1998). The expression of these genes of defense is regulated by transcription factors, which also regulate gene expression in other physiological processes such as development. In higher plants, WRKY factors constitute one of the largest families of transcriptional regulators with key features against biotic stress caused by fungi, bacteria and nematodes (Rushton *et al*., 2010). These proteins are encoded by a multigene family, for instance, 74 and 81 *WRKY* isogenes were reported in *Arabidopsis thaliana* (L.) Heynh.*,* and *Solanum lycopersicum* L. (Rushton *et al*., 2010; Huang *et al.,* 2012); whereas for *Capsicum annuum* L., the *WRKY-a*, *WRKY1*, *WRKYb*, *WRKYd*, *WRKY58, WRKY40*, *WRKY30*, and *WRKY2* genes, were described (Park *et al*., 2006; Oh *et al*., 2006; Oh *et al*., 2008; Lim *et al*., 2011; Jingyuan *et al.,* 2011; Huh *et al.,* 2012; Wang *et al*., 2013; Dang *et al.,* 2013). The WRKY isogenes act as positive or negative regulators by activating or repressing genes in interactions of plants with their environment (Rushton *et al*., 2010).

The chilli pepper line "Criollo de Morelos" (CM334) is highly resistant to the oomycete *Phytophthora capsici* Leonian (Fernández-Pavía, 1997) and to the three main species of root-knot nematodes [*Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949; *M. arenaria* (Neal, 1889) Chitwood, 1949; and *M. javanica* (Treub, 1885) Chitwood, 1949] (Pegard *et al.,* 2005). However, this pepper line is susceptible to the "false root-knot" nematode *Nacobbus aberrans* Thorne & Allen, 1944 (Trujillo-Viramontes *et al*., 2005). The resistance to the oomycete and to the *Meloidogyne* species already mentioned is expressed as a hypersensitive response (Fernández-Pavia, 1997; Pegard *et al.,* 2005). Among the defenses expressed by pepper genotypes resistant to *P. capsici,* are the accumulation of transcripts of genes encoding for pathogenesis-related proteins (PRs) as *PR-1* (Fernández-Herrera *et al.,* 2012), *GLU* and *POX* (Silvar *et al.,* 2008). Over-expression of other defense genes such as *OPR-3,* which encodes for 12-oxophytodienoate reductase associated with jasmonic acid biosynthesis (Ueeda *et al*., 2006), and those encoding for hydroxymethylglutaryl-CoA reductase (*HMG2*), sesquiterpene cyclase (*SC*) and 5-epiaristolochene synthase (*EAS*), all involved in sesquiterpene phytoalexin biosynthesis like capsidiol, have also been reported (Zavala-Páramo *et al*., 2000*;* Ha *et al.,* 2003; Silvar *et al.,* 2008; Fernández-Herrera *et al.,* 2012). Furthermore, in certain genotypes of chilli,

resistance to *M. incognita* is associated with increases in expression of *WRKY30*, *WRKY2* and *WRKY-a* genes (Li *et al.,* 2008; Jingyuan *et al.,* 2011) and a marked accumulation of chlorogenic acid (Pegard *et al.,* 2005). In contrast to the above, in compatible CM334-*N. aberrans* interaction, the expression of *PR-1*, *POX*, *GLU* and *EAS* genes recorded in whole roots at 21 days after inoculation with the nematode was reduced considerably (Fernández-Herrera *et al.,* 2012); these alterations could favor the successful establishment and reproduction of *N. aberrans* in this genotype.

Through the induction of specialized feeding sites (giant cells and syncitia), root-knot nematodes like *Meloidogyne* spp and *N. aberrans*, and cyst nematodes like *Globodera* spp and *Heterodera* spp establish an intimate and sophisticated interaction with their susceptible hosts for the successful completion of its life cycle. The formation of specialized sites involves up-regulation of genes whose products favor them and down-regulation of those whose products restrict their establishment and reproduction (Sijmons *et al*., 1994). Thus, for example, in soybean roots (*Glycine max*, cv. Williams 82) inoculated with a compatible race of *Heterodera glycines*, the expression of genes involved in jasmonic acid biosynthesis (*OPR1* and *OPR2*) was locally repressed; jasmonic acid is a signaling molecule involved in defense responses against biotic or abiotic stress (Ithal *et al.,* 2007).

The transcriptional alterations that *N. aberrans* induces in their hosts has been only assessed in whole root system of CM334 chilli plants, but the magnitude of these changes at the local level (*i. e.* galls) is unknown. Therefore, in this study, the expression levels of defense genes (*WRKY-a*, *WRKY1*, *POX*, *PR-1* and *EAS*) were determined in galls induced by this nematode, and compared with those recorded in the incompatible CM334-*P. capsici* interaction.

Materials and Methods

Assay establishment

In this study, the reference for the incompatible interaction was the interaction CM334-*P. capsici.* The following treatments were evaluated: 1) CM334 plants inoculated with *N. aberrans* only (Na), 2) CM334 plants without nematode inoculation (Control *Na*), 3) CM334 plants inoculated with *P. capsici* only (Pc) and 4) CM334 plants without oomycete inoculation (Control *Pc*). Control samples (Control *Na* or Control *Pc*) were taken at different times (days or hours) after inoculation (dai or hai) according to the pathogen that was used, *N. aberrans* at 50 dai and *P. capsici* at 24 hai. Since the galls induced by *N. aberrans* in CM334 chilli roots are well defined at 50 dpi, this time was considered. Twenty plants for treatments one and two, and 15 plants for the other two treatments were established. A larger number of plants in treatments one and two had the purpose of having a greater amount of root galls from the inoculated plants. In order to assess the gene expression in plants with the same phenological stage, the pathogens were inoculated at different times; thus, in the case of treatment one, plants were inoculated with *Na* at the moment of assay establishment and in treatment three the inoculation with *Pc* was at 49 days after the inoculation with nematodes in treatment one and 24 h before harvesting the root tissue from plants of all treatments. The experiment was repeated once.

Chilli plants and inoculation with N. aberrans and P. capsici

Seeds of chilli "serrano type" CM334 resistant to *P. capsici* were germinated under aseptic conditions and once emerged, they were individually transplanted into pots containing 25 cm^3 of sterile sand and maintained in growth chambers at 28±1 °C, and with a 14-h photoperiod at a luminous intensity of 6768 lux (fluorescent light) and 10 h of darkness. Plants were irrigated with sterile water and fertilized weekly with nutrient solution (3.15 g of Nitrofoska™ 12-12-12 by liter of sterile water). The assay was established when the plants showed four to five true leaves.

The inoculum of *N. aberrans* was obtained from galled tomato roots collected from a naturally infested plot. The extraction of eggs was performed following the methodology described by Vrain (1977) and second-stage juveniles (J_2) were obtained according to Villar-Luna *et al*., (2009). Plants with four to five true leaves were inoculated with 3 000 J_2 per plant. *P. capsici* inoculum was prepared as indicated by Villar-Luna *et al.* (2009) and each plant was inoculated with 500 000 zoospores. As a reference of the pathogenicity of *P. capsici* and inoculation efficacy, chili plants cv. Yolo Wonder susceptible to the oomycete were also inoculated.

Table 1. Real-time PCR primers used in gene expression analysis

Protein	Gene	Accession number†		Primer sequence (5' → 3')	PCR product (bp)
GAPDH	*GAPDH*	AJ246011	‡Fw	GGCCTTATGACTACAGTTCACTCC	217
			Rv	GATCAACCACAGAGACATCCACAG	
EAS	*EAS*	AJ005588	‡Fw	GCTCAAGAAATTGAACCGCCGAAG	200
			Rv	TCTTCATTATAGACATCGCCCTCG	
WRKY-a	*WRKY-a*	AY391747	‡Fw	CATTACCGTCAACCATCTCA	377
			Rv	TCAACATCCTCATCTCCAAA	
WRKY1	*WRKY1*	EF468464	‡Fw	CAGCAGCAAGAGGAAAGAAGT	335
			Rv	GGCAGTAGAACCCTGATTTAGC	
POX	*POX*	AF442386	‡Fw	CCAGTACGTGCCCAAGAGCTG	560
			Rv	GGATGCGTCGATTGAAGGGTC	
PR-1	*PR-1*	AF053343	ΔFw	GTTGTGCTAGGGTTCGGTG	301
			Rv	CAAGCAATTATTTAAACGATCCA	

†NCBI (National Center for Biotechnology Information); GAPDH: glyceraldehyde-3-phosphate dehydrogenase; EAS: 5- epiaristolochene synthase. POX: peroxidase. PR-1: pathogenesis-related protein 1. WRKY transcription factors. ‡Primers designed by our research group. ΔGayoso *et al.,* (2007).

At 50 days after inoculation with *N. aberrans* (dain), the galls from plant roots of treatment one were separated and for treatment two, root sections were collected. Roots from plants of treatments three and four were sampled at 24 h after inoculation with the oomycete (haio), that is, at 50 days after the assay was established. The pooled samples from 15 or 20 plants (galls, root sections or whole roots) were immediately frozen in liquid N_2 and stored at -80 °C.

RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted from frozen root tissues (galls, root sections or whole roots) using the RNeasy™ Plant mini kit and including a DNase treatment (Qiagen) following the manufacturer's instructions. The purity and integrity of RNA was verified by spectrophotometry (ND-1000, Nanodrop Technologies) and by 1.2 % denaturing agarose gel electrophoresis, respectively. First-strand cDNA was synthesized from 2 µg of total RNA using the oligo dT_{12-18} (Invitrogen) and M-MLV reverse transcriptase (Promega) following the manufacturer's instructions.

Gene expression levels were determined by real-time PCR on the ABI7500™ system (Applied Biosystems). The reaction mixture consisted of buffer 10X, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 0.2 mM dNTPs, amplificase (Biotecmol), SYBR™ Green I (1:75000) (Molecular Probes, Eugene, OR) as the reporter fluorophore, 10 nM fluorescein as passive reference, 2 µL cDNA, and nuclease-free water were added to a final volume of 25 µL. The determinations were done with three technical replicates. The characteristics of the primers used in this study are shown in Table 1. In order to check the amplification efficiency, a validation of the qRT-PCR assay with each primers pair was performed. Amplification conditions consisted of

an initial denaturation at 95 °C for 3 min, followed by 30 cycles at 95 °C for 15 s, annealing for 35 s, and extension at 72 °C for 35 s; annealing temperature for *WRKY-a* primers was at 55 °C, whereas for the rest of the primers was at 60°C. The data were collected during the extension step. Dissociation curve analysis was performed to rule out amplification of non-specific products. PCR products were purified with QIAquick™ PCR purification Kit (Qiagen) according to the manufacturer's instructions and sequenced to confirm their identity. Glyceraldehyde-3-phosphate dehydrogenase gene was used as internal reference to normalize expression level, and control plants to calibrate the transcript levels of the gene of interest. Transcript levels were expressed as fold-change due to treatment in relation to the transcript basal levels in control plants (1x). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008). The data from each sampling time were submitted to analysis of variance in a completely randomized model, and when significant differences were detected, the means of treatments were compared using Tukey's test ($P \leq 0.05$). All statistical procedures were performed in SAS version 9.0 (SAS Institute Inc., 2002).

Results

In the CM334 plants infected by the nematode, the levels of all studied genes were significantly ($P \leq 0.05$) lower (*WRKY1*, *POX*, and *PR-1*) or down-regulated (*WRKY-a* and *EAS*); in contrast, in the incompatible interaction (CM334-*P. capsici*) all genes were significantly ($P \leq 0.05$) up-regulated. The expression levels of *WRKY1*, *POX* and *PR-1* genes in plants infected by *N. aberrans* were in a range from 1.02 – 2.31, 1.11 – 2.49, and 1.27 – 1.72-fold, respectively. However, in plants inoculated with *P capsici* all genes were significantly up-regulated ($P \leq 0.05$), increases were in the range of 15.35 – 18.89-fold for *WRKY-a*, 3.09 – 4.76-fold for *WRKY1*, 20.82 – 24.76-fold for *POX*, 16.34 – 20.11-fold for *PR-1* and 28.25 – 31.12-fold for *EAS* (Fig. 1).

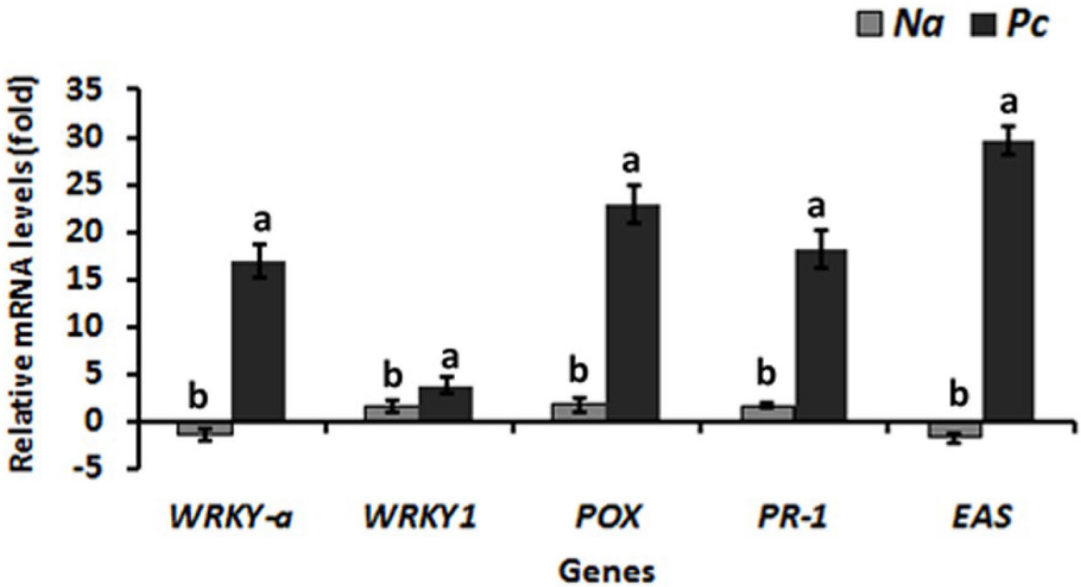

Fig. 1. Accumulation of *WRKY-a, WRKY1, POX, PR-1* and *EAS* transcripts in CM334 chilli roots resistant to *Phytophthora capsici. Na*: plant roots inoculated with *Nacobbus aberrans* (compatible interaction)*; Pc*: plant roots inoculated with *Phytophthora capsici* (incompatible interaction). In plants of *Na* treatment, the transcript levels were determined in root galls, whereas in plants of *Pc* treatment the whole root system was considered. Each bar represents the average of three technical replicates and the line in each bar the standard deviation. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Glyceraldehyde-3-phosphate dehydrogenase gene was used as internal reference to normalize expression, and control plants to calibrate the transcript levels of the gene of interest, which were expressed as fold-change due to treatment in relation to the basal level of transcripts in control plants (1x). Bars with the same letter are not significantly different (Tukey, $P \leq 0.05$). The experiment was repeated once.

Discussion

WRKY transcription factors, unique to plants, are encoded by a multigene family and whose members are largely over-expressed in response to biotic stress (Rushton *et al*., 2010). Park *et al.* (2006) reported that in pepper plants (*Capsicum annuum*), *WRKY-a* gene was over-expressed during the hypersensitive response triggered by *Tobacco mosaic virus* (TMV) and *Xanthomonas campestris* pv. *Vesicatoria,* and they suggested its involvement in the activation of defense mechanisms against pathogens. In the present study, over-expression of *WRKY-a* gene in CM334 chilli roots inoculated with *P. capsici* (*Pc*, incompatible interaction) was much higher than that recorded in plants infected by *N. aberrans* (*Na*, compatible interaction). In the incompatible interaction *C. annuum* (cv. HDA149)-*Meloidogyne incognita*, there was also an over-expression of *WRKY-a* and *WRKY2* genes (Li *et al.,* 2008). The *WRKY1* gene was over-expressed in both interactions (CM334-*N. aberrans* and CM334-*P. capsici*); however, gene expression was lower in the compatible interaction compared to incompatible (Fig. 1). The differential activation of this gene by pathogens has been documented, for instance, Oh *et al*., (2008) found that in the incompatible interaction *C. annuum* (cv. ECW-20R)-*Xanthomonas axonopodis* pv. *vesicatoria* (*Xcv*), the *WRKY1* expression was earlier and more intense as compared to the compatible [*C. annuum* (cv. ECW)-*Xcv*]; in both interactions the *PR-1* gene was also over-expressed in a similar trend, but with higher intensity than the *WRKY1* gen.

In the compatible interaction (CM334-*N. aberrans*), the expression of *PR-1* and *POX* genes was of lower magnitude compared to that in the incompatible (CM334-*P. capsici*). Previously, Fernández-Herrera *et al*. (2012) reported the same behavior for *PR-1, POX*, y *GLU* (β-1, 3-glucanase) genes in the whole root system in the CM334-*P. capsici* and CM334-*N. aberrans* interactions. In addition, Silvar *et al*. (2008) found that in the incompatible pepper CM331-*P. capsici* interaction, genes encoding for peroxidase, β-1, 3-glucanase and PR-1 protein were over-expressed. It is often mentioned that over-expression of *PR-1* gene might be involved in the strengthening of cell walls of the host plant in response to pathogen attack (Vidhyasekaran, 2008). Based on this information, perhaps the strong over-expression of *PR-1* recorded in incompatible CM334-*P. capsici* interaction could contribute to the confinement of the oomycete during the hypersensitive response (HR) in CM334 roots (Fernández-Pavía, 1997). The expression of *PR-1* gene was not turn-off in galls induced by *N. aberrans* and its lower over-expression could be associated with profound changes and reorganization of cell walls that take place during the differentiation of the specialized feeding site (syncytium). On the other hand, peroxidases appear to contribute to the strengthening of cell walls by lignin and suberin depositions, constituting physical barriers that can restrict the advance and establishment of the pathogen (Van Loon, 1997). In addition, these enzymes are associated with the generation of H_2O_2 during the HR (Do *et al*., 2003). Thus, the strong over-expression of *POX* gene in the incompatible CM334-*P. capsici* interaction, perhaps contributed to the restriction of the pathogen advance by lignification and H_2O_2 generation. The lower over-expression of *POX* gene that occurred in the compatible interaction could be necessary for restructuring the cell wall of the syncytium, since differentiation of the feeding site is a prerequisite for the development and reproduction of the nematode. In the compatible *A. thaliana-M. incognita* interaction, the expression of various defense-related genes as *PR-4* (pathogenesis-related protein 4), *PAD4* (lipase-like protein, implied in salicylic acid signaling), and 17 *WRKY* genes, was down-regulated; thus, the successful establishment of the nematode was associated with an effective repression of defense mechanisms (Jammes *et al.,* 2005). In root galls induced by *M. javanica* in *Solanum lycopersicum*, the expression of genes encoding pathogenesis-related proteins (PRs) as peroxidases was down-regulated, whereas two *WRKY* isogenes were over-expressed (Bar-Or *et al.,* 2005); these researchers suggest that the down-regulation of genes encoding PRs was promoted by WRKY proteins. It is well documented that several WRKY proteins regulate the expression of defense genes by binding to a consensus sequence known as W box [(T) GACC/T] in the promoter region of the target gene (Rushton *et al*., 2010). The W box is common in defense genes as those encoding for PRs (Eulgem *et al*., 2000) and others defense genes such as *EAS* (5-epiaristolochene synthase) (Maldonado-Bonilla *et al.,* 2008). Thus, in this study, the modulation of the *WRK-a* gene induced by *N. aberrans* and *P. capsici* in CM334 plant roots, perhaps favored the over-expression of defense genes *WRKY-a*, *PR-1*, *POX* and *EAS* in the incompatible interaction (*Pc* treatment) and its reduced expression in the compatible interaction (*Na* treatment).

EAS gene expression in galls induced by *N. aberrans* was considerably reduced; this gene encodes for 5-epiaristolochene synthase (sesquiterpene cyclase, SC) involved in capsidiol biosynthesis, a phytoalexin often associated with resistance to *P. capsici* in certain pepper genotypes (Egea *et al*., 1996; Zavala-Páramo *et al*., 2000) and possibly also to nematodes, because, there is evidence that capsidiol is toxic to the second-stage juveniles of *N. aberrans* (Godínez-Vidal *et al*., 2010). In contrast, in the incompatible CM334-*P. capsici* interaction, a strong over-expression of this gene was recorded. These results are similar to those reported in CM334 whole roots infected by *P. capsici* or *N. aberrans* (Fernández-Herrera *et al.,* 2012). Except for the *EAS,* the gene expression levels of *PR-1* and *POX* genes recorded in galls, these were similar to the results obtained by Fernández-Herrera *et al.,* (2012) in CM334 whole roots.

In the present study, the expression of defense genes *WRKY-a*, *WRK1*, *PR-1*, *POX* and *EAS* genes in the compatible chilli CM334-*N. aberrans* interaction was significantly reduced; while in the incompatible CM334-*P. capsici*, maximum transcript levels were recorded. The transcriptional modulation carried out by *N. aberrans* could be necessary to ensure the successful completion of its life cycle in CM334 chilli roots, while that induced by *P. capsici* would restrict its establishment.

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