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Molecular Characterization of Chitinase (*chi18-5*) and its Expression in *Trichoderma viride:* Role on Nematode Egg Parasitism

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A B S T R A C T

Studies on molecular mechanism of *Trichoderma viride* in the pathogenesis action on nematode eggs mostly focus on the transcriptional activity of selected genes. However, studies investigating the exact role of a specific gene induction in *T. viride* on nematode egg parasitism are scant. The molecular interface between *T. viride* and *Meloidogyne incognita* eggs was investigated through the qPCR gene expression. The resultant gene *chi18-5* of *T. viride* played a vital role in the induction of mycosis in the nematode eggs. Real-time PCR revealed that *chi18-5* started up regulating at 2 hours post-inoculation (hpi) with maximum expression at 5 hpi, and the up regulation of the gene gradually decreased until 19 hpi. The chitinolytic enzyme *chi18-5* plays a major role in egg parasitism in transcriptional activity in *T.viride* was characterized. Our present study proved that *chi18-5* encodes one of the lytic enzymes required by *T. viride* to parasitise nematode eggs.

Introduction

Root-knot nematodes are major pests of agri-horticultural crops and cause severe yield loss to a wide range of vegetables in tropical and subtropical countries (Dababat *et al.*, 2005; Khalil, 2012). Several fungal and bacterial pathogens gain easy entry into the plant root system affected by nematodes and lead to disease complexes (Taylor, 1990). Naturally occurring soil organisms that show antagonistic activity against plant parasitic nematodes are successfully used as biocontrol agents (Dong and Zhang, 2006). Of the many efficient biocontrol agents identified, only a few of them are successfully commercialized (Larkin *et al.*, 1998; Meyer and Roberts, 2002). Among the several bacterial and fungal biocontrol agents used, *Trichoderma viride* is extensively used to control a wide range of plant parasitic nematodes (Al-Hazmi and Tariq, 2016; Kerry, 2000; Meyer *et al.*, 2001; Shamalie *et al.*, 2012). *T. viride* is an effective biocontrol agent against *Meloidogyne* spp. (Saeedizadeh, 2016). It significantly reduced the number of egg masses of *M. incognita* (Dababat *et al.*,

2007) and was effective against several fungal pathogens (Kapoor *et al.,* 2010). Many successful reviews on the use of *T. viride* to control plant-parasitic nematode infestations on various crops have been reported (Rajinikanth *et al.,* 2013; Rao *et al.,* 2007; Sahebani and Hadavi, 2008; Sharon *et al.,* 2001; Spiegel *et al.,* 2007; Yang *et al.,* 2010). Mycoparasitism exhibited by *Trichoderma* spp. has been well documented by many researchers (Howell, 2003; Savazzini *et al.,* 2009; Szabo *et al.,* 2012; Verma *et al.,* 2007; Vinale *et al.,* 2008) and applied for the biocontrol of phytopathogens.

Trichoderma spp. are highly competitive in root, soil, and foliar environments. It produces several lytic enzymes such as chitinases, proteases, lipase, and glucanases to degrade the cell wall components of pathogenic fungi (Blaszczyk *et al.,* 2014; Chet *et al.,* 1997; GajeraandVakharia, 2012; Geraldine *et al.,* 2013; Parmar *et al.,* 2015). Among all the enzymes, chitinase plays the most vital role in nematode egg parasitism by *Trichoderma* spp. by which the fungus ruptures the egg shell of the nematodes (Gortariand Hours, 2008). Mycoparasitism involves morphological changes, such as formation of appressorium-like structures and coiling, to penetrate a host (McIntyre *et al.,* 2004).

Increase in chitinase activity through direct egg parasitism by *Trichoderma* spp. can reduce nematode infestation (Sharon *et al.,* 2001; Suarez *et al.,* 2004). Chitinase genes, such as *chi18-5*(chit42), *chi18-12* (chit33), *chi18-15* (chit36) (Viterbo *et al.,* 2001, 2002), and *nag,* (Brunner *et al.,* 2003) and their effective regulation against mycoparasitism have been extensively studied. Relative gene expression data was analysed using real-time qPCR and the $2-\Delta\Delta CT$ method (Livak and Schmittgen, 2001).

In this study, experiments were conducted to evaluate chitinase *chi18-5*(chit42) gene expression, and variation in activity was determined at different time intervals using real-time PCR. The chitinase enzyme assay was performed to measure the activity in control and test samples under various conditions.

Materials and Methods

Collection of the bioagent culture and nematode egg masses

T. viride strain (ITCC No. 6889) was isolated and maintained at ICAR-Indian Institute of Horticultural Research on potato dextrose agar (PDA) using the cryopreservation method (Sudheer, 2010). A subculture of test inoculants of *T. viride* was prepared by placing a 5-mm culture disc on plates with solidified PDA and incubated for 3 days at 27±2°C.Pure culture of the pathogenic root-knot nematode *M. incognita* was collected from nematode-infected cauliflower plants at Doddaballapur (Bengaluru Rural, Karnataka, India), and presence of *M. incognita* was confirmed by the perineal cuticular pattern observed under a stereomicroscope (Hussey and Barker, 1973). The identified *M. incognita* was used for further studies.

Assay of egg parasitism

T. viride was pre cultured in PDA medium for 3 days. Approximately 5–8 egg masses of each carrying approximately 150–250 eggs of *M. incognita* were used for the assay. *T. viride* culture was used to observe endochitinase gene expression upon interaction with *M. incognita*. The egg masses were placed near the periphery of *T. viride* (10^8 spores/mL) mycelia in a 5-mm-wide zone in each PDA plate *in vitro*. To maintain sterile conditions, the PDA plates

were sealed and incubated at 25°C in the dark and replicated thrice. Observations were recorded at various time intervals [0, 1, 2, 3, 5, 7, 9, 11, 13, 15, 17, and 19 h post-inoculation (hpi)] to monitor the variation in chitinase gene expression. At each interval, *M. incognita* egg mass-colonised *T. viride* mycelia were collected and pooled from 10 plates. They were then flash-frozen in liquid nitrogen and stored at −80°C for further downstream applications.

RNA extraction, cDNA synthesis, and real-timePCR

RNA extraction was performed from 25mg of stored fresh fungal mats using a NucleoSpin Tissue extraction kit (Macherey-Nagal, Germany), following the supplier's instructions. After DNase treatment, RNA concentrations quantified at 260/280 nm were estimated using a NanoDrop spectrophotometer (Thermo Scientific-Nano drop light, USA). RNA was reverse-transcribed into a single-stranded cDNA using a Verso cDNA synthesis kit (Thermo Scientific, USA) following the supplier's instructions. Primers were designed using a web-based online primer design service (RozenandSkaletsky, 1999), Primer3 (http://fokker.wi.mit.edu/primer3). The endochitinase gene (*chi18-5*) was used for analyzing chitinase gene expression, and alfa-tubulin (Szabo *et al.,* 2012) was used as a reference gene. PCR was performed using a Step One real-time PCR system (ABI-7500, TR-PCR-Applied Biosystems) with SYBR green master mix (Takara Bio Inc, Japan) to examine DNA synthesis. Final reaction was performed in 25 µL volumes, according to the manufacturer's instructions, containing 1:10 volume of cDNA and 1 µL (0.5µM) of gene-specific forward and reverse primers (Table 1). All reactions were triplicated including a control with no template. Single gene amplification was confirmed by the presence of melting curves. The variation levels of *chi18-5* expression were estimated using log2RQ values with a 0 hpi steady state chitinase transcript at all remaining time points (0,1,2,3,5,7,9,11,13,15,17, and 19).

Each experimental setup of qPCR was designed and performed according to the Minimum Information for Publication of qPCR Experiments guidelines (Bustin *et al.,* 2009). Alfa-tubulin was used as a normalisation control.

Chitinase enzyme assay

One gram of *T. viride* mycelium was homogenised in 2 mL of 0.1 M sodium acetate buffer (pH5.0). The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C, and the supernatant was used for the chitinase enzyme assay. The protein concentration was estimated by the method of Lowry *et al*. (1951). *T. viride* chitinase activity was assayed with 200 µL of colloidal chitin (5 mg/mL) and 200 µL of enzyme solution. The mixture was incubated for 60 min at 40°C, and the reaction was terminated by adding 1 mL of 1% NaCl and centrifuged at 6000*g* for 5 min. The supernatant was boiled with 100 µL of potassium tetraborate buffer for 3 min. To this reaction, 3 mL of DMAB reagent [10 g of dimethyl amino benzaldehyde in 100 mL of glacial acetic acid (12.5%) and 10 M chloridric acid (87.5%)] was added. The mixture was incubated at 40°C for 20 min, and the amount of N-acetylglucosamine (GLcNAc) produced in the supernatant was determined by a previously described method (Zeilinger *et al.,*1999) and using GLcNAc as a standard. One unit of enzyme activity was defined as the amount of enzyme that catalysed the release of 1 µmolGLcNAc in 60 min at 40°C.

Results and Discussion

Effect of chitinase (chi18-5) on eggs of *M. incognita*

Chitinase (*chi18-5*) gene expression pattern was studied at various time intervals as shown in Fig1. At 1 hpi, the expression gradually increased (log2RQ fold change to 5.079) and at 2 hpi to 5.201. The expression was observed at 3 and 5 hpi (log2RQ fold change to 5.690 and 6.261) with a decrease in the expression observed at 7 hpi to 5.923. It was followed by gradual decrease in expression from 9 hpi (log2RQ fold change to 5.217) and at 11, 13, and 15 hpi (log2RQ fold change to 3.874, 3.516, and 3.162). It was continued at 17 hpi (log2RQ fold change to 2.314)and then final decrease to 1.392 at 19 hpi (Fig.1). Chitinase (*chi18-5*) is mainly involved in early stages of mycoparasitic process, which establishes a hyphal attachment with the host (Zeilinger *et al.*, 1999). Results indicated that chitinase gene (*chi18-5*) played an important role in the *M. incognita* egg parasitism.

Chitinase assay

At room temperature, chitinase specific activity was 0.018µmol/min/mL, whereas the chitinase activity increased to 0.038 µmol/min/mL *invitro* at 5 hpi in the presence of *M. incognita*. The enzyme activity was measured at 5 hpi since the maximum chitinase (*chi18-5*) expression was observed.

Table.1 Primers for Endochitinase (Chi18-5) and Alfa-tubulin genes

S.No	Gene	Forward Primer	Reverse Primer	NCBI AC Numbers
1	*Chi18-5*	CGGTATCTGGGATTACAAGG	GCCTCCCAGAACATGCTACC	KP271024
2	Alfa-tubulin	CTGGTCTTCCCTCTCCCTCA	GGCAGCAACCTCCTCGTAAT	KP271025

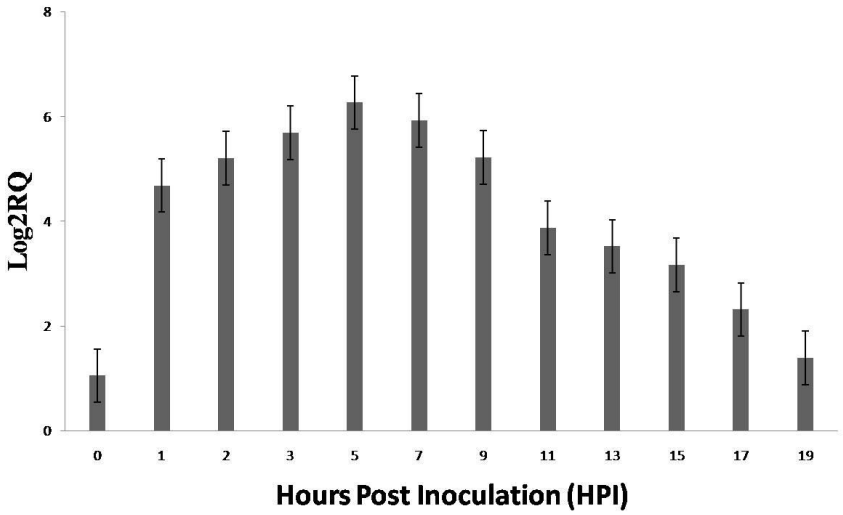

Fig 1. Chitinase (*chi18-5*) gene expression pattern at various Hours Post Inoculation (HPI)

T. viride has a parasitic and symbiotic association with plants and other microbes (Kubicek and Harman, 1998; Perveen and Bokhari, 2012). This fungus was also found to be more resistant to natural and human-made chemicals and toxins (Harman *et al.,* 2004). Nematophagous fungi use specialised mycelial structures to entrap and thus kill and assimilate motile nematodes, eggs, and cysts (Nordbring-Hertz *et al.,* 2006; Szabo *et al.,* 2012). *Trichoderma* spp. utilize chitinase along with other enzymes (proteases, lipase, and glucanases) to degrade the cell wall of the pathogens (Boller and Mauch, 1988; Duo-chuan, 2006; Gortariand Hours, 2008; Seidl *et al.,* 2005; Szabo *et al.,* 2012).

Hence, *T. viride* is a major source of chitinolytic enzymes, and combinations of these fungal hydrolytic enzymes function effectively in chitin hydrolysation. The synergistic effect of chitin hydrolysis is one of the major factors contributing to the biocontrol ability of *T. viride* against a broad spectrum of chitin-containing plant pathogens (Brunner *et al.,* 2003; Carsolio *et al.,* 1994; Chet *et al.,* 1998; Haran *et al.,* 1995; Inbarand Chet, 1995; Kulling *et al.,* 2000a; Viterbo *et al.,* 2001).

In mycoparasitism, *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1, 3-glucanases, and proteases (Haran *et al.,* 1996). Therefore, chitinase gene expression on nematode egg parasitism was clearly established at various time intervals. *Trichoderma* has the potential to produce cellwall-degrading enzymes in the presence of chitin in the growth medium. *Trichoderma* β-1,3-glucanases are responsible for hydrolysis of phytopathogenic fungi during a mycoparasite attack (Matroudi *et al.,* 2009). Colonisation by *Trichoderma* strains results in increased levels of defence-related plant enzymes, including various peroxidases, chitinases, β-1,3-glucanases (Harman *et al.,* 2004).

Chitinase gene (*Chi18-5*) expression studies revealed that *T. viride* possesses excellent biocontrol abilities against *M. incognita.* It is involved in direct nematode egg parasitism. Chitinase activity varied depending on the influence by the expression of chitinase. Using the fundamental knowledge of the present study, we aim at developing new strains with *chi18-5* over expression that may help in controlling nematodes effectively and confirm the role of chitinase in *T. viride* on egg parasitism. Additional studies on the mode of action of other enzymes antagonistic to nematodes are needed.

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