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Exposure to double-stranded RNA mediated by tobacco rattle virus leads to transcription up-regulation of effector gene *Mi-vap-2* from *Meloidogyne incognita* and promotion of pathogenicity in progeny

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ABSTRACT

Meloidogyne spp. are economically important plant parasites and cause enormous damage to agriculture world-wide. These nematodes use secreted effectors which modify host cells, allowing them to obtain the nutrients required for growth and development. A better understanding of the roles of effectors in nematode parasitism is critical for understanding the mechanisms of nematode-host interactions. In this study, *Mi-vap-2* of *Meloidogyne incognita*, a gene encoding a venom allergen-like protein, was targeted by RNA interference mediated by the tobacco rattle virus. Unexpectedly, compared with a wild type line, a substantial up-regulation of *Mi-vap-2* transcript was observed in juveniles collected at 7 days p.i. from *Nicotiana benthamiana* agroinfiltrated with TRV::*vap-2*. This up-regulation of the targeted transcript did not impact development of females or the production of galls, nor the number of females on the TRV::*vap-2* line. In a positive control line, the transcript of *Mi16D10* was knocked down in juveniles from the TRV::*16D10* line at 7 days p.i., resulting in a significant inhibition of nematode development. The up-regulation of *Mi-vap-2* triggered by TRV-RNAi was inherited by the progeny of the nematodes exposed to double-stranded RNA. Meanwhile, a substantial increase in *Mi*-VAP-2 expression in those juvenile progeny was revealed by ELISA. This caused an increase in the number of galls (71.2%) and females (84.6%) produced on seedlings of *N. benthamiana* compared with the numbers produced by control nematodes. Up-regulation of *Mi-vap-2* and its encoded protein therefore enhanced pathogenicity of the nematodes, suggesting that *Mi-vap-2* may be required for successful parasitism during the early parasitic stage of *M. incognita*.

1. Introduction

Plant parasitic nematodes (PPNs) are important pathogens of agricultural crops and cause billions of dollars of yield losses world-wide (Nicol et al., 2011). Root-knot nematodes (RKNs, *Meloidogyne* spp.) create a large proportion of these losses and one of the most damaging species, *Meloidogyne incognita*, can infect over 2000 plant species (Bird et al., 2009). RKNs have evolved intimate parasitic relationships with host plants. The motile second-stage juveniles (J2s) penetrate root tips behind the elongation zone and migrate intercellularly to the vascular cylinder. Secreted proteins introduced into the cytoplasm and apoplast of vascular cells modify the development of the cells into the feeding cells-giant cells (GCs), which are crucial for nematodes to obtain nutrients and sustain growth and development (Gheysen and Mitchum, 2011).

The formation of GCs requires suppression of host defences and disturbance of the normal cell cycle. These processes are thought to be mediated by secretions from nematode organs such as the pharyngeal glands, the amphids, the excretory/secretory (E/S) system and the rectal glands (Vanholme et al., 2004). The proteins expressed exclusively in the pharyngeal gland cells and secreted through the stylet are regarded as the effectors which play key roles in nematode parasitism (Mitchum and Hussey, 2013). Enzymes that modify the host cell wall are among the most well-studied effectors of RKNs. Enzymes such as beta-1,4-endoglucanase, cellulases, pectate lyase, xylanase, polygalacturonases and expansins can directly degrade and modify the cell wall, thereby allowing J2s to penetrate the epidermis or migrate within the roots (Rosso et al., 1999; Dautova et al., 2001; Doyle

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and Lambert, 2002; Jaubert et al., 2002; Huang et al., 2005a; Ledger et al., 2006; Abad et al., 2008; Ibrahim et al., 2011). Other effectors have also been identified that are important in the compatible interaction with a host plant. A secreted chorismate mutase (CM) may manipulate host biochemistry by reducing the pool of chorismate available for conversion to salicylic acid via the shikimate pathway in plants and thus prevent the normal activation of host defences (Doyle and Lambert, 2003; Huang et al., 2005b; Long et al., 2006). The *16D10* sequence from *M. incognita*, encoding a 13-amino acid secretory peptide, interacts in planta with two putative transcription factors that belong to the SCARECROW-like (SCL) family, and may function in the extensive transcriptional reprogramming responsible for feeding cell ontogenesis (Huang et al., 2006a,b). Targeting this sequence by RNA interference (RNAi) in transgenic *Arabidopsis* causes a failure of parasitism, demonstrating the importance of this protein to RKNs (Huang et al., 2006b). More recently a secreted calreticulin from *M. incognita* has been identified that suppresses host defences; overexpression of the *Mi-crt* in *Arabidopsis thaliana* suppressed the induction of defense marker genes and callose deposition after treatment with the pathogen-associated molecular pattern elf18 (Jaubert et al., 2005; Jaouannet and Magliano, 2013). Furthermore, other effectors including *Mi*-GSTS-1 (Dubreuil et al., 2007), *Mi*-EFF1 (Jaouannet et al., 2012), *Mj*-NULG1a (Lin et al., 2013) and 8D05 (Xue et al., 2013) have been identified that may be involved in parasitism by RKNs. However, the functions of many other effectors from RKNs still remain to be elucidated, including the venom allergen-like proteins (VAPs).

The VAPs belong to the SCP/TAPS protein family which is found in many eukaryotes (Chalmers et al., 2008). A number of SCP proteins characterised from animal helminths are known to be involved in diverse immunomodulatory effects in host-parasite inter-relationships (Tawe et al., 2000; Del Valle et al., 2003; Bower et al., 2008). VAP effectors have also been identified from PPNs, including *Hg*-VAP-1 and -2 from *Heterodera glycines* (Gao et al., 2001), *Da*-VAP-1 from *Ditylenchus africanus* (Haegeman et al., 2009), *Bx*-VAP-1, -2 and-3 from *Bursaphelenchus xylophilus* (Lin et al., 2011; Kanga and Koh, 2012) and *Gr*-VAP-1 from *Globodera rostochiensis* (Lozano-Torres et al., 2012). *Gr*-VAP-1 was shown to interact with the apolastic cysteine protease Rcr3pim and to activate the plant defense response mediated by the resistance gene *Cf-2*, therefore selectively suppressing the host immunity mediated by the surface-localised immune receptors (Lozano-Torres et al., 2012, 2014). Two VAP genes, *Mi-vap-1* and *Mi-vap-2*, have been identified from *M. incognita* and shown to be expressed in the subventral oesophageal gland cells (Ding et al., 2000; Wang et al., 2007), although the functions of these proteins remain uncertain.

RNAi is a powerful tool for gene function analyses of PPNs. Gene silencing by RNAi can be realised through soaking nematodes in double-stranded (ds)RNA solutions or feeding of dsRNA expressed in transgenic plants (Bakhetia et al., 2005; Dalzell et al., 2010; Dinh et al., 2014a, 2014b). In addition, dsRNA can be delivered to feeding nematodes via tobacco rattle virus (TRV) mediated gene silencing (Valentine et al., 2007; Dubreuil et al., 2009). Here we report the functional analysis of *Mi-vap-2* of *M. incognita* using TRV-mediated RNAi. *Mi16D10* was selected as a positive control gene, as this is known to be an effective target for RNAi in RKNs (Huang et al., 2006b; Yang et al., 2013). We found substantial up-regulation of the *Mi-vap-2* transcript in 7 days p.i. juveniles on the root of *Nicotiana benthamiana* agroinfiltrated with TRV::*vap-2*, whereas *Mi16D10* showed the expected down-regulation compared with the wild type (WT) line. The up-regulation of the *Mi-vap-2* transcript was transmitted to *M. incognita* progeny, which resulted in increased protein expression and promotion of pathogenicity. This suggests that *Mi-vap-2* may be required for the successful parasitism of *M. incognita* in plants during the early parasitic stages.

2. Materials and methods

2.1. Biological materials

The population MIJS3 of *M. incognita* was maintained on susceptible tomato plants (variety Sufen No.8) in a greenhouse at 25 °C. The egg masses were harvested from the root 8 weeks after inoculation. After treatment with 0.5% NaOCl and rinsing with distilled water, the egg masses were put on sieves with 25 μm openings suspended over deionized water in plastic bowls and preparasitic second-stage juveniles (pre-J2s) were hatched (Hussey and Barker, 1973). Seedlings of *N. benthamiana* were grown in a greenhouse under 16:8 light (22 °C):dark (20 °C), and the four-leaf stage was used for inoculation tests.

2.2. Viral constructs and infections of N. benthamiana

Total RNA of *M. incognita* pre-J2s was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and the first-strand cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's instructions. A fragment of target gene *Mi-vap-2* (GenBank accession number EF370395) was amplified from the cDNA with the specific primer pair VAPF/R (Table 1). The region amplified by these primers is marked in Supplementary Fig. S1. The gene *Mi16D10* (DQ087264) was chosen as a positive control to evaluate the silencing effect mediated by TRV, while the *gfp* gene was used as a negative control. The fragments of *Mi16D10* and *gfp* were amplified from the cDNA and pBin-*gfp* vector with the primers 16D10F/R and GFPF/R, respectively. All primers used in this study are listed in Table 1.

The construction of vectors is illustrated in Fig. 1. The amplified fragment was inserted into the vector pTRV2 digested with *Xma*I and the constructs pTRV2::*vap-2*, pTRV2::*16D10* and pTRV2::*gfp* were generated. Construct pTRV2::*pds* was used as a control; this construct targeted the endogenous phytoene desaturase (PDS)

Table 1
Primers used in this study.

Name	Sequences (5′→3′)[a]	References
16D10F	CCC GGG CCT CAA AAA TAC CAT AAA G	Current study
16D10R	CCC GGG GAA ATT AAC AAA GGA AAC	Current study
VAPF	CCC GGG TGA AAA GCA GGC ACA GGA ATG	Current study
VAPR	CCC GGG GGC AAT CTG TAC AAG GCT CAC	Current study
GFPF	CCC GGG AGG TGA AGT TCG AGG GCG	Current study
GFPR	CCC GGG CTT GTA CAG CTC GTC CAT	Current study
TRV1F	ACT CAC GGG CTA ACA GTG CT	Current study
TRV1R	GAC GTA TCG GAC CTC CAC TC	Current study
16D10RTF	TTA TTT AAT GCC TTT AAT GGT TAC TTT	Huang et al., 2006b
16D10RTR	CAA TTA TTT CCT CCA GG	Huang et al., 2006b
VAPRTF	TGA GCC TTG TAC AGA TTG CC	Current study
VAPRTR	TGT TGT TGG AGT AAT AGG AGC TTT	Current study
MIAF	GAT CCT CAC TGA ACG TGG TTA TTC	Huang et al. (2006b)
MIAR	CTT GAT GTC ACG GAC ATC TC	Huang et al. (2006b)
NbAF	GCA ACT GGG ATG ATA TGG AG	Current study
NbAR	TCA CGT GTA AGC GAG TTT TTC	Current study
VAPatF	CGG ATC CAT GCC ACC TGG AAA TGT TTA TAC	Current study
VAPatF	CGC TCG AGG TCC TCA TCA GAA CTA CTA GA	Current study

[a] Restriction sites are underlined.