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RESEARCH PAPER

Tomato root exudates induce transcriptional changes of *Meloidogyne hispanica* genes

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Summary. *Meloidogyne hispanica* is a polyphagous root-knot nematode of emerging importance that has the ability to infect a broad range of plants, and tomato (*Solanum lycopersicum* L.) crops can be severely damaged. This study investigated whether tomato root exudates regulate the expression of five candidate parasitism genes previously identified and sequenced in *M. hispanica*. These were calreticulin (*crt-1*), cathepsin L cysteine protease (*cpl-1*), β-1,4 endoglucanase-1 (*eng-1*), fatty acid retinol binding protein (*far-1*) and venom allergen-like protein (*vap-1*). One thousand *M. hispanica* second-stage juveniles (J2) were exposed overnight to tomato root exudates, obtained from the root systems of 4-week-old plants during 4 h of agitation in sterilized distilled water, and the relative expression of the parasitism genes was determined by quantitative real-time PCR. The *cpl-1*, *crt-1*, *far-1* and *vap-1* genes were differentially up-regulated ($P<0.05$) in the pre-parasitic J2 after exposure to tomato root exudates, while expression of *eng-1* was largely unaffected ($P=0.05$) by the treatment. This results suggests that tomato root exudates induce changes in the expression of candidate parasitism genes. Gene silencing by RNAi will elucidate the exact function of these candidate parasitism genes in nematode penetration and survival. Identification of the plant signal molecules in the tomato root exudates responsible for the up-regulation of these parasitism genes may lead to the development of novel approaches for the management of *M. hispanica*.

Key words: effectors, plant-nematode interactions, root-knot nematodes, secretions.

Introduction

The sedentary endoparasitic root-knot nematodes (RKN), *Meloidogyne* spp., are among the most economically damaging plant-parasitic nematodes (PPN), and these parasites constitute major obstacles to agricultural production in developing countries (Hussey and Janssen, 2002; Moens *et al*., 2009). *Meloidogyne* species have co-evolved with their hosts to develop mechanisms that optimise the chances of successful root invasion. Infective nematode stages rely on responses to plant signals originating from root exudates or sites of previous nematode penetration to find their hosts, and when a nematode finds a root, the root surface is explored for a suitable penetration site (reviewed in Curtis, 2008).

Plant chemicals originating from root exudates attract nematodes to the roots or result in repellence, motility inhibition or even death (Curtis *et al*., 2009). A combination of signals emanating from different areas of the roots affects nematode behaviour in a given plant-nematode interaction (Prot, 1980). Pre-parasitic J2 of *Meloidogyne* spp. are attracted to the zones of elongation in growing root tips and display characteristic exploratory behaviour at the root surfaces, including stylet thrusting, release of secretions in preparation for root penetration, aggregation and an increase in mobility (Von Mende,

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1997). This exploratory behaviour has been induced *in vitro* by compounds present in root exudates, and a number of plant compounds, such as catechol and caffeic acid, induced nematode stylet thrusting and production of secretions (reviewed in Curtis, 2007). Root exudate components, such as tannic acid, flavonoids, glycosides and fatty acids, may regulate chemotaxis of pre-parasitic J2 by repulsion or attraction (Chitwood, 2002; Bais *et al*., 2006). Many crops naturally release nematotoxic compounds from their roots that induce PPN behavioural changes (Bais *et al*., 2006; Curtis *et al*., 2006; Curtis, 2007; Dutta *et al*., 2011). Dutta *et al*. (2012) showed that semiochemicals such as small lipophilic molecules emitted by root exudates of tomato (*Solanum lycopersicum* L.) and rice (*Oryza sativa* L.) affected stylet thrusting and motility of RKN J2 and might exert repellent or allelopathic effects on these nematodes. Until recently, little was known about RKN gene expression and the signaling mechanisms occurring before nematodes penetrate host roots. Teillet *et al*. (2013) and Dong *et al*. (2014) showed that RKNs respond to root signals by changing their behaviour and gene expression. Several *M. incognita* genes were differentially expressed after incubation in *Arabidopsis thaliana* root exudates, and lauric acid (found in root exudates of *Chrysanthemum coronarium* L.) down-regulated the expression of the *Mi-flp-18* gene.

The RKN *M. hispanica* Hirschamnn, 1986 (*Mhi*), detected for the first time in Spain from peach rootstocks, has a worldwide distribution, and is associated with a wide range of plants including tomato cultivars (Maleita *et al*., 2012a). The potential impact of *M. hispanica* in agriculture warrants research towards developing new control strategies based on improved understanding of the host-pathogen interactions.

Several putative effector genes have been considered as likely to play important roles in plant-RKN interactions, and they are strongly associated with potential nematode digestion, plant cell wall degradation and suppression or manipulation of plant basal defences (Haegeman *et al*., 2012). Five of these putative effector genes, calreticulin (*crt-1*), cathepsin L cysteine protease (*cpl-1*), β-1,4 endoglucanase-1 (*eng-1*), fatty acid retinol binding protein (*far-1*) and venom allergen-like protein (*vap-1*), were previously identified and sequenced in *M. hispanica* (Duarte *et al*., 2014a; 2014b). The transcripts of the *Mhi-far-1* and *Mhi-vap-1* genes, associated with suppression or manipulation of plant basal defences, were localized in the oesophageal subventral gland cells of *M. hispanica* J2 (Kang *et al*., 2012; Iberkleid *et al*., 2013; Duarte *et al*., 2014a). The objective of the present research was to investigate whether tomato root exudates regulated the expression of the five candidate parasitism genes.

Materials and methods

Root knot nematode isolate

Meloidogyne hispanica isolate PtHi3, which was obtained from fig tree (*Ficus carica* L.) roots in Portugal (Abrantes *et al*., 2008) and characterized, was re-confirmed by specific esterase phenotype (Maleita *et al*., 2012b). The isolate was reared on tomato cv. Easypeel, and freshly hatched J2 were obtained from egg masses placed on a 25 μm mesh sieve.

Root exudates and J2 incubation

Root exudates were obtained from the root systems of three 4-week-old tomato cv. Easypeel plants. The roots were washed gently and transferred to an Erlenmeyer with 250 mL of sterilized distilled water, with agitation during 4 h. The root exudates were filtered using Whatman filter paper grade 1. Afterwards, 1000 *M. hispanica* J2 were exposed overnight to tomato root exudates or to water (control).

RNA extraction and reverse transcription

After exposure to tomato root exudates or water (control), the J2 were centrifuged at 8000 g for 2 min at room temperature, washed three times with RNase free water and stored at -80ºC until use. RNA was isolated according to the Affymetrix (Santa Clara, USA) standard protocol with Trizol reagent (Invitrogen Life Technologies). Afterwards, the RNA was purified using the RNeasy Mini Kit including RNase-Free DNase Set (QIAGEN). Total RNA (50 ng) was reverse transcribed into cDNA using the Omniscript RT Kit (QIAGEN), according to the manufacturer's instructions. The concentration of the cDNA was determined using a Nanodrop ND-1000 Spectrophotometer.

Gene expression analysis by quantitative RT-PCR

To ascertain the effect of tomato root exudates on the expression of the *Mhi-cpl-1*, *Mhi-crt-1*, *Mhi-*

Table 1. *Meloidogyne hispanica* (MHI) primers used in qRT-PCR for amplification of several candidate parasitism genes, with β-actin as control.

Gene name	Primer name	Primer sequence 5′→3′
Calreticulin (*crt-1*)	MHIq-CRT-1f	AGACTTGAGCTGATTGGGAGTTG
	MHIq-CRT-1r	TCTTTGCGTCAGGGTCCTTAA
Cathepsin L cysteine protease (*cpl-1*)	MHIq-CPL-1f	TTGGATACGGCACAGATGACA
	MHIq-CPL-1r	TTCCCCCCAACTATTTTTAACAAG
β-1,4-endoglucanase-1 (*eng-1*)	MHIq-ENG-1f	CGTTCTCGGTACAACAACATGGT
	MHIq-ENG-1r	TGTGCCGCTTACAGGATTGTTA
Fatty acid and retinol binding protein	MHIq-FAR-1f	ATTGACCGAGGACGACAAGA
(*far-1*)	MHIq-FAR-1r	TCAGTTGCATATTCACTGTGCTTCT
Venom allergen-like protein-1 (*vap-1*)	MHIq-VAP-1f	CCTTATCCTGGCCAAGACTGC
	MHIq-VAP-1r	TTGTGTCCAATGTCCAATACCTCT
β-actin (control)	MHIq-ACTINf	TGTATCCAGGCATTGTGATCGT
	MHIq-ACTINr	CATTGTTGATGGTGCCAAAGC

eng-1, *Mhi-far-1* and *Mhi-vap-1* genes, quantitative real time PCR (qRT-PCR) was carried out with SYBR Green PCR Master Mix according to the manufacturer's instructions (Applied Biosystems 7500/7500 Fast Real-Time PCR System). The qRT-PCR primers (Table 1) for *M. hispanica cpl-1*, *crt-1*, *eng-1*, *far-1* and vap-1 gene fragments and the β-actin gene fragment were designed, respectively, from the *M. hispanica* sequences KF030974, KF679110, KF679121, KF679116 and KF030969 and *M. incognita* BE225475.1 sequences, obtained from Genbank, using the software Primer Express v3.0 (Applied Biosystems). The β-actin gene was used as a reference for normalization. The concentration of qRT-PCR primers sets was optimized for maximal specificity and efficiency. Efficiency of the PCR was also determined according to Pfaffl (2001), using Applied Biosystems software to confirm that only a single PCR product was obtained. All primer pairs had efficiency greater than 86%. Nonspecific PCR products were confirmed by the PCR negative control, without cDNA template.

The qRT-PCR reaction mix (20 µL) contained 3 µL of cDNA in 1 × Fast SYBR Green Master Mix and 200 nM of primers for *Mhi-vap-1* and β-actin, 100 nM for *Mhi-cpl-1*, *Mhi-crt-1* and *Mhi-eng-1* or 80 nM for *Mhi-far-1* (Table 1). The qRT-PCR was performed with the following conditions: 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 58ºC for 1 min. Three replicates were performed for each run of the qRT-PCR and the mean Ct values determined. The relative expression of the candidate genes was calculated by the Comparative CT method according to Applied Biosystems 7500 Fast Real-Time PCR System (software version 2.0.4). The experiment was performed three times and the results analysed using the iteration test (REST 2009 Software).

Results and discussion

The tomato root exudates induced changes in gene expression of candidate parasitism genes in *M. hispanica* J2, before root penetration. The *M. hispanica cpl-1*, *crt-1*, *far-1*, and *vap-1* genes were differentially up-regulated ($P<0.05$) after exposure of pre-parasitic J2 to tomato root exudates, while the expression of gene *eng-1* was largely unaffected ($P=0.05$, Figure 1). Gene expression was validated and confirmed in all cases. The *crt-1* and *vap-1* genes showed approximately six-fold increase when compared with the control and approximately two-fold for *cpl-1* and *far-1* (Figure 1).

It has been extensively reported that plant signal molecules present in root exudates induce behavioural changes in nematodes. The regulation of nem-

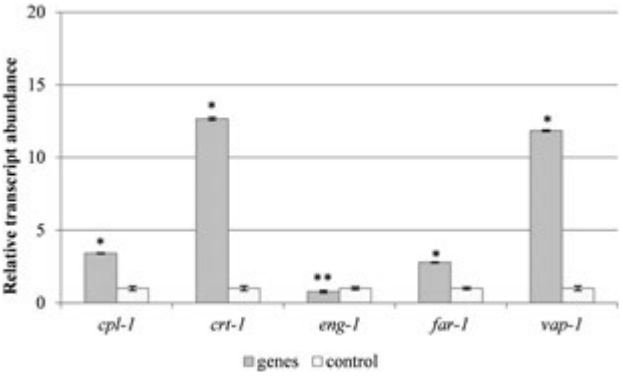

Figure 1. Relative transcript abundance of *Meloidogyne hispanica cpl-1*, *crt-1*, *eng-1*, *far-1* and *vap-1* genes determined by qRT-PCR after exposure of second-stage juveniles to tomato cv. Easypeel root exudates. Values are means of three replicates, and bars represent standard deviation. Significant differences (* $P<0.05$ and ** $P=0.05$) were assessed by iteration test (REST 2009 Software).

atode gene expression by root signal molecules has been studied specifically in relation to the hatching of cyst nematodes (reviewed in Curtis, 2008). Teillet *et al*. (2013) were the first to show that a number of genes of *M. incognita* J2 were differentially expressed in response to signals present in *A. thaliana* root exudates, and that these genes continued to be up-regulated post penetration, during migration and feeding site initiation. The genes considered in the present study were expressed during migration and feeding site formation (Duarte *et al*., 2014a; 2014b). However, this is the first report showing that RKN genes potentially involved in parasitism are up-regulated by tomato root exudates prior to nematode infection.

Understanding the complexity of the molecular signal exchange and response during the early stages of host-parasite interactions is important to identify vulnerable points in the parasite life cycle that can be used to target disruption of nematode host recognition. Dong *et al*. (2014) demonstrated the importance of such studies and showed that a possible novel control strategy could be devised by implementing a tomato and crown daisy intercropping system, in which crown daisy root exudate down-regulated the expression of the *M. incognita Mi-flp-18* gene. This negatively affected nematode motility and led to decreased infection of the tomato plants.

Our data showed that *M. hispanica* pre-parasitic J2 recognized signal molecules present in root exudates that triggered a change in gene expression in juveniles. The expression of *Mhi-crt-1* and *Mhi-vap-1* genes (Figure 1) suggests that these two genes, associated with the suppression of host defence, may be involved in the early events of recognition between the host plants and the nematodes (Haegeman *et al*., 2012; Jaouannet and Rosso, 2013). In previous studies, the *crt-1* gene was abundantly secreted into the apoplasm by *M. incognita* sedentary stages during induction and maintenance of the giant cells (Jaubert *et al*., 2005). The expression of the *Mhi-cpl-1* and *Mhi-far-1* genes was only slightly up-regulated after exposure to tomato root exudates (Figure 1). The *M. incognita* CPL-1 is a digestive enzyme expressed in the nematode intestine whilst FAR-1 detected in preparasitic J2 cuticle surfaces has an important function in the infection process, through the manipulation of the jasmonate-dependent defense response that induces susceptibility to RKN (Haegeman *et al*., 2012; Iberkleid *et al*., 2013).

These candidate parasitism genes have a strategic function in the early events of the infection of plants by nematodes and their up-regulation prior to root infection contributes to successful parasitism. Gene silencing should indicate whether these genes are vital for penetration and survival by nematodes inside host roots. The identification of the plant signal molecules in the tomato root exudates responsible for up-regulation of these parasitism genes may lead to novel approaches to RKN management. Further work should be conducted to assess whether it is possible to modify host rhizosphere and interfere with host recognition processes. This could be achieved by blocking or inactivating the molecules present in the root exudates which act as signals to up-regulate parasitism genes, or by adding compounds which are repellent. These could impede detection of plants by nematodes.

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