Plant Science 180 (2011) 276–282

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Ectopic expression of a *Meloidogyne incognita* dorsal gland protein in tobacco accelerates the formation of the nematode feeding site

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ARTICLE INFO

Article history:
Received 19 November 2009
Received in revised form 6 September 2010
Accepted 9 September 2010
Available online 16 September 2010

Keywords:
Root-knot nematode
Parasitic infection
Nematode's life cycle
Ectopic protein expression

ABSTRACT

Meloidogyne spp., plant-parasitic nematodes present worldwide, are intensively studied because of the damage caused to a large variety of agronomically important crops. Several reports indicate that proteins from the *Meloidogyne* spp. dorsal gland might play an important role to allow proper establishment of a functional nematode feeding site. The precise role of these proteins in the process of feeding cell development is unknown. To gain insights into the function of these secreted *M. incognita* proteins, we constitutively (ectopically) expressed the nematodes dorsal gland protein 7E12 in tobacco plants. It was found that the number of galls at 8 and 16 days after nematode infection was significantly higher in transgenic plants compared to control plants. Eggs from nematodes in transgenic plants hatched faster than those in control plants. Histological analysis of nematode induced galls in transgenic plants clearly shows a different morphology. Giant feeding cells harbor more vacuoles and an increased amount of cell wall invaginations, while neighboring cells surrounding feeding cells are more numerous. These results suggest that the presence of the 7E12 protein in tobacco accelerates gall formation. This assumption is supported by our data illustrating faster gall formation and egg eclosion in transgenic plants.

1. Introduction

Plant-parasitic nematodes encompass a diverse group of sophisticated pathogens that feed on plant organs like leaves, fruit, flowers, seeds and roots as obligate parasites. Damages caused by phytonematodes such as the root-knot nematodes *Meloidogyne* spp., cyst nematodes *Heterodera* spp. and *Globodera* spp., reach annual rates around US$125 billion globally [1]. In this scenario, *M. incognita*, a typical root-knot nematode (RKN), appears as the most harmful plant-parasitic nematode, infecting more than 1700 plant species due to its polyphagous habit [2].

Several adaptations are essential for all phytopathogenic nematodes that are successful parasites, including specialized organs such as, cuticular structures, amphids, the stylet, esophageal glands and their secretions [3]. These structures are essential to allow the root-knot nematode to establish parasitism, by locating the root tip, penetrating the root and finally inducing the formation of a specialized feeding site in the vicinity of the vascular cylinder of the root.

In the beginning of the parasitism cycle, the RKN eggs hatch releasing motile juveniles (J2) along the surface of the root. These J2s move towards the root tip of susceptible roots guided by cuticular structures and secretions from the amphid [4]. These authors used monoclonal antibodies against cuticle and amphidial proteins and confirmed that nematode motility can be affected and cause

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doi:10.1016/j.plantsci.2010.09.003

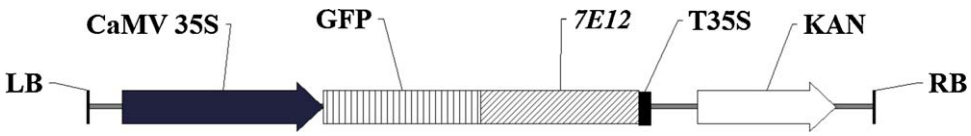

Fig. 1. Expression cassette showing CaMV 35S promoter followed by green fluorescent protein gene (GFP) fused to *7E12* from *M. incognita* esophageal gland. Kanamycin resistance gene (Kan) was used as selection marker. LB = left border; RB = right border; T35S = terminator.

a delay in root penetration as observed for cyst nematodes. After locating the root tip, worms invade the root by wall-piercing using their stylet and release a mix of hydrolases such as glucanases, xylanases and pectinases from their esophageal subventral glands to digest the plant cell wall and middle lamella polymers [5–7]. The importance of these enzymes during root invasion was clearly demonstrated by Chen et al. [8]. They showed that the knock-down of the cyst nematode *Globodera rostochiensis* β-1,4-endoglucanase using RNA interference (RNAi), resulted in the reduced ability of the nematode to invade *Solanum tuberosum* roots. Despite the obvious role of hydrolytic enzymes for nematode penetration, no information is available on the mechanism of nematode migration or feeding cell establishment [3].

Several studies contributed to the identification of potential signal molecules involved in the mechanisms of RKN parasitism. The first described and well characterized protein was the *M. javanica* chorismate mutase I [9]. This metabolic enzyme secreted by the nematode was ectopically expressed in transgenic soybean roots, and induced a phenotype of reduced and aborted lateral roots. It was speculated that this phenotype is caused by chorismate degradation in the cytoplasm reducing plastid auxin levels, leading to alterations in the shikimate pathway [9]. Another regulator described in *Heterodera glycines* esophageal gland was designated Hg-SYV4, a secretory peptide related to the CLAVATA3/ESR family. Hg-SYV4 down-regulates the expression of the transcription factor WUSCHEL, upon constitutive expression in transgenic Arabidopsis plants [10]. These lines were characterized by a reduction of stem cell population, a premature termination of the shoot apical meristem and the development of flowers lacking the central gynoecium [10]. Furthermore, the expression of a secreted peptide from *M. incognita* called 16D10, stimulates root growth in Arabidopsis, supposedly via an interaction with SCARECROW-like transcription factors [11]. Taken these studies together, it is feasible to conclude that nematode secreted proteins can affect plant cell functions and to suggest an active role of nematode compounds in the development of feeding sites.

It is generally accepted that phytopathogenic nematodes release signals in plant tissues during their parasitism cycle, called parasitism effectors. The role of these parasitism effectors in the adaptation of plant cells to allow nematode development was addressed by Zinov'eva et al. [12]. The induction of multinucleated giant cells is also mediated by the secretion of these parasitism effectors and by yet unknown mechanisms of plant gene expression reprogramming [13].

Our report focuses on nematode parasitism factors and the process by which these factors might contribute to the differentiation of plant host cells and the establishment of the parasitism.

Due to the obvious implications of the nematode esophageal glands in parasitism, subventral and dorsal gland products are intensively studied [14,15]. cDNA libraries from the cytoplasmic content of these glands revealed that the majority of the potential parasitism genes have no similarities with known proteins and are therefore referred to as novel genes [16].

We chose the 7E12 protein from the *M. incognita* cDNA library produced by Huang et al. [16], to gain insight about its role, because it possibly functions as an effector of *M. incognita* parasitism like the 16D10 protein, for example, as described by Huang et al. [11]. Here we present data illustrating that the ectopic expression of *7E12*, is able to stimulate faster feeding cell development in transgenic tobacco plants and results in an overall faster hatching of the RKN eggs. These data open new possibilities to engineer resistance against nematodes in agricultural important crops.

2. Methods

2.1. cDNA amplification and whole-mount in situ hybridization

First, cDNA was prepared using RNA extracted from *M. incognita* females according Gao et al. [15], using the SuperScript™ II (Invitrogen™) kit, following the manufacturer's instructions. Then, primers to clone the 7E12 cDNA were designed using the 7E12 mRNA sequence deposited in GeneBank (http://www.ncbi.nlm.nih.org) under the accession number AF531166. The cDNA was cloned into the pGEM-T easy vector (Promega - Madison, WI, USA).

To evaluate the spatial distribution of the gene expression, we used the in situ hybridization technique. Briefly, sense and antisense RNA probes were labeled with digoxigenin using the DIG RNA labeling kit (SP6/T7) (Roche). Probes were made by reverse transcription of cloned fragments in pGEM-T easy vector. Probe labeling was performed following the manufacturer's instructions. *M. incognita* females were extracted from roots according to Hussey and Barker [17], separated by individual pipetting and fixed with 2% paraformaldehyde solution. The in situ hybridization was performed as described by de Boer et al. [18].

2.2. Cloning of M. incognita 7E12 cDNA for expression in plants

The *7E12* cDNA was cloned from *M. incognita* females. The RNA was extracted and amplified as previously described. For cloning purposes, we used the Gateway system (Invitrogen™) following the manufacturer's instructions. Briefly, cDNA from the *M. incognita 7E12* gene was amplified without the signal peptide, using the primers Mi7F (5′ GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AGC AGG CGA TCG AAA TGC ATC 3′) and Mi7R (5′GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTA AAC AGT ATT AGC TCT TCC 3′). The PCR product was purified, subcloned in the pDONR™ 221 vector (Invitrogen™), and subsequently subcloned in the binary vector pK7WGF2 [19] for expression of GFP fusions in plants. The expression construct was composed of the GFP gene fused with the 7E12 gene driven by the CaMV 35S promoter. The expression cassette also contains the 35S terminator and the nptII gene as a Kanamycin selection marker (Fig. 1). All constructs were checked by sequencing.

2.3. Plant transformation and selection

The construct for ectopic expression of the *7E12* cDNA in tobacco plants was electroporated into *Agrobacterium tumefasciens* strain EHA105. The transformed *A. tumefasciens* cells were co-cultured with *Nicotiana tabacum* (var. Xantii) leaf discs. The material was subsequently multiplied *in vitro* according to the method described by Horsch et al. [20]. DNA and RNA extracted from *N. tabacum* (T1) plants was used to perform PCR and RT-PCR analysis in order to

verify the presence of GFP:7E12 fusion in the genomic DNA and transcripts, respectively, in the transgenic plants.

2.4. Nematode inoculum and infection

The *M. incognita* inoculum was carried out in *N. tabacum* (var. Xantii) transgenic (two independent events of transformation were selected with 16 repetitions each) and control plants (16 repetitions) cultivated in a greenhouse. *M. incognita* eggs were extracted according Hussey and Barker [17]. The egg suspension was submitted to a modified Baermann funnel technique in a distilled water recipient for hatching of second stage juveniles (J2) at room temperature during 48 h. J2 stages were harvested by centrifugation at 700 × *g* for 5 min and quantified in a Peters chamber. Each plant was inoculated with 600 J2.

In order to verify the eclosion of J2 nematodes at the end of its life cycle, forty egg masses were harvested 45 days after infection (DAI) from transgenic and non-transgenic *N. tabacum* plants and transferred to individual eclosion chambers. After 24 and 48 h the J2 were counted in a light microscope.

2.5. Sample preparations and microscopy analysis

The *N. tabacum* roots from transgenic and non-transgenic plants were assayed at 8, 16 and 28 DAI with five samples from the apical meristem and the secondary root internodes collected from each plant. Root segments were fixed with 2% glutaraldehyde, dehydrated in ethanol at 10, 30, 50, 70, 90 and 100%, and infiltrated with Technovit 7100 (EMS cat. no. 14655) resin according to the manufacturer. Polymerized blocks were cut using a microtome (2–4 μm) and subsequently stained with toluidine blue for microscopic visualization.

2.6. Protein extraction and immunodetection by dot blot

Aqueous protein extracts were prepared from 1 g of roots from both transgenic and non-transgenic plants of *N. tabacum*, 16 days after inoculation with *M. incognita* J2, triturated in a final volume of 1.5 ml of extraction buffer (100 mM sodium phosphate, 20 mM sodium chloride, 2 mM DTT, 0.1% PMSF, pH7.0). Root protein extracts were kept under soft agitation at 4 °C for 2 h. The samples were then centrifuged at 8000 × *g* for 30 min. Protein concentration of the supernatants was determined by the Bradford method [21] and the protein profile was determined via 12% SDS-PAGE [22]. Root aqueous extracts were acetone precipitated as described in [23] with final amounts ranging between 50 and 400 μg for each plant. Samples were resuspended in 50 μL PBS buffer (1×) and transferred to an Imobilon-P membrane, using a vacuum concentrator (BioRad). Protein transfer was carried out overnight at continuous agitation at 4 °C employing 0.09 V/30 mA in a Bio-Rad wet transfer system. The membrane was placed on a 50 ml tube covered with aluminum foil and blocked with a PBS solution for 2 h at 4 °C. Afterwards, the membrane was washed three times with PBS buffer (pH 7.0) containing 5 mM Tris–HCl, 15 mM NaCl, Tween 20 (1%), and milk (1%) and incubated overnight with a 1:5000 dilution of an anti-GFP Rabbit IgG antibody conjugated to Alexa Fluo 594 (Alexa - Invitrogen) under continuous agitation at 4 °C. Finally the membrane was washed three times with PBS buffer (pH 7.5) containing 5 mM Tris–HCl, 15 mM NaCl and Tween 20 (1%) and analyzed with a fluorescence microscope (Zeiss AxioImager).

2.7. Statistical analysis

In order to compare and evaluate the number of galls, egg masses, hatched J2's and root dry weight of control and 7E12 plants, ANOVA and Tukey's mean comparison test from SPSS software (SPSS Inc., Chicago, IL) were used for statistical analysis.

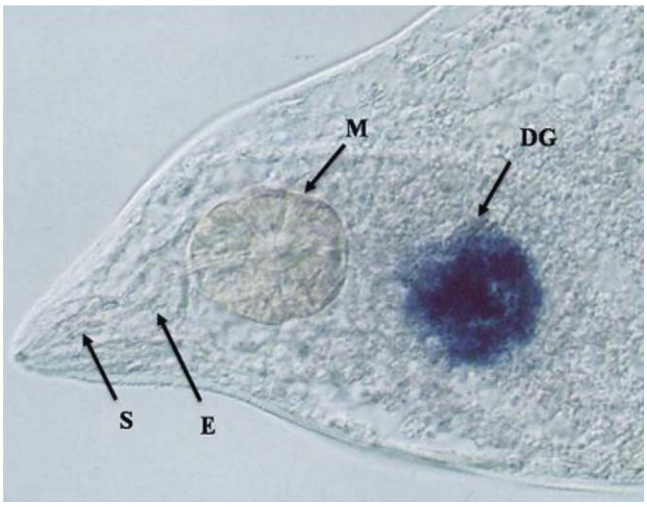

Fig. 2. *In situ* localization of *7E12* mRNA within female adults dorsal esophageal gland cells of *M. incognita*. DG, dorsal esophageal gland; S, stylet; E, esophagous; M, metacorpus.

3. Results

3.1. In situ hybridization

In order to study the expression profile of 7E12 in *M. incognita*, we performed *in situ* hybridization using the 7E12 itself as the probe. Fig. 2 presents a tissue slice of an adult nematode female. The stylet (S), esophagus (E), metacarpus (M) and dorsal esophageal gland (DG) are depicted. *In situ* hybridization of the *7E12* probe shows a strong and specific signal at the dorsal gland, confirming similar results obtained by Huang et al. [16] that the 7E12 protein is specifically transcribed in the *M. incognita* esophageal dorsal gland.

3.2. Dot blot analysis confirms the presence of 7E12:GFP fusion protein

PCR positive and kanamycin-resistant T1 plants (data not shown) were selected to verify the expression of the gene construct *7E12*, by dot blot. In a first assay, aliquots of 50 μg of root protein extracts were used from transgenic and non-transgenic plants and probed with anti-GFP antibody. Fig. 3 shows that under these conditions, only the positive control shows a positive immunoreaction. However in a dot blot assay using an 8-fold concentrated root protein extract (400 μg), the presence of a faint GFP signal can also be observed in the 7E12 event. Negative controls were completely negative, whereas GFP controls showed a clear positive immunoreaction (Fig. 3). All the other events showed the same signal in dot blot assays (data not shown).

Based on these results, we conclude that although present in root cells, the cellular concentration of the fusion protein is far to low for proper imaging of its fluorescence in living roots.

3.3. Macroscopic and microscopic analyses of transgenic roots phenotype

We performed our analyses in tobacco plants because it is a well established plant model in our laboratory. It also has the advantage over *Arabidopsis* plants when we consider the number and weight of the roots, which facilitates the macroscopic analysis of the nematode infection assays.

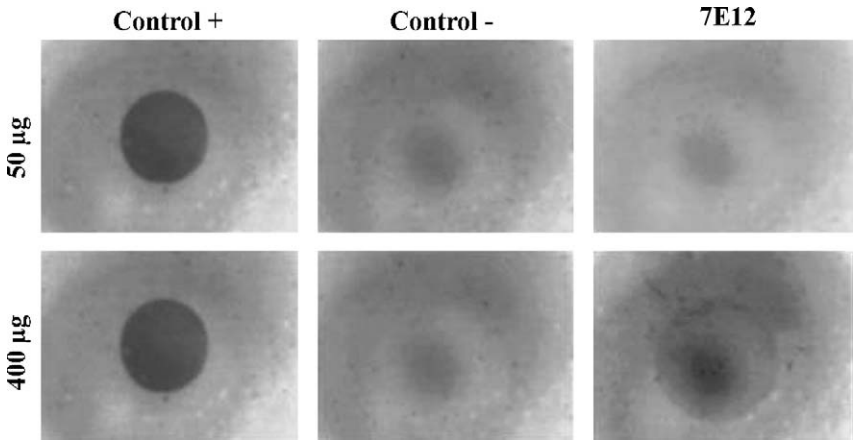

Fig. 3. Dot blot using a fluorescent labeled anti-GFP antibody. Positive controls represent 50 and 400 μg of a transgenic *Nicotiana tabacum* expressing free GFP. The negative controls represent 50 and 400 μg of a non-transgenic *N. tabacum* root galls protein extract. Dot blots from 7E12 plant extracts were made with 50 and 400 μg of protein obtained from plants 15 DAI.

For the phenotypic evaluation, we used the two independent transformation events of *N. tabacum*, 7E12-1 and 7E12-2, which were produced by *Agrobacterium* transformation. These events were selected for their kanamycin resistance due to the presence of the marker gene *nptII* in the pK7WGF2 vector. The seeds from these two events (T0) were also selected in the presence of kanamycin because the T1 generation is segregating. Those seeds that were able not only to germinate, but also to develop roots in the presence of kanamycin, were selected and submitted to PCR and RT-PCR in order to assess the presence of the 7E12 gene and transcript, respectively (data not shown). We used non-transformed plants as negative controls. We used 16 replicates for each treatment, including the two 7E12 events, all checked for the presence of GFP:7E12, and control plants.

The macroscopic analyses were performed 25 days after germination. Our results showed that the plants do not show any apparent phenotypic differences in relation to the non-transformed plants when not infected by nematodes (data not shown). However, the comparison of roots from transgenic and non-transformed *N. tabacum* infected with *M. incognita* J2 and collected 8, 16 and 28 DAI, clearly illustrated a different morphology and size of the RKN galls (Fig. 4). Eight DAI, events 7E12-1 and 7E12-2 showed larger and more elongated galls as compared to control plant roots (Fig. 4). In 16 DAI plants, galls from events 7E12-1 and 7E12-2 were significantly bigger than control galls. It was observed an obvious difference in gall morphology between 7E12 transgenic events and control galls in all analyzed stages. These results suggest that galls developed faster in transgenic plants than in control plants.

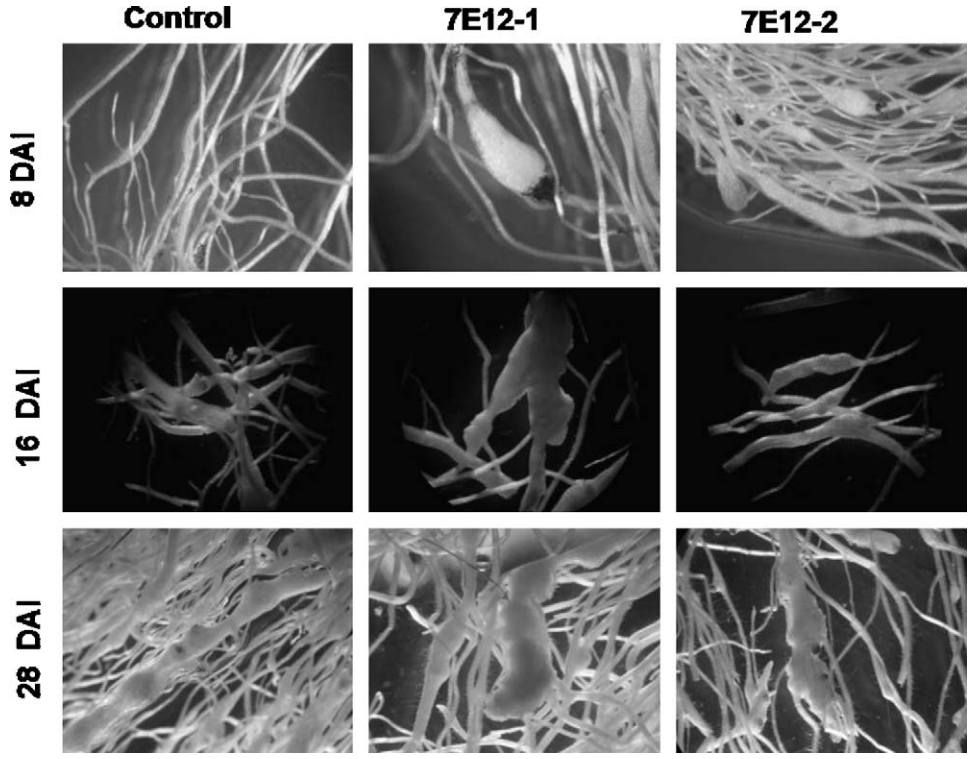

Fig. 4. *Nicotiana tabacum* roots inoculated with second stage juvenile (J2) of *Meloidogyne incognita*. The roots were collected after 08, 16 and 28 DAI.

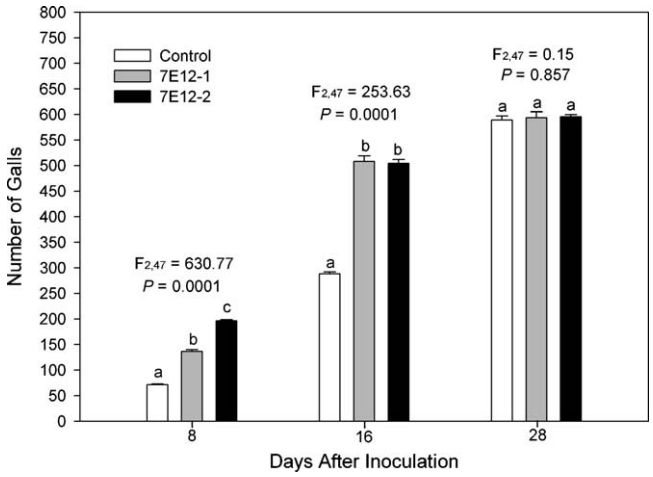

Fig. 5. Number of galls after 8, 16 and 28 DAI. The number of galls were counted and compared with control plants. Sixteen replicate plants were counted. Bars indicate the standard error of each group and different letters mean statistical difference between groups by Tukey's means comparison test.

Statistical analysis showed that the number of galls between control and transformed events, both harvested at 8 and 16 DAI, was significantly different (Fig. 5). The average number of galls observed for each plant at 8 and 16 DAI was almost twice the number in control plants. However, at 28 DAI, the number of galls was quite similar (Fig. 5). Both 7E12 plants showed a higher number of galls with modified morphology of the largest galls. This observation suggests that the process of gall formation is accelerated in the 7E12 events, corroborating our morphological data.

Microscopic analysis of tissue sections showed that giant cells are slightly different in size and morphology when comparing control with transgenic events at 8 DAI. Galls from both 7E12 events contain giant cells with more undulated walls and a larger number of small sized vacuoles (Fig. 6). Furthermore, it was observed that the neighboring cells surrounding the feeding cells are much more numerous in the transgenic events than in control galls (Fig. 6).

Resistance tests carried out on samples 45 DAI showed that the number of egg masses of the two 7E12 events and control plants were statistically not different (data not shown) yielding an average of 500 egg masses per plant. Additionally, the dry weight of roots from the 7E12 events was around 120 mg and similar to those from control plants (data not shown). Although an accelerated gall formation could be observed in 7E12 plants until 28 DAI, the final number of galls at 45 DAI was not different between 7E12 events and control plants.

The analysis of egg hatching carried out with eggs harvested 45 DAI from 7E12 and control plants showed a higher number of hatched J2's for the 7E12-1 and 7E12-2 eggs than control eggs after 24 and 48 h (Fig. 7). The number of hatched J2's from event 7E12-2 was also higher than the number for event 7E12-1 (Fig. 7). This result suggests that egg maturation in 7E12 transformed plants at 45 DAI is faster in comparison to eggs in control plants, as a result of an accelerated female development.

4. Discussion

This study evaluates the role of the novel gene *7E12* in the *M. incognita* parasitism process. To do so, a construct containing a fusion of *7E12* cDNA with GFP was generated to ectopically overexpress the 7E12 protein in tobacco plants. Protein 7E12 was first isolated and described by Huang et al. [16] using a cDNA library from *M. incognita* esophageal glands. To identify and characterize differentially expressed nematode genes in phytopathogenic

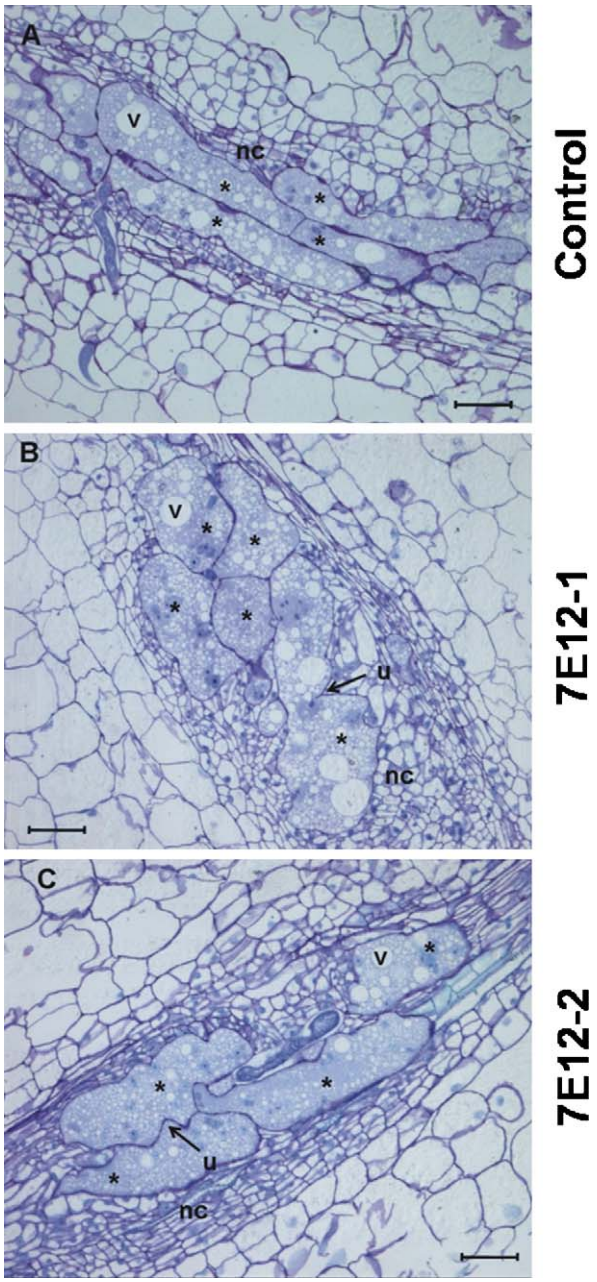

Fig. 6. Bright-field images from giant cells after toluidine blue-staining of control and 7E12 transformed *N. tabacum* galls, eight DAI with *M. incognita*. (A) Control plant; (B) and (C) 7E12-1 and 2 plants. nc, neighboring cells; v, vacuoles; u, undulations; *, giant cell; bar, 100 μm.

and non-phytopathogenic nematodes is an important strategy to study the vital biological processes involved in the nematode's life cycle such as feeding, development, signaling, reproduction and longevity [24,25] as well as for the better understanding of the host–parasite interactions [26].

In this work, we used a gene construct in fusion with a GFP gene reporter in order to localize the 7E12 protein in plant roots. The very low expression level of this fusion protein, however, prevented us from detecting GFP fluorescence of the 7E12:GFP fusion protein in transgenic events using confocal microscopy.

Dot blot analysis, on the other hand, confirmed the presence of the GFP protein in extracts from the 7E12 transgenic events. Despite of the low concentration of GFP and probably 7E12 protein in roots, its presence causes a profound effect on the development

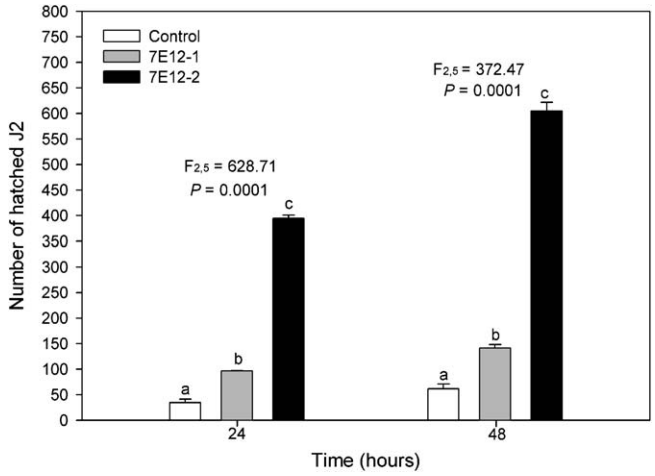

Fig. 7. Evaluation of J2 eclosion from control and transformed *N. tabacum* plants, 45 DAI. The number of hatched J2 was determined 24 and 48 h after egg mass removal. Bars indicate the standard error of each group and different letters represent statistical differences between the groups using the Tukey's means comparison test.

of giant cells. As controls, we used non-transformed plants. It is well established in the literature that neither the CaMV35S promoter nor GFP protein expression affect the life cycle of *M. incognita* [27]. It was observed that galls were induced earlier in transgenic plants than in the control plants. Moreover, transgenic events expressing 7E12 always showed an increased number of galls mainly between 8 and 16 DAI. Histological analysis reveal a clear morphological difference between giant cells from the transgenic and the control plants. Transgenic events giant cells contain more numerous, but smaller vacuoles, and are surrounded by a higher number of neighboring cells. Also, giant cells in the transgenic events were characterized by more undulated cell walls. These histological data suggest a faster maturation of feeding cells in plants that ectopically express the 7E12 protein. Finally at 28 DAI, no significant difference of the number of galls was observed when compared with wild type plants. These data suggest that *M. incognita* dorsal gland 7E12 protein has an important role in establishing a compatible interaction between the plant and the nematode at an early stage. The 7E12 protein probably acts early during the infection process by stimulating giant cell formation which, in turn, leads to a faster development of the nematode feeding site. This hypothesis is supported by the observation that a higher number of hatched J2 nematodes were found in the transgenic events at 24 and 48 h DAI, indicating faster hatching when compared to control plants.

To our knowledge, this is the first report describing a *M. incognita* dorsal gland protein that upon ectopic expression in a plant is able to accelerate feeding site development. The nematode secreted 7E12 protein responsible for this phenotype shows no identity with any protein of known function using BLASTn and BLASTp (http://www.ncbi.nlm.nih.gov/BLAST) [28]. As such, the potential function for this protein remains unknown. However, it is known that the *7E12* gene is exclusively transcribed in the *M. incognita* dorsal gland [16] at a time point between host root penetration and nematode feeding site induction, which corresponds with proteinaceous granule accumulation in the developing dorsal gland of the parasite. This fact provides evidence that the dorsal gland of sedentary endoparasite phytonematodes is probably a major site responsible for the synthesis of essential parasitism factors related to host cell control [3].

Another finding that supports this hypothesis is described by Huang et al. [11], in which it is showed that a small peptide named 16D10, expressed by the *M. incognita* dorsal gland, was ectopically expressed in Arabidopsis and resulted in an increase of the cell division rate in root meristems. The mature 16D10 peptide was similar to the C-terminal conserved motif of the plant CLE protein family [29,30]. Moreover, two SCARECROW-like transcription factors of the GRAS protein family were suggested as the putative targets of this nematode protein.

Taking these results together, we hypothesize that the 7E12 protein has a similar interaction with some protein involved in the cell division process. However, the lack of information about 7E12, which has no similarity with any protein of known function, neither a motif nor domain identified in its amino acid sequence, prevented us to derive a more solid hypothesis concerning its function.

To validate our results and to gain insight into the function of the 7E12 protein, we are currently generating knockouts and knockdowns of this gene using the RNAi strategy. In addition, it will be important to identify the target(s) of 7E12 in plants. The understanding of the mechanism of nematode parasitism proteins will undoubtedly aid in the development of better strategies to control resistance against phytopathogenic nematodes.

Acknowledgments

We are grateful to CNPq, CAPES, FAP-DF, Embrapa Genetic Resources and Biotechnology (Brazil) and INRA (France) for grants, scholarships and financial support to this study.

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